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ORIGINAL PAPER

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Identification of gadoid fish species using DNA-based techniques

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Abstract The polymerase chain reaction (PCR) technique was employed to obtain a 464 bp amplicon from the mitochondrial cytochrome *b* gene from gadoid species to study its ability to differentiate them. The sequences of this fragment from 16 species were analysed using a genetic distance method, and polymorphic sites were determined. The fragment was shown to be moderately polymorphic (151 sites), and this permitted the differentiation of most of the species. A phylogenetic tree construction using Tamura-Nei distances was employed to allow the identification of Gadidae species, each species resulted in a well-differentiated clade, with the exception of *Gadus ogac* and *Gadus macrocephalus*, which could not be differentiated. Based on the sequences obtained, three restriction enzymes, *Dde* I, *Hinc* II and *Nla* III, were selected to provide specific restriction profiles, which allowed the differentiation of 15 species of gadoids in a faster and less expensive way than sequencing. The PCR-restriction fragment length polymorphism methodology was also tested using commercial samples.

Keywords Polymerase chain reaction · Authentication · Gadoids · Mitochondrial DNA · Cytochrome *b* gene

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Introduction

Gadoids is the common name used for a group of bony fish, included in the taxonomical order Gadiformes, comprising several families of great commercial interest. The family Gadidae comprises mostly commercial species, including Atlantic cod (*Gadus morhua*), pollack (*Pollachius pollachius*) and haddock (*Melanogrammus aeglefinus*). The over-exploitation of cod has led to the collapse of some stocks, epecially in the western Atlantic, and this has prompted the issuing of a moratorium in this fishery. Obviously, the consumer interest in some of these species has not decreased and therefore the market price has increased according to demand. Gadoid species can be found in markets in different presentations, depending on the country. Presentation may include fresh or frozen fillets, smoked, salted, surimi-based products, fish cakes etc. European Union labelling regulations (EC No 104/2000) specify that the commercial and scientific names should be included on the label of seafood products; however, most of these processes involve the removal of morphological characteristics hindering the process of species identification.

The use of biochemical markers, such as proteins and nucleic acids, has provided a tool for controlling the compliance of fish products belonging to different families with labelling regulations [1, 2]. In the case of protein analysis, the usefulness of the techniques developed is restricted to fresh or frozen products, since heat or desiccation treatments denature proteins, hindering their analysis. DNA analysis has overcome these difficulties

and became the method of preference for the analysis of a wide range of seafood products [3].

DNA analysis methods include several techniques with different levels of applicability and resolution. Forensically informative DNA sequencing (FINS) as a method for species identification in seafood products was first described by Bartlett and Davidson [4] and provides the highest level of resolution, depending on the type of DNA fragment studied. The use of this technique for the identification and differentiation of gadoid species has not been described so far, in spite of the great commercial value of this group of fish. Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) methodologies constitute an affordable and reliable alternative to the use of FINS, and have also been described as useful techniques for the identification of fish species [5, 6, 7, 8, 9, 10].

The objective of this work was to study the applicability of FINS and PCR-RFLP for the discrimination and identification of commercial gadoid species, and to validate this methodology using commercial gadoid samples.

Material and methods

Authentic gadoid species and commercial fish samples. Whole specimens of authentic species were obtained fresh in the local fish market; other specimens were obtained frozen from fish and food industrial companies (Pez Austral, Vigo, Spain and Hero, Alcantarilla, Spain) (Table 1). Both types of samples were stored frozen (–80 °C) until analysed.

Six samples of commercial salted cod, labelled as cod, were obtained in the local market. These samples were prepared as raw muscle with a prior rinse with sterile distilled water.

DNA extraction. DNA extraction from frozen or salted muscle, previously thawed, was carried out using the standard Wizard DNA Clean-up System (Promega, Madison, Wis., USA). For the DNA extraction, 150 mg of tissue sample was placed into an Eppendorf tube and suspended in 860 μl of lysis buffer containing 2 mM of EDTA, 150 mM of NaCl, 1% of SDS and 10 mM Tris-HCl (pH 8). To this, 120 μl of guanidium thiocyanate (Sigma-Aldrich, St. Louis, Mich., USA) and 40 μl of proteinase K solution (20 mg/ml) (Gibco, Prat de Llobregat, Spain) were added and incubated in a waterbath at 56 °C. After 2 h, an extra 40 μl of proteinase K was added to the solution and left overnight in the waterbath at 56 °C. Then, the resulting digest was centrifuged, and the supernatant collected.

For the isolation of the DNA, 500 μl of the collected solution was placed in a syringe barrel attached to a Wizard Minicolumn, to which 1 ml of Wizard DNA Clean-up Resin (Promega) were added. Then, vacuum was applied to draw the solution through the minicolumn. The column was washed using 2 ml of 80% isopropanol and re-application of vacuum. Then, the column was transferred to a clean microfuge tube and spun for 2 min. The DNA was eluted from the column by adding 50 μl of water prewarmed at 70 °C and centrifuging, after 1 min, at 10,000×*g* for 20 s. The DNA solution was collected and stored at −20 °C.

DNA quantitation. DNA content in the extracts was measured by a fluorescence assay based on the dye Hoechst 33258 (Molecular Probes, Eugene, Ore, USA) [11] in a LS-3B fluorescence spectrometer (Perkin-Elmer, Rockville, Md., USA). Calf-thymus DNA (Sigma-Aldrich) was used as a standard to construct the DNA standard curve.

PCR amplification of DNA samples. The primers used amplify a region of 464 bp of the cytochrome *b* previously described by Kocher et al. in 1989 [12]. The sequence of the primers is: H15149AD: 5'-GCICCTCARAATGAYATTTGTCCTCA-3' for the forward primer, and for the reverse L14735: 5'-AAAAAC-CACCGTTGTTATTCAACTA −3'.

PCR reactions were performed in volumes of 25 μl using Ready-to-Go PCR beads (Amersham Biosciences, Freiburg, Germany) which contain, when reconstituted, 200 μM of each dNTP in 50 mM KCl, 1.5–2.0 mM $MgCl_2$, 10 mM Tris-HCl (pH 9)at room temperature, and 1.5 U of Taq Polymerase. To the reaction, 2 μl of each primer (10 μM) was added, and 125 ng of the template DNA. Amplifications were carried out in a GeneAmp 2400 PCR system (Applied Biosystems, Foster City, Calif., USA) with a preheating step of 5 min at 94 °C, then 35 cycles of 90 s at 94 °C, 90 s at 50 °C, 90 s at 72 °C, and a final extension step of 7 min at 72 °C.

Sequencing of PCR fragments. Prior to the sequencing reactions, 20 μl of PCR product was treated with 2 μl of Exonuclease I and 2 μl of Shrimp Alkaline Phosphatase (Amersham Biosciences). The mixture was incubated at 37 °C for 30 min and then at 80 °C for

Table 1 Authentic species used in the present study. The *specimens* column indicates each of the individuals (each number correspond to different individuals) used for performing the analysis. Sequences obtained from GenBank are marked by *GB* and followed by accession number in parentheses

Keys	Common Name	Scientific name	Specimens[a]
C	Atlantic Cod	*Gadus morhua*	1, 2, 46, 208, GB (X76365)
GM	Pacific cod	*Gadus macrocephalus*	GB (AF081683)
GO	Greenland cod	*Gadus ogac*	GB (AF081684)
AP	Alaska pollack	*Theragra chalcograma*	1, 2, 214, 215, 216, GB (AF081685)
BS	Polar Cod or Artic cod	*Boreogadus saida*	GB (AF081686)
MM	Whiting	*Merlangius merlangus*	GB (AF081688)
MAE	Haddock	*Melanogrammus aeglefinus*	GB (AF165075)
EN	Navaga	*Eleginus navaga*	GB (AF081690)
MP	Pacific tomcod	*Microgradus proximus*	GB (AF081691)
MT	Atlantic tomcod	*Microgadus tomcod*	GB (AF081692)
PV	Pollock, Saithe or Coley	*Pollachius virens*	GB (AF469634)
P	Pollack	*Pollachius pollachius*	189, 350, 226, 175
L	Ling	*Molva molva*	131, 207
BW	Blue whiting	*Micromesistius poutassou*	289, 294, 297
TE	Norway pout	*Trisopterus esmarkii*	GB (AF081695)
TM	Poor cod	*Trisopterus minutus*	GB (AF081693)
TL	Bib, Whiting pout or Pounting	*Trisopterus luscus*	1, 2, 3, 4, 5 GB (AF081694)

[a] Fresh/frozen. Samples obtained at the local market or from the fish and food industry (Pez Austral, Hero). Arabic numerals indicates different specimens obtained fresh or frozen in Spain

another 15 min. The purified PCR products were quantified with the Hoechst 33258 method described above.

Sequencing reactions of both strands of the 464 cytochrome *b* fragment were prepared with the ABI Prism dRhodamine Terminator cycle sequencing ready reaction kit (Applied Biosystems). To 4 μl of Terminator mix from the kit, 90–200 ng of treated PCR product, 6.4 pmol of the corresponding primer (H15149AD and L14735) and distilled water up to 10 μl were added. The components were all mixed and the tube loaded in the thermal cycler. The conditions of the reaction were: 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. The extension products were purified using an ethanol/magnesium chloride precipitation procedure for the removal of the non-incorporated dye terminators. The pellet was dried at 30 °C with a centrifuge with a vacuum device and stored at −20 °C

Once the extension products were purified, electrophoresis was carried out in an automated ABI PRISM 377 DNA Sequencer (Applied Biosystems) using 6% polyacrylamide gels for fluorescent DNA sequencing.

Prior to sample loading, the pooled and dried reaction products were suspended in loading buffer (Applied Biosystems) containing 5 parts of deionized formamide to 1part of 25 mM EDTA (pH 8.0) and 50 mg/ml Dextran Blue (Applied Biosystems). The DNA was then denatured at 94 °C for 3 min. Finally, the gel was electrophoresed for 5 h at 50 °C. The collected data was processed using the software CHROMAS and VISED to get the sequence.

Data analysis. Sequences on the fragment studied available in public databases, such as the GenBank, and the sequences obtained in our laboratory were analysed using the program CLUSTAL to align the sequences [13], and the program MEGA to calculate genetic distances using the Tamura-Nei method [14]. Phylogenetic trees for FINS were constructed using the calculated distances using the neighbor-joining method [15], and a bootstrap test was performed for each tree using MEGA program.

RFLP analysis of the fragment. A search for restriction sites was done using the sequences obtained from the fragment (DNASIS V2.1 Hitachi, Berlin, Germany), with a set of enzymes selected on the basis of the predictable specific pattern they would produce.

Two PCR reactions from each sample were concentrated to a volume of 10 μl by using a Microcon-30 microconcentrator (Millipore, Madrid, Spain). Aliquots from concentrated PCR amplicons were digested separately with the enzymes *Dde* I (New England Biolabs, Beverly, Mass., USA), *Hinc* II (New England Biolabs), and *Nla* III (Amersham Biosciences). The volumes of each component of the reactions were 2.5 μl of PCR product, 1 μl of buffer 10x (supplied by the manufacturer with the respective enzyme), and 0.5 μl of the enzyme (5 U). All digestions were carried out at 37 °C for 6 h in 10 μl volumes using reaction conditions specified by the manufacturer.

Separation of DNA fragments was carried out in a GeneGel Excel 12.5 (T 12.5%, C 2%) (Amersham Biosciences), using 6.5 μl of the digestion products on the gels. Anode buffer was 0.4% SDS and 0.45 M Tris-acetate (pH 8.3); the cathode buffer was 0.6% SDS and 0.08 M tricine. Electrophoresis was carried out on a GenePhor (Amersham Biosciences) with a temperature of the cooling plate of 15 °C, and a voltage of 200 V. The run was stopped when the tracking dye reached the anode edge of the gel. DNA restriction fragments were visualized by silver staining using the method of Heuskeshoven and Dermick [16].

Results and discussion

DNA sequences and genetic analysis

Cytochrome *b* fragments (H15149-L14735) from the specimens and gadoids shown in Table 1 were sequenced and analysed; sequence data for the same fragment obtained from the GenBank data base (marked as GB in Table 1) was also included in the analysis. Table 2 shows the variable positions (151 positions) for all the species and specimens used in this study; 124 of these positions have two variants, 21 have three and only 6 have four variants. Figure 1 shows the constructed tree with the bootstrap test results. Each family studied is grouped in a separate clade, thus allowing the adequate differentiation of most studied species, including those from GenBank. All the species studied belong to the order Gadiforme, and most of them are from the family Gadidae, except for L (*Molva molva*), which is a member of the family Lotidae.

Table 2 Position of variable sites for the studied DNA fragment of gadoids (see Table 1 for species keys)

	1223344 5555566667 7777888899 1485362514 3678935681 4578036927
	1111111111 1111111111 1111111111 1703690258 1245670347 9568127036
	1111111122 2222222222 2222222222 9025814703 6902581473 6935814703
	2222222222 2222222222 2223333333 6925814703 6792584703 6925789147
	3333333333 3333333333 3333333344 3679123581 4570625801 2478013
	4555666777 7888899900 0011112223 3344455566 6677788899 9990001122
	2233333444 5555666667 7778899900 0000111
	0011112222 3333334444 4555666777 7888899900 0011112223 3344455566
	6677788899 9990001122 2233333444 5555666667 7778899900 0000111
ENGB	???gtgctca ttggttagcc cccgccctct acccatcact ctaatttacc gacacttacc ctgccgatcc ccgacctttt cactcgtacc ctctccttct taatcccctt ccctttgaaa tcgattgttc ctagattatc gcccc
MPGB	???..... ...a..... .....t... ....g..... .g..... t.a.ta...g .t..t..... ...a.a.... ....ct..t.c ....g. .t...g.... t.....g. t.t..t
MTGB	???..... ...a..... .....t... ..t..c.... .c..... a.t..c..t. t.att....g t.....c.cc tt.c.ac.t. tct.t....c c...t....c .t.....g. ....ac. t..a..c.gt t.t..t
PVGB	cgc..... ...acc...tt ...a..t... ..t.c.t... .... a...tcc... t.a.t..ctg tg.tttc... .ctata.g.. tct..... ..g.ttat.c .ttc..... ...g....c. t..t..c.gt t.t..c
P189	cgc.....t. .c.a.c...t t.....t.t. ..t.t.t... .....c.... a...tc.... t.a.ta.c.a tg.tttc... .ctg.a.... tc.....c ....ttat.c .ttc....g. ...c....c. t..c..c.gt t.t..c
P350	cgc.....t. .c.a.c...t t.....t.t. ..t.t.t... .....c.... a...tc.... t.a.ta.c.a tg.tttc... .ctg.a.... tc.....c ....ttat.c .ttc....g. ...c....c. t..c..c.gt t.t..c
P226	cgc.....t. .c.a.c...t t.....t.t. ..t.t.t... .....c.... a...tc.... t.a.ta.c.a ta.tttc... .ctg.a.... tc.....c ....ttat.c .ttc....g. ...c....c. t..c..c.gt t.t..c
P175	cgc.....t. .c.a.c...t .....t.t. ..t.t.t... .....c.... a...tc.... t.a.ta.c.a ta.tttc... .ctg.a.... tc.....c ....ttat.c .ttc....g. ...c....c. t..c..c.gt t.t..c
GMGB	???..... ..a.c...tt ....t. .tt.t..... .... a...tc.... ..a....c.a .a.c..... .t.ct...tt .....t..tc ....t.t... .tt..... c......c.. t..a..c.gt t.....t
GOGB	???..... ..a.c...tt ....t. .tt.t..... .... a...tc.... ..a....c.a .a.c..... .t.ct...tt .....t..tc ....t.t... .tt..... c......c.. t..a..c.gt t.....t
C208	cgc..... ..a.c...tt .... .tt.t..... .... a...tcc... ..a....c.a .a.c.....c .c.at...tt t....tc.t. ..gct.t.. t.t.....g. c..g...c.. t..a..c.gt tt...c
C46	cgc..... ..a.c...tt .... .tt.t..... .... a...tcc... ..a....c.a .a.c.....c .c.at...tt t....tc.t. ..gct.t.. t.t.....g. c..g...c.. t..a..c.gt tt...c
C2	cgc..... ..a.c...tt .... .tt.t..... .... a...tcc..t ..a....c.a .a.c.....c .c.at...tt t....tc.t. ..gct.t.. t.t.....g. c..t...c.. t..a..c.gt tt...c
CGB	cgc..... ..a.c...tt .... .tt.t..... .... a...tcc..t ..a....c.a .a.c.....c .c.at...tt t....tc.t. ..gct.t.. t.t.....g. c..g...c.. t..a..c.gt tt...c
C1	cgc....c.. ..aac...tt ..a..... .tt.t..... ......c... a...tcc..t ..a..a.c.a .a.c.....c .c.at...tt t..a.tc.t. ..gct.tt.. t.t.....g. ...g...c.. t..a..ctgt t....c
AP2	cgc..... ..a.c...tt ....t. .t..t..... .... a...tcc..t ..at.agc.a .a.t..... .c.at...tt t....tc.t. c.g.t.t..c .tt.....g. c......... t..ag.c.gt t.t.tc
AP216	cgc..... ..a.c...tt ....t. .t..t..... .... a...tcc..t ..at.agc.a .a.t..... .c.at...tt t....tc.t. c.g.t.t..c .tt.....g. c......... t..ag.c.gt t.t.tc
AP214	cgc..... ..a.c...tt ....t. .t..t..... .... a...tcc..t ..at.agc.a .a.t..... .c.at...tt t....tc.t. c.g.t.t..c .tt.....g. c......... t..ag.c.gt t.t.tc
AP215	cgc..... ..a.c...tt ....t. .t..t..... .... a...tcc..t ..at.agc.a .a.t..... .c.at...tt t....tc.t. c.g.t.t..c .tt.....g. c......... t..ag.c.gt t.t.tc
AP1	cgc..... ..a.c...tt ....t. .t..t..... .... a...tcc..t ..at.a.c.a .a.t..... .c.at...tt t....tc.t. c.g.t.t..c .tt.....g. c......... t..ag.c.gt t.t.tc
APGB	???..... ..a.c...tt ....t. .t..t..... .... a...tcc..t ..at.a.c.a .a.t..... .c.at...tt t....tc... c.g.t.t..c .tt.....g. c......... t..ag.c.gt t.....t
BSGB	???..... ..a.....tt .... .tt.t..... ....g.t a.t.tcc... .gat.a.c.a .a.c..... tctata..tt t....t..t. ...c..tt.c .tt.....g. c......c.t tc.a..c.gt t.t.tt
MAEGB	cgt...t... ...a..c.tt t..at...t. .tttt...t. tc.....a .t..t.cg.t t.a.ta.cta .a.tt.....c .c.a.a..tt .ct....ct. ....t.tt.. t.t..... g c..g...c.. t.ga..c.g. t.t.tc
MMGB	???..... ...a....tt .... .ttgt..... t......... a...t.cg.. a.a.t..c.a .a..tt..c .c.ata.... t......t. ......t... ...c....g. c..g..... t..a..c.g. tt...t
L131	tgt.ca.ct. c..acc...at ..a..t.ct. .t..tc.g.c ....at a..gt.a..t ..at.a.cta tt..... t..a.cc.t. ..t..... ....t.a.c. t...a..... .t...c.... .c.agcc.g. t.a.tc
L207	tgt.ca.ct. c..acc...at ..a..t.ct. .t..tc.g.c ....at a..gt.a..t ..at.a.cta tt..... t..a.cc.t. ..t..... ....t.a.c. t...a..... .t...c.... .c.agcc.g. t.a.tc
TEGB	???....ctc ...ac...t. ..t..... g .tt.t.t... ......cg.t ac.ct.g.tt ..a...a...g t..cttca.. .ctatacg.t .gt..t..t. ....cc .a....a.g. .t.gc.a.c. a..a..cgga tt.t.t
TMGB	???....c.c ...ac...tt ..t...t... .tt.t.t... t.....cg.. ac.ct.cgt. t.att....a tt.cttcg.. .c.ata...t ..t..t..t. ......gtcc ta...c..g. .....ga.c. a.gag..gg. t.....t
TLGB	???a...ctc c..acc.... ....t.t.t. gt...t..g.c ......c... ac.ct.c.t. a.at.....a ..ac.tc... tctatac..t ..a..t..tc cc....g.cc ..tc..... ct....a.c. gcga..c.g. t.t..t
BW289	cgt....c.. ...accc.tt ......tc.. ..t.tc.... ..ggc..g.. ag.ctcc..t t.at.a...g .a.c..cc.. .cta.a.g.t ....g.cc .tt....gg. .... tc.a..c.g. t.....c
BW294	cgc....c.. ...acc...tt ......tc.. ..t.tc.... ..ggc..g.. ag.ctcc..t t.at.a...g .a.c..cc.. .cta.a.g.t ....g.cc .tt....gg. .... tc.a..c.g. t.....c
BW297	cgt....c.. ...acc...tt ......tc.. ..t.tc.... ..ggc..g.. ag.ctcc..t t.at.a.... .a.c..cc.. .cta.a.g.t ....g.cc .tt....gg. .tt..... tc.at.cgg. t.....c
TL2	caca...ctc c..acc.a.. .a..t.t.t. gt...t..g.c ......c... ac.ct.c.t. a.at.....a ..ac.tc... tctatac..t ..a..t..tc cc....g.cc ..tc..... ct....a.c. gcga..c.g. t.t..a
TL4	caca...ctc c..acc.a.. ....t.t.t. gt...t..g.c ......c... at.ct.c.t. a.at.....a ..ac.tc... tctatac..t ..a..t..tc cc....g.cc ..tc..... ct....a.c. gcga..c.g. t.t..a
TL1	caca...ctc c..acc.a.. ....t.t.t. gt...t..g.c ......c... at.ct.c.t. a.at.....a ..ac.tc... tctatac..t ..a..t..tc cc....g.cc ..tc..... ct....a.c. gcga..c.g. t.t..a
TL3	caca...ctc c..acc.a.. ....t.t.t. gt...t..g.c ......c... at.ct.c.t. a.at.....a ..ac.tc... tctatac..t ..a..t..tc cc....g.cc ..tc..... ct....a.c. gcga..c.g. t.t..a
TL5	caca...ctc c..acc.a.. ....t.t.t. gt...t..g.c ......c... at.ct.c.t. a.at.....a ..ac.tc... tctatac..t ..a..t..tc cc....g.cc ..tc..... ct....a.c. gcga..c.g. t.t..a

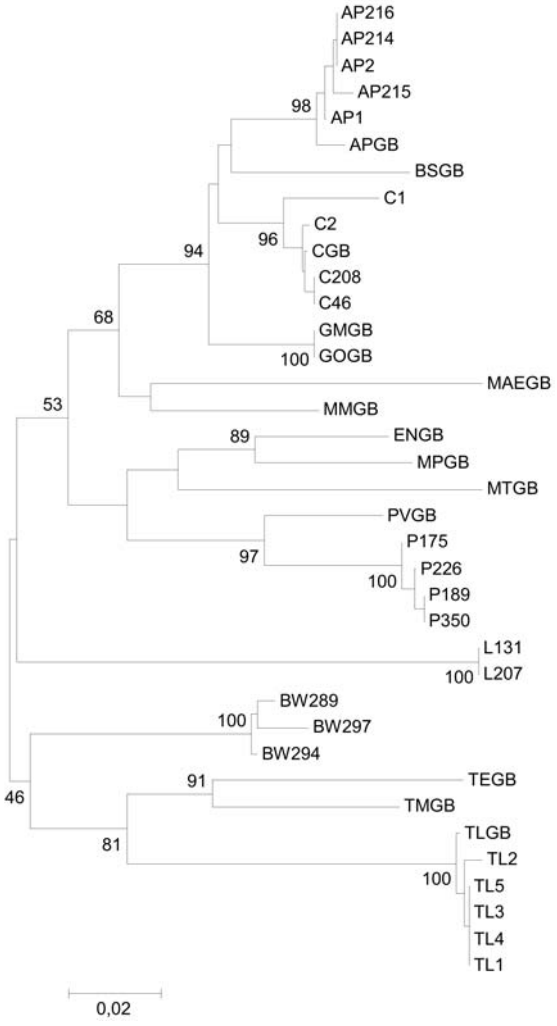

Fig. 1 Phylogenetic tree of 464 bp fragment sequences from 17 gadoid species. See keys for species in Table 1

The phylogenetic tree presented in Figure 1 reflects this fact except for the clear separation of one major branch with the species belonging to the genus *Trisopterus*, and the branch with the *M. molva* species. However, most commercial fraud with gadoids include the substitution of cheaper species for Atlantic cod, depending on the product. The GenBank sequences for Pacific cod (*Gadus macrocephalus*) and Greenland cod (*Gadus ogac*) were identical; since the fragment showed enough variation to differentiate species belonging to the same genus, this finding made us question the reliability of these two sequences. In other cases, sequences from GenBank were very similar to the ones obtained in our laboratory.

Development of a PCR-RFLP identification method

The sequences obtained were used for searching for restriction targets using the program DNASIS. Restriction enzymes were selected to allow the differentiation of all studied species. Table 3 shows the expected size of restriction fragments determined with DNASIS with the 464 bp amplicon sequence belonging to 17 species of Gadiformes with three restriction enzymes, *Dde* I, *Hinc* II, and *Nla* III. Based on these patterns, the haplotypes for the species are also shown in Table 3. The haplotypes obtained with the three enzymes allowed the differentiation of all species except for *Gadus ogac* and *Gadus macrocephalus*, which sequences were identical. The selected restriction targets showed no intraspecific variability, and only two of them can lead to misinterpretation due to similar molecular weights of the generated fragments (B and B*, F and F*), but still permit the correct identification of the species.

Figure 2 shows the RFLP patterns obtained after digesting the 464 bp amplicon of some gadoid species (Theragra *chalcogramma*, *Micromesistius poutassou*, *Gadus morhua*, *M. molva*, *Pollachius pollachius*, *Trisopterus luscus*) with *Dde* I, *Hinc* II and *Nla* III and the haplotypes

Table 3 Theoretical fragment size expected after digestion with restriction enzymes *Dde* I, *Hinc* II and *Nla* III of 464 pb amplicon of gadoid species. *H* Haplotype for each enzyme, *F** this pattern is similar to F, *B*[§] this pattern is similar to B, GB Genbank. See Table 1 for species key

	Dde I	H	*Hinc* II	H	*Nla* III	H
Gadidae						
AP	204, 234, 18, 8	A	464	O	286, 38, 50, 90	A
BSGB	117, 47, 274, 18, 8	B	464	O	286, 88, 90	B
BW	117, 47, 274, 18, 8	B	464	O	374, 90	C
C	117, 87, 234, 18, 8	C	464	O	286, 88, 90	B
ENGB	117, 69, 213, 57, 8	D	93, 333, 38	A	374, 90	C
GMGB	204, 234, 18, 8	A	265, 199	B	286, 88, 90	B
GOGB	204, 234, 18, 8	A	265, 199	B	286, 88, 90	B
L	204, 234, 18, 8	A	464	O	286, 88, 90	B
MAEGB	438, 18, 8	E	464	O	195, 91, 178	D
DMMGB	117, 321, 18, 8	F	464	O	195, 179, 90	E
MPGB	117, 69, 270, 8	G	93, 371	C	374, 90	C
MTGB	117, 339, 8	F*	93, 172, 199	D	286, 88, 90	B
P	438, 18, 8	E	464	O	374, 90	C
PVGB	117, 321, 18, 8	F	464	O	374, 90	C
TEGB	117, 87, 252, 8	H	93, 371	C	282, 92, 90	B[§]
TL	456, 8	I	464	O	374, 66, 24	F
TMGB	117, 339, 8	F*	464	O	195, 90, 179	E

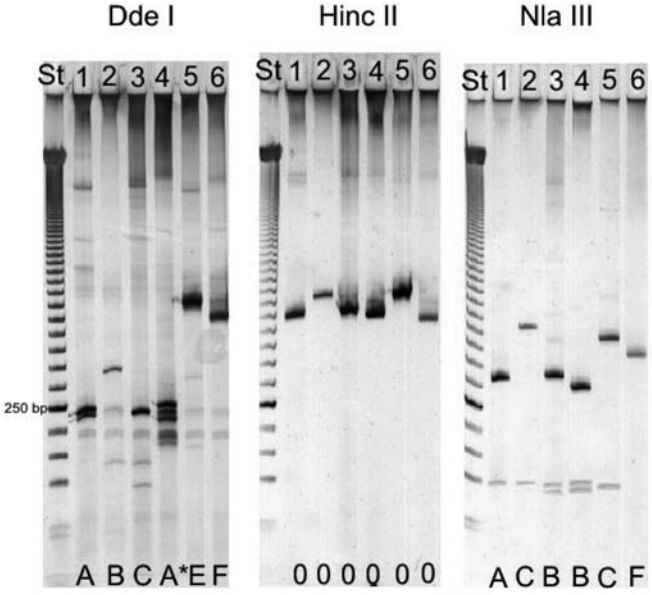

Fig. 2 Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) patterns of gadoid species following digestion with restriction enzymes *Dde* I, *Hinc* II and *Nla* III. *1 Theragra chalcogramma*, *2 Micromesistius poutassou*, *3 Gadus morhua*, *4 Molva molva*, *5 Pollachius pollachius*, *6 Trisopterus luscus*. Haplotypes corresponding to Table 3 are indicated at the bottom of each track. *A** This pattern is similar to *A*

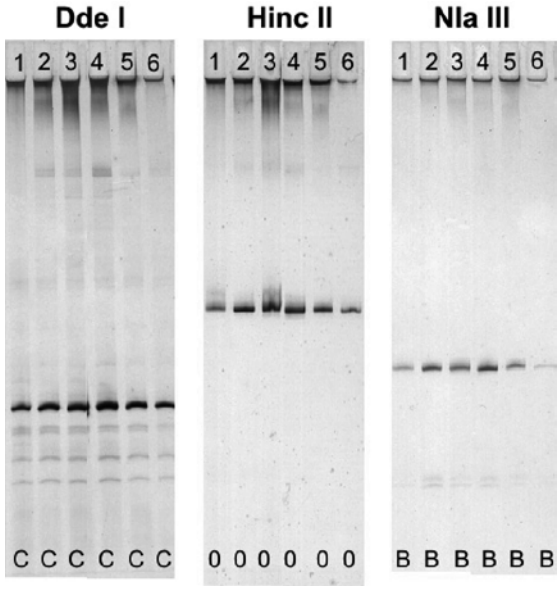

Fig. 3 PCR-RFLP patterns of commercial salted cod following digestion with restriction enzymes *Dde* I, *Hinc* II and *Nla* III. Haplotypes corresponding to Table3 are indicated at the *bottom of each track*

obtained. The restriction patterns did not always agree with those predicted, as previously described by Hold et al. (2001) [7], and although low molecular weight bands were weak and mobility shifts were observed in some cases, probably due to differences in mobility due to sequence differences, major bands corresponded with those predicted, thus allowing the differentiation of the species under study. In the case of ling (*M. molva*), a modified A pattern was obtained (A*) with *Dde* I.

Six commercial samples labelled as "salted cod" were analysed using the PCR-RFLP method developed here. Figure 3 shows the RFLP and haplotypes obtained with these samples. All samples presented the same haplotype of cod (*G. morhua*), C0B for the *Dde* I, *Hinc* II and *Nla* III enzymes respectively. Although, the *Nla* III presented very weak bands for the 88 and 90 fragments, the identification of cod relies on the pattern obtained with *Dde* I, which is unique for this species. The sequence analysis of these commercial samples confirmed the results obtained by PCR-RFLP.

Identification of gadoid species in seafood products, using DNA methodologies, has scarcely been studied. Some studies using DNA analysis were aimed at the study of population structure, using sequence analysis [17], RFLP [18] or microsatellites [19]. However, the identification of gadoid species in seafood products has been addressed only by using protein analyses [20, 21, 22, 23, 24], and although with these techniques gadoid species are differentiated, their applicability is restricted to fresh or frozen seafood products. Another drawback is the need for running reference species in most of these methods. DNA analysis allows the use of a single method for the whole range of products, and although reference species should be also obtained and analysed, this is only necessary once, and sequences are also available in public databases and may be used for the development of the methodology, especially in the case of commercial species difficult to obtain.

The PCR is a rapid, sensitive and specific technique that has been widely used for food identification. Several methodologies based on PCR have been successfully applied in the identification of commercial fish species [4, 6, 7, 10, 24, 25, 26, 27] and PCR-RFLP usually constitutes an affordable method in quality control and industry laboratories.

Sequence analysis and genetic distance measurement allowed the identification of the 17 species of gadoids, with the exception of *G. ogac* and *G. macrocephalus*, which showed the same sequence (sequences obtained from EMBL). Unknown sample sequences, from commercial species, were easily identified with their level of genetic similarity measured against the pool of reference sequences.

We have demonstrated that RFLP patterns can be used for the reliable identification of commercial samples, in this case a salted fish, labeled as cod, was analysed, indicating that PCR-RFLP allows identification in the instance that sequencing is not an affordable option. The cytochrome *b* fragment analysed presented very low intraspecific variability, thus permitting the unequivocal identification of fish species contained in the commercial products. A set of three restriction enzymes was enough for the differentiation of the species under study. These enzymes were selected from all the enzymes available on

the market with a software analysis, narrowing the selection to three enzymes: *Dde* I, *Hinc* II, and *Nla* III. Wolf et al. [28] had shown that three gadoid species, *G. morhua*, *Pollachius virens* and *Melanogrammus aeglefinus*, could be differentiated using three restriction enzymes; however they employed only one specimen per species, not knowing exactly the level of expected intraspecific variability. Another important issue is the number of species included in the study: when developing RFLP methods for species identification, the larger the number of species included, the higher the reliability of the method developed. As an example we can take the enzyme *Dde* I, which can differentiate between *G. morhua* and *G. macrocephalus/ogac* and pollack (Table 3). However if a new species is included in the analysis, for instance *M. molva*, the RFLP pattern obtained will look exactly as *G. macrocephalus/ogac*.

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