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Sparid species discrimination by COIBar-RFLP in commercial products

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ABSTRACT

Processed seafood products are subject to fraudulent species substitution practices that pose problems not only economically but also for human health due to the risk of allergies. DNA-based molecular techniques offer an undoubted contribution to unraveling commercial fraud in processed seafood products, and several investigations have been conducted to develop specific and rapid assays for the identification of fish species. In this context, we hypothesize that the Cytochrome Oxidase I (COI) Barcode-Restriction Fragment Length Polymorphisms (COIBar-RFLP) strategy may be a useful molecular tool to quickly assess the authenticity of seafood products because (i) it takes advantage of the specific discriminatory power of the COI gene as a barcode and of the robust Restriction Fragment Length Polymorphism methodology and (ii) the interspecific variation in the digestion pattern obtained using the restriction enzymes allows bypassing the gene sequencing step. The *Sparidae* family includes species of high commercial value and many sparid species are difficult to recognize considering only their morphological features; thus, misidentifications are frequent. The aim of this work was to identify sparid species in processed products using the COIBar-RFLP strategy with the *MspI* restriction enzyme which yielded differential digestion patterns and unveiled two cases of species substitution. The proposed methodology could be used in food control laboratories to combat the widespread habit of fraudulent species substitution in the fishing industry.

1. INTRODUCTION

Recent investigations suggest that the illegal practice of species substitution and mislabeling is the most common fraud worldwide detected in the fish trade [1,2]. In the fish industry, intentional fraud is generally motivated by economic gain based on the substitution of one valuable species for another of lower value. Other motivations have also been highlighted such as the need to sell illegally caught species by hiding them under a specific false name on the label [1]. However, this type of fraud is feasible if the commercial product has undergone processing that has removed the morphological characteristics useful to recognize the species declared on the label. On the other hand, unintentional commercial fraud may be due to the difficulty of distinguishing species morphologically. An overview of fish fraud extracted from four databases (the European Union's Rapid Alert System for Food and Feed, the Food Fraud Database, HorizonScan, and Nexis) was recently published [3]. Interestingly, the authors found that the scientific literature showed a higher level of species substitution than those found in the questioned databases. This result could indicate both a low level of attention by regulatory bodies to this important issue facing the seafood industry, but also the urgency of using, common guidelines to address the problem along the seafood production chain in a global market. In this regard, the need for greater coordination between research and policy actions has recently been claimed with the aim of minimizing mislabeling, decreasing its negative impacts, and improving transparency [4].

Polymerase chain reaction (PCR) is the most modern practical technology in diagnosing and compared with classical techniques [5,6]. It has been shown to be more rapid, with results obtained in a few hours, and also more reliable [7]. Moreover, PCR allows a faster identification directly from samples [8]. Genotyping, which is based on a more stable marker, DNA, is not dependent on gene expression [6,9]. In this context, DNA-based molecular techniques and in particular DNA barcoding offer an undoubted contribution to unraveling the fraudulent practice of species substitution in processed fish products [2]. More specifically, the standard region of approximately 650 bp of mitochondrial cytochrome oxidase I (COI) has been proposed as a reliable barcode gene for species discrimination [10] and could currently be considered the best barcode for animals. COI shows high interspecific and low intraspecific divergence (barcoding gap) and a very large number of sequences are available in public databases (that is, the Barcode of Life Database or BOLD, and GenBank accessible at the National Center for Biotechnology Information or NCBI). On the other hand, besides being based on reliable molecular markers, a method for the

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Table 1: Reference sparid species identified on morphological characters.

Sample Code	Scientific name	Common name	GenBank Accession Number	Matched GenBank Accession from BLAST	% identity with 100% coverage
S1	*Diplodus sargus*	*white seabream*	OQ353062	LC203132	99.10
S2	*Diplodus vulgaris*	*common two-banded seabream*	OQ353063	LC195195	98.92
S3	*Pagellus acarne*	*axillary seabream*	OQ353065	KJ012382	99.24
S4	*Pagellus erythrinus*	*common pandora*	OQ353066	KX586210	99.54
S5	*Pagrus pagrus*	*red porgy*	OQ353067	KJ012417	99.85
S6	*Spondyliosoma cantharus*	*black seabream*	OQ353069	KJ012436	99.54

Table 2: Sparidae sampling in Sicilian fish market. In bold, misdescription cases.

Code	Retail point	Retail format	Label Description	Scientific name of declared species	Identified species by DNA barcoding and BLAST search	GenBank Accession Number	Matched GenBank Accession from BLAST	% identity with 100% coverage
X1	Street fish market	whole	*white seabream*	*D. sargus*	*S. cantharus*	OR268681	KJ012436	99.39
X3	Street fish market	fillet	*common pandora*	*P. erythrinus*	*P. pagrus*	OR268682	OQ865598	99.54
X4	supermarket	fillet	*common pandora*	*P. erythrinus*	*P. erythrinus*	OR268683	KX586210	99.23
X5	fish market	fillet	*red porgy*	*P. pagrus*	*P. pagrus*	OR268684	OQ865598	99.69
X6	supermarket	whole	*red porgy*	*P. pagrus*	*P. pagrus*	OQ359508	KJ012417	99.85
X7	supermarket	fillet	*white seabream*	*D. sargus*	*D. sargus*	OR268685	LC203132	98.80
X8	Street fish market	whole	*common pandora*	*P. erythrinus*	*P. pagrus*	OQ359510	KJ012417	99.54
X9	Street fish market	whole	*white seabream*	*D. sargus*	*D. sargus*	OQ359511	LC203131	99.85
X10	Street fish market	fillet	*axillary seabream*	*P. acarne*	*P. acarne*	OR268686	KJ012382	98.93
X12	Street fish market	whole	*common pandora*	*P. erythrinus*	*P. erythrinus*	OQ359514	KM538475	98.78
X13	Street fish market	whole	*white seabream*	*D. sargus*	*S. cantharus*	OQ359515	K5J012439	99.54
X14	fish market	fillet	*red porgy*	*P. pagrus*	*P. pagrus*	OR268687	OQ865598	99.54
X15	Street fish market	whole	*common two-banded seabream*	*D. vulgaris*	*D. vulgaris*	OQ359517	LC195196	99.69
X16	Street fish market	fillet	*common two-banded seabream*	*D. vulgaris*	*D. vulgaris*	OR268688	LC203527	99.06
X17	fish market	whole	*red porgy*	*P. pagrus*	*P. pagrus*	OQ359519	KJ012417	99.24
X18	fish market	whole	*common pandora*	*P. erythrinus*	*P. erythrinus*	OQ359520	KX586210	99.39
X19	supermarket	fillet	*white seabream*	*D. sargus*	*D. sargus*	OR268689	LC203132	98.80
X21	Street fish market	whole	*white seabream*	*D. sargus*	*S. cantharus*	OR268690	KJ012436	99.39
X24	Street fish market	Fillet	*axillary seabream*	*P. acarne*	*P. acarne*	OR268691	KJ012382	98.71
X26	Street fish market	Fillet	*axillary seabream*	*P. acarne*	*P. acarne*	OQ359528	KM538461	99.69
X29	fish market	fillet	*red porgy*	*P. pagrus*	*P. pagrus*	OQ359531	KF461214	99.24

authentication of processed products should also be easy to apply, quick, inexpensive, and easy to use in food control laboratories. Interestingly, the PCR-RFLP (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) method is based on the amplification of a DNA region and the subsequent digestion of the amplicon by restriction endonucleases that recognize restriction sites and cut the DNA sequence into fragments of different lengths [11,12]. The species-specific pattern of DNA fragments obtained has been widely used and validated for the genetic authentication of species in both fish and meat products using various nuclear and mitochondrial genes [13-18]. In particular, PCR-RFLP of the COI gene has been successfully used to identify tuna species in raw and cooked tuna products [13] and recently for the recognition and discrimination of 9 of 25 meat species [17]. However, it should be noted that in the studies just mentioned, at least two restriction enzymes are used simultaneously to increase the specific discriminatory power of the methodology. Therefore, the main advantage of PCR-RFLP, which is considered to be a cheap and fast method, is called into question.

Table 3: List of the enzymes used in this study, their restriction sites (*), and their temperatures of use.

Enzymes	Restriction sites	Temperature (°C)/time
HinfI	G*ANT*C	37°C/1 h
AluI	AG*CT	37°C/1 h
MboI	*GATC	37°C/1 h
MspI	C*CGG	37°C/1 h

Table 4: Fragments greater than 100bp produced by the digestion of the selected enzymes (lengths in base pair) for each of the investigated species in this study.

	Diplodus sargus	*Diplodus vulgaris*	*Pagellus acarne*	*Pagellus erythrinus*	*Spondilosoma cantharus*	*Pagrus pagrus*
*Hinf*I	376; 258	ND	564	ND	564	303; 282
*Alu*I	252; 195; 165	187; 165; 158	244; 207; 159	252; 155; 144; 102	347; 187	182; 146; 102
*Mbo*I	376; 207	393; 170	244; 207; 204	421; 207	448; 207	484; 101
*Msp*I	270; 153; 107	270; 246	210	338; 228	246; 234	293; 228

* ND, not digested.

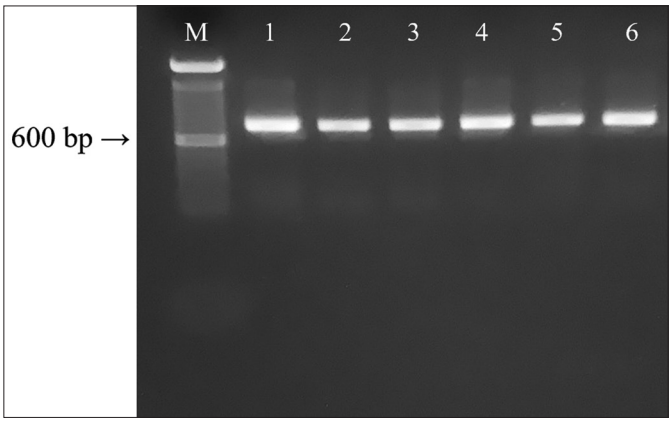

Figure 1: Amplification products of the species examined in this study. M= 100bp ladder. Lane 1 = *Diplodus sargus*, Lane 2 = *Diplodus vulgaris,* Lane 3 = *Pagellus acarne,* Lane 4 = *Pagellus erythrinus,* Lane 5 = *Spondyliosoma cantharus,* Lane 6 = *Pagrus pagrus.*

Among the teleosts affected by the species substitution fraud is the family *Sparidae* which comprises 39 genera and 164 species currently listed in the catalog of fishes [19] and includes commercially and ecologically very important fishes. The ecomorphology of several sparid species has been studied based on the correlation between feeding behavior and body morphology [20]. Indeed, body shape provides relevant information on the biology and ecology of species, and geometric morphometric methods have proven to be an efficient way to explore various biological traits in fish species [21-23]. In particular, through a geometric morphometric approach conducted on 92 sparid species, it was found that several morphological traits show similarity between distinct species with respect to a common feeding and habitat utilization strategy [24]. As a result, many sparid species are difficult to recognize based on their morphological features, which increase the likelihood and frequency of misidentifications. Furthermore, the presence of hybridization between sparid species has been noted [25], which further confuses morphological identification.

Therefore, due to (i) the increase in the marketing of sparid species over the past decade, (ii) the high selling price of the most valuable species, and (iii) the morphological similarity between species, sparids are often subject to intentional or unintentional substitution fraud. In this regard, several investigations have recently been conducted focusing on the search for specific molecular markers and the development of specific and rapid tests for the identification of sparid species [26-28]. However, COI-DNA barcoding is still confirmed as a reliable molecular methodology for the identification of fish species [29-33] and for sparids in particular [26]. In this context, the aim of the present study was to identify sparid species in processed products using the COIBar-RFLP strategy, which exploits the specific discriminatory power of the *COI* gene (DNA barcode) and of the interspecific variation of the restriction enzymes digestion pattern. For this purpose, a single restriction enzyme will be selected *in silico* to simultaneously discriminate the sparid species examined in this study.

2. MATERIALS AND METHODS

2.1. Samples and DNA Extraction

Twenty-seven fish specimens belonging to the *Sparidae* family were purchased from several local markets (fish market, street fish market, and supermarket) in Southern Italy in 2021/2022. Among them, six fresh whole samples of the most commonly sold sparids were identified by morphological inspection using analytical keys [34] and the detected species were confirmed by molecular approach. The specimens were then used as reference samples for the remaining 21 fish products labeled with the names of the six reference species [Table 1]. To verify the correctness of the specific name given on the label, these samples, 11 fillets and 10 whole specimens, were identified only through the molecular approach [Table 2]. Genomic DNA was isolated from muscle tissue (25–30 mg) following the extraction protocol of the DNeasy tissue kit (Qiagen, Hilden, Germany).

2.2. Barcode Amplification and Sequencing

The *COI* gene (~650 bp) was amplified using the universal primers VF2_t1 and FishR2_t1 [35]. A total of 25 µL of PCR amplification mix were prepared as described in [36], including negative controls in each run. All obtained amplicons were verified by 0.8% agarose gel electrophoresis and subsequently purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). Sequencing was performed by Eurofins Genomics using M13 forward and reverse primers [37]. All sequences were checked and edited using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and only those with a quality score above 20 were considered and deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) [Table 1]. The NCBI GenBank database was queried for the consensus sequences of each PCR product using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence similarity >98% and query coverage =100% were used as thresholds to identify species. ClustalX software [38] was used to align edited sequences. A *COI* reference library was constructed using six sparid species sequences obtained by us and six downloaded from GenBank [Table 1]. Subsequently, twenty-one unchecked (Xn) sequences were added to the reference *COI* sequences and a Maximum Likelihood (ML) tree was built by MEGA X software [39]. The bootstrap method was applied using 1000 non-parametric replicates [40].

2.3. *In silico* Restriction Analysis and COIBar-RFLP

COI reference sequences of each investigated species were aligned using MEGA X [39] and then were uploaded to NEBcutter 3.0.15 (https://nc3.neb.com/NEBcutter/) to choose the most suitable restriction enzyme. The enzymes *Hinf*I, *Alu*I, *Mbo*I, and *Msp*I (New England Biolabs, Inc.) were selected to perform COIBar-RFLP (Table 3). The fragments produced by the *in silico* digestion in each species are shown in Table 4. Only one enzyme was selected and used

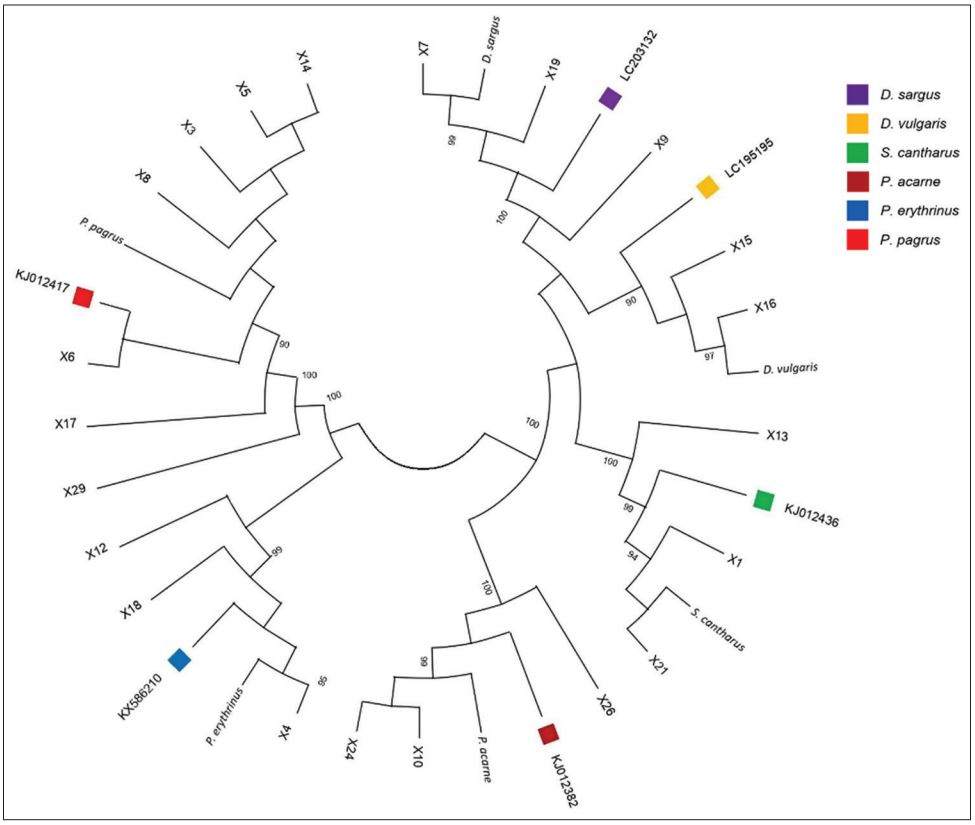

Figure 2: Circular maximum likelihood (ML) tree constructed using *COI* sequences from sparid specimens and those downloaded from GenBank for each targeted species. Only bootstrap values greater than 70% are showed.

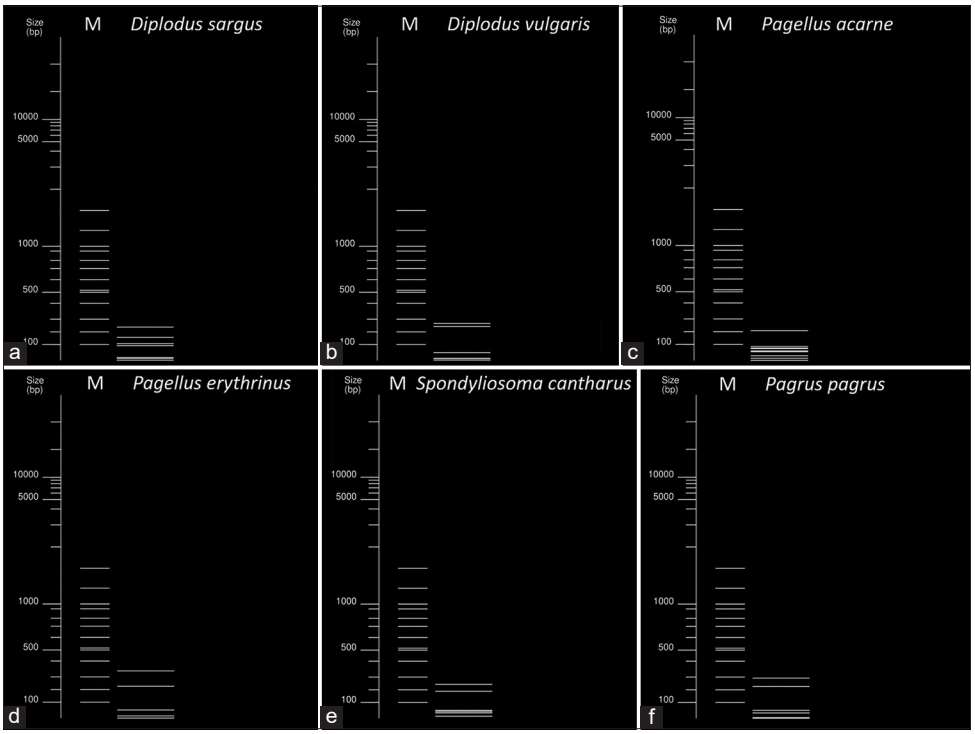

Figure 3: *In silico Msp*I restriction pattern of COI amplicons for sparid reference species in this study. "M" indicates the 100bp ladder used as a reference. Following are the sizes of each restriction band obtained in each species. (a) *Diplodus sargus*: 250,153,107,90,34 bp. (b) *Diplodus vulgaris*: 280,250, 74,50 bp. (c) *Pagellus acarne*: 210, 95, 85, 77, 70, 57, 40, 20 bp. (d) *Pagellus erythrinus*: 340, 220, 64, 30 bp. (e) *Spondyliosoma cantharus*: 260, 194, 70, 60, 50, 20 bp. (f) *Pagrus pagrus*: 290, 234, 70, 50, 10 bp. In each case, the sum of the individual band sizes is 654 bp.

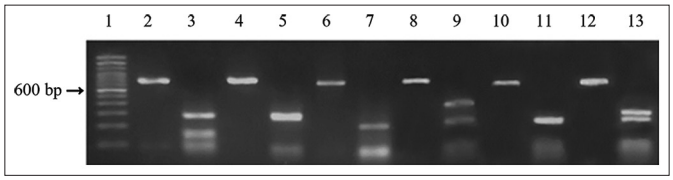

Figure 4: *In vitro Msp*I restriction pattern of *COI* amplicons for each sparid species in this study. Lane 1 = 100 bp ladder. Lane 2 = *D. sargus* (not digested amplicon, 654 bp). Lane 3 = *D. sargus* (digested amplicon, 270-153-107 bp). Lane 4 = *D. vulgaris* (not digested amplicon, 654bp). Lane 5 = *D. vulgaris* (digested amplicon, 280-250 bp). Lane 6 = *P. acarne* (not digested amplicon, 654 bp). Lane 7= *P. acarne* (digested amplicon, 210 bp). Lane 8 = *P. erythrinus* (not digested amplicon, 654 bp). Lane 9 = *P. erythrinus* (digested amplicon, 340-220 bp). Lane 10 = *S. cantharus* (not digested amplicon, 654 bp). Lane 11 = *S. cantharus* (digested amplicon, 250-220 bp). Lane 12 = *P. pagrus* (not digested amplicon, 654 bp). Line 13 = *P. pagrus* (digested amplicon, 290-250 bp). The fragments smaller than 100 bp were not taken into consideration for species identification.

to digest the *COI* barcode amplicons obtained from the reference and unchecked samples. The digestion reaction and the visualization of the obtained fragments were carried out as performed by Ferrito *et al.* [41].

3. RESULTS

3.1. COI Barcode

All *COI* gene fragments of 654 bp [Figure 1] resulted from functional mitochondrial sequences and not pseudogenes, not including insertions, deletions, or stop codons [42]. The *COI* sequences of the reference samples allowed to identify six sparid species confirming their morphological identification [Table 1]. The BLAST search in the GenBank database revealed 98.71 to 99.85% sequence identity for the PCR products to *Pagellus erythrinus*, *Pagrus pagrus*, *Diplodus sargus*, *Spondyliosoma cantharus*, *Diplodus vulgaris,* and *Pagellus acarne* [Table 2]. The COI sequences of the samples morphologically unidentified yielded the detection of the following six species by BLAST search in the GenBank database: *P. erythrinus* (n = 3), *P. pagrus* (n = 7), *D. sargus* (n = 3), *S. cantharus* (n = 3), *D. vulgaris* (n = 2), and *P. acarne* (n = 3) [Table 2]. Two cases of mislabeling were observed: *P. pagrus* in place of *P. erythrinus* and *S. cantharus* in place of *D. sargus*.

To confirm the species revealed by BLAST, an ML tree was built using the 27 COI sequences of all aforementioned species and six additional sequences downloaded from GenBank. All species clustered into different groups corresponding to the six species matched from the BLAST search [Figure 2].

3.2. COIBar-RFLP

In silico analysis allowed to selection of the enzyme able to generate a differential restriction pattern for all tested sparid species: the fragments greater than 100 bp were reported in Table 4. In addition, Figure 3 shows the *in silico Msp*I restriction patterns with all the virtual bands (greater and smaller than 100bp) visualized for each sparid species.

The *in silico* patterns were confirmed by the *in vitro* digestion with *Msp*I visualized in Figure 4. COI amplicon restriction yielded three fragments of 270, 153, and 107 bp in *D. sargus.* A single fragment of about 200 bp was observed in *P. acarne*, while in the remaining species, two fragments of different lengths were detected. In particular, *P. erythrinus* yielded a longer fragment of 340 bp and a shorter one of 220 bp while two fragments of 290 bp and 234 bp were produced by digestion of COI amplicon of *P. pagrus*. Finally, the two species *D. vulgaris* and *S. cantharus* shared the same fragment of 250 bp but the second fragment, which appears almost overlapping in the gel, was species-diagnostic being 280 bp for *D. vulgaris* and 220 bp for *S. cantharus.* The same specific pattern was obtained when COIBar-RFLP was applied in all samples identified only through a molecular approach.

4. DISCUSSION

The *COI* barcode sequencing of fresh whole samples of sparid species commonly sold in several local fish markets and supermarkets in Southern Italy, confirmed the detection of six species previously identified through morphological inspection using analytical keys. The COIBar-RFLP strategy applied to the *COI* amplicons of these reference samples allowed us to obtain different digestion patterns of the restriction enzyme *Msp*I useful to discriminate all species simultaneously. In addition, the COI amplicons of the samples (11 fillets and 10 whole specimens), selected on the basis of the reference sparid species reported on the label, were analyzed by COIBar-RFLP using the *Msp*I restriction enzyme that successfully discriminated all species. Considering that (i) no morphological identification was conducted for these specimens and (ii) the restriction pattern obtained confirmed that of the reference specimens, it can be stated that the advantage of the COIBar-RFLP strategy could be twofold: First, it is possible to quickly verify the correctness of the species declared on the label of the commercial products examined and Second, it is possible to achieve this by skipping the sequencing phase of the amplicons obtained, thus saving time and money. Five cases of mislabeling (24% of cases) were detected in which *P. pagrus* was found instead of *P. erythrinus* (two cases) and *S. cantharus* instead of *D. sargus* (three cases). The percentage of mislabeling we detected is slightly lower than the overall weighted rate of mislabeling (28.4%) in fish products sold on the Italian market (Giusti *et al*. 2023). Based on the sales prices of sparid species, we sampled (see Table S1 in Supplementary Materials) the substitution of *S. cantharus* in place of *D. sargus*, can be considered intentional, due to the obvious economic gain from the fraudulent replacement, while the substitution of *P. pagrus* for *P. erythrinus* can be considered unintentional since the two species were sold at the same price and are morphologically very similar. Moreover, as very similar sparid species are often distinguished on the basis of dentition alone [43], substitution fraud is hardly detectable by the consumer. This issue has been addressed in several studies that explored the efficacy of various molecular markers in discriminating sparid species. More specifically, the efficacy of DNA Inter-simple Sequence Repeat markers was tested in the identification of four species of Mediterranean sea bream and the Mediterranean common snapper [44]. Authentication of sparid fish species has also been achieved by sequencing the PCR products, Polymerase Chain Reaction–Single Strand Conformation Polymorphism (PCR-SSCP), and IsoElectric Focusing (IEF) [45]. More recently, the complete mitochondrial DNA of *Dentex gibbosus* [46], *P. acarne* [47], *Dentex dentex* [48], *P. erythrinus* [49], and *Diplodus puntazzo* [50] has been sequenced to provide useful genetic information for species identification. In particular, the comparison of the known sequences of the complete mitochondrial genome of sparid species [27,28,51] allowed to obtain new barcode genes to be used in place of the classic *COI* and *Cytb* which were considered less effective for the identification of sparid species. However, it should be emphasized that new barcode genes would have to be tested on a large number of species before being used in the forensic field. In contrast,

the efficacy of *COI* barcode sequences in discriminating 75 sparid species was demonstrated by Armani *et al*. [26] in a comprehensive study aimed at testing the complete *COI* barcode for the identification of sparid species, also highlighting the effectiveness of the *COI* mini barcode for discriminating species in highly processed commercial products.

The simplicity and ease of use of PCR-RFLP together with its low cost are certainly the most advantageous aspects of this technique which is widely used in food control laboratories [52]. Indeed, a high degree of specialization or experience in the application of the molecular biology technique is not required to perform it, nor the use of expensive and sophisticated instruments. However, the disadvantages of this strategy cannot be ignored either, which lie in the choice of the specific restriction enzyme and processing conditions, the poor performance of incorrectly stored enzymes, and the need to use more than one enzyme in the same reaction to increase the discriminating power for all the species studied. This particular problem increases the processing times and costs of the methodology [53]. For these reasons, the criterion we used for our COIBar-RFLP was to select a single restriction enzyme, which simultaneously and successfully discriminated all target species both in the present study and in similar studies we had previously conducted [54-58].

5. CONCLUSION

The need to protect consumers from the adulteration of seafood products is explicitly stated in Annex II of the European Commission Recommendation (12.3.2015), according to which in each Member states "*Competent authorities should carry out official controls in order to establish whether fish species found in unprocessed or processed fishery and aquaculture products complies with the species that is declared on the label or in other means of information accompanying the food product.*"

In this context, our contribution focused on the COIBar-RFLP method as a reliable molecular strategy to identify fish species in processed products. Six sparid species were recognized using the differential digestion pattern produced by the restriction enzyme *Msp*I.

The proposed methodology could be used in food control laboratories to combat the widespread habit of fraudulent species substitution in the fishing industry. At least two important implications derive from this problem, the first is purely economic to the detriment of consumers and the second concerns consumer health due to the risk of allergic reactions as demonstrated by the existence of cases of monosensitivity to single species of sparids [59].

6. ACKNOWLEDGMENT

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7. AUTHOR CONTRIBUITIONS

Venera Ferrito: Conceptualization, writing original draft, reviewing, editing, and funding acquisition. Marta Giuga: methodology, analyzing data, reviewing and editing. Giada Santa Calogero: methodology and analyzing data. Anna Maria Pappalardo: conceptualization, methodology, analyzing data, writing original draft, reviewing, editing, and supervision.

8. FUNDING

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVAL

In this study, no *in vivo* experiments on animals were performed. Tissues used for DNA extraction were pieces of processed fish products (fillets or whole specimens) purchased at fish markets and supermarkets. Therefore, approval from the Ethical Committee is not required.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

12. PUBLISHER'S NOTE

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13. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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