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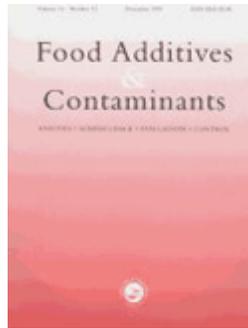
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**Application of an indirect ELISA and a PCR technique to detect grouper (*Epinephelus marginatus*) adulteration in the fish market**

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3 **Application of an indirect ELISA and a PCR technique to detect**  
4 **grouper (*Epinephelus marginatus*) adulteration in the fish**  
5 **market**  
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**Abstract**

An indirect enzyme-linked immunosorbent assay (ELISA) and a multiplex polymerase chain reaction (PCR) procedure has been applied for the detection of grouper (*Epinephelus marginatus*) mislabelling in the fish market. An indirect ELISA (microtiter plate format) using two monoclonal antibodies (3D12 and 1A4) has been assayed in this study. Moreover, the multiplex PCR has been performed by using species-specific primers of the 5S rDNA gene for the rapid authentication of grouper. In this work, 70 commercial fish fillets samples collected from local markets and supermarkets, which were labelled as grouper, have been analysed by these techniques; 12 out of 70 samples were confirmed to be grouper. The PCR technique permitted the detection of the Nile perch (*Lates niloticus*) in the commercial fish fillets while it was not possible using ELISA. These results suggested that both ELISA and PCR may provide specific and reliable tools for the detection of grouper adulteration, and an accurate implementation of the traceability for successful regulatory food controls.

**Keywords:** grouper, adulteration, multiplex PCR, ELISA

## Introduction

Fresh and frozen fish fillets, where only a portion of flesh is available, are difficult to identify since many of the distinguishing morphological features are no longer detectable. This opens the possibility of fraudulent adulteration and substitution of the expected fish species with others of less value.

In this context, grouper (*Epinephelus marginatus*) is a highly appreciated and expensive fish in the Spanish fish market. Due to its high cost, popularity and demand, grouper fillets are subjected to substitution using cheaper fish species such as Nile Perch (*Lates niloticus*). Also, grouper can be substituted by closely related species such as wreck fish (*Polyprion americanus*); this fish species is renamed as grouper to make it sound more appealing to consumers. Therefore, analytical approaches for the specific identification of commercial fish species are required for detection of inappropriately labelled products (Woolfe and Primrose 2004).

Different protein-based methods have been reviewed for fish species identification, such as electrophoretic, chromatographic and immunological techniques (Dreyfuss et al. 1997; Knuutinen and Harjula 1998; Berrini et al. 2006; Martinez et al. 2007). Nevertheless, in food analysis, the Enzyme-Linked ImmunoSorbent Assay (ELISA) has been the most widely used technique for regulatory purposes in detecting food authenticity because of its specificity, simplicity and sensitivity, among other advantages (Asensio et al. 2008).

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3 However, work relating to fish species identification is scarce, partly due to the  
4 variety of fish species that are commercialized. To date, enzyme immunoassays  
5 have been used for rock shrimp (An et al. 1990), sardines (Taylor and Jones  
6 1992), red snapper (Huang et al. 1995), flat fish (Céspedes et al. 1999), or  
7 grouper identification (Asensio et al. 2003a), among others.  
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17 In recent years, significant attention has been turning towards DNA-based  
18 approaches, which have proven to be reliable, sensitive and fast for many  
19 aspects of food authentication. Among them, polymerase chain reaction (PCR)  
20 based techniques are undoubtedly the most common genetic methods used for  
21 tracing the species origin in food (Lockley and Bardsley 2000). PCR-based  
22 methods commonly used for fish species identification include random amplified  
23 polymorphic DNA (RAPD) (Bartfai et al. 2003), PCR-restriction fragment length  
24 polymorphism (PCR-RFLP) (Santaclara et al. 2006), PCR-single strand  
25 conformational polymorphism (PCR-SSCP) (Colombo et al. 2005), multiplex-  
26 PCR (Infante et al. 2006) and PCR lab-on-a-chip (Dooley et al. 2005), among  
27 others.  
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46 On the basis of this information, this paper reports the application of two  
47 alternatives for the detection of inappropriately labelled grouper fillets in the  
48 marketplace: a multiplex PCR technique relying on the 5S rDNA gene (Asensio  
49 et al. 2001), and an indirect ELISA assay that uses two monoclonal antibodies  
50 against grouper muscle proteins (Asensio et al. 2003a; Asensio et al. 2003b).  
51 Both methodologies provide a specific, sensitive and reliable means to detect  
52 adulteration of grouper, and may assist the regulatory agencies to enforce  
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3 national and trans-national laws and regulations (Council Regulation (EC)  
4 104/2000; Resolution of 27<sup>th</sup> of February 2007 of the Spanish Secretary  
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8 General of Maritime Fishing).  
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## 10 11 12 **Materials and methods**

### 13 *Fish samples collection*

14  
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17 Grouper (*Epinephelus marginatus*), wreck fish (*Polyprion americanus*), Nile  
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19 perch (*Lates niloticus*) and other fish species (*Table 1*) were obtained from local  
20  
21 markets from Madrid. Every species was morphologically identified according to  
22  
23 the keys of Muus et al. (1998) and Froese and Pauly (2007).  
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26  
27 In addition, 70 commercial fish fillets samples (*Table 2*) labelled as grouper  
28  
29 were collected at random in different local markets and supermarkets from  
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31 Madrid.  
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### 33 *Preparation of fish samples for ELISA analysis*

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37 Fish muscle samples (1 g) were homogenized in 5 mL of saline solution (8.5 g  
38  
39 of NaCl/liter) and maintained for 10 min in a stomacher. Extracts containing  
40  
41 soluble muscle proteins were filtered through a Whatman no. 1 filter paper.  
42  
43 Protein extracts were diluted 1/50 in phosphate buffered saline (PBS) before  
44  
45 they were analyzed by the ELISA technique described bellow.  
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### 49 *Indirect ELISA*

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52 Flat-bottomed micro-ELISA plates (Costar, Corning, NY) were filled with 0.1 mL  
53  
54 of the diluted muscle protein extracts and incubated for 1 h at 37 °C. After  
55  
56 washing with PBST (PBS containing 1% Tween 20), 40 µL of the 3D12 and 1A4  
57  
58 monoclonal antibodies (MAbs) developed previously (Asensio et al. 2003a;  
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3 Asensio et al. 2003b) were diluted in 60  $\mu\text{L}$  of PBS and the total volume (100  
4  $\mu\text{L}$ ) was added to the wells. The plates were shaken for 1 h at room  
5  
6 temperature and, after washing with PBST, 0.1 mL aliquots of peroxidase-  
7  
8 conjugated rabbit antimouse immunoglobulins (DAKO, Glostrup, Denmark)  
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10 diluted 1/2000 in PBST were added to the wells. The plates were incubated with  
11  
12 shaking for 1 h at room temperature and the wells were washed with distilled  
13  
14 water before addition of 0.05 mL of a ready-to-use substrate solution of  
15  
16 3,3',5,5',-tetramethylbenzidine (Boehringer Mannheim GmbH, Mannheim,  
17  
18 Germany). After 10 min of incubation, the reaction was stopped by addition to  
19  
20 each well of 0.025 mL of 1 M  $\text{H}_2\text{SO}_4$ . The yellow color developed by conversion  
21  
22 of the substrate was measured at 450 nm with an iEMS Reader MF  
23  
24 Spectrophotometer (Labsystems, Oy, Helsinki, Finland).  
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### 32 *DNA extraction procedure*

33  
34 Genomic DNA was extracted from fish samples using a Wizard DNA cleanup kit  
35  
36 (Promega, Madison, WI, USA). The extraction procedure was performed as  
37  
38 follows:  
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42 Samples (0.2 g) were digested in 860  $\mu\text{L}$  of extraction buffer, pH 8.0 (10 mM  
43  
44 Tris, 150 mM NaCl, 2 mM EDTA and 1% SDS), 100  $\mu\text{L}$  of 5 M guanidine  
45  
46 hydrochloride and 40  $\mu\text{L}$  of 20 mg  $\text{mL}^{-1}$  proteinase K (Boehringer Mannheim  
47  
48 GmbH, Mannheim, Germany). Digests were incubated overnight at 55  $^\circ\text{C}$  with  
49  
50 shaking at 70 x g, and were left to cool at room temperature. 500  $\mu\text{L}$  of  
51  
52 chloroform were added to the lysate before centrifugation at 10.000 x g for 10  
53  
54 min. The clear aqueous supernatant obtained after the centrifugation (500  $\mu\text{L}$ )  
55  
56 was used to purify the DNA using the Wizard DNA cleanup system kit  
57  
58 (Promega) with a vacuum manifold, according to the manufacturer's  
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3 instructions. Finally, the DNA was eluted in 100  $\mu$ L of sterile deionized water  
4  
5 and its concentration was determined by spectrophotometry at 260 nm  
6  
7  
8 (Eppendorf Biophotometer, Hamburg, Germany).  
9

### 10 *Polymerase Chain Reaction (PCR)*

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13 The set of primers used for PCR amplification of DNA were the forward primer  
14  
15 5S1 (5'-TACGCCCGATCTCGTCCGATC-3'), designed by Pendás et al. (1994),  
16  
17 and the specifically reverse primers 5SG (5'-  
18  
19 CTTAATGCACATATGCTCACTGAC-3'), 5SW (5'-  
20  
21 CCTCTGTGCTATAAGTTGGACCT-3') and 5SP (5'-  
22  
23 TACGCTGACGTGCAGATGCA-3') designed by Asensio et al. (2001). These  
24  
25 primers were mixed in the same ratio and used together for the multiplex PCR  
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27  
28 of this study.  
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33 Double-stranded amplifications were carried out in a final volume of 25  $\mu$ l  
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35 containing 2 mM MgCl<sub>2</sub>, 10 pmol of each primer (5S1, 5SG, 5SW and 5SP), 20  
36  
37 ng of template DNA, and 2 U of DNA polymerase (Roche, Mannheim,  
38  
39 Germany) in a reaction buffer containing 75 mM Tris-HCl, pH 9.0, 50 mM KCl,  
40  
41 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 0.001% bovine serum albumin (BSA).  
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46 PCR amplification was performed in a Progene Thermal Cycler (Techne Ltd,  
47  
48 Cambridge, UK). Thirty-five cycles of amplification with the following step-cycle  
49  
50 profile were carried out: strand denaturation at 94 °C for 45 s, primer annealing  
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52 at 66 °C for 45s and primer extension at 72 °C for 45 s. An initial denaturation at  
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54 94 °C for 3 min, and a final extension at 72 °C for 5 min, improved the product  
55  
56 yielded.  
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3 To avoid false negative PCR results and verify positive amplification of fish  
4 species analysed in this study we designed an eukaryote-specific primer pair:  
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6 18Seu-fw (5'- GGTAGTGACGAAAAATAACAATACAGGAC-3')/18Seu-inv (5'-  
7  
8 ATACGCTATTGGAGCTGGAATTACC-3') flanking a 141 bp fragment from 18S  
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10 rRNA gene was used as an internal control of the assay. Amplification was  
11  
12 performed with the following steps: a denaturation step of 93 °C for 2 min,  
13  
14 followed by 35 cycles with a strand denaturation at 93 °C 30 s, primer annealing  
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16 at 60 °C for 30 s, and primer extension at 72 °C for 45 s. The final extension  
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18 step was performed at 72 °C for 5 min.  
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25 PCR results were determined by visualization of amplicons on 2% agarose gels  
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27 stained with ethidium bromide (1 µg/mL). The sizes of the DNA fragments were  
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29 estimated by comparison with a commercial 1Kb plus DNA ladder (Gibco BRL,  
30  
31 Life Technologies Inc., Rockville, MD).  
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### 37 **Results and discussion**

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39 Food quality and safety are priority research areas that include traceability  
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41 along the production chain. Then, it is necessary to develop reliable traceability  
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43 techniques to establish the origin and mode of manufacturing fishery products  
44  
45 needed to prevent fraud and consequently to assure consumer confidence and  
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47 to enable rapid product withdrawal (Santaclara et al. 2006). In this context,  
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49 analytical tools such as immunoenzymatic assays (ELISA) are being  
50  
51 increasingly demanded by law enforcement agencies for fish and fishery  
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53 products identification (Asensio et al. 2008). Moreover other approaches include  
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55 PCR-based methods, which have also proved successful for fish species  
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57 authentication (Mackie et al. 1999; Asensio Gil, 2007). In the present study, we  
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3 describe the application of an indirect ELISA by using two MAbs and a multiplex  
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5 PCR assay relying on the 5S rDNA gene, to detect grouper adulteration in the  
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7 fish shops.  
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### 10 *ELISA analysis*

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12 Among immunological methods, the enzyme-linked immunosorbent assay  
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14 (ELISA) has been the most widely used technique for regulatory purposes in  
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16 detecting fish species adulteration, because of its simplicity, specificity and  
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18 sensitivity (Céspedes et al. 1999).  
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25 The pre-requisite for an ELISA is the availability of amounts of antibodies  
26  
27 sufficient to detect analytes. Both polyclonal and monoclonal antibodies (MAbs)  
28  
29 can be used in ELISA methods for fish identification. Monoclonal antibodies,  
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31 compared to polyclonal antibodies, are a homogeneous population of antibodies  
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33 produced by hybridoma technology that have defined biological activity,  
34  
35 consistent specificity and their production is not limited (Goding, 1996).  
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42 In two previous publications, we reported the development of two monoclonal  
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44 antibodies specific to grouper (3D12 MAb) (Asensio et al. 2003a) and grouper  
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46 and wreck fish (1A4 MAb) (Asensio et al. 2003b). Firstly, to study the specificity  
47  
48 of these MAbs, other fish species were analysed by ELISA using these MAbs.  
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50 As it can be seen in the *Table 1*, 3D12 MAb recognized specifically the grouper  
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52 and did not show cross-reactivity with the other species analysed in this work.  
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54 Furthermore, 1A4 MAb only recognized grouper and wreck fish specimens. The  
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56 absorbance values of negative samples were similar to those of the background  
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3 readings (0.084 to 0.150), while positive samples reached values higher than  
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5 1.0.  
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10 In addition, 70 commercial fillets samples labelled as grouper were also  
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12 analysed by this methodology. As can be observed in the *Table 2*, 3D12 MAb  
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14 recognized specifically 12 samples of grouper out of 70. Alternatively, 1A4 MAb  
15  
16 recognized grouper and wreck fish species. When results obtained with the two  
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18 MAbs were compared, we could distinguish wreck fish from grouper samples,  
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20 so that, we could identify specifically 13 samples of wreck fish out of 70 (*Table*  
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22 *2*).  
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29 This ELISA procedure, which can be performed in less than 4 h, has proved to  
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31 be specific, sensitive, cheap and very simple to develop. It could be very useful  
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33 as routine analysis tools in food control laboratories for enforcing labeling  
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35 regulations in the authentication of these fish species.  
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#### 40 41 *Multiplex PCR*

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43 Detailed sequence information has become available for many species and  
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45 consequently phylogenetically informative single base polymorphisms may be  
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47 identified that enable species-specific primers to be designed. Under suitably  
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49 stringent reaction conditions, such primers generate a product only in the  
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51 presence of DNA from a given species. Complete sequence information permits  
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53 the size of the product to be predicted, so that identification is confirmed if an  
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55 appropriately sized amplicon is seen on a gel (Lockley and Bardsley 2000).  
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3 Recent works have reported the application of specific primers for fish and  
4 fishery products authentication (Taylor et al. 2002; Hsieh et al. 2004).  
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10 In this context and in agreement with the results obtained in a previously  
11 reported work (Asensio et al. 2001), the combination of different primers specific  
12 for grouper (5SG), Nile perch (5SP) and wreck fish (5SW), along with the 5S1  
13 oligonucleotide (forward primer), allowed the amplification of specific regions of  
14 the 5S rDNA gene: a 323 bp fragment from grouper DNA, a 471 bp fragment  
15 from wreck fish DNA and a 185 bp fragment from Nile perch DNA (*Figure 1*).  
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27 To determine the specificity of this procedure, other fish species were analysed  
28 by using these markers, and no amplification was obtained (*Table 1*). *Figure 1*  
29 shows an example of this specificity study. Moreover, to avoid false negative  
30 PCR results and verify positive amplification we used an internal control based  
31 on the 18S rRNA gene. All fish species studied in this work were amplified by  
32 this marker (*Table 1*), which is eukaryotes specific.  
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43 Also, the commercial samples collected from the fish shops were analysed by  
44 using the multiplex PCR. PCR amplifications results matched unequivocally with  
45 ELISA results showing a complete agreement of both methods allowing the  
46 authentication of grouper (*Table 2*). In addition, the multiplex PCR allowed to  
47 detect 34 samples of Nile perch out of the 70 fish samples. Nile perch is the fish  
48 species most used as a substitute for grouper in Spain. Likewise, 11 samples  
49 analysed were unknown: no amplification was obtained. Probably other cheap  
50 fish species could be used to substitute grouper. Also, to avoid false negatives  
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3 these unknown samples amplified the internal control used in this study (data  
4 not shown). *Figure 2* shows an example of electrophoresis of various  
5 commercial samples analysed by this technique.  
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12 The application of this multiplex PCR is a powerful approach for the specific  
13 identification of grouper fillets. Since it is very specific, rapid, reliable and easy  
14 to perform, this methodology is an useful alternative for detection of grouper  
15 adulteration in the market of fish fillets.  
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## 22 **Conclusions**

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25 Results obtained in this work suggest that both the ELISA and PCR techniques  
26 may provide a reliable means for the specific detection of inappropriately  
27 labelled grouper fillets. PCR-based methods are the most specific and sensitive  
28 methods for fish species identification, although they require some expensive  
29 laboratory equipment and a certain degree of knowledge. On the contrary,  
30 ELISA, being sensitive and specific, are quicker than genetic methods for  
31 routine analysis of large sample numbers. In spite of ELISA technique was  
32 unable to recognise Nile perch from other unknown species used to adulterate  
33 the grouper, both procedures reported here may represent two effective and  
34 straightforward tools for grouper authentication and this work is a good example  
35 of applied research and its direct impact on the lives and health of consumers.  
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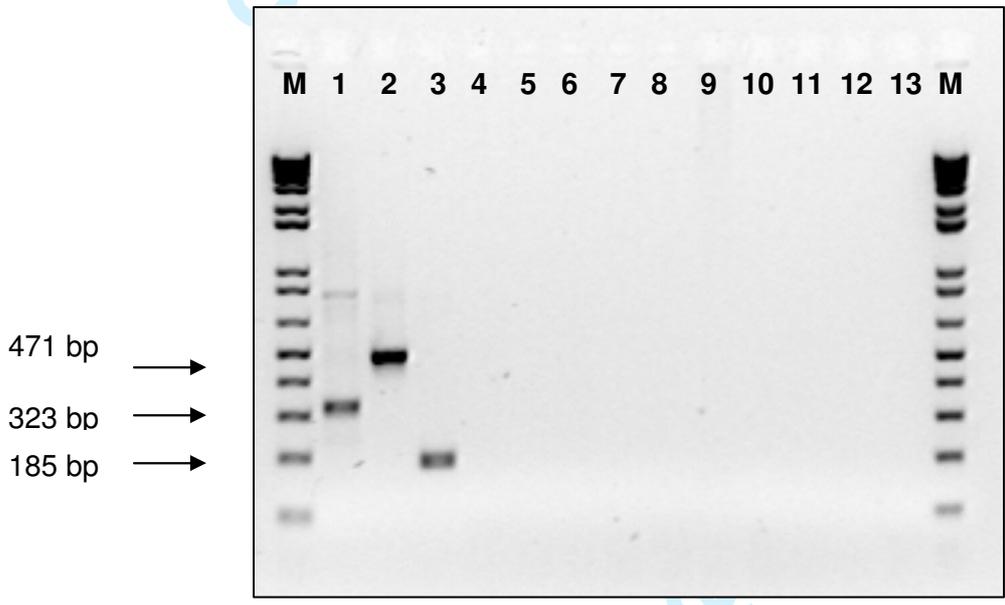
### 43 **Figure captions**

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46 **Figure 1.** Electrophoretic analysis of the 5S rDNA amplicons from the multiplex  
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48 PCR. Samples are: 1= *Epinephelus marginatus*; 2= *Polyprion americanus*; 3=  
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50 *Lates niloticus*; 4= *Prionace glauca*; 5= *Solea solea*; 6= *Pleuronectes platessa*;  
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52 7= *Auxis rochei*; 8= *Sardina pilchardus*; 9= *Platichthys flesus*; 10= *Engraulis*  
53  
54 *encrasicholus*; 11= *Trisopterus luscus*; 12= *Xiphias gladius*; 13= *Diplodus*  
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56 *sargus*. M indicates kb plus DNA ladder for molecular weight marker.  
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3 **Figure 2.** Electrophoretic analysis of the 5S rDNA amplicons from the multiplex  
4 PCR. Lanes 1-11 corresponds to samples 60-70 of the study. M indicates kb  
5 plus DNA ladder for molecular weight marker and N corresponds negative  
6 control.  
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Figure 1.

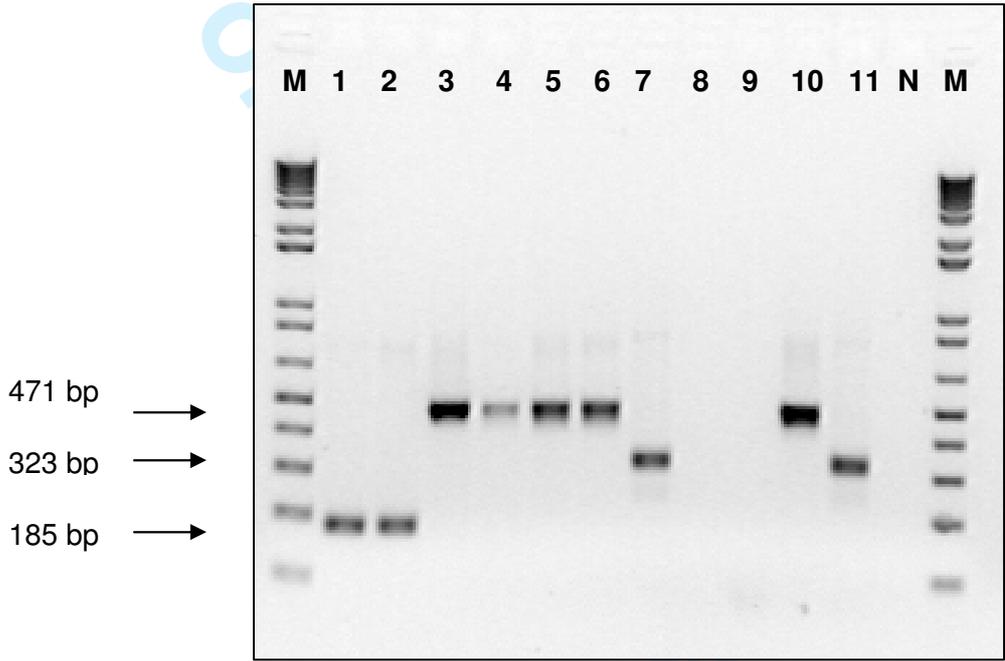


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Figure 2.



**Table 1.** Different fish species analysed by ELISA and PCR techniques.

Fish species analysed	ELISA by 1A4 MAb	ELISA by 3D12 MAb	Amplification by Multiplex PCR	Internal control amplification
<i>Epinephelus marginatus</i>	+	+	323 bp	141 bp
<i>Polyprion americanus</i>	+	-	471 bp	141 bp
<i>Lates niloticus</i>	-	-	185 bp	141 bp
<i>Argyrosomus regius</i>	-	-	-	141 bp
<i>Auxis rochei</i>	-	-	-	141 bp
<i>Brama brama</i>	-	-	-	141 bp
<i>Chamelea gallina</i>	-	-	-	141 bp
<i>Clupea harengus</i>	-	-	-	141 bp
<i>Conger conger</i>	-	-	-	141 bp
<i>Dicentrarchus labrax</i>	-	-	-	141 bp
<i>Diplodus sargus</i>	-	-	-	141 bp
<i>Eledone cirrosa</i>	-	-	-	141 bp
<i>Engraulis encrasicolus</i>	-	-	-	141 bp
<i>Ensis arquatus</i>	-	-	-	141 bp
<i>Gadus morhua</i>	-	-	-	141 bp
<i>Galeorhinus galeus</i>	-	-	-	141 bp
<i>Genypterus blacodes</i>	-	-	-	141 bp
<i>Lepidorhombus boscii</i>	-	-	-	141 bp
<i>Lepidorhombus whiffiagonis</i>	-	-	-	141 bp
<i>Limanda limanda</i>	-	-	-	141 bp
<i>Littorina littorea</i>	-	-	-	141 bp
<i>Liza aurata</i>	-	-	-	141 bp
<i>Loligo vulgaris</i>	-	-	-	141 bp
<i>Lophius budegassa</i>	-	-	-	141 bp
<i>Melanogrammus aeglefinus</i>	-	-	-	141 bp
<i>Merluccius merluccius</i>	-	-	-	141 bp
<i>Micromesistius poutassou</i>	-	-	-	141 bp
<i>Mullus barbatus</i>	-	-	-	141 bp
<i>Mytilus edulis</i>	-	-	-	141 bp
<i>Necora puber</i>	-	-	-	141 bp
<i>Nephrops norvegicus</i>	-	-	-	141 bp
<i>Oncorhynchus mykiss</i>	-	-	-	141 bp
<i>Oreochromis niloticus</i>	-	-	-	141 bp
<i>Pagellus bogaraveo</i>	-	-	-	141 bp
<i>Pagellus erythrinus</i>	-	-	-	141 bp
<i>Pangasius hypophthalmus</i>	-	-	-	141 bp
<i>Parapenaeus longirostris</i>	-	-	-	141 bp
<i>Platichthys flesus</i>	-	-	-	141 bp
<i>Pleuronectes platessa</i>	-	-	-	141 bp
<i>Pollicipes pollicipes</i>	-	-	-	141 bp
<i>Prionace glauca</i>	-	-	-	141 bp
<i>Salmo salar</i>	-	-	-	141 bp
<i>Sarda sarda</i>	-	-	-	141 bp
<i>Sardina pilchardus</i>	-	-	-	141 bp

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4	<i>Scomber scombrus</i>	-	-	-	141 bp
5	<i>Scophthalmus maximus</i>	-	-	-	141 bp
6	<i>Scorpaena scrofa</i>	-	-	-	141 bp
7	<i>Solea solea</i>	-	-	-	141 bp
8	<i>Sparus aurata</i>	-	-	-	141 bp
9	<i>Thunnus alalunga</i>	-	-	-	141 bp
10	<i>Thunnus thynnus</i>	-	-	-	141 bp
11	<i>Trachurus trachurus</i>	-	-	-	141 bp
12	<i>Trisopterus luscus</i>	-	-	-	141 bp
13	<i>Xiphias gladius</i>	-	-	-	141 bp
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**Table 2.** Commercial fish fillets analysed by ELISA and PCR techniques.

Sample designation	ELISA by 1A4 MAb	ELISA by 3D12 MAb	ELISA authenticity	Amplification by Multiplex PCR	Multiplex PCR authenticity
1	-	-		-	
2	-	-		185 bp	Nile perch
3	-	-		185 bp	Nile perch
4	-	-		-	
5	-	-		185 bp	Nile perch
6	-	-		185 bp	Nile perch
7	+	+	Grouper	323 bp	Grouper
8	+	+	Grouper	323 bp	Grouper
9	+	+	Grouper	323 bp	Grouper
10	+	+	Grouper	323 bp	Grouper
11	-	-		-	
12	-	-		-	
13	-	-		-	
14	-	-		185 bp	Nile perch
15	-	-		185 bp	Nile perch
16	-	-		185 bp	Nile perch
17	-	-		185 bp	Nile perch
18	-	-		185 bp	Nile perch
19	-	-		185 bp	Nile perch
20	-	-		185 bp	Nile perch
21	-	-		185 bp	Nile perch
22	-	-		185 bp	Nile perch
23	+	-	Wreck fish	471 bp	Wreck fish
24	-	-		185 bp	Nile perch
25	+	-	Wreck fish	471 bp	Wreck fish
26	-	-		185 bp	Nile perch
27	+	-	Wreck fish	471 bp	Wreck fish
28	-	-		185 bp	Nile perch
29	-	-		185 bp	Nile perch
30	-	-		185 bp	Nile perch
31	-	-		185 bp	Nile perch
32	-	-		185 bp	Nile perch
33	-	-		185 bp	Nile perch
34	-	-		185 bp	Nile perch
35	-	-		185 bp	Nile perch
36	-	-		185 bp	Nile perch
37	-	-		185 bp	Nile perch
38	-	-		185 bp	Nile perch
39	-	-		185 bp	Nile perch
40	-	-		185 bp	Nile perch
41	+	+	Grouper	323 bp	Grouper
42	+	+	Grouper	323 bp	Grouper
43	+	+	Grouper	323 bp	Grouper
44	+	+	Grouper	323 bp	Grouper

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45	+	+	Grouper	323 bp	Grouper
46	+	+	Grouper	323 bp	Grouper
47	+	-	Wreck fish	471 bp	Wreck fish
48	+	-	Wreck fish	471 bp	Wreck fish
49	+	-	Wreck fish	471 bp	Wreck fish
50	+	-	Wreck fish	471 bp	Wreck fish
51	-	-		-	
52	-	-		-	
53	-	-		-	
54	-	-		-	
55	-	-		185 bp	Nile perch
56	-	-		185 bp	Nile perch
57	-	-		185 bp	Nile perch
58	-	-		185 bp	Nile perch
59	+	-	Wreck fish	471 bp	Wreck fish
60	-	-		185 bp	Nile perch
61	-	-		185 bp	Nile perch
62	+	-	Wreck fish	471 bp	Wreck fish
63	+	-	Wreck fish	471 bp	Wreck fish
64	+	-	Wreck fish	471 bp	Wreck fish
65	+	-	Wreck fish	471 bp	Wreck fish
66	+	+	Grouper	323 bp	Grouper
67	-	-		-	
68	-	-		-	
69	+	-	Wreck fish	471 bp	Wreck fish
70	+	+	Grouper	323 bp	Grouper