DNA barcoding reveals fraudulent substitutions in shark seafood products: The Italian case of "palombo" (*Mustelus* spp.)

M. Barbuto [a,1], A. Galimberti [a,1], E. Ferri [a], M. Labra [a], R. Malandra [b], P. Galli [c], M. Casiraghi [a,*]

[a] *Dipartimento di Biotecnologie e Bioscienze, ZooPlantLab, Università di Milano Bicocca, P.zza della Scienza 2, 20126 Milano, Italy*
[b] *Mercato Ittico all'Ingrosso, ASL Città di Milano, Viale Molise, 20, 20137 Milan, Italy*
[c] *Dipartimento di Biotecnologie e Bioscienze, Water Ecology Lab, Università di Milano Bicocca, P.zza della Scienza 2, 20126 Milano, Italy*

ARTICLE INFO

ABSTRACT

In food safety and traceability, consumers are more and more demanding about composition and provenance of processed seafood products. In the trade of many species, manufacturing alterations usually bring to the loss of any morphological diagnostic features of the species, enhancing the possibility of fraudulent substitutions and incorrect product labeling. In this study, we used a DNA barcoding approach to identify species substitutions cases in shark slices sold in Italy under the vernacular name of "palombo" (that is referred to the triakiids *Mustelus mustelus* and *Mustelus asterias* for the Italian regulation). We produced the *coxI* barcode sequence (550 bp long) for all the analysed specimens, and we compared them with reference sequences from different databases (GenBank and BOLD), using two bioinformatic identification methods, one of them developed in our laboratory. Results showed a high amount of commercial frauds rising the 80% of analysed "palombo" slices and highlighting a relevant economical impact for consumers.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Nowadays consumers pay more and more attention to food quality and safety topics. Science communication through media increases the consumers knowledge, but in the meantime could drive not supported news leading to a suspicious relation to science and in particular, biotechnologies. Consumers are susceptible to any form of food alteration that may occur during the standard manufacturing processes and usually they pay attention to food ingredients in case of dietary nutritional requirements or medical conditions. Due to these reasons, the consumer is becoming more and more demanding on food quality, in particular for product traceability and for the use of detailed product labels.

In the last years, media attention has repeatedly focused on seafood commercial frauds (Marko et al., 2004; Smith, McVeagh, & Steinke, 2008; Wong & Hanner, 2008) because of the difficult in species recognition of these kinds of products, that are usually highly processed to meet consumer requirements (Blanco, Pérez-Martìn, & Sotelo, 2008). For example, many fish species are similar in taste and texture and it is very difficult to identify the right species when the fish is delivered without diagnostic body parts (e.g. skin, entails, head and fins), or when it is processed on fillets or slices. It has also to be taken into account that substituted or mislabeled fishes offered in markets, fisheries and restaurants may be potentially dangerous, due to the presence of unknown toxic or allergenic substances or hurtful in the case of commercial of endangered species (Holmes, Steinke, & Ward, 2009; Ward, Holmes, White, & Last, 2008; Wong & Hanner, 2008).

Another problem involved in fish traceability is linked to the fact that on our tables it is more and more common to eat fish caught not only in different countries, but even in other continents. Nevertheless, the nomenclature of commercialized fishes is not globally standardized; in fact, different species may be identified by the same vernacular name or a single species may be labeled differently depending on the region (even within the same country). To resolve these matters the US Food and Drug Administration (FDA) in 1988 (USDHHS, 1988) and the EU in 2001 (CE, 2065/2001) have published official guides to the scientific and related commercial nomenclature for seafood products labeling. Notwithstanding, locally, at the consumers level, there is a very little perception of the regulations reported above. Moreover, these guidelines remark the need for using adequate tools to confirm species authenticity, products labeling and avoid commercial frauds.

A recent promising method in species identification is DNA barcoding (Hebert, Cywinska, Ball, & deWaard, 2003). This technique is based on the analysis of variability in a short nucleotide sequence (in animals usually belonging to the mitochondrial subunit

1 of cytochrome c oxidase, *coxI*) to evaluate differences between species (Hebert, Ratnasingham, & deWaard, 2003). DNA barcoding has been successfully tested on different taxa, from invertebrates (Hajibabaei, Janzen, Burns, Hallwachs, & Hebert, 2006; Hebert, Penton, Burns, Janzen, & Hallwachs, 2004; Hogg & Hebert, 2004) to vertebrates (Clare, Lim, Engstrom, Eger, & Hebert, 2007; Hebert, Stoeckle, Zemlak, & Francis, 2004; Yoo et al., 2006), allowing the discrimination of different species, often coherently with tradi-tional morphological approaches (Ferri et al., 2009; Ward, Zemlak, Innes, Last, & Hebert, 2005).

In 2005, as a consequence of the increasing use of DNA barcod-ing approach in the identification of fish species (Ward et al., 2005; Wong & Hanner, 2008), a new research project was launched under the auspices of the Consortium for the Barcoding of Life (http://www.barcoding.si.edu/): the Fish Barcode of Life initiative (FISH-BOL; http://www.fishbol.org), which data are included into a main unique database called BOLD (Barcode of Life Data System, http://www.barcodinglife.org/views/login.php; Ratnasingham & Hebert, 2007). Nowadays, a total of 48.724 DNA barcode sequences from 7.205 fish species had been archived in FISH-BOL and consequently in BOLD as of November 13, 2009.

The Barcode of Life Data System also includes a tool for the characterization of unknown specimens, based on the analysis of their DNA barcode sequence: the Identification System resource (IDS; http://www.barcodinglife.org/views/idrequest.php). In par-ticular, this application works on three nested *coxI* libraries which the most useful to the correct identification of species is the Refer-ence Barcode Database containing only DNA barcode sequences that satisfy some specific conditions (see Ratnasingham & Hebert, 2007 for more details). It is important to underline that the gener-ation of integrated systems for interactive retrievals, in which a multiple level of data will be stored and made available (e.g. BOLD), is the future trend for DNA barcoding research projects. These are not to be considered as "stand alone" tools, but they should be integrated with taxonomical data (Ferri et al., 2009) and with other resources (http://www.keytonature.eu/wiki/).

In the present study we focused on species substitution cases concerning the superorder Selachiomorpha (Chondrichthyes: Elasmobranchii), commonly named "sharks", in the Italian fish trade. It is estimated that every year more than 38 million sharks are killed in commercial fishing (Clarke, 2004) due to the increas-ingly request in food industry (FAO, 2004; Holmes et al., 2009). Some studies (De Maddalena & Piscitelli, 2001) indicate that every month, in Italy, about fifteen tons of sharks are sold in one of the most important national fish market in Europe (Merca-to Ittico di Milano-Milan, Italy). In particular, we studied sharks that are placed in the "smooth-hound" complex (*Mustelus* spp., family Triakidae), species daily sold in all Italian markets and fisheries. Four shark species out of the 19 included in the Italian Regulation (G.U. No 45, February 22, 2008) belong to this group: *Mustelus mustelus*, *Mustelus asterias*, *Mustelus schmitti* and *Mustelus punctulatus*. Following this regulation, the first two, are labeled under the same vernacular name "palombo", and they are the most requested by consumers, and consequently, they are also the most subjected to commercial frauds and species substitution (De Maddalena & Piscitelli, 2001). In fact, "palombo" is typically sold as slices because of its length (usually between one and two meters) and after this kind of handling the organ-isms do not retain the diagnostic morphological details useful for the identification of the whole fish (Farrell, Clarke, & Mariani, 2009).

The objective of this work is to verify the reliability of DNA bar-coding in the recognition of commercialized shark species and to evaluate, through this approach, the amount of commercial frauds in the trading of shark slices labeled as "palombo" in Italian markets.

2. Materials and methods

2.1. Biological samples, DNA extractions, PCR conditions, DNA sequencing

In this study a total of 59 biological samples from commercial-ized sharks were collected in different Italian fish markets. In par-ticular, 14 of them were isolated from nine shark species commonly sold in Italy and included in the Italian regulation (see Supplementary Material 1 for details). These were sampled at the Milan fish market (Milan, Italy) and morphologically identified at the species level by a veterinary surgeon of our group (RM). The remaining 45 samples were isolated from slices or fillets (i.e. white muscular tissue) of sharks sold as "palombo" (we checked the name reported on the label) in different Italian fish markets and supermarkets. Due to the strong manufacturing processes a species identification under morphological criteria was not possible and we named these specimens as blind samples (see Table 1 for more details).

DNA extracts were prepared from muscle tissue using the Guanidinium Thiocyanate and Diatomaceous earth method (Gerl-off et al., 1995). *coxI* amplification and sequencing were obtained using the primer pair Fish R2 (5′-ACTTCAGGGTGACCGAAGAATCA-GAA-3′) and Shark-int (5′-ATCTTTGGTGCATGAGCAGGAATAGT-3′) (Ward et al., 2005). PCRs were performed in a volume of 20 μl un-der the following final conditions: $1\times$ buffer including 2.5 mM $MgCl_2$ (MasterTaq kit, Eppendorf™), 0.2 mM of each dNTP, 1 μM of each forward and reverse primers and 1U of DNA polymerase (MasterTaq kit, Eppendorf™). The thermal profile consisted of 35 cycles at 94 °C for 50 s, 54 °C 50 s and 72 °C for 1 min. The ampli-cons obtained were about 550 bp long.

PCRs products were gel purified (using the Perfectprep Gel Cleanup, Eppendorf™) and directly sequenced using ABI technol-ogy. Sequences were checked by eye with Bioedit sequence align-ment editor (version 7.0.5; Hall, 1999), using GenBank sequences as reference sequences (see Supplementary Material 1) and unam-biguously aligned using Clustal X (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). After checked for the presence of pseudogenes and numts (i.e. nuclear mitochondrial pseudogenes), the sequences obtained have been deposited in the EMBL Data Li-brary under the accession numbers listed in Table 1 and Supple-mentary Material 1).

2.2. DNA barcoding dataset definition and OT calculation

Nucleotide distances have been calculated using MEGA 4.0 (Tamura, Dudley, Nei, & Kumar, 2007) – options: nucleotide, Kim-ura 2-parameter, complete deletion, standard error computation by bootstrapping 500 replicates. To perform a DNA barcoding anal-ysis based on an integrated approach involving both morphological and molecular evidences, an optimum threshold value of genetic divergence (OT) must be calculated.

To obtain this parameter, we analyzed the most comprehensive molecular dataset that included 482 *coxI* sequences from more than 100 shark species already present in GenBank and here con-sidered as morphologically identified (see Supplementary Material 1 for details). In addition, we integrated this dataset with the 14 *coxI* sequences from nine morphologically identified shark species produced in this study. The optimum threshold (OT) represents the value that maximizes the coherence between morphological and molecular identification and minimizes, at the same time, the cumulative error. As stated in Ferri et al. (2009), this last parameter is the sum of those errors given to the presence of discrepancies in combining morphological and molecular evidences (i.e. false posi-tives: single morphospecies showing a molecular variability higher

Table 1
List of all identification results of 45 blind samples collected in Italian market and labeled as "palombo". The identifications were performed using the IDS (identification engine on BOLD System) and the OT (Ferri et al., 2009) approaches.

Voucher	Accession number	Identified as (IDS)	Identified as (OT)
MIB:zpl:00004	FM164426	*Squalus brevirostris; Squalus cf. megalops*	*Squalus brevirostris* *Squalus megalops*
MIB:zpl:00005	FM164427	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00006	FM164428	*Prionace glauca*	*Prionace glauca*
MIB:zpl:00009	FM164429	*Galeorhinus galeus*	*Galeorhinus galeus*
MIB:zpl:00010	FM164430	*Mustelus antarticus; Mustelus asterias; Mustelus lenticulatus; Mustelus manazo; Mustelus schmitti; Mustelus stevensi; Mustelus sp.2*	*Mustelus antarticus* *Mustelus asterias* *Mustelus lenticulatus*
MIB:zpl:00012	FM164431	*Alopias superciliosus*	*Alopias superciliosus*
MIB:zpl:00013	FM164432	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00014	FM164433	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00015	FM164434	–	*Mustelus mustelus*
MIB:zpl:00016	FM164435	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00018	FM164436	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00019	FM164437	*Mustelus antarticus; Mustelus asterias; Mustelus lenticulatus; Mustelus manazo; Mustelus schmitti; Mustelus stevensi; Mustelus sp.2*	*Mustelus antarticus* *Mustelus asterias* *Mustelus lenticulatus*
MIB:zpl:00022	FM164438	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00023	FM164439	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00024	FM164440	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00025	FM164441	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00027	FM164442	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00028	FM164443	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00029	FM164444	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00030	FM164445	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00031	FM164446	*Mustelus antarticus; Mustelus asterias; Mustelus lenticulatus; Mustelus manazo; Mustelus schmitti; Mustelus stevensi; Mustelus sp.2*	*Mustelus antarticus* *Mustelus asterias* *Mustelus lenticulatus*
MIB:zpl:00032	FM164447	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00033	FM164448	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00034	FM164449	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00035	FM164450	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00036	FM164451	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00037	FM164452	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00038	FM164453	*Mustelus antarticus; Mustelus asterias; Mustelus lenticulatus; Mustelus manazo; Mustelus schmitti; Mustelus stevensi; Mustelus sp.2*	*Mustelus antarticus* *Mustelus asterias* *Mustelus lenticulatus*
MIB:zpl:00039	FM164454	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00040	FM164455	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00041	FM164456	*Mustelus antarticus; Mustelus asterias; Mustelus lenticulatus; Mustelus manazo; Mustelus schmitti; Mustelus stevensi; Mustelus sp.2*	*Mustelus antarticus* *Mustelus asterias* *Mustelus lenticulatus*
MIB:zpl:00042	FM164457	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00043	FM164458	*Prionace glauca*	*Prionace glauca*
MIB:zpl:00044	FM164459	*Prionace glauca*	*Prionace glauca*
MIB:zpl:00046	FM164460	–	*Mustelus mustelus*
MIB:zpl:00047	FM164461	–	*Mustelus mustelus*
MIB:zpl:00050	FM164462	*Isurus oxyrinchus*	*Isurus oxyrinchus*
MIB:zpl:00053	FM164463	*Isurus oxyrinchus*	*Isurus oxyrinchus*
MIB:zpl:00055	FM164464	*Squalus acanthias*	*Squalus acanthias*
MIB:zpl:00057	FM164465	*Isurus oxyrinchus*	*Isurus oxyrinchus*
MIB:zpl:00058	FM164466	–	–
MIB:zpl:00059	FM164467	*Isurus oxyrinchus*	*Isurus oxyrinchus*
MIB:zpl:00063	FM164468	*Isurus oxyrinchus*	*Isurus oxyrinchus*
MIB:zpl:00064	FM164469	*Mustelus antarticus; Mustelus asterias; Mustelus lenticulatus; Mustelus manazo; Mustelus schmitti; Mustelus stevensi; Mustelus sp.2*	*Mustelus antarticus* *Mustelus asterias* *Mustelus lenticulatus*
MIB:zpl:00065	FM164470	*Isurus oxyrinchus*	*Isurus oxyrinchus*

than threshold and false negatives: different morphospecies showing a molecular variability under the threshold). For the calculation of OT we followed the bioinformatic approach developed by Ferri et al. (2009).

To give a schematic view of the relationships among the shark species treated in our *coxI* molecular dataset (blind samples sold as "palombo" included), a phenetic tree has been generated (see Supplementary Material 2). The tree has been obtained using MEGA 4.0 (Tamura et al., 2007) – options = tree inference method: neighbor-joining; phylogeny test and options: bootstrap (100 replicates); gaps/missing data: pairwise deletion; codon positions: 1st + 2nd + 3rd + non-coding; substitution model: K2P; substitutions to include: transitions + transversions; pattern among lineages: same (homogeneous); rates among sites: uniform rates.

2.3. Identification of blind samples sold as "palombo" and estimation of commercial frauds

The identification of the 45 blind samples was conducted with two approaches at DNA barcoding: the Identification Engine tool (IDS) on BOLD (searching on the Reference Barcode Database and considering only matches up to 98% of specimen similarity) and

the species recognition based on the OT as described in Ferri et al. (2009).

The top species matches (highest percentage of similarity) obtained with these two approaches were compared to the labeled name recorded at the market in order to determine the percentage of species substitution in the acquiring of "palombo" slices.

3. Results

3.1. DNA barcoding dataset

The molecular dataset generated in this study comprises a total of 496 *coxI* barcode sequences 551 bp long, no insertion/deletion (indel) were introduced during the alignment, and no numts were detected. Sequences belong to 110 shark species representative of 47 genera, 22 families and 8 orders. Fourteen *coxI* sequences relative to 9 morphospecies (identification were performed by morphological experts, see above) were produced in this study and in four cases they represent the first entries in GenBank (i.e. *Lamna nasus*, *Scyliorhinus stellaris*, *M. mustelus* and *M. asterias*, see Supplementary Material 1).

The average number of barcoded specimens per species is 4.51 (standard deviation 3.83, range 1–28). The high value for standard deviation is due to the over-representation of specimens for some species (i.e. 28 *coxI* sequences of *Squalus acanthias*).

3.2. DNA barcoding analyses and optimum threshold

Data obtained from a classical DNA barcoding analysis, based on K2P distance matrix, showed the following results: *coxI* mean nucleotide intraspecific distance 0.19% (standard error: 0.34%; range: 0–2.60%); *coxI* mean nucleotide interspecific distance 19.55% (standard error: 5.62%; range: 0.40–30.00%) and *coxI* overall mean diversity 19.20% (standard error: 1.40%).

The OT obtained from the dataset assumes the value of 2.0% and generates a minimum cumulative error of 1.7%, where the cumulative error is the percentage of mismatched identifications (see Ferri et al., 2009). In summary, the identification approach based on molecular divergence threshold (OT) for *coxI* is coherent with morphological approach for 79 species out of 110 (71.8%) while for almost all the remaining 31 species, values of interspecific K2P distances are lower than OT (false negatives *sensu* Ferri et al., 2009).

3.3. Identification of blind specimens sold as "palombo" through DNA barcoding

DNA barcoding performed with the IDS allowed to recognize at the species level 34 species out of 45 (75.6%) belonging to five shark families (see Table 1 for details). Six cases of indecision among species of the genus *Mustelus* and another case of doubtful identification relative to two species of the genus *Squalus* were found. Additionally, four blind specimens did not reach any match with IDS.

The DNA barcoding approach performed with OT method allowed to identify shark species in 37 cases out of 45 (82.2%). Six different species belonging to five families were unequivocally recognized, and, similarly to the IDS search previously described, we found the same seven indecision cases (concerning genus *Mustelus* and *Squalus*). For one blind specimen (a.n. FM164466), no species correspondence for a genetic divergence value lower than the threshold was found (see Table 1). Moreover, it is important to underline that the OT approach allowed the identification of three specimens as *M. mustelus* that IDS was unable to identify due to the

absence of *coxI* sequences for this species in the BOLD Reference Database.

Comparing the efficacy of the two approaches, we found that a coherent identification at the species level was possible in 34 cases out of 45. Moreover, both methods cannot discriminate in six cases out of 45 among three different species of *Mustelus* (*Mustelus antarcticus*, *M. asterias* and *Mustelus lenticulatus*). The Identification System Engine (IDS) shows for these indecision cases a positive match for other four species (*Mustelus manazo*, *M. schmitti*, *Mustelus stevensi* and an indeterminate species called *Mustelus* sp. 2) not present in our dataset but stored in BOLD (the sequences are not directly accessible). Another indecision case refers to a blind sample (MIB:zpl:00004, a.n. FM164426) for which two different species have been suggested: *Squalus brevirostris* and *Squalus megalops*. Although this result is shared between the two identification tools, IDS underlines that the *coxI* reference sequence of *S. megalops* from BOLD belongs to a specimen with an uncertain identification (is reported as *S.* cf. *megalops*). Finally, the two methods were coherently unable to identify sample MIB:zpl:00058 (a.n. FM164466) that remains unknown.

3.4. Estimation of commercial frauds

Both IDS and OT approaches revealed that out of the 45 blind specimens analyzed, only three (6.7%) can be unequivocally assigned to "palombo" and in particular to the species *M. mustelus* (see Table 2 for details). Further, six cases out of 45 (13.3%) refer to species belonging to the genus *Mustelus*. Altogether, we identified 35 cases of species substitution out of 45 (77.8%) collected samples. Concerning these cases, all the specimens belong to four families different from Triakidae and all but one of them (a.n. FM164426, see previous paragraph) were identified at the species level. In addition, one sample (a.n. FM164466) was not included in the count of species substitution cases due to the absence of any identification match with the two approaches. It is important to underline that among the blind samples identified at the species level and coherently between two methods, one of these was identified as *Alopias superciliousus*: a species not included in the Italian regulation.

4. Discussion

Coherently to previous published studies (Holmes et al., 2009; Ward et al., 2005, 2008), DNA barcoding is clearly a useful tool for the species identification. The optimum threshold value (OT), calculated following the method proposed by Ferri et al. (2009), showed a rather high strength of coherence between morphological and molecular identification of the species analyzed even if some inconsistencies were detected. In fact, 31 species (belonging to 6 families) out of the 110 included in our highly comprehensive *coxI* dataset, have been marked as "problematic" (see also Supplementary Material 2). In almost all these cases, we found an interspecific K2P genetic divergence lower than OT (false negatives

Table 2
Summary of the identification at the species level through IDS and OT approach. The prices of species, relative to Mercato Ittico di Milano, are reported.

OT/IDS	Number of specimens	Family	Italian regulation	Price (€/kg)
Mustelus mustelus	3	Triakidae	Included	7.26
Squalus acanthias	23	Squalidae	Included	3.90
Prionace glauca	3	Carcharhinidae	Included	2.99
Galeorhinus galeus	1	Triakidae	Included	3.00
Alopias superciliosus	1	Alopiidae	Not included	–
Isurus oxyrinchus	6	Lamnidae	Included	5.50

sensu Ferri et al. (2009)), while, only in the case of *Pristiophorus nudipinnis* we found an intraspecific K2P genetic divergence higher than OT (false positive *sensu* Ferri et al. (2009)). Most of these situations were already reported in other studies (Ward et al., 2005, 2008) and explained by different causes like species misidentification (cryptic species, uncertain taxonomy), wrong labeling of the specimens or mistakes during sequences submission to GenBank. Moreover, concerning the target of our work, five "problematic" cases referring to species belonging to genus *Mustelus* were detected. In particular, the OT value calculated on our molecular dataset, allows the identification through DNA barcoding of *M. mustelus* only, while *M. asterias* is included in a MOTU (together with *M. antarcticus, M. schmitti, M. lenticulatus,* and *M. manazo*) characterized by a K2P genetic divergence lower than the threshold (see Supplementary Material 2).

These cases had been previously discussed by Lopez, Ryburn, Fedrigo, and Naylor (2006), in a phylogenetic study based on mitochondrial and nuclear genes. The authors highlighted a strong correlation between genetics and bioecological aspects for several *Mustelus* species, leading to the identification of two species complexes named "asterias clade" and "mustelus clade" respectively. In particular, low levels of genetic divergence among species were detected for the two clades and the authors pointed out for hybridization and/or incomplete lineage sorting phenomena as causes.

The fact that *Mustelus* is the most problematic genus among Triakidae (in terms of systematics and taxonomy) is known (Farrell et al., 2009; Heemstra, 1997) and the cases of false negative identified in our dataset using DNA barcoding approach confirm the need for a thorough taxonomical revision of this species complex.

Referring to the 19 shark species reported in the Italian regulation, the *coxI* molecular dataset here presented, includes 12 of them. It should be noted that barcode data for the lacking species are available in the BOLD (Barcode Reference Database) except for *Squatina squatina* and *M. punctulatus*. Notwithstanding, none of these seven species has been recovered in the identification of the blind specimens collected as "palombo".

Our results show that the two identification approaches based on DNA Barcoding (IDS on BOLD and OT on our dataset) are highly coherent to characterize the fish products sold as "palombo" in Italian fish markets (blind specimens) at the specific level. As shown before, only *M. mustelus* is clearly recognizable through a DNA barcoding approach, while, *M. asterias*, is not discernible from others congeneric species. In any case, our system is highly efficient to discriminate *Mustelus* spp. from other sharks. Most of the case of uncorrected species labeling is not due to problems within the genus *Mustelus*. In conclusion, excluding these particular situations, the percentage of commercial frauds about "palombo" on our sampling remains high, rising the 78%.

Some consideration is needed about the following economical aspects: "palombo" is valued by Italian fisheries at 7.26 €/kg (data relative to Mercato ittico Milano, July 2009). As expected, it is usually substituted with less valuable species, for example *S. acanthias* (more than 50% of substitution cases in our blind specimens sampling are indeed relative to this species) which price is fixed at 3.90 €/kg. The prices discrepancy is evident, and the economic impact on sellers and consumers is clear. However, besides economic aspects, one of the emerging warning from our study is relative to the potential problems for human health (allergens, parasites) due to species substitutions in fish trade. However, they cannot be considered secondary, particularly in a global world fish market. According to this latter consideration, conservation themes involved in the management of commercialized fish populations (especially for endangered species or in the case of harvesting of unnamed species) emerge as a dramatic concern (Holmes et al., 2009; Ward et al., 2008; Wong & Hanner, 2008). Even if further studies are necessary to quantify the entity of these problems, it

is clear that DNA barcoding can be a valuable tool for addressing this topics.

5. Conclusion

In this study we have confirmed the reliability of DNA barcoding approach for food traceability, in particular for fish trade. The dataset produced by our group and the identification system engine on BOLD allowed to recognize commercial frauds in the trade of "palombo", especially in the case of *M. mustelus*.

Although both *coxI* databases are incomplete for the genus *Mustelus*, it is clear that the high molecular similarity between *M. asterias* (one of the two species saleable as "palombo" as reported in the Italian regulation) with others congeneric species could lead to the development of economical problems due to cases of species substitution. In this context, the development of molecular techniques for food traceability and identification represents a bridge towards a better integration of science in the society. Our results, clearly show the need for the use of molecular tools like DNA barcoding to assist in the accurate identification of fish species marketed in Italy, as yet proposed by Yancy and colleagues (2008) concerning the generation of the USA Regulatory Fish Encyclopedia.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodres.2009.10.009.

References

Blanco, M., Pérez-Martìn, R. I., & Sotelo, C. G. (2008). Identification of shark species in seafood products by forensically informative nucleotide sequencing (FINS). *Journal of Agricultural and Food Chemistry, 56*, 9868–9874.

CE, 2065/2001. Laying down detailed rules for the application of Council Regulation (EC) No. 104/2000 as regards informing consumers about fishery and aquaculture products.

Clare, E. L., Lim, B. K., Engstrom, M. D., Eger, J. L., & Hebert, P. D. N. (2007). DNA barcoding of neotropical bats: Species identification and discovery within Guyana. *Molecular Ecology Notes, 7*, 184–190.

Clarke, S. (2004). *Shark product trade in Mainland China and Hong Kong and implementation of the CITES shark listings.* Hong Kong, China: TRAFFIC East Asia.

De Maddalena, A., & Piscitelli, L. (2001). Analisi preliminare dei selaci registrati presso il mercato ittico di Milano (aprile-settembre 2000). *Bollettino del Museo Civico di Storia Naturale di Venezia, 52*, 129–145.

Farrell, E. D., Clarke, M. W., & Mariani, S. (2009). A simple genetic identification method for Northeast Atlantic smoothhound sharks (*Mustelus* spp.). *ICES Journal of Marine Science, 66*, 561–565.

Ferri, E., Barbuto, M., Bain, O., Galimberti, A., Uni, S., & Guerriero, R., et al. (2009). Integrated taxonomy: traditional approach and DNA barcoding for the identification of filarioid worms and related parasites (Nematoda). *Frontiers in Zoology* 6.

Food and Agriculture Organization (FAO). (2004). Fisheries Circular No. 990, World markets and industry of selected commercially exploited aquatic species with an international conservation profile.

Gerloff, U., Schlötterer, K., Rassmann, I., Rambold, G., Hohmann, B., Fruth, B., et al. (1995). Amplification of hypervariable simple sequence repeats (microsatellites) from excremental DNA of wild living bonobos (*Pan paniscus*). *Molecular Ecology, 4*, 515–518.

Hajibabaei, M., Janzen, D. H., Burns, J. M., Hallwachs, W., & Hebert, P. D. N. (2006). DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Science of the United States of America, 103*, 968–971.

Hall, T. A. (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series, 41*, 95–98.

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B, 270*, 313–321.

Hebert, P. D. N., Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. *Proceedings of the National Academy of Science of the United States of America, 101*, 14812–14817.

Hebert, P. D. N., Ratnasingham, S., & deWaard, J. R. (2003). Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B, 270*, S596–S599.

Hebert, P. D. N., Stoeckle, M. Y., Zemlak, T. S., & Francis, C. M. (2004). Identification of birds through DNA barcodes. *PloS Biology, 2*, 1657–1663.

Heemstra, P. C. (1997). A review of the smooth-hound sharks (genus *Mustelus*, family Triakidae) of the western Atlantic Ocean, with description of two new species and a new subspecies. *Bulletin of Marine Science, 60*, 894–928.

Hogg, I. D., & Hebert, P. D. N. (2004). Biological identification of springtails (Collembola: Hexapoda) from the Canadian Arctic, using mitochondrial DNA barcodes. *Canadian Journal of Zoology, 82*, 749–754.

Holmes, B. H., Steinke, D., & Ward, R. D. (2009). Identification of shark and ray fins using DNA barcoding. *Fisheries Research, 95*, 280–288.

Italian Regulation from Ministro delle Politiche Agricole Alimentari e Forestali (G.U. No. 45, February 22, 2008).

Lopez, J. A., Ryburn, J. A., Fedrigo, O., & Naylor, G. J. P. (2006). Phylogeny of sharks of the family Triakidae (Carcharhiniformes) and its implications for the evolution of carcharhiniform placental viviparity. *Molecular Phylogenetics and Evolution, 40*, 50–60.

Marko, P. B., Lee, S. C., Rice, A. M., Gramling, J. M., Fitzhenry, T. M., McAlister, J. S., et al. (2004). Fisheries: Mislabelling of a depleted reef fish. *Nature, 430*, 309–310.

Ratnasingham, S., & Hebert, P. D. N. (2007). BOLD: The barcode of life data system. *Molecular Ecology Notes, 7*, 355–367. <www.barcodinglife.org>.

Smith, P. J., McVeagh, S. M., & Steinke, D. (2008). DNA barcoding for the identification of smoked fish products. *Journal of Fish Biology, 72*, 464–471.

Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA 4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution, 24*, 1596–1599.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The ClustalX Windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 24*, 4876–4882.

USDHHS, 1988. "The fish list" FDA guide to acceptable market names for food fish sold in interstate commerce. Department of Health and Human Service, FDA Center for Food Safety and Applied Nutrition, available from Sup. Documents, US Govt. Printing Office, Washington, DC.

Ward, R. D., Holmes, B. H., White, W. T., & Last, P. R. (2008). DNA barcoding Australasian chondrichthyans results and potential uses in conservation. *Marine and Freshwater Research, 59*, 57–71.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., & Hebert, P. D. N. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society B, 360*, 1847–1857.

Wong, E. H. K., & Hanner, R. H. (2008). DNA barcoding detects market substitution in North American seafood. *Food Research International, 41*, 828–837.

Yancy, H. F., Zemlak, T. S., Mason, J. A., Washington, J. D., Tenge, B. J., Nguyen, N. T., et al. (2008). Potential use of DNA barcodes in regulatory science: Applications of the Regulatory Fish Encyclopedia. *Journal of Food Protection, 71*, 210–217.

Yoo, H. S., Eah, J. Y., Kim, J. S., Kim, Y. J., Min, M. S., Paek, W. K., et al. (2006). DNA barcoding Korean birds. *Molecules and Cells, 22*, 323–327.