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DNA barcoding approaches for fishing authentication of exploited grouper species including the endangered and legally protected goliath grouper *Epinephelus itajara*

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SUMMARY: Fishing strategies are constantly changing to meet the needs for new or alternative food sources. Consequently, management of fishing activities regarding rates of exploitation is essential, as a number of resources have reached situations of overexploitation. The aim of the present study was to use DNA barcoding from the goliath grouper and other exploited epinephelids in order to provide procedures for DNA authentication to be used as evidence for combating putative illegal fishing. The species studied were *Epinephelus adscensionis, Mycteroperca bonaci, Mycteroperca interstitialis, Epinephelus itajara*, *Mycteroperca venenosa, Epinephelus mystacinus*, *Dermatolepis inermis, Alphestes afer, Cephalopholis fulva, Mycteroperca acutirostris*, *Rypticus saponaceus*, *Mycteroperca marginata* and *Epinephelus morio*. Four of these species are the main epinephelids fished in the Atlantic Ocean. Differential patterns of polymerase chain reaction–restriction fragment length polymorphism were obtained from the species and additional single nucleotide polymorphisms were also detected among the four main epinephelids studied. The procedures proved very efficient and we suggest their applicability to the other fish groups as a way to control illegal capture and retail around the world, especially in cases in which filleting and other forms of de-characterization cause a lack of morpho-anatomical key characters.

Keywords: DNA authentication, PCR-RFLPs, SNPs, groupers, goliath grouper, epinephelids.

RESUMEN: Autenticación mediante *DNA barcoding* de especies de meros legalmente protegidas y en peligro de extinción, sometidas a explotación pesquera, incluyendo el mero Goliat *Epinephelus itajara*. – Las estrategias de pesca cambian constantemente para satisfacer las necesidades de fuentes de alimento nuevas o alternativas. En consecuencia, a medida que los recursos alcanzan situaciones de sobreexplotación, resulta esencial establecer procedimientos de inspección de las actividades relacionadas con las tasas de explotación pesquera. El objetivo de este estudio es proponer el uso de la técnica de *DNA barcoding* para establecer la verificación de la identidad del mero Goliat y otros epinefélidos, a fin de utilizarlo como evidencia para combatir la pesca ilegal cuando se sospeche su ocurrencia. Las especies aquí estudiadas fueron *Alphestes afer, Cephalopholis fulva, Dermatolepis inermis, Epinephelus adscensionis, E. itajara, E. morio, E. mystacinus, Mycteroperca acutirostris, M. bonaci, M. interstitialis, M. marginata, M. venenosa y Rypticus saponaceus* de las cuales cuatro constituyen las más pescadas en el Océano Atlántico. Fueron encontrados patrones diferenciables de PCR-RFLPs para todas las especies y, además, fue posible detectar SNPs adicionales entre las cuatro especies más explotadas. Los procedimientos aquí empleados fueron muy eficaces por lo que sugerimos su aplicabilidad a otros grupos de peces como medida de control de la captura y comercialización ilegal a nivel mundial, particularmente en aquellos casos en los que el fileteado y otras formas de procesamiento que alteran las características anatómicas y morfológicas impiden su identificación.

Palabras clave: verificación mediante ADN, meros, mero Goliat, PCR-RFLPs, SNPs, epinefélidos.

INTRODUCTION

Molecular DNA markers are now sufficiently sophisticated to recognize genetic variants of a species. The low cost of the techniques has resulted in a recent increase in the number of studies using this approach. For example, one of the simplest, lowest-cost methods uses polymerase chain reaction–restriction fragment length polymorphisms (PCR-RFLPs) (Schlötterer 2004).

PCR-RFLP markers are commonly used and highly repeatable (Meyer *et al*. 1995) for the identification of a variety of species in several phyla (Wolf *et al.* 1999, McDonald *et al*. 2005, Torres 2006, Santaclara *et al*. 2007, Spergser and Rosengarten 2007, Fernandez-Tajes and Méndez 2007, Sowmya *et al*. 2007, Kang *et al.* 2008). Although DNA sequencing and analysis is accurate and authentic, it is costly, time-consuming and not suitable for routine species identification studies. PCR-RFLP has been proven to be a practical, simple and rapid technique (Meyer *et al*. 1995, Partis *et al*. 2000) and a high level of expertise in molecular genetics is not necessary for interpreting results obtained in agarose gels. The technique allows mislabeled or fraudulent fish products to be detected easily (Hsieh *et al*. 2010). Additionally, this technique has recently been used as a forensic tool for solving crimes ("forensic genetics", Butler 2005, Jobling and Gill 2004). For example, it has been used to determine the origin of cattle meat (Verkaar *et al*. 2002) and illegal wildlife trading (Dubey *et al*. 2010).

Because of the abundance of mutations in the genomes of many species (Morin *et al*. 2004), single nucleotide polymorphisms (SNPs) are very effective markers for resolving similar issues. Using SNPs has become one of the most recent innovations for ecological and conservation management, especially that of the fishery industry (Morin *et al*. 2004, Hauser and Seeb 2008). Technological advances in fisheries have resulted in an industrial level of fishing with a much greater and widespread impact. As a consequence, regulation is required to control the exploitation of fish and prevent depletion of stocks (Pauly *et al*. 2002). Following the worldwide trend for marine fisheries (Pauly *et al*. 2005, Pauly 2009), most grouper fisheries are sharply declining, collapsing or already depleted (Morris *et al*. 2000, Frédou and Ferreira 2005, Mitcheson *et al*. 2012).

As a management tool, accurate identification of these species based on molecular authentication (e.g. DNA barcoding) is very important because species may be identified from tissue samples in the absence of morphological characters (Frézal and Leblois 2008). In addition, several molecular techniques have been used to distinguish between legal and illegal products, to relocate animals for their natural populations, and to mark and track DNA profiles (Alacs *et al*. 2012). Often, fish fillets are mislabeled as different species for marketing purposes or to disguise illegal capture and retail (Jacquet and Pauly 2008, Miller and Mariani 2010, Carvalho *et al*. 2011a, b). DNA authentication through DNA barcoding has applications in ecology, medicine, epidemiology, evolutionary biology, biogeography and conservation biology (Frézal and Leblois 2008, Alacs *et al*. 2012), such as the use of cytochrome C oxidase for species delimitation and fishing authentication (Hebert *et al*. 2003, 2004a, Sass *et al*. 2007, Hajibabaei *et al*. 2007, Linacre and Tobe 2011, Carvalho *et al*. 2011b).

The grouper family (Epinephelidae) comprises 62 genera and 449 species (Heemstra and Randall 1993, Nelson 2006), many of which are exploited throughout the world (Morris *et al*. 2000, Mitcheson, *et al*. 2008, Mitcheson *et al*. 2012) and comprise some of the most important fishery resources in the tropical west Atlantic (Heemstra and Randall 1993, Claro *et al*. 2001). Due to over-fishing, the goliath grouper (*Epinephelus itajara*) is critically endangered and consequently its capture is prohibited in several countries, including Brazil (IBAMA 2007, IUCN 2008). Also, because of its slow development in brackish waters, it requires many years to recover (Sadovy and Eklund 1999, Frias-Torres 2007). Traditional knowledge about the ecology and habits of the grouper among local, small-scale fishermen, has been very important for the conservation of the goliath grouper in Brazil (Hostim-Silva *et al*. 2005, Gerhardinger *et al*. 2009). Other species of grouper, including the dusky (formerly *Mycteroperca marginata sensu* Craig and Hastings 2007), red (*Epinephelus morio*) and black (*M. bonaci*), are also exploited, yet their conservation receives less attention worldwide (Gimenez-Hurtado *et al*. 2005, Machado *et al*. 2003, Teixeira *et al*. 2004, Gerhardinger *et al*. 2006, Freitas *et al*. 2011). Therefore, they may suffer even greater extinction risks.

Taxonomic identification of fish is a difficult task because the way in which the fish are processed leads to a lack of diagnostic morphological characters. This favours the illegal trade of prohibited species because the fish can be sold under the name of similar, legally caught fish (Wong and Hanner 2008). In the case of *E. itajara* in Brazil, knowledge about the fish ban is widespread, so illegal traders also use filleting to trade it mislabeled as other species of grouper. In these cases, environmental law enforcement is often hampered by absence of proof. Thus, DNA-based procedures are valuable tools for law enforcement since they can attest the species in which is marketed. We adopted PCR-RFLP because it is less time-consuming and more cost-effective than DNA sequencing. Such methodology also requires equipment readily available in most molecular laboratories, and it has proven its utility in species identification (Palo and Merila 2003, Rohilla and Tiwari 2008, Dubey *et al*. 2010). Here, we examined the variability of the barcode region (*COI* mtDNA gene) in groupers to develop DNA authentication tools (PCR-RFLPs and SNPs), specifically the genetic signatures of the 13 commercially important

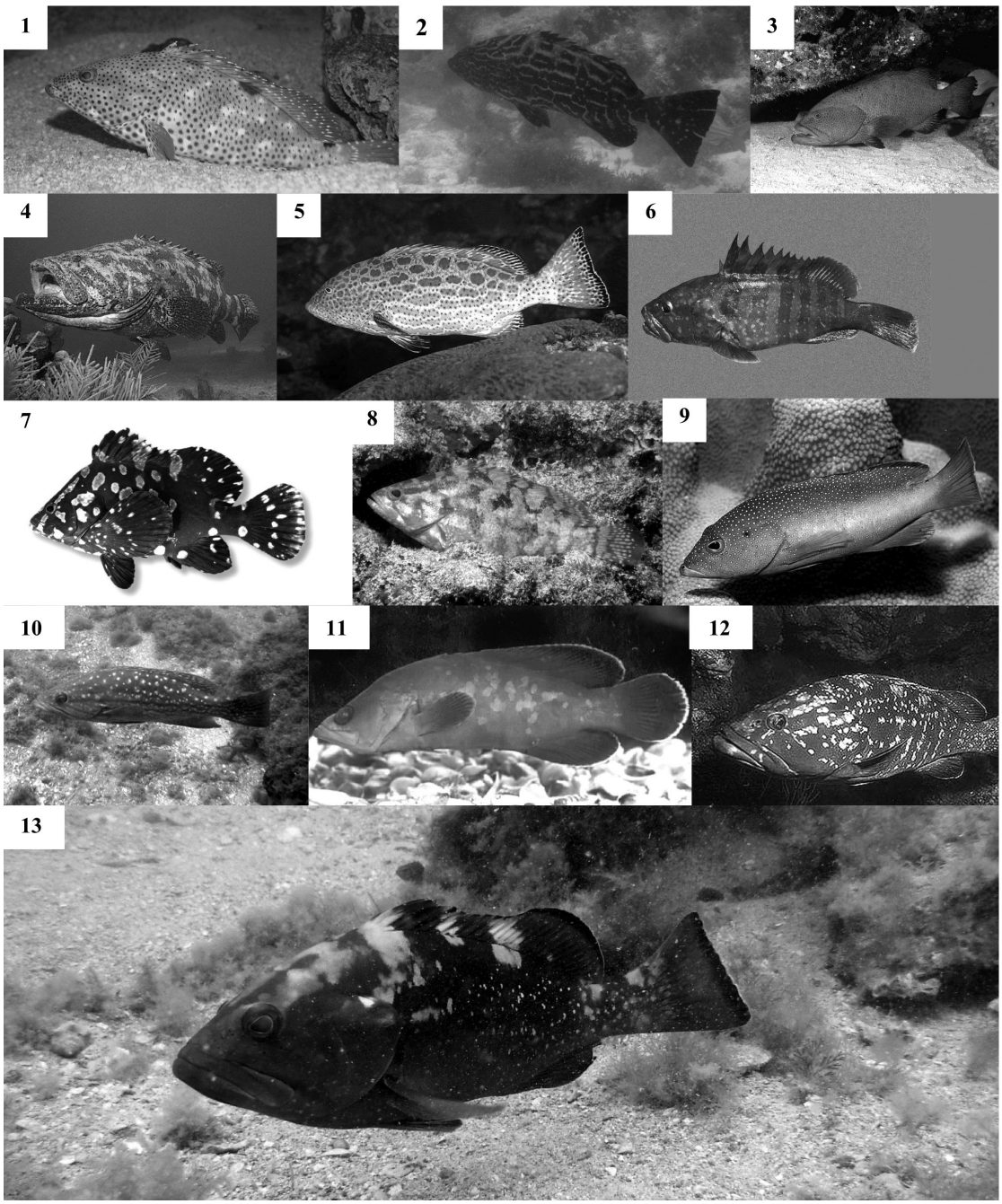

FIG. 1. – The 13 species of grouper in this study: *Epinephelus adscensionis* (1), *Mycteroperca bonaci* (2), *Mycteroperca interstitialis* (3), *Epinephelus itajara* (4), *Mycteroperca venenosa* (5), *Epinephelus mystacinus* (6), *Dermatolepis inermis* (7), *Alphestes afer* (8), *Cephalopholis fulva* (9), *Mycteroperca acutirostris* (10), *Rypticus saponaceus* (11), *Mycteroperca marginata* (12) and *Epinephelus morio* (13); Numbers 1-13 indicate species-specific PCR-RFLP profiles in Table 2.

species, especially the goliath (*Epinephelus itajara*), red (*E. morio*), dusky (*Mycteroperca marginata*) and black (*M. bonaci*) groupers.

MATERIALS AND METHODS

Thirteen valid species of southern Atlantic grouper were collected (*Epinephelus adscensionis*, *E. itajara*, *E. mystacinus*, *E. morio*, *Mycteroperca bonaci*, *M. interstitialis*, *M. venenosa*, *M. acutirostris*, *M. marginata*, *Dermatolepis inermis*, *Alphestes afer*, *Cephalopholis fulva, Rypticus saponaceus*) (Fig. 1). All the specimens were identified by keys following Heemstra and Randall (1993). The specimens were purchased from artisanal fishermen and, because of the taxonomic validity and rarity of the species, one specimen from each species was analysed. While intra-species variation is substantially less than inter-species variation, allowing

accurate identification among species levels (Ward *et al*. 2008), COI variations were not considered. In addition, negligible nucleotide variation is expected in the barcode region between several geographically different marine fish populations (F_{ST}<0.01/p>0.24; Ward *et al*. 2008).

We collected fin, liver and muscle samples that were stored in 96% ethanol (Merck) at –20°C. DNA extraction through the DNEasy Blood and Tissue kit (QIAGEN) followed the instructions of the manufacturer. Templates were dissolved in Tris+EDTA (pH 8.0) and diluted at 1:20 for PCR.

PCR-RFLPs

PCR was carried out in a final volume of 25 μL containing 2.5 μL of 10× PCR buffer, 1.25 μL of $MgCl_2$ (50 mM), 0.5 μL of dNTP mix (10 mM), 0.2 μL of Taq polymerase (1 U), 0.26 μL of each primer (forward FishF1 5' TCAACCAACCACAAAGACATTGGCAC 3' and reverse FishR1 5' TAGACTTCTGGGTGGCCAAAGAATCA 3'), 2.0 μL of DNA template and 18.0 μL of ultrapure water. For *Mycteroperca bonaci, M. interstitialis* and *Epinephelus itajara*, FishR2 (5'ACTTCAGGGTGACCGAAGAATCAGAA 3') was used as the reverse primer (Ward 2005). Reactions had an initial step of 2 min at 95°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 54°C and 1 min at 72°C, with a final extension step of 10 min at 72°C.

COI RFLP profiles were obtained through reaction with a final volume of 20 μL containing 5-10 μL of the PCR products, 20 μL of the 10× buffer from each enzyme (as indicated by the manufacturer), 10 units of each enzyme and ultrapure water to reach the final volume (Table 1). When necessary, 0.2 μL of 100× bovine serum albumin was used.

RFLP reactions occurred in 90 min at the temperatures recommended by the manufacturer of each enzyme. Resultant products were resolved by electrophoresis in 1.5% agarose gel immersed in TBE buffer, stained with GelGreen™ (Biotium), and then photographed. The molecular weight of the fragments was estimated using a 100-bp molecular marker ladder (New England Biolabs). Few different reagent brands were tested and no different RFLP profiles were detected.

Sequencing and SNP detection

PCR fragments of ~700 bp of COI from *Mycteroperca bonaci*, *M. marginata*, *Epinephelus morio* and *E. itajara* were amplified using the primers FishF1 and FishR1 (Ward *et al*. 2005). The 25-μL PCR mixes comprised 19.5 μL of ultrapure water, 2.5 μL of PHT IB, 10X PCR buffer (2.5 mM $MgCl_2$), 0.35 μL of each primer (10 mM), 2.5 μL of dNTP (1 mM), 0.25 μL of PHT Taq polymerase (5U/μl) and 1.0 μL of DNA template (50-100 ng/μL). Purified PCR products (1-2 μl) were sequenced bi-directionally using the BigDye Terminator v.3.1 Cycle Sequencing Kit (www.appliedbiosystems.com), following the manufacturer's instructions. Reactions were analysed in an automated DNA sequencer (ABI 310, Applied Biosystems). The obtained sequences were deposited in Genbank under ascension numbers (JF421452-455). Sequences were then aligned with BioEdit version 5.0.9 (Hall 1999) using ClustalW multiple alignment, with gap opening at 15 and gap extension costs at 0.3 (Hall 2001). Ambiguous extremities of the sequences were trimmed after alignment. Sequence differences were measured by the Kimura 2-parameter (K2P) model using Paup* v.4.0b10 through its graphic interface PaupUp (Calendini and Martin 2005). Detection of SNPs was performed visually, with special attention to the detection of multiple sites capable of differentiating taxa within genera and between genera and species.

TABLE 1. – The list of the enzymes used in this study, their restriction sites (*), and their temperatures of use.

Enzymes	Restriction sites	Temperature (°C)/time
Alu I	AG * CT	37/1 hour
Bam HI	G * GATCC	37/1 hour
Bts CI	GGATGNN *	50/1 hour
Eco RI	G * AATTC	37/1 hour
Hae III	GG * CC	37/1 hour
Hha I	GC * GC	37/1 hour
Mbo I	GATC *	37/1 hour
Taq$^{\alpha}$ I	T * CGA	65/1 hour

RESULTS

PCR-RFLPs

COI for all species was successfully amplified using the primers FishF1 and FishR, yielding ~700 bp of PCR products (Fig. 2a). In *Mycteroperca bonaci, M. interstitialis* and *Epinephelus itajara* the reverse primer was substituted with FishR2 (5'ACTTCAGGGTGACCGAAGAATCAGAA3'; Ward *et al*. 2005) because it provided better results (Fig. 2b).

Apart from BamHI and EcoRI (data not shown), other enzymes yielded diagnosable RFLP profiles (Figs 3-5). A single undigested PCR fragment (undigested control lane) was observed with BamHI. Experiments with EcoRI also revealed a single band in all species that was slightly smaller than the undigested control lane. The remaining enzymes allowed different unique or shared PCR-RFLP profiles to be identified. For example, AluI resulted in ten species-linked profiles (Fig. 3, lanes 1-5, 9, 11 and 12) and two shared profiles (Fig. 3, lanes 6-7 and 8-10). Despite lower resolution, enzymes MboI and BtsCI allowed eight PCR-RFLP profiles to be identified (Fig. 4a, b). HaeIII, HhaI and Taq$^{\alpha}$I gave seven, six and five profiles, respectively (Fig. 5a, b, c).

Restriction experiments with AluI yielded two bands in *E. itajara* of ~200 and 220 bp (Fig. 3, Table 2). The BtsCI experiment yielded two bands of ~300 and 400 bp each. The HaeIII experiments yielded a

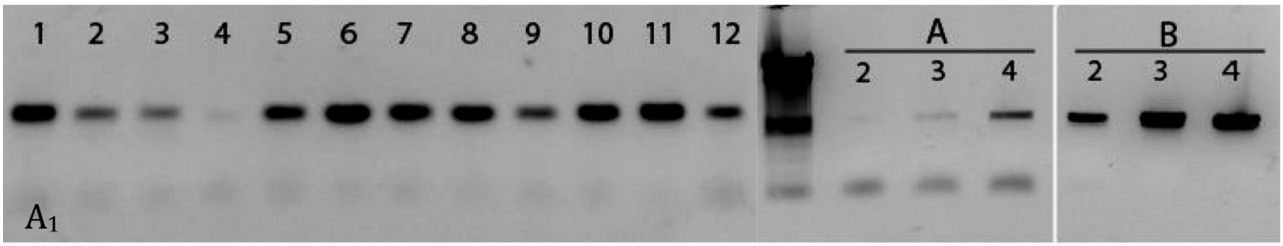

Fig. 2. – Results of amplification of the barcode regions: (A) primary PCR products (~700 bp) from 12 grouper species using primers F1 and R1; (A_1) weak PCR products obtained with primers F1 and R2 for (2) *Mycteroperca bonaci,* (3) *M. interstitialis* and (4) *Epinephelus itajara* (note similar profiles in lanes 2, 3 and 4); (B) optimized PCR reactions for species using primers F1 and R2 (note three clear bands of COI for (2) *Mycteroperca bonaci,* (3) *M. interstitialis* and (4) *Epinephelus itajara*); numbers 1-12 indicate species as listed in Figure 1.

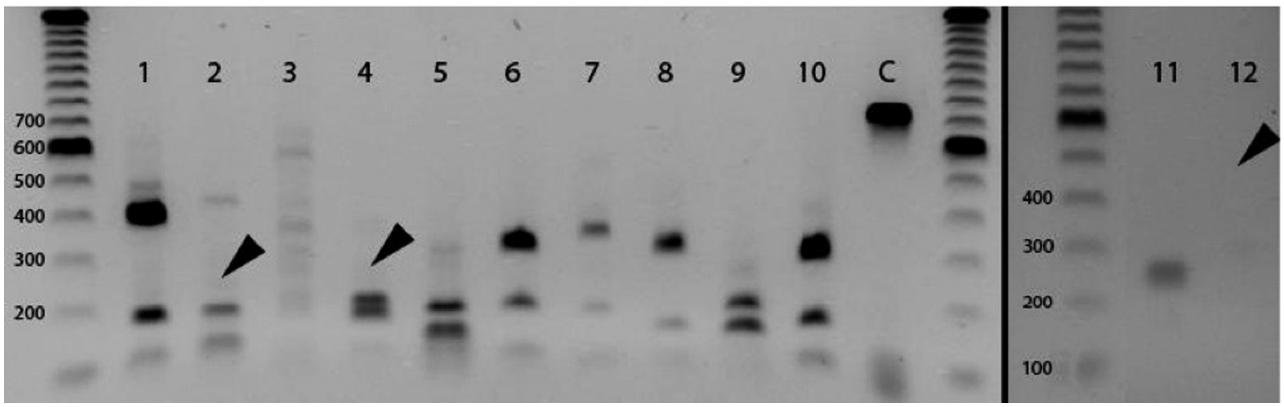

Fig. 3. – PCR-RFLPs by Alu I; Numbers 1-12 indicate species in Figure 1; "C" is undigested control lane; arrows indicate species profiles: *Epinephelus itajara* (4), *Mycteroperca bonaci* (2) and *M. marginata* (12) (note other unique and shared profiles).

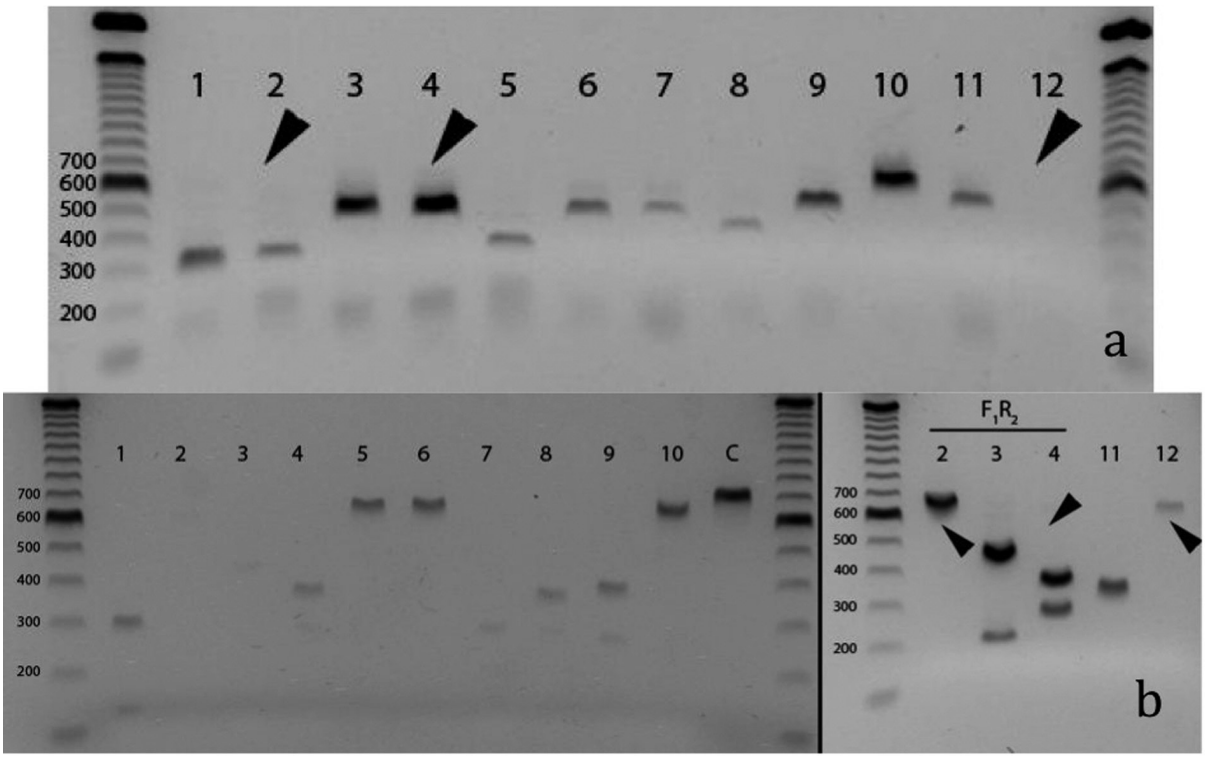

Fig. 4. – PCR-RFLPs with MboI (a) and BstCl (b); 2 and 4 indicate *Mycteroperca bonaci* and *Epinephelus itajara* respectively. Note (a), shared profile with others, except *M. marginata*, without bands; in (b), 2 and 4 shared RFLP profiles with other species, while *Mycteroperca marginata* without bands; on right, clear pattern of *M. bonaci* (2), *M. interstitialis* (3) and *E. itajara* (4) and shared profile of *Mycteroperca marginata* (12).

Table 2. – PCR-RFLP profiles (in base pairs of DNA fragments) of the barcode region of the groupers in this study (species numbers as in Fig. 1).

	Species number											
	1	2	3	4	5	6	7	8	9	10	11	12
Alu I	400 200	180	250 200 150	220 200	210 180	350 220	380 220	350 200	210 180	330 200 120	250	
Bam HI	700	700	700	700	700	700	700	700	700	700	700	700
Bts CI	300 150	650	450 250	400 300	650	650	300	380 300	400 270	650	350	650
Eco RI	600	600	600	600	600	600	600	600	600	600	600	600
Hae III	400 300	550	600	650	350 280	600	300	350 280	600	600	550	600
Hha I	700	700	700	500 300	700	700	550	700	650	300 250	450	400 300
Mbo I	350	350 250	500 200	500 200	400 200	500 200	500 200	450 200	550	600	550	
Taq$^{\alpha}$ I	400 270	450 250	450 250	450 250	450 250	450 200	600	600	450 250	450 200	600	400

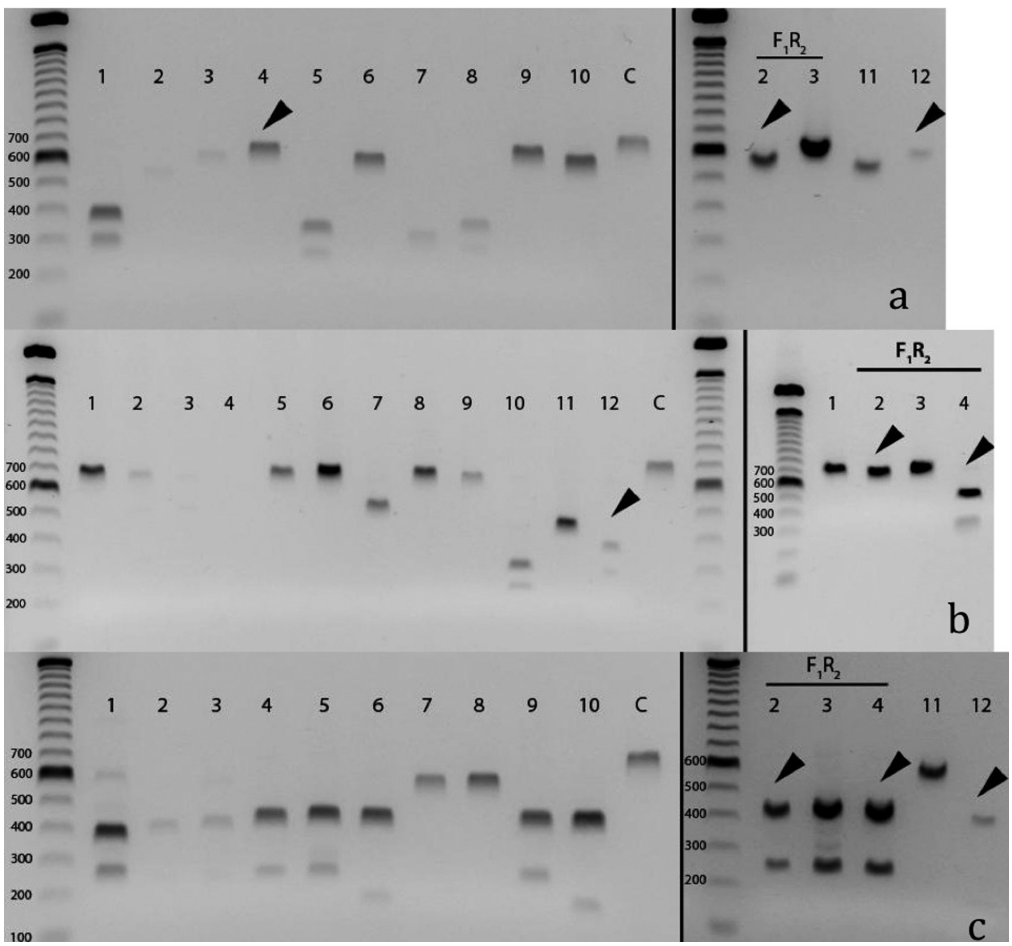

Fig. 5. – PCR-RFLPs with HaeIII (a), HhaI (b), and Taqꟾ (c). Numbers are species in Figure 1; "C" is COI fragment without enzyme digestion; arrows indicate *Mycteroperca bonaci* (2), *Epinephelus itajara* (4) and *Mycteroperca marginata* (12); on right, optimized PCR reactions for species with F1 and R2. Note in (a) slightly different COI genetic profiles; in (b) clearly different COI genetic profiles and in (c) genetically unique *Mycteroperca marginata.*

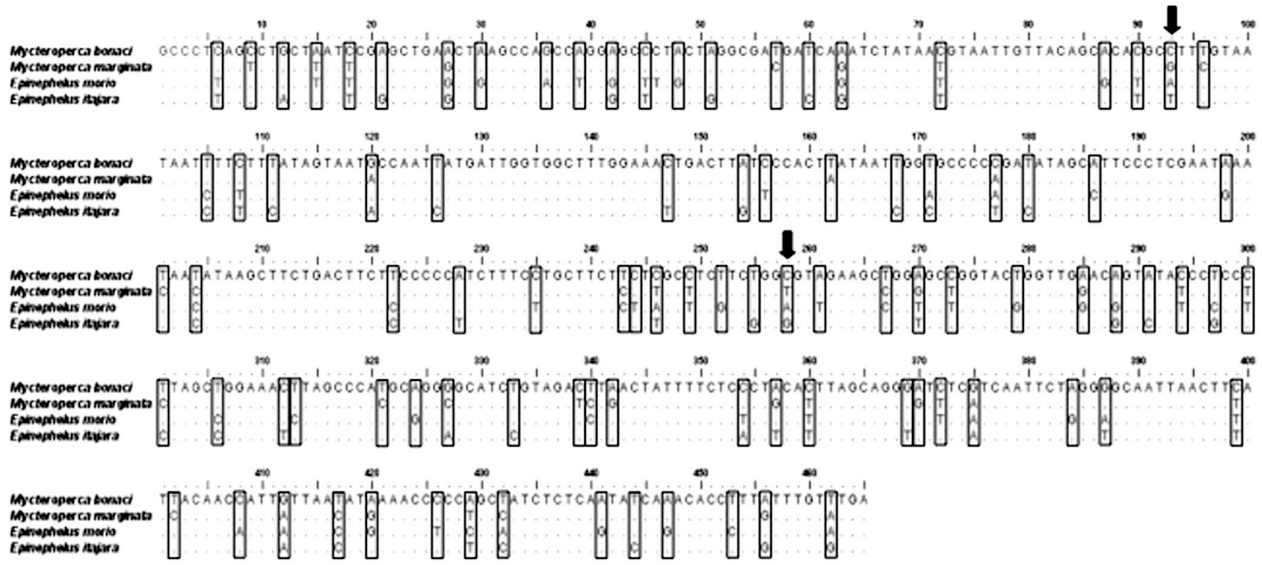

FIG. 6. – Aligned sequences from *Mycteroperca bonaci*, *M. marginata*, *Epinephelus morio* and *E. itajara*. Boxes indicate the SNP positions among the analyzed species; arrows indicate two SNP positions that uniquely identify all species.

TABLE 3. – Observed K2P COI differences (%) among the four groupers analysed.

	1	2	3	4
1 *Mycteroperca bonaci*	-			
2 *Mycteroperca marginata*	10.3	-		
3 *Epinephelus itajara*	12.6	13.9	-	
4 *Epinephelus morio*	15.5	13.4	16	-

single band of 650 bp. Two bands each resulted from enzymes MboI (200 and 500 bp) and Taq$^{\alpha}$I (250 and 450 bp; Figs 4a, 5c; Table 2).

Genetic profiles had lower resolution in *M. marginata*. Enzymes AluI and MboI gave no visible bands (Figs 3 and 4a, Table 2). The restriction experiments with BtsCI, EcoRI, HaeIII and Taq$^{\alpha}$I each yielded a single band (650, 600, 600 and 400 bp, respectively; Figs 3b, 5b, 6a, c, Table 2). Experiments with HhaI revealed two distinguishable bands (400 and 300 bp; Fig. 5b, Table 2).

In *M. bonaci,* the restriction experiments with enzymes AluI, BtsCI, HaeIII, and HhaI each revealed a single band (180, 650, 550 and 700 bp, respectively; Figs 3, 4b, 5a, b, Table 2). Two bands each resulted from enzymes MboI (250 and 350 bp) and Taq$^{\alpha}$I (250 and 450 pb; Figs 4a, 5c, Table 2).

TABLE 4. – SNP positions for species and genera identification. The numbers indicate sites in the alignment (Fig. 7).

Species	Identify species	Identify genera	Shared between both genera
Mycteroperca bonaci	18, 72, 93, 204, 246, 258, 270, 360, 375, 399, 412, 417, 429, 432, 462.	9, 15, 18, 57, 63, 72, 93, 96, 162, 177, 201, 204, 243, 246, 249, 258, 267, 270, 273, 285, 294, 300, 301, 321, 327, 339, 340, 342, 357, 360, 370, 372, 375, 399, 402, 412, 420, 429, 432, 456, 462.	
Mycteroperca marginata	9, 57, 93, 96, 162, 201, 258, 270, 321, 327, 339, 342, 357, 370, 402.		
Epinephelus morio	30, 36, 39, 46, 47, 87, 93, 156, 171, 186, 198, 235, 244, 246, 252, 258, 261, 279, 297, 313, 324, 354, 384, 387, 408, 429, 432, 441, 447.	12, 15, 21, 30, 36, 39, 46, 48, 51, 60, 87, 93, 111, 120, 126, 147, 154, 156, 168, 171, 177, 180, 186, 198, 228, 235, 244, 246, 252, 255, 258, 267, 273, 279, 285, 291, 294, 297, 300, 301, 312, 313, 324, 327, 333, 340, 354, 357, 369, 372, 384, 387, 408, 420, 426, 429, 432, 441, 444, 447, 453, 456, 462.	
Epinephelus itajara	12, 21, 51, 60, 93, 126, 111, 147, 154, 168, 171, 177, 180, 228, 255, 258, 291, 297, 312, 327, 333, 354, 357, 369, 387, 444, 462.		
Mycteroperca - Epinephelus			6, 42, 45, 90, 105, 108, 222, 288, 306,

Sequencing and SNPs

An edited/aligned block comprising 465 homologous sites was obtained after sequence editing and alignment (Fig. 6) in which no insertions, deletions or stop codons were observed. This last observation prevented the use of nuclear *COI* pseudogenes (nuclear mitochondrial sequences – NUMTs; Song *et al*. 2008). Among the sequences the percentage differences (K2P) ranged from 10.3 to 16 (Table 3) and 94 SNPs were useful for diagnosis of within- and between-genera and among-species comparisons (Fig. 6; gray boxes). Of these sites, autapomorphic SNPs support clear identification for several species: 15 identify *M. bonaci* (black grouper; Table 4), 15 identify *M. marginata* (dusky grouper; Table 4), 29 identify *E. morio* (red grouper; Table 4), and 27 identify *E. itajara* (goliath grouper; Table 4). A total of 41 SNPs supported differentiations between *Mycteroperca* spp. (Table 4), 63 supported differentiations between *Epinephelus* spp. (Table 4), two simultaneously supported among-species identification (Fig. 6; black arrows), and nine supported among-genera identification (*Mycteroperca* × *Epinephelus*) (Table 4).

DISCUSSION

PCR-RFLPs

Our PCR-RFLP analyses allowed us to correctly identify 12 species of grouper, including the endangered goliath grouper, using individual or combined genetic profiles obtained with a few restriction enzymes (Table 2, Figs 3, 4a, b, 5a-c). Thus, our results provide useful DNA authentication tools for identifying fishing of goliath (*Epinephelus itajara*), dusky (*Mycteroperca marginata*), and black (*Mycteroperca bonaci*) groupers without morphological characters in hand, as in fish markets. Considering the extreme risk of extinction of the analysed species (Mitcheson *et al*. 2008, Mitcheson *et al*. 2012), our finding are very welcome for grouper fishery management. While laws protect endangered fish such as goliath grouper, these laws have been futile due to the lack of an effective means of identifying illegal catches. Thus, our results provide the resolution of this important problem, especially for the goliath, dusky (*M. marginata*) and black (*M. bonaci*) groupers.

With a properly amplified barcode segment for all species (Fig. 2), a small adjustment improved resolution for those three species. That is, the replacement of the reverse primer (Fig. 2) improved resolution by avoiding the formation of nonspecific PCR products (possibly from *COI*). Thus, F1 and R1/R2 primers are specific for *COI* amplifications, and homology was shown by identical molecular weight for these three species with F1 and R2 primers (Ward *et al*. 2005).

In considering the use of the eight enzymes in procedures for DNA authentication of fishing (PCR-RFLPs), the enzymes BamHI and EcoRI yielded very similar RFLP profiles with a single band for each of the 12 species (data not shown). It is possible that a priori these enzymes were not used because the terminal *COI* region in all 12 species may have been cut into tiny fragments of low molecular weight. Nonetheless, these enzymes may be effectively used to prove that a putative fish sample was not a grouper when more than a single band appears.

Clear authentication of illegal fishing of these fish can be resolved through the use of five enzymes. The goliath grouper had RFLP profiles shared by two of the six enzymes (MboI and Taq$^{\alpha}$I; Figs 3 and 5, respectively), yet had unique RFLP profiles for AluI, BtsCI and HhaI (Figs 3, 4b, 5b). Thus, these data show that a combination of these profiles will provide clear forensic evidence for the goliath grouper.

Similarly, the black grouper (*Mycteroperca bonaci*) had a RFLP profile shared with the goliath grouper in the enzyme Taq$^{\alpha}$I (Fig. 5c). With enzymes AluI and MboI, identification is much better, with a single band of ~180 bp (AluI) and two bands of ~250 and 350 pb (MboI, Figs 3 and 4a). Also, the dusky grouper (*M. marginata*) is identified by the combined RFLP profiles of AluI (Fig. 3), HhaI (Fig. 5b), and Taq$^{\alpha}$I (Fig. 5). By using the genetic signatures of each species (Table 2), the necessary steps of using a single or combined genetic profile are shown for DNA authentication of the black (*Mycteroperca bonaci*), dusky (*M. marginata*) and goliath (*E. itajara*), and other groupers.

SNPs

A total of 94 SNPs were observed for correct identification both among and within genera, and among species (Fig. 6). Previously, a minimum of 60 SNPs was suggested as necessary for good forensic/authentication diagnostics (Sobrino *et al*. 2005). The evidence provided herein (94 SNPs) indicate the robustness of the genetic features observed regarding a DNA authentication method. Traditional (as shown herein) and alternative barcoding regions have been used to examine the authentication and traceability of cattle meat (Fontanesi *et al*. 2010), wildlife (Sato *et al*. 2010, Ferreira *et al*. 2011) and fish (Baker and Palumbi 1994, Comi *et al*. 2005, Ogden 2008, Rasmussen and Morrissey 2008, Holmes *et al*. 2009, Ardura *et al*. 2010, Supernault *et al*. 2010, Carvalho *et al*. 2011a). Thus, correct species identification by SNP positions (Table 4) illustrates that the current barcode regions are very useful for fishery management. A clear example is the red grouper (*Epinephelus morio*), which has 29 autapomorphic SNPs (Table 4, Fig. 6) and which now has an important tool to prevent its exploitation (Morris *et al*. 2000, Sadovy 2001, Olavo *et al*. 2005, Freitas *et al*. 2011).

In addition, the barcode distinctiveness observed between the two *Mycteroperca* and *Epinephelus* spp. (Table 3) indicates the efficiency of the presented protocol. According to Ward *et al*. (2009) and Carvalho

et al. (2011b), congeneric fish species are genetically (*COI*) different at a minimum rate of 8.4%. Thus, the minimum *COI* difference of 10.3% (*Mycteroperca marginata* X *M. bonaci*, Table 3) seems to be high enough to state that the presented SNP-based protocol is an accurate DNA authentication procedure. In practical terms, sites 93 and 258 (Fig. 6, arrows) support this statement because they allow the simultaneous identification of those four overexploited and legally protected species as *E. itajara* by presenting a different nucleotide in each of the species.

In summary, we developed and tested the DNA barcoding as an effective tool for monitoring fisheries and illegal trade in groupers, including the endangered goliath grouper.

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