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Multiplex PCR Method for Use in Real-Time PCR for Identification of Fish Fillets from Grouper (*Epinephelus* and *Mycteroperca* Species) and Common Substitute Species

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Mitochondrial 16S rRNA sequences from morphological validated grouper (*Epinephelus aeneus*, *E. caninus*, *E. costae*, and *E. marginatus*; *Mycteroperca fusca* and *M. rubra*), Nile perch (*Lates niloticus*), and wreck fish (*Polyprion americanus*) were used to develop an analytical system for group diagnosis based on two alternative Polymerase Chain Reaction (PCR) approaches. The first includes conventional multiplex PCR in which electrophoretic migration of different sizes of bands allowed identification of the fish species. The second approach, involving real-time PCR, produced a single amplicon from each species that showed different T_m values allowing the fish groups to be directly identified. Real-time PCR allows the quick differential diagnosis of the three groups of species and high-throughput screening of multiple samples. Neither PCR system cross-reacted with DNA samples from 41 common marketed fish species, thus conforming to standards for species validation. The use of these two PCR-based methods makes it now possible to discriminate grouper from substitute fish species.

KEYWORDS: Multiplex PCR; 16S rRNA; *Epinephelus* spp.; *Mycteroperca* spp.; *Lates niloticus*; *Polyprion americanus*; real-time PCR; species identification

INTRODUCTION

At present, commercial fish products in Europe come from all parts of the world, meaning that accurate species identification is not always easy. The introduction of exotic species and the increased number of processed products, such as fish fillets, impair their identification at the species level. Mislabeling of fish such that less valuable species are sold under the names of more expensive ones is a growing problem in the production and distribution chain. In U.S. fish markets, a recent analysis of the red snapper served to identify the mislabeling of 77% of samples (*1*). Grouper from the genera *Epinephelus* and *Mycteroperca* are a highly appreciated and expensive group of fishes from the Serranidae family (*2*). Their high demand and popularity have led to the substitution of grouper fillets with those of closely related species. In markets, grouper are frequently misidentified as Nile perch (*Lates niloticus*) or the wreck fish (*Polyprion americanus*) (*3*). When morphological characters are preserved, species identification is feasible, but these characteristics are lost during processing and the species are not longer recognizable. The main features of grouper, in particular, completely disappear, and their identity cannot be established on the basis of morphological features. Identification procedures generally include the analysis of proteins by electrophoretic techniques such as isoelectric focusing (IEF) (*4*−*6*), capillary electrophoresis (CE), or immunoassay techniques such as ELISA (*7*, *8*). Nevertheless, fish proteins can be denatured or degraded during food processing or storage, to the extent that they lose their natural biological properties, rendering them unsuitable for comparative analyses (*9*, *10*). In addition, immunological methods may be compromised by cross-reactions among proteins from closely related species (*11*). Moreover, these methods, useful in some cases, are inappropriate for routine high-throughput sample analysis as they are time-consuming, expensive, and complex to perform. Hence, fish species identification methods based on DNA analysis are now being developed as an alternative to morphological analyses (*12*−*16*). In particular, Polymerase Chain Reaction (PCR) has become routine in many standard methods for species identification (*17*−*19*). Mitochondrial DNA (mtDNA) sequences have been commonly used for the purpose of species identification by PCR, because mtDNA is multicopy DNA existing in every cell. This facilitates its amplification from small tissue samples. The high rate of evolution of mtDNA also makes it easier to resolve differences between closely related species. A further advantage is that the complete mtDNA sequence of a large number of

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representative fish species is known (*20*, *21*), allowing the design of generic and specific primers to identify sequence differences between species, and areas showing high mutation rates among individuals and populations of a given species can be avoided (*12*, *14*, *22*). Sequencing and restriction fragment length polymorphism (RFLP) of mtDNA PCR fragments have been used to identify different tuna species in processed food (*23*, *24*). Also, sequencing and phylogenetic mtDNA analysis have been used to check for mislabeling of the red snapper (*1*). PCR amplification as a prelude to techniques such as RFLP, PCR-SSCP, and RAPD analysis has been reported for the differentiation of a single grouper species from its common substitute species, but the whole procedure is time-consuming and not easy for routine checks (*3*, *25*, *26*). The use of specifically designed primers under restrictive conditions of PCR amplification could, nevertheless, allow the direct and specific identification of PCR-amplified mtDNA fragments, avoiding subsequent sequencing (*1*) or RFLP identification (*27*). The present paper deals with a simple, reliable, and quick method that is useful for routine, high-throughput analysis. Our proposed method was able to rapidly and accurately identify and differentiate six grouper species from their common substituted species in fish markets. The method has two PCR options for differentiating genera after amplification: multiplex conventional PCR or the duplex real-time SYBR Green I fluorescent PCR assay. The method can be applied to fresh, frozen, or processed fillets to detect the fraudulent or unintentional mislabeling of grouper.

MATERIALS AND METHODS

Fish Samples. Grouper (*Epinephelus caninus*, *E. costae*, *E. marginatus*; *Mycteroperca fusca* and *M. rubra*) and wreck fish (*P. americanus*) samples were collected from European seawaters and taxonomically identified by an ichthyologist at the Canarian Institute of Marine Sciences. Nile perch (*L. niloticus*) and grouper (*E. aeneus*) were purchased from the Naples fish market and taxonomically identified by the Official Veterinary Service. Also, fillets prepared from *L. niloticus* and grouper were obtained from local markets in Madrid to test the reliability of the analyses. Muscle samples from duplicate or triplicate specimens of each species were obtained fresh and immediately processed by aseptically cutting small muscle portions (1−2 g) and subsequently preserving them in ethanol 70% at −20 °C until use.

DNA Extraction. Total genomic DNA extraction was performed from muscle tissue. Frozen pieces of tissue (25 mg) were ground in liquid N_2 and homogenized in 600 μL of extraction buffer (0.14 M NaCl, 1.5 mM magnesium acetate, 5 mM KCl, 1% SDS). The resulting homogenate was extracted using standard proteinase K/phenol−chloroform protocols (*28*). DNA was resuspended in ultrapure water and used for further analysis.

Amplification and Sequencing of Part of the Mitochondrial 16S rRNA Gene. PCR was used to amplify a 590 bp fragment of the mitochondrial 16S rRNA gene in *P. americanus*, *E. aeneus*, *E. costae*, and *E. marginatus* using 16SbrH and 16SarL primers (*29*). PCR amplification reactions were carried out in a total volume of 25 μL of a solution containing 15 ng of DNA template, 2 μL of PCR buffer (100 nM Tris-HCl, pH 8.3, 500 mM KCl); 320 nmol of each primer, 0.4 mM dNTP, 2.5 mM of $MgCl_2$, and 0.6 unit of Ampli Taq Gold (Applied Biosystems). After an initial denaturation step at 94 °C for 5 min, 40 cycles were performed as follows: denaturation at 94 °C (90 s), annealing at 52 °C (180 s), and extension at 72 °C (90 s), with a final extension of 7 min at 72 °C. PCR was carried out in a Master CyclerGradient (Eppendorf). DNA automatic sequencing in an Applied Biosystems 3730 sequencer was performed on both strands of the PCR fragments, employing the same primers as for PCR amplification. Sequences were aligned by Sequence Navigator software (PE Applied Biosystems) and phylogenetically validated.

Design of Species-Specific PCR Primers. Clustal X alignment (*30*) of 88 fish 16S rRNA sequences, including GenBank sequences of 84 species from the Centropomidae, Moronidae, Percidae, Sparidae, and Serranidae (**Table 1**) and the sequences of four species obtained here (*E. aeneus*, *E. costae*, *E. marginatus*, and *P. americanus*), allowed us to design specific primers for each of the three groups of fish. Four specific primers were designed to amplify specific fragments of different sizes of the mitochondrial 16S rRNA gene: a universal forward primer (GenFor) and three group-specific reverse primers (EpiRev, LatRev, and PolRev), resulting in three different primer pairs (**Table 2**). Each primer pair was first tested individually on samples of the species for which the specific reverse primer was designed according to the following groups: *Epinephelus* spp. and *Mycteroperca* spp. (GenFor and EpiRev), *L. niloticus* (GenFor and LatRev), and *P. americanus* (GenFor and PolRev).

Multiplex PCR. Multiplex PCR reactions were run using 10−15 ng of DNA template in a total volume of 25 μL containing PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 320 nmol of each primer, 100 nmol of each dNTP, 2.5 mM $MgCl_2$, and 0.6 unit of Ampli Taq Gold (Applied Biosystems). The PCR cycling conditions were as follows: an initial denaturation step at 95 °C for 5 min and then 40 cycles of denaturation at 94 °C (1 min), annealing at 59 °C (30 s), and extension at 72 °C (20 s), with a final extension of 7 min at 72 °C.

Specific primers were designed to produce single amplicons of different sizes to be identified after electrophoretic migration. PCR amplification products (5 μL) were analyzed by electrophoretic separation in 1.5% agarose gel stained with ethidium bromide in TAE buffer (Tris, acetic acid, EDTA, pH 8).

Real-Time PCR. An ABI PRISM 7000 sequence detection system (Applied Biosystems) was used for all real-time PCRs, using the same primers as described above (**Table 2**). For fluorescence detection of amplicons, a SYBR Green PCR Master Mix (Applied Biosystems) was used. The cycling conditions included preincubation at 50 °C for 2 min and incubation at 95 °C for 10 min. This was followed by 37 cycles at 95 °C (15 s) and 63 °C (1 min). Optimization of PCR reactions was carried out with different DNA concentrations (30, 15, 3, 0.6, 0.3, 0.03, and 0.003 ng/μL) to determine the detection range and precision of the melting peaks. Optimal peaks were obtained in the range from 0.3 to 0.6 ng/μL of DNA template. Fluorescence signals were measured once in each cycle at the end of the extension step. After PCR amplification, T_m curve analysis was performed. The duplex PCR products were cooled to 60 °C and then heated to 90 °C at a rate of 0.2 °C/s. The T_m peaks of the products were calculated for 10 or more assays on different samples for each species and were based on the initial fluorescence curve (F/T) by plotting the negative derivative of fluorescence over temperature versus temperature ($-dF/dT$ versus T).

RESULTS

The aim of this study was to develop a reliable method for the routine identification and discrimination of grouper (*Epinephelus* spp. and *Mycteroperca* spp.) from wreck fish (*P. americanus*) and Nile perch (*L. niloticus*). Primer pair 16Sarl and 16Sbrh (*28*) amplified a 590 bp fragment of the 16S rRNA gene in all samples, both fresh and frozen, of *E. aeneus*, *E. costae*, *E. marginatus*, and *P. americanus*. These sequences have been deposited under GenBank accession numbers AY31176 to AY31179. By aligning these sequences alongside 84 other Perciformes 16S rRNA sequences, we were able to design four primers to differentiate three groups of Perciformes utilizing a common forward primer to all three groups of species, and a specific reverse primer for each group of fish (grouper, Nile perch, and wreck fish). This set of four primers used in a multiplex PCR assay amplified three specific fragments of different sizes for each group of fish. The quick identification of the three groups was therefore possible, given the different amplicon sizes detected through agarose gel electrophoresis: 140 bp for *P. americanus*, 230 bp for *L. niloticus*, and 300 bp for *Epinephelus* spp. and *Mycteroperca* spp. (**Figure 1**). Sequencing of the bands confirmed primer specificity.

Real-Time PCR. Multiplex PCR in a real-time PCR instrument also allowed us to amplify specific bands for each fish

Table 1. Sources of the 16S rRNA Sequences Used for Alignment and Primer Design

species	GenBank accession no.	species	GenBank accession no.
Alphestes immaculatus	AF297290	*Epinephelus tauvina*	AJ496735
Alphestes multiguttatus	AF297305	*Epinephelus undulosus*	AF297326
Anyperodon leucogrammicus	AF297306	*Etheostoma radiosum*	AY341348
Centropomus armatus	U85010	*Gymnocephalus cernuus*	AY141443
Centropomus ensiferus	U85008	*Holanthias chrysostictus*	AY141436
Centropomus medius	U85019	*Hypoplectrus aberans*	AY015057
Centropomus mexicanus	U85017	*Hypoplectrus guttavarius*	AY015059
Centropomus nigrescens	U85015	*Hypoplectrus puella*	AY015058
Centropomus parallelus	U85016	*Hypoplectrus unicolor*	AY072680
Centropomus pectinatus	U85018	*Lateolabrax japonicus*	AY141439
Centropomus poeyi	U85014	*Lateolabrax japonicus*	AF247438
Centropomus robalito	U85011	*Lateolabrax latus*	AF247439
Centropomus undecimalis	U85012	*Lates calcarifer*	AY141441
Centropomus unionensis	U85009	*Lates niloticus*	U85007
Centropomus viridis	U85013	*Mycteroperca bonaci*	AF297315
Cephalopholis cruentatus	AF297323	*Mycteroperca jordani*	AF297329
Cephalopholis fulva	AF297292	*Mycteroperca microlepis*	AF297312
Cephalopholis miniatus	AF297321	*Mycteroperca olfax*	AF317512
Cephalopholis panamensis	AF297313	*Mycteroperca phenax*	AF297303
Cephalopholis sonnerati	AF297307	*Mycteroperca rosacea*	AF297300
Cephalopholis urodeta	AF297325	*Mycteroperca venenosa*	AF297291
Dermatolepis dermatolepis	AF297317	*Pagrus auratus*	AF247424
Epinephelus acanthistius	AF297318	*Pagrus auriga*	AJ247275
Epinephelus adscensionis	AF297314	*Pagrus auriga*	AF247425
Epinephelus aeneus	AY141437	*Pagrus auriga*	NC_005146
Epinephelus analogus	AF297302	*Pagrus coeruleostictus*	AJ247276
Epinephelus areolatus	AF297316	*Pagrus pagrus*	AJ247278
Epinephelus bleekeri	AJ496736	*Pagrus pagrus*	AJ247277
Epinephelus cifuentesi	AF297295	*Pagrus pagrus*	AF247426
Epinephelus drummondhayi	AF297308	*Paranthias colonus*	AF297301
Epinephelus fasciatus	AF297319	*Perca fluviatilis*	AY141442
Epinephelus flavolimbatus	AF297293	*Perca fluviatilis*	U87422
Epinephelus guttatus	AF297299	*Perca* sp.	AF488469
Epinephelus itajara	AF297294	*Plectropomus leopardus*	AF297298
Epinephelus labriformis	AF297296	*Plectropomus maculatus*	AF297320
Epinephelus morio	AF297324	*Pogonoperca punctata*	AF297322
Epinephelus multinotatus	AY428594	*Pogonoperca punctata*	AY141438
Epinephelus mystacinus	AF297304	*Pronotogrammus multifasciatus*	AF297330
Epinephelus nigritus	AF297297	*Rypticus saponaceus*	AF297327
Epinephelus niphobles	AF297309	*Serranus accraensis*	AY141435
Epinephelus niveatus	AF297310	*Serranus tabacarius*	AY015056
Epinephelus striatus	AF297311	*Serranus tigrinus*	AY015060

group. At the end of the reaction, fluorescence signals, continuously monitored during a slow warm-up gradient, produced a curve with a sharp peak in the double-negative derivative plot corresponding to the amplicon denaturation temperature (melting temperature, T_m). Every PCR product melts at a characteristic temperature that depends on its sequence. This T_m serves to distinguish between different PCR fragments (*31*). Therefore, the use of real-time PCR should allow simultaneous amplification and T_m analysis, thus increasing the speed of analysis and adapting the procedure for a high-throughput.

T_m values obtained for the specific PCR fragments ranged between 79.8 and 82.1 °C. Given the closeness of the T_m values for the Nile perch and wreck fish, 80.3 ± 0.2 and 80.0 ± 0.2 °C, respectively, two independent duplex PCR runs would be required to distinguish between these two species. Hence, using real-time PCR, optimum identification of the three groups required two simultaneous duplex PCR reactions. For this purpose two sets of primers, GEL (GenFor, EpiRev, and LatRev) and GEP (GenFor, EpiRev, and PolRev), allowed the differential T_m curve analysis identifying the three groups of fish. Through this procedure, different T_m values were obtained for each species (**Figure 2**), and the fish groups were directly identified without the need for gel electrophoresis of the amplicons. Dissociation curve plots (melting temperature analysis) showed only one peak for each sample, with T_m values of 80.3 ± 0.2 °C for *L. niloticus*, 80.0 ± 0.2 °C for *P. americanus*, and 81.6 ± 0.5 °C for *E.* spp. To validate the specificity of the real-time PCR, the amplification products were examined by both agarose gel electrophoresis and dissociation curve analysis, demonstrating that the real-time PCR protocol used produced the specific product without nonspecific amplifications or primer−dimer formation. It is, therefore, proposed that melting curve analysis should be sufficient to establish the success of the reaction.

Cross-Reactions. To verify the suitability and reliability of any diagnostic procedure for fish species, the method has to be tested on many species of the different groups that could interfere in the analysis, or cross-react. To this end, the standard multiplex PCR and real-time PCR procedures were conducted using as template DNA from common fish species frequently commercialized in European fish markets. **Table 3** shows the fish species tested for cross-reactivity using the PCR method in standard multiplex PCR and real-time PCR. No cross-reactivity was observed using our method on 41 species belonging to 28 different fish families, indicating the high specificity of this new procedure.

DISCUSSION

The differentiation of grouper from their common substitute species is of great interest to the fish-processing industry. To

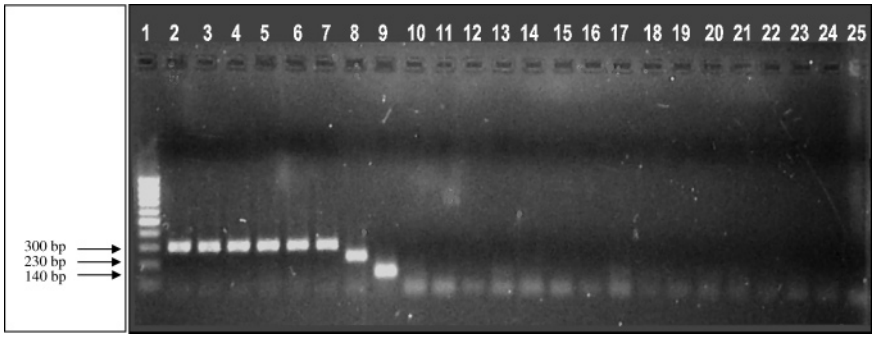


Figure 1. Agarose gel electrophoresis of the multiplex PCR products using, as template, DNA from grouper, substitute species, and selected species tested for cross-reactivity. Arrows on the left indicate the size of the bands obtained. Lane 1, control ladder 100 bp; lane 2, *E. aeneus*; lane 3, *E. caninus*; lane 4, *E. costae*; lane 5, *E. marginatus*; lane 6, *M. fusca*; lane 7, *M. rubra*; lane 8, *L. niloticus*; lane 9, *P. americanus*; lane 10, *Beryx decadactylus*; lane 11, *Dentex dentex*; lane 12, *Diplodus sargo*; lane 13, *Engraulis encrasicholus*; lane 14, *Merluccius merluccius*; lane 15, *Muraena helena*; lane 16, *Pagellus bellotii*; lane 17, *Pagrus pagrus*; lane 18, *Sardina pilchardus*; lane 19, *Scomber scombrus*; lane 20, *Salmo salar*; lane 21, *Solea lascaris*; lane 22, *Sparus aurata*; lane 23, *Xiphias gladius*; lane 24, *Zeus faber*; lane 25, negative control.

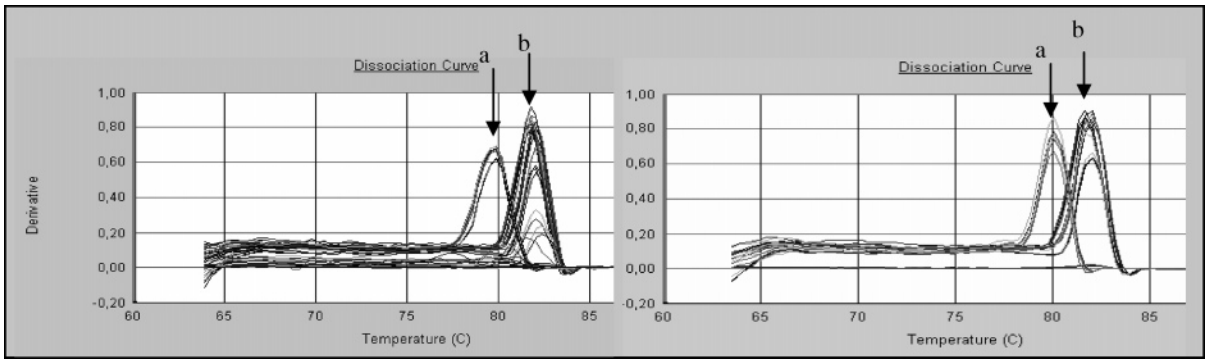
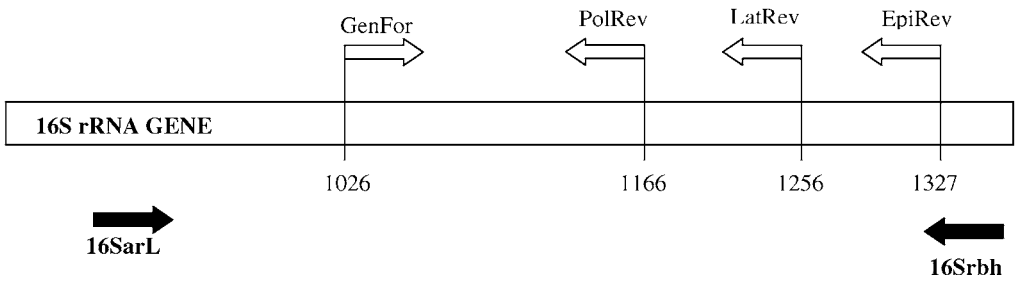


Figure 2. T_m plots of the products of duplex RT-PCR: (left) RT-PCR performed with primers (GenFor–PolRev–EpiRev) [(a) $T_m = 80.0 \pm 0.2$ °C (*P. americanus*); (b) $T_m = 81.6 \pm 0.5$ °C (*Epinephelus* spp.)]; (right) RT-PCR performed with primers (GenFor–LatRev–EpiRev) [(a) $T_m = 80.3 \pm 0.2$ °C (*L. niloticus*); (b) $T_m = 81.6 \pm 0.5$ °C (*Epinephelus* spp.)].

Table 2. (Top) Sequences and Positions of the Four Primers Used and the Amplicon Sizes Generated by Each Primer Pair; (Bottom) Schematic Representation of the 16S rRNA Gene Showing Annealing Sites and Orientation of the Primers Used in the PCR Assays

Primer name	Primer sequence	Position at 16S rRNA	Amplicon size obtained with primer GenFor
GenFor	5'-AAGGTAGCGYAATCACTTGTCT-3'	nt 1026-1047	----
PolRev	5'-TAGGGTCTTCTCGTCTTATGTACA-3'	nt 1166-1146	140 bp
LatRev	5'-CCCAACCGAAGACATTAGAAT-3'	nt 1256-1238	230 bp
EpiRev	5'-AGCTGTCGCTCTTGGTTGTGAAT-3'	nt 1327-1306	300 bp



this end, several analytical methods have been described including DNA analysis by RFLP and SSCP of the 12S rRNA gene (*3*, *25*) and random amplified polymorphisms (*26*), which have recently switched to immunological procedures (*8*). All of these procedures focus on a single species, *E. guaza* [According to FishBase (www.fishbase.org), *E. guaza* is not a

Table 3. Fish Species from 30 Different Fish Families Tested Using the Multiplex and Real-Time PCR Method

species	amplification 300 bp	amplification 230 bp	amplification 140 bp	no amplification	fish family
Epinephelus aeneus	X				Serranidae
Epinephelus caninus	X				Serranidae
Epinephelus costae	X				Serranidae
Epinephelus marginatus	X				Serranidae
Mycteroperca fusca	X				Serranidae
Mycteroperca rubra	X				Serranidae
Lates niloticus		X			Centropomidae
Polyprion americanus			X		Polyprionidae
Balistes carolinensis				X	Balistidae
Beryx decadactylus				X	Berycidae
Brama brama				X	Bramidae
Brotula barbata				X	Ophidiidae
Conger conger				X	Congridae
Dentex dentex				X	Sparidae
Dicentrarchus labrax				X	Moronidae
Diplodus cervinus				X	Sparidae
Diplodus sargus				X	Sparidae
Engraulis encrasicholus				X	Engraulidae
Katsuwonus pelamis				X	Scombridae
Lepidorhombus boscii				X	Scophthalmidae
Lophius piscatorius				X	Lophiidae
Merluccius merluccius				X	Merlucciidae
Molva molva				X	Lotidae
Mullus surmuletus				X	Mullidae
Muraena augusti				X	Muraenidae
Muraena helena				X	Muraenidae
Pagellus acarne				X	Sparidae
Pagellus bellotii				X	Sparidae
Pagellus bogaraveo				X	Sparidae
Pagrus pagrus				X	Sparidae
Phycis phycis				X	Phycidae
Pollachius pollachius				X	Gadidae
Ranzania laevis				X	Molidae
Ruvettus pretiosus				X	Gempylidae
Salmo salar				X	Salmonidae
Sardina pilchardus				X	Clupeidae
Scarus hoefleri				X	Scaridae
Scomber scombrus				X	Scombridae
Seriola dumerili				X	Carangidae
Seriola fasciata				X	Carangidae
Serranus atricauda				X	Serranidae
Serranus hepatus				X	Serranidae
Solea lascaris				X	Soleidae
Sparus aurata				X	Sparidae
Thunnus thynnus				X	Scombridae
Trachurus mediterraneus				X	Carangidae
Umbrina canariensis				X	Sciaenidae
Xiphias gladius				X	Xiphiidae
Zeus faber				X	Zeidae

valid synonym for *E. marginatus* (Lowe, 1834).], as the only marketed grouper species, although other *Epinephelus* spp. and *Mycteroperca* spp. are also widely sold as grouper in European markets. In addition, ELISA-based DNA analysis of the 5S rRNA gene has been also developed for the detection of Nile perch in fish muscle mixtures (*32*). Nevertheless, a definitive analytical procedure for the differential diagnosis of all species defined by the European markets as grouper (*E. aeneus*, *E. costae*, *E. marginatus*, *M. fusca*, and *M. rubra*) and their common substitute species has not yet been described. Moreover, the close phylogenetic relationships among teleost species and the large number of marketed fish species means that any diagnostic technique should be tested for cross-reactivity with many common species to avoid false positives.

In the present paper, the four primers designed to discriminate fish species-specific sequences in the 16S rRNA gene were used as the basis for a reliable PCR diagnostic system for the origin of fish fillets. Two alternative PCR approaches were developed to quickly and accurately identify and differentiate three groups of fish, the fillets of which are frequently misidentified as grouper.

Both approaches, multiplex PCR and real-time PCR, rendered the expected discrimination of the DNA origin without the need for any additional analytical steps such as sequencing (*1*), enzyme digestion (*25*), conformational analysis (*3*), or ELISA detection (*32*). These tests are time-consuming and laborious and, besides increasing costs, do not allow for scale-up of the diagnostic system for routine high-throughput analysis.

Thus, combining the four primers in a multiplex PCR assay simplifies the identification process in that only a single PCR amplification is needed to simultaneously distinguish among the three groups of species according to three different diagnostic amplicon sizes, easily detected by agarose gel electrophoresis. This design has clear advantages over individual tests on unknown samples using species-specific primer pairs in separate reactions.

The proposed method was also used in a real-time PCR assay to speed the differential diagnosis of the three groups of species, avoiding gel electrophoresis. The benefit of the duplex real-time PCR design was the overall speed of the procedure (~2 h). This speed means several 96-well plates can be assayed in a working day, allowing the routine high-throughput screening of multiple samples. Additional advantages of the real-time PCR assay were the high reproducibility of the T_m analysis of the amplified fragments, with standard errors below 1% (in different runs), and the lower cost of the non-sequence-specific SYBR Green assay compared to fluorescent probe-based assays performed using Taqman probes or molecular beacons (*33*), providing equivalent efficiency and specificity.

Standardized methods of species validation should provide proof that the method does not cross-react with samples from close origins. This is especially true of many PCR methods used in bacterial (*34*−*36*) and viral (*37*, *38*) diagnostic systems and is also a condition for tests targeting teleost species, given the large number of marketed and processed fish species. Here, we provide evidence that the PCR system did not cross-react with DNA samples from 41 different fish species, thus conforming to standards for species validation. Finally, the new method here presented and those already described (*3*, *8*, *25*, *26*) could well complement each other in the precise diagnosis of fish fillets from grouper.

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