Research Article

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Universal primers for species authentication of animal foodstuff in a single polymerase chain reaction

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Abstract

BACKGROUND: There are many DNA-based systems for detecting animal species present in food and food products, applicable for food quality control and authentication. However, most (if not all) methods require more than one pair of primers and cannot be applied over a wide taxonomic range, e.g. identifying vertebrates and invertebrates with the same primers and protocols.

RESULTS: A pair of primers is described here that allows in a single polymerase chain reaction the identification of animal species in food and processed (precooked, canned or smoked) food products over a wide taxonomic range.

CONCLUSION: These primers permit the identification of most animal taxa employed in human nutrition, from invertebrates such as molluscs to higher vertebrates, distinguishing between species of the same genus. The short fragment amplified within the 16S rDNA exhibits phylogenetic value and could be considered universal based on the wide taxonomic range assayed. The primers are easy to use and accessible for laboratories with a modest budget, as well as being valuable for consumer information and to reveal food fraud.
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Keywords: universal primers; species identification; animal foodstuff; 16s rDNA

INTRODUCTION

The fraudulent misdescription of food contents on product labels is a widespread problem[1–4] that poses serious risks to human health (e.g. allergies)[5,6] as well as problems for resource conservation.[7,8] Detection of food constituents can be carried out by a plethora of methods.[9] Many of them are based on DNA: various molecular markers used to physically map genomes have now been successfully adapted for detection of food substitution, allowing the speciation of meats, fish and fruits in processed food products.[10] Species identification through DNA sequences, particularly the cytochrome oxidase (COI) gene, has been proposed as a barcode for identification of all living organisms.[11]

Variation in DNA sequences can be revealed by applying many different techniques. Genetic markers employed in food analysis have been reviewed by various authors,[9,10,12] and seafood identification has been a subject of special attention[13,14] owing to the high level of mislabelling detected worldwide.[1] Most markers use polymerase chain reaction (PCR) methodology because it permits a large number of copies of target sequences to be obtained from a minimal amount of sample tissue, and positive results can be obtained from degraded (e.g. precooked, canned or smoked) samples. It is based on designing a pair of primers that flank the DNA region to be amplified. The target DNA is denatured to allow primer annealing by nucleotide match on the complementary sequences, then the region between the primers is replicated by a DNA polymerase (Taq polymerase) in successive cycles of denaturing/annealing/replication. The obtained fragment can be sequenced or the genotype revealed in different ways (fragment size, presence/absence of restriction sites, etc). Sequencing and genotyping protocols are technically simple and do not require expensive equipment.

However, despite considerable development of DNA-based methods for food identification, the perfect universal PCR marker has not been found to date. Theoretically, a good pair of universal PCR primers should amplify target DNA regions from a vast range of species (from plants to invertebrates such as shrimps and mussels to vertebrates such as fish, pigs and cows), and such DNA regions should be short enough to enable amplification from samples with degraded DNA (cooked, processed food), generally less than 200 nucleotides.[10,15] Identifying DNA regions with some parts extremely conserved (to design primers that can anneal in a wide range of species) and other short zones with high phylogenetic value (i.e. variable among species but not within species) is thus a requisite for designing true universal primers useful for food identification at species level over a wide phylogenetic range of species.

16S rDNA fulfils these requirements: it contains some internal regions strongly conserved across taxa, suitable for designing

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primers that would amplify in a diverse variety of species, alternated with other short regions less conserved that exhibit species-specific differences and confer phylogenetic value to this mitochondrial gene.[16] The objective of the present study was to design primers within 16S rDNA for amplification of short species-specific DNA segments containing enough variability to solve speciation in both distant and closely related taxa, and to test their validity in a large variety of food samples from different species and origins.

MATERIALS AND METHODS

Baseline sequences for designing primers

Groups of animals of interest in food sciences were pre-targeted: vertebrates such as mammals, birds and fish and invertebrates such as gastropods. In total, 40 16S rDNA sequences of representative species of main classes (Table 1), covering a wide phylogenetic range, were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/).

Design of universal primers

The construction of primers was based on the nucleotide sequences of the 16S rDNA of the 40 animal species listed in Table 1. For this, sequences were aligned using the ClustalW application[17] included in the BioEdit Sequence Alignment Editor software.[18] A region in the 16S rRNA gene of less than 100 nucleotides having a sequence that was different between species but highly conserved within each species and being flanked on both sides by conserved regions was searched for, so that the primers could specifically hybridise to the flanking regions.

Table 1. Sequences of 16S rDNA gene retrieved from GenBank that were employed for developing new marker: species (scientific and common name), class and GenBank Accession Number (AN)

No.	Scientific name	Common name	Class	GenBank AN
1	*Merluccius merluccius*	European hake	Actinopterygii	DQ274032
2	*Salmo salar*	Atlantic salmon	Actinopterygii	HQ167673
3	*Merluccius australis*	Southern hake	Actinopterygii	FJ215162.1
4	*Oncorhynchus clarkii*	Cutthroat trout	Actinopterygii	AY886762.1
5	*Pomatomus saltatrix*	Bluefish	Actinopterygii	FJ374805.1
6	*Salvelinus fontinalis*	Brook trout	Actinopterygii	NC_000860.1
7	*Dissostichus eleginoides*	Patagonian toothfish	Actinopterygii	FJ647635.1
8	*Salmo trutta*	Brown trout	Actinopterygii	GU214390.1
9	*Sprattus sprattus*	European sprat	Actinopterygii	AM911201.1
10	*Thunnus obesus*	Bigeye tuna	Actinopterygii	GU256525
11	*Merlangius merlangus*	Whiting	Actinopterygii	NC_007395.1
12	*Ammodytes personatus*	Pacific sandlance	Actinopterygii	EF042197.1
13	*Clupea harengus*	Atlantic herring	Actinopterygii	GU324147.1
14	*Engraulis ringens*	Peruvian anchovy	Actinopterygii	AM911216.1
15	*Gadus macrocephalus*	Pacific cod	Actinopterygii	GU581267.1
16	*Patella rustica*	Rustic limpet	Gasteropoda	JF758500.1
17	*Patella vulgata*	Common limpet	Gasteropoda	AB238445
18	*Petromyzon marinus*	Sea lamprey	Cephalaspidomorphi	U11880
19	*Triturus marmoratus*	Marbled newt	Amphibia	NC_015795.1
20	*Alytes obstetricans*	Common midwife toad	Amphibia	DQ283112.1
21	*Bufo bufo*	European common toad	Amphibia	GQ380402.1
22	*Lissotriton helveticus*	Palmate newt	Amphibia	GQ380399.1
23	*Carcharodon carcharias*	Great white shark	Chondrichthyes	EF081281.1
24	*Alectorys chukar*	Chukar partridge	Aves	FJ752426
25	*Coturnix coturnix*	Common quail	Aves	AF302070.1
26	*Cairina moschata*	Muscovy duck	Aves	EU755254.1
27	*Gallus gallus*	Chicken	Aves	AY235571
28	*Meleagris gallopavo*	Wild turkey	Aves	NC_010195.2
29	*Anas platyrhynchos*	Mallard	Aves	AB238445
30	*Phasianus colchicus*	Common pheasant	Aves	NC_015526.1
31	*Bos taurus*	Cattle	Mammalia	AB511049
32	*Oryctolagus cuniculus*	European rabbit	Mammalia	NC001913
33	*Ovis aries*	Sheep	Mammalia	HM236185.1
34	*Sus scrofa*	Pig	Mammalia	AP003428.1
35	*Felis silvestris*	Wildcat	Mammalia	DQ334822.1
36	*Canis lupus*	Grey wolf	Mammalia	DQ334813.1
37	*Rattus norvegicus*	Common rat	Mammalia	AC_000022.2
38	*Delphinus delphis*	Short-beaked common dolphin	Mammalia	EU685096.1
39	*Balaena mysticetus*	Bowhead whale	Mammalia	NC_005268.1
40	*Eubalaena australis*	Southern right whale	Mammalia	AP006473.1

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Table 2. Species with control samples in which target partial 16S rDNA sequence was amplified with new primers 16S-HF and 16S-HR: common and scientific name of species, class, geographic origin of samples analysed, family and GenBank Accession Number (AN) of target partial sequence

Common name	Scientific name	Class	Geographic origin	Family	GenBank AN
Pacific sandlance	*Ammodytes personatus*	Actinopterygii	Pacific Ocean, Japan	Ammodytidae	HQ623638
Peruvian anchovy	*Engraulis ringens*	Actinopterygii	Pacific Ocean, Peru	Engraulidae	HQ623641
European sprat	*Sprattus sprattus*	Actinopterygii	Atlantic Ocean, Norway	Clupeidae	HQ623649
Grey cod	*Gadus macrocephalus*	Actinopterygii	Pacific Ocean, USA	Gadidae	HQ623642
Atlantic herring	*Clupea harengus*	Actinopterygii	Atlantic Ocean, UK	Clupeidae	HQ623640
Whiting	*Merlangius merlangus*	Actinopterygii	Atlantic Ocean, Spain	Gadidae	HQ623646
Cape hake	*Merluccius paradoxus*	Actinopterygii	Atlantic Ocean, South Africa	Gadidae	HQ623630
European hake	*Merluccius merluccius*	Actinopterygii	Atlantic Ocean, Spain	Gadidae	HQ623628
Southern hake	*Merluccius australis*	Actinopterygii	Pacific Ocean, New Zealand	Gadidae	HQ623626
Black prochilodus	*Prochilodus nigricans*	Actinopterygii	Continental waters, Brazil	Curimatidae	HQ623625
Brycon	*Brycon orbignyanus*	Actinopterygii	Continental waters, Brazil	Characidae	HQ623627
Cutthroat trout	*Oncorhynchus clarkii*	Actinopterygii	Continental waters, USA	Salmonidae	HQ623629
Bluefish	*Pomatomus saltatrix*	Actinopterygii	Atlantic Ocean, USA	Pomatomidae	HQ623631
Brook trout	*Salvelinus fontinalis*	Actinopterygii	Continental waters, USA	Salmonidae	HQ623632
Atlantic salmon	*Salmo salar*	Actinopterygii	Aquaculture, Norway	Salmonidae	HQ623624
Brown trout	*Salmo trutta*	Actinopterygii	Continental waters, Spain	Salmonidae	HQ623639
Cape snoek	*Thyrsites atun*	Actinopterygii	Indian Ocean	Gempylidae	HQ623635
Stripped trumpeter	*Latris lineata*	Actinopterygii	Indian Ocean	Latridae	HQ623634
Patagonian toothfish	*Dissostichus eleginoides*	Actinopterygii	Indian Ocean	Nototheniidae	HQ623633
Domestic pig	*Sus scrofa*	Mammalia	Domestic, Spain	Suidae	HQ623650
European rabbit	*Oryctolagus cuniculus*	Mammalia	Domestic, Spain	Leporidae	HQ623647
Cattle	*Bos taurus*	Mammalia	Domestic, Spain	Bovidae	HQ623639
Sheep	*Ovis aries*	Mammalia	Domestic, Spain	Bovidae	HQ623648
Domestic turkey	*Meleagris gallopavo*	Aves	Domestic, Spain	Phasianidae	HQ623645
Chicken	*Gallus gallus*	Aves	Domestic, Spain	Phasianidae	HQ623644
Muscovy duck	*Cairina moschata*	Aves	Domestic, Spain	Anatidae	HQ623643
Philby's partridge	*Alectoris philbyi*	Aves	Domestic, Spain	Phasianidae	HQ623637
Lamprey	*Petromyzon marinus*	Agnatha	River estuary, Spain	Petromyzontidae	HQ623651
Common limpet	*Patella vulgata*	Gasteropoda	Atlantic Ocean, Spain	Patellidae	HQ623652

Samples analysed

Control samples were obtained from individuals identified visually (Table 2) for amplification of the short target sequence within the 16S rDNA with the newly designed primers and for confirmation of species specificity. At least ten samples were collected per species for checking possible within-species polymorphisms.

Samples of animal-based foodstuffs (processed meats, fish and shellfish) commercialised in Europe were purchased directly from Spanish, French and German markets. Species from America, Asia and Africa were obtained from importers. Different processed products were considered: canned, smoked and precooked foods.

DNA extraction

For control samples, owing to their good state of conservation and the absence of DNA mixture, we decided to follow a Chelex-based protocol (low price and high efficiency). Briefly, 1 mg of tissue was introduced into an Eppendorf tube with 500 μL of 10 mg mL^{-1} Chelex® resin (Bio-Rad Laboratories, Hercules, USA) and 7 μL of 400 U mL^{-1} Proteinase K. This mixture was incubated first at 55 °C for 90 min with agitation and then at 100 °C for 20 min to inactivate the enzyme.[19] Tubes were stored at 4 °C or frozen at −20 °C for long-time preservation.

For processed food samples, 10 mg of precooked or processed product was introduced into a solution of methanol/chloroform/water (2 : 1:0.8 v/v/v) for 2 h, followed by washing in distilled water and finally in 1× phosphate-buffered saline. The DNA was extracted using a QIAamp® DNA Mini Kit (QIAGEN, Venlo, Netherlands).

PCR amplification and sequencing

The PCR with the primers designed in this study was performed in a total volume of 40 μL containing 1× Promega buffer (Promega, Madison, WI, USA), 2.5 mmol L^{-1} $MgCl_2$, 0.25 mmol L^{-1} dNTPs, 20 pmol of each 16S-H primer (described below), 20 ng of template DNA and 1 U of DNA Taq polymerase (Promega). PCR conditions were as follows: initial denaturation at 95 °C for 5 min, then 35 cycles of denaturation at 95 °C for 20 s, annealing at 61 °C for 20 s and extension at 72 °C for 30 s, followed by final extension at 72 °C for 20 min and post-extension at 20 °C for 1 min.

PCR products were visualised in agarose gels with 3 μL of 10 mg mL^{-1} ethidium bromide. DNA was purified using an Eppendorf PerfectPrep Gel CleanUp® Kit (Eppendorf, Hamburg, Germany) prior to the sequencing reaction. The resulting purified amplification products were precipitated by standard 2-propanol precipitation and resuspended in formamide. DNA samples (50 ng) were sequenced using 5 pmol of each primer. Sequencing was performed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, USA) with a BigDye 3.1 terminator system.

Sequence editing

Sequences were visualised and edited manually using the BioEdit Sequence Alignment Editor software.[18] Comparison of

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sequences with those contained in public databases (GenBank, http://www.ncbi.nlm.nih.gov/) was done using the BLAST program within NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). BLAST finds regions of local similarity between sequences and can therefore be employed for identifying the species of unknown sequences, provided that sequences from voucher specimens of such species exist in public databases. Specifically, a Megablast search was carried out for evaluation of the degree of similarity between the sequences obtained with the new primers and those included in public databases.

Species identification of samples

Sequences obtained from DNA extraction, PCR amplification and sequencing were aligned with the control sequences (obtained from reference samples or from GenBank) using the software CLUSTAL W[17] or the ClustalW application included in BioEdit.[18]

Phylogeny construction

The phylogenetic value of the sequence amplified with the new primers was explored by constructing a phylogenetic tree with the software MEGA 4.0.[20] Maximum parsimony (MP) methodology was followed for reconstructing the tree, and the robustness of the MP topology was assessed using 10 000 bootstrap replicates. The MEGA program was also employed for calculating pairwise distances with the Maximum Composite Likelihood model and uniform rates among sites between sequences.

RESULTS

Universal primers

The 16S rDNA sequences retrieved from GenBank to develop the marker (Table 1) were of variable length. Once aligned with ClustalW, two regions were identified in the 16S ribosomal gene that were sufficiently conserved between species to allow the design of universal primers (Fig. 1, where position 1 is position 174 in the *Merluccius merluccius* sequence, GenBank Accession Number DQ274032). Some positions within the conserved region marked in black in Fig. 1 were polymorphic and this was taken into account in designing the reverse primer, which consisted of a mixture of two primers differing in one nucleotide. The downstream conserved region was identical for all species considered. These two conserved regions flank a region that is variable between species (Fig. 1) but conserved within individuals of the same species. The primers designed for amplifying the variable region were as follows:

- 16S-HF: 5′-ATAACACGAGAAGACCCT-3′;
- 16S-HR1: 5′-CCCACGGTCGCCCCAAC-3′;
- 16S-HR2: 5′-CCCGCGGTCGCCCCAAC-3′.

The primers described above were submitted for patent (on 4 August 2010) with the reference P201031217.

The sequences obtained with these primers for the species in Table 1 allowed identification of all those species. The nucleotide sequences of different species differed according to their taxonomic position (Table 3); distances between members of the same taxonomic group (e.g. mammals, i.e. between rabbit and cattle) were smaller than distances between members of different taxonomic groups (i.e. between invertebrates and vertebrates).

Species identified and phylogenetic value of target sequence

Using the new primers, amplicon sizes between 80 and 125 nucleotides were obtained for all tested species (Table 2). The sequencing analysis of the amplified fragments confirmed that they were different among species, whereas the sequences

Figure 1. Alignment of five 16S rDNA partial sequences showing highly conserved (shaded in black) and least conserved (non-shaded) sections: 1–40, sequences of species employed for developing new marker (see Table 1).

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Table 3. Pairwise distances between DNA sequences amplified with new primers 16S-HF and 16S-HR within 16S rDNA for food and seafood animal species from a wide phylogenetic range

	Rabbit	Cattle	Chicken	Mallard	Tuna	Salmon	Hake	Lamprey	Lobster	Prawn	Squid	Snail	Abalone
Cattle	0.025												
Chicken	0.105	0.078											
Mallard	0.105	0.078	0.001										
Tuna	0.052	0.079	0.108	0.108									
Salmon	0.052	0.079	0.108	0.108	0.001								
Hake	0.080	0.108	0.079	0.079	0.025	0.025							
Lamprey	0.141	0.141	0.111	0.111	0.110	0.110	0.081						
Lobster	0.332	0.371	0.336	0.336	0.288	0.288	0.328	0.379					
Prawn	0.499	0.499	0.505	0.505	0.440	0.440	0.491	0.429	0.142				
Squid	0.325	0.363	0.446	0.446	0.355	0.355	0.397	0.451	0.292	0.467			
Snail	0.408	0.453	0.360	0.360	0.311	0.311	0.269	0.413	0.425	0.473	0.456		
Abalone	0.282	0.318	0.315	0.315	0.312	0.312	0.274	0.318	0.250	0.288	0.170	0.379	
Limpet	0.171	0.203	0.204	0.204	0.139	0.139	0.170	0.141	0.379	0.473	0.388	0.392	0.340

obtained for different samples of the same species were identical (100% identity). The sequences obtained from control samples were checked online against those of the same species available in GenBank using the BLAST program. Total match between the partial (short) sequences within the 16S rDNA obtained in the sequencing reactions and the corresponding part of larger 16S rDNA sequences of the same species available in GenBank was obtained. Once their species specificity was confirmed, the sequences were submitted to GenBank, where they are available as Accession Numbers HQ623624–HQ623652.

The marker could be considered truly universal for animal food. The primers designed here annealed in DNA obtained from species of a wide phylogenetic range from molluscs to vertebrates. These species represent most animal taxa consumed by humans worldwide, from domestic (husbandry, poultry, aquaculture) and wild (fisheries) origin (Table 2). The sequence obtained, although short, was highly informative. It allowed us to distinguish even closely related species within the same genus, e.g. different hakes of the genus *Merluccius*, salmonids of the genus *Salmo* and others (Fig. 2). Species of the same taxonomic group tended to cluster together, conferring phylogenetic value to the target sequence.

Processed food and value of target sequence

Simple PCR yielded positive amplification of the target DNA fragment from all processed products assayed. Clear bands were visualised in agarose gels (Fig. 3), and sequences obtained directly from PCR products were clean. Weaker amplification bands were yielded by sardine pâté and canned sardine (lanes 3 and 4 respectively in Fig. 3), but the DNA extracted from them was sufficient in quantity and quality to obtain sequence chromatogram profiles allowing the unambiguous identification of all nucleotides (Fig. 4).

The species contained in all products was identified by comparing the sequence obtained from each product with those of control samples and, when 100% match was not obtained with any of our references (the sequences obtained from canned tuna, canned tuna pâté, sardine pâté and fish fingers), checking them online against GenBank nucleotide sequences using the BLAST program. In all cases it was possible to retrieve only one match with 100% similarity, allowing us to identify the species (Table 4). The species was consistent with the product name in all cases except sardine pâté, which contained pollock instead of sardine.

DISCUSSION AND CONCLUSIONS

The marker designed here allows species identification of food and seafood of animal origin over a wide range of species. There are plentiful methods for species identification in food,[9] but this one exhibits some advantages that could make it one of the most useful in food sciences in order to avoid economic fraud as well as prevent some health problems, e.g. allergies.[6]

The main advantage of the technique described here is that the target fragment is very short and can be PCR amplified even in degraded DNA. It represents an improvement in comparison with other techniques such as barcoding[11,21] that amplify longer DNA fragments, which can be fragmented in degraded DNA, thus impeding PCR amplification. In such cases, e.g. in processed food, it is possible to use a cocktail of primers[22,23] that amplify shorter sequences (mini-barcodes). This latter strategy complicates somewhat the analysis of the obtained fragments, which are mixed and cannot be directly sequenced in a simple two-step process like when only one primer pair is employed. Instead, with our technique the species can be retrieved from a single sequence. This may be not important when high-throughput sequencing technologies are available,[24] but for laboratories without access to such equipment a method based on a single PCR can be very useful. Further technical developments of the present marker could be e.g. DNA chips,[25] SNPs[26] and many others.

An additional asset provided by the proposed marker results from the observation that the flanking primers appear to amplify DNA over a wide phylogenetic range. In most current barcoding projects, not all taxonomic groups can be identified using a single and universal pair of primers,[27] and most DNA fragments employed for species identification require taxon-specific primers. For example, there are primers that serve to identify most fish species,[21] but different primers and even DNA regions are targeted for identifying other taxa.[28–30] Employing only one pair for all routine assays simplifies everyday life in the laboratory.

Our primers can also be employed on mixed samples containing traces of species from different taxa.[31] If more than one species is present in the same sample, different fragments of different origin will be present in the final PCR product. These cannot be sequenced directly, otherwise there will be a non-sense chromatogram due to overlapping of different sequences. In this case the solution is to clone the pool of PCR fragments and sequence the different clones to identify every species present in the analysed product.

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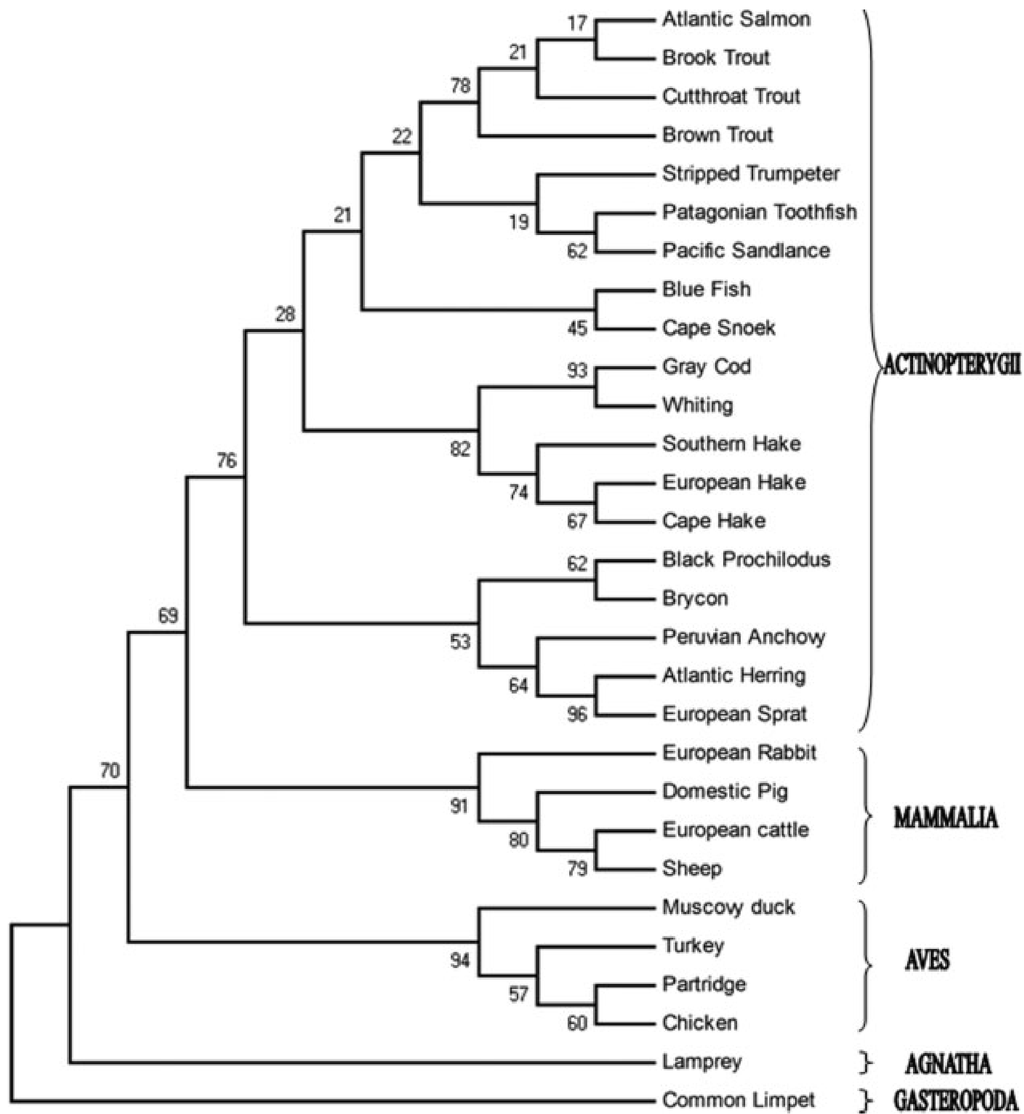

Figure 2. Phylogenetic value of 16S rDNA fragment amplified with new primers 16S-HF and 16S-HR: maximum parsimony tree of species from different levels of taxonomic proximity (class, family, genus). Bootstrapping values are given on the branches.

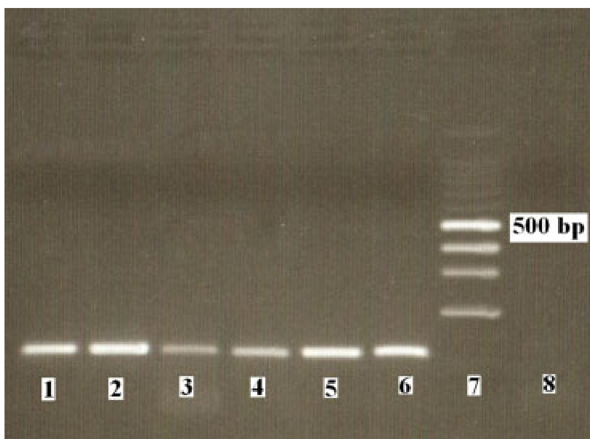

Figure 3. Agarose gel showing PCR amplification products obtained with primers 16S-HF and 16S-HR from processed food: lane 1, smoked salmon; lane 2, fish fingers; lane 3, sardine pâté; lane 4, canned sardine; lane 5, York pork; lane 6, canned turkey; lane 7, DNA size marker (BenchTop 100 bp DNA Ladder, Promega); lane 8, negative control.

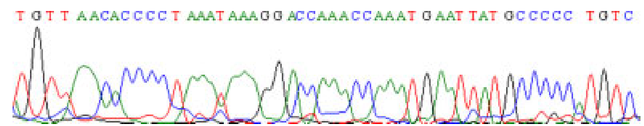

Figure 4. Partial chromatogram obtained from PCR amplification of canned tuna pâté employing primers 16S-HF and 16S-HR designed in this study.

Using this methodology, Ardura *et al.*[31] have employed the primers developed in this work to analyse commercial samples of three types of dry fish meal employed to feed aquarium cichlids, farmed salmonids and aquarium marine fish in Spain. They identified a minimum of eight different species in their composition and confirmed the value of the primers for amplification of extremely degraded and processed samples such as as dry fish meals.

The region amplified is very short; even so, it has some phylogenetic value (Fig. 2), comparable to longer mitochondrial DNA regions within 16S rDNA,[16] the mitochondrial COI gene region employed for barcoding,[32] and cytochrome b, which is

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Table 4. Processed meat and seafood products analysed and species identified by sequence obtained with primers 16S-HF and 16S-HR

Product	Species	Common name
Smoked salmon	*Salmo salar*	Atlantic salmon
Canned anchovy (in oil)	*Engraulis ringens*	Peruvian anchovy
Surimi	*Merluccius merluccius*	European hake
Smoked salmon pâ té	*Salmo salar*	Atlantic salmon
York pork	*Sus scrofa*	Domestic pig
Canned tuna	*Thunnus obesus*	Bigeye tuna
Canned sardines	*Sardina pilchardus*	Sardine
Canned tuna pâ té	*Katsuwonus pelamis*	Skipjack tuna
Sardine pâ té	*Pollachius virens*	Pollock
Canned turkey	*Meleagris gallopavo*	Turkey
Fish fingers	*Merluccius hubbsi*	Argentine hake

the main target for FINS methodology.[33] In food sciences this is not particularly important, but the result points out the diagnostic power of the marker, because it can differentiate not only between higher taxa but also between closely related species belonging to the same genus (e.g. different hake species of *Merluccius*, Fig. 2). Phylogenetically informative sequences generally have higher diagnostic power than other techniques,[33–35] and unknown species likely possess sequences different from other species in those regions. Therefore it can be reasonably forecasted that this technique will be of application in most cases of food authentication, regardless of geographical area. It is simple, relatively cheap and does not require expensive equipment. This point is important for application worldwide.[36] Consumers have the right to know what they are really buying and eating, and the authorities can establish more control measures employing, for example, this kind of robust and easy methodology for ascertaining seafood products.

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REFERENCES

1 Jacquet JL and Pauly D, Trade secrets: renaming and mislabeling of seafood. *Mar Pol* **32**:309–318 (2008).
2 Wong EHK and Hanner RH, DNA barcoding detects market substitution in North American seafood. *Food Res Int* **41**:828–837 (2008).
3 Filonzi L, Chiesa S, Vaghi M and Marzano FN, Molecular barcoding reveals mislabelling of commercial fish products in Italy. *Food Res Int* **43**:1383–1388 (2010).
4 Miller DD and Mariani S, Smoke, mirrors, and mislabeled cod: poor transparency in the European seafood industry. *Front Ecol Environ* **8**:517–521 (2010).
5 Sheth SS, Waserman S, Kagan R, Alizadehfar R, Primeau MN, Elliot S, *et al*, Role of food labels in accidental exposures in food-allergic individuals in Canada. *Ann Allergy Asthma Immunol* **104**:60–65 (2010).
6 Triantafyllidis A, Karaiskou N, Perez J, Martinez JL, Roca A, Lopez B, *et al*, Fish allergy risk derived from ambiguous vernacular fish names: forensic DNA-based detection in Greek markets. *Food Res Int* **43**:2214–2216 (2010).
7 Marko PB, Lee SC, Rice AM, Gramling JM, Fitzhenry TM, McAlister JS, *et al*, Fisheries: mislabelling of a depleted reef fish. *Nature* **430**:309–310 (2004).
8 Garcia-Vazquez E, Horreo JL, Campo D, Machado-Schiaffino G, Bista I, Triantafyllidis A, *et al*, Mislabelling of commercial North American hakes suggests underreported exploitation of *Merluccius albidus*. *Trans Am Fish Soc* **138**:790–796 (2009).
9 Primrose S, Woolfe M and Rollinson S, Food forensics: methods for determining the authenticity of foodstuffs. *Trends Food Sci Technol* **104**:60–65 (2010).
10 Woolfe M and Primrose S, Food forensics: using DNA technology to combat misdescription and fraud. *Trends Biotechnol* **22**:222–226 (2004).
11 Hebert P, Cywinska A, Ball S and deWaard J, Biological identification through DNA barcodes. *Proc R Soc B* **270**:313–321 (2003).
12 Rasmussen RS and Morrissey MT, DNA-based methods for the identification of commercial fish and seafood species. *Compr Rev Food Sci Food Saf* **7**:280–295 (2008).
13 Teletchea F, Molecular identification of fish species; reassessment and possible applications. *Rev Fish Biol Fish* **19**:265–293 (2009).
14 Teletchea F, Maudet C and Hänni C, Food and forensic molecular identification: update and challenges. *Trends Biotechnol* **23**:359–366 (2005).
15 Unseld M, Beyermann B, Brandt P and Hiesel R, Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. *PCR Meth Appl* **4**:241–243 (1995).
16 Parker A and Kornfield I, An improved amplification and sequencing strategy for phylogenetic studies using the mitochondrial large subunit rRNA gene. *Genome* **39**:793–797 (1996).
17 Thompson JD, Higgins DG and Gibson TJ, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. *Nucl Acids Res* **22**:4673–4680 (1994).
18 Hall TA, BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**:95–98 (1999).
19 Estoup A, Largiader CR, Perrot E and Chourrout D, Rapid one-tube DNA extraction protocol for reliable PCR detection of fish polymorphic markers and transgenes. *Mol Mar Biol Biotechnol* **5**:295–298 (1996).
20 Tamura K, Dudley J, Nei M and Kumar S, MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**:1596–1599 (2007).
21 Ward RD, Hanner R and Hebert PDN, The campaign to DNA barcode all fishes, FISH-BOL. *J Fish Biol* **74**:329–356 (2009).
22 Ivanova NV, Zemlak TS, Hanner RH and Hebert PDN, Universal primer cocktails for fish DNA barcoding. *Mol Ecol Notes* **7**:544–548 (2007).
23 Meusnier I, Singer GA, Landry JF, Hickey DA, Hebert PD and Hajibabaei M, A universal DNA mini-barcode for biodiversity analysis. *BMC Genom* **9**:214 (2008).
24 Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, *et al*, Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**:376–380 (2005).
25 Kochzius M, Nölte M, Weber H, Silkenbeumer N, Hjörleifsdottir S, Hreggvidsson GO, *et al*, DNA microarrays for identifying fishes. *Mar Biotechnol* **10**:207–217 (2008).
26 Machado-Schiaffino G, Martinez JL and Garcia-Vazquez E, Detection of mislabeling in hake seafood employing mtSNPs-based methodology with identification of eleven hake species of the genus *Merluccius*. *J Agric Food Chem* **56**:5091–5095 (2008).
27 Waugh J, DNA barcoding in animal species: progress, potential and pitfalls. *Bioessays* **29**:188–197 (2007).
28 Barlett SE and Davidson WS, FINS (Forensically Informative Nucleotide Sequencing): a procedure for identifiying the animal origin of biological specimens. *Biotechniques* **12**:408–411 (1992).
29 Vences M, Meike T, Van Der Meijden A, Chiari Y and Vieites DR, Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. *Front Zool* **2**:5 (2005).
30 Fajardo V, González I, López-Calleja I, Martín I, Rojas M, Hernández PE, *et al*, Identification of meats from red deer (*Cervus elaphus*), fallow deer (*Dama dama*), and roe deer (*Capreolus capreolus*) using polymerase chain reaction targeting specific sequences from the mitochondrial 12S rRNA gene. *Meat Sci* **76**:232–240 (2007).
31 Ardura A, Horreo JL, Hernandez E, Jardon A, Pola I, Martinez JL, *et al*, Forensic DNA analysis reveals use of high trophic level marine fish in commercial aquaculture fish meals. *Fish Res* **115–116**:115–120 (2012).
32 Hajibabaei M, Singer GAC, Hebert PDN and Hickey DA, DNA barcoding: how it completes taxonomy, molecular phylogenetics and population genetics. *Trends Genet* **23**:167–172 (2007).

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33 Sotelo CG, Calo-Mata P, Chapela MJ, Perez-Martin RI, Rehbein H, Hold GL, *et al*, Identification of flatfish (Pleuronectiformes) species using DNA-based techniques. *J Agric Food Chem* **49**:4562–4569 (2001).
34 Calo-Mata P, Sotelo CG, Perez-Martin PI, Rehbein H, Hold GL, Russell VJ, *et al*, Identification of gadoid fish species using DNA-based techniques. *Eur Food Res Technol* **217**:259–264 (2003).
35 Chapela MJ, Sotelo CG and Perez-Martin RI, Molecular identification of cephalopod species by FINS and PCR-RFLP of a cytochrome b gene fragment. *Eur Food Res Technol* **217**:524–529 (2003).
36 Ardura A, Pola IG, Lind AR and Garcia-Vazquez E, DNA-based methods for species authentication of Amazonian commercial fish. *Food Res Int* **43**:2295–2302 (2010).

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