Running title: DNA barcoding in support of seafood companies self-check

DNA barcoding for the verification of supplier's compliance in the seafood chain: how the lab can support companies in ensuring traceability

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Abstract

[Food Business Operators (FBOs)] rely on laboratory analysis to ensure seafood traceability. DNA barcoding and [Forensically Informative Nucleotide Sequencing (FINS)] may represent a support within self-checking programs finalized to suppliers' qualification and products identity certification. The present study aimed at verifying the usefulness of a decisional procedure (decision tree) set up at the FishLab (Department of Veterinary Sciences, University of Pisa) for seafood species identification by DNA analysis, to cope with FBOs' needs. The decision tree was applied to the analysis of 182 seafood (fish and molluscs) products, conferred to the FishLab by different FBOs between 2014 and 2015 as result of their self-checking activities. The analysis relied on a standard *COI* gene fragment eventually integrated by the analysis of alternative or supportive molecular targets (*cytb* and *16S rRNA*). It also included a mini-DNA barcoding approach for processed products. Overall, 96.2% of the samples were unambiguously identified at species level using the elective target alone (92.4%) or a multi-target approach (3.8%). The lack of species identification (3.8%) was attributable to the absence of reference sequences or to the low resolution of the molecular targets. Nonetheless, all the molecular results were deemed adequate to evaluate the sample's compliance to the label information. Non-compliances were highlighted in 18.1% of the products. The protocol was proven as an effective supportive tool for the seafood identity verification within the supply chain self-checking activities. In addition, a considerable fraud rate was confirmed and the species most frequently involved in substitution were pointed out.

1. Introduction

Economically Motivated Adulterations (EMA) of food, or more simply frauds, are a growing phenomenon due to the relevant gains that can be achieved by substituting high quality with less valuable products (Spink et al., 2016). In the fishery sector frauds are favored by the high complexity of the globalized supply chain, which exposes seafood trade to safety and quality challenges (http://oceana.org/sites/default/files/reports/Seafood_Traceability_Report_FINAL).

At the European level, the Regulation (EU) n. 1379/2013 establishes some mandatory information (commercial and scientific name, production method, origin, category of fishing gear used for the catching) that must accompany seafood all along the supply chain (D'Amico *et al*., 2016). Thus, ensuring seafood traceability represents a priority of [Food Business Operators (FBOs)] that are responsible for all the information provided to the downstream operators or to the consumers. However, concerns about the effective maintenance of seafood traceability arise considering that the seafood supply chain operating at national and international level usually involves many intermediaries between the primary production (catch or aquaculture) and the final delivering (Thorpe and Benneth, 2004). Moreover, the lack of an international standardized labeling and seafood naming system, the inappropriate training of fishery operators and the removal of morphological features during processing affect the correct identification of seafood commodities (Cawthorn *et al*., 2015; Leal *et al.*, 2015). In addition, the seafood industry is the second food sector most exposed to adulteration and the replacement of low-value seafood species for greater value species (*aliud pro alio* fraud), represents a frequent illegal practice (D'amico *et al*., 2016). Fraudulent behaviors expose consumers not only to commercial but also to health issues and undermine stock conservation efforts (Stawitz *et al.*, 2016; Armani *et al.*, 2015a; Warner *et al*., 2013).

By the years, many initiatives have been proposed to provide FBOs with guidelines to comply with the mandatory EU traceability (Aung and Chang, 2014). These were furtherly implemented by the ISO 12875:2011 and ISO 12877:2011 on the information to be recorded for captured and farmed

79 finfish distribution chains, respectively (https://www.iso.org/obp/ui/#iso:std:iso:12875:ed-1:v1:en;
80 https://www.iso.org/obp/ui/#iso:std:iso:12877:ed-1:v1:en). However, these standards do not specify
81 the analytical instruments for the acquisition and the management of traceability data, delegating to
82 FBOs the selection of the most performant tool according to the implemented traceability plan (Tamm
83 et al., 2016). Therefore, seafood traceability procedures applied in the supply chain consist of record-
84 keeping methodologies represented by simple, paper-based records or by complex electronic data
85 systems and radio frequency identification (RFID) (Aung and Chang, 2014). Moreover FBOs have
86 begun to adopt specific supplier's monitoring systems which are usually based on customer
87 complaints, direct controls (such as visual inspections, control of transport temperatures) and
88 laboratory analysis (Barach, 2016, Aung and Chang, 2014).
89 Analytical tools may offer a significant support to the fishery sector companies, especially those
90 dealing with import/export and large distribution, for the verification of the accompanying
91 documents. Laboratory activities are especially intended to checkup incoming goods at the time of
92 acceptance as well as monitor suppliers' reliability (Barach, 2016).
93 DNA-based methods, particularly DNA barcoding and *Forensically Informative Nucleotide*
94 *Sequencing* (FINS) are nowadays the most routinely utilized since they fully address the requirements
95 stated by rules and can be applied throughout the whole supply chain (Handy *et al.*, 2011; Aung and
96 Chang, 2014). Therefore, they can be used to support companies' self-checking program for goods'
97 verification . However, to cope with the huge gamma of seafood products currently traded worldwide
98 and the need to quickly respond to FBOs, a suitable workflow protocol is required (Griffiths *et al.*,
99 2014).
100 At the FishLab of the Department of Veterinary Sciences (University of Pisa) an identification
101 protocol based on DNA analysis, operating like a systematic decision-making tool, generally known
102 as decision tree analysis, was developed to sort out the most appropriate lab pathways in the light of
103 the features of the samples to be analyzed. This study aimed at checking the usefulness of the
104 aforesaid protocol to verify the identity of seafood sent to our lab by FBOs during 2014 and 2015

105 within the self-checking programs of companies. The ability to respond to the needs of FBOs is
106 discussed in the light of the results obtained.

107 **2. Materials and Methods**

108 **2.1** ***Reception of the samples***

109 A total of 182 tissue samples from fishery products were sent to our lab from 2014 to 2015 by
110 distinct Italian wholesaler distributors (WD) and seafood retail delivery platforms (RP): 133 were
111 sent to the laboratory from WD and the remaining 49 from RP. All the samples were collected in the
112 framework of self-check programs. The samples included 162 fish (159 fresh and 3 salted products),
113 16 fresh cephalopods and 4 frozen and precooked mollusk bivalves, that were conferred to our
114 laboratory accompanied by the trade name and the scientific name recovered from the tracking
115 documents (Table 1). Each sample was registered and submitted to the decision tree analysis
116 graphically described in Figure 1. Then it was analyzed following a four steps identification protocol.

117 ***2.2 Application of the four-steps protocol for the samples analysis***

118 *1) DNA extraction and quality assessment*. Total DNA extraction was performed from 100 mg of
119 tissue using the protocol proposed by Armani *et al*. (2014). DNA concentration and purity were
120 assessed with Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE,
121 US) by two subsequent measurements of the absorbance value at 260 nm and the calculation of the
122 absorbance ratios A260/A280 and A260/A230. All the DNA samples extracted from processed
123 products were submitted to gel electrophoresis and classified according to their degree of
124 fragmentation as described in Armani *et al*. (2017).

125 *2) DNA amplification.* The selection of the molecular target follows the decision tree reported in
126 Figure 1. The Cytochrome C oxidase subunit I (*COI*) gene was selected as the "first choice" target
127 except for tuna products for which the *cytb* gene was chosen as elective target. Two primer pairs
128 proposed by Folmer *et al*. (1994) and Handy *et al*. (2011) were selected for obtaining a *COI* fragment
129 of 655-658 bp [Full Length Barcode (FLB)] from mollusks and fish DNA, respectively. The primers
130 designed by Sevilla *et al.* (2009) were used for the amplification of the entire *cytb* gene (1140 bp) or

for a 854 bp fragment. In the presence of medium-highly DNA fragmentation (fragment <500 bp) two primer pairs were used for the amplification of a shorter DNA targets: one proposed by Armani *et al*. (2015b) for obtaining a 139 bp *COI* fragment and one designed by Kocher *et al.* (1989), for the amplification of a 464 bp *cytb* fragment. Each sample was initially analyzed using one target gene (long or short fragment). Alternative targets such as the entire *cytb* target and/or a fragment of ~ 550 bp of the *16S rRNA* obtained by the primer pair designed by Palumbi (1996) were further amplified if the post sequencing analysis of the elective target alone (*COI*) did not allow species identification (Figure 1). The PCR amplification and PCR products purification were set according to the protocol described in Armani et al. (2017) except for the amplification of DNA samples belonging to bivalves for which the standard Taq was substituted with a 5′-exonuclease deficient Taq polymerase KlenTaq LA (DNA Polymerase technology, Inc, USA).

3) *DNA sequencing and evaluation of the sequencing products quality.* DNA sequencing was carried out by GATC Biotech AG, European Custom Sequencing Centre (Cologne Germany). Forward and reverse sequences were aligned with Clustal W program as described in Armani et al., (2017).

4) Post sequencing analysis. Final sequences were queried against the reference sequences available on GenBank and BOLD databases by the means of [Basic Local Analysis Search Tool (BLAST)] (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and [Identification System (ID's)] (Ratnasingham & Hebert, 2007). The Barcoding identity score cut-off and the parameters of the Neighbour Joining analysis used for species identification were set as reported in Armani et al. (2017). The results obtained by the analysis of the molecular target were compared to the commercial designation and scientific name provided by the different suppliers. The samples were declared non-compliant if the commercial designation provided by the Ministry of agricultural food and forestry policies (https://www.politicheagricole.it/flex/cm/pages/ServeBLOB.php/L/IT/IDPagina/156) for the molecularly identified species, did not match the declared one.

3. Results

157 ***3.1 Samples composition***. The self-checking programs of supplier were plausibly driven by the
158 European consumer's buying preferences (EUMOFA,
159 2015:https://www.eumfa.eu/documents/20178/66003/EN_The+EU+fish+market_Ed+2015.pdf/4cb
160 d01f2-cd49-4bd1-adae-8dbb773d8519) and during the two-year survey the sampling was mainly
161 focused on fish (88%), followed by mollusks (12%), of which 10% cephalopods (squid, cuttlefish
162 and octopus) and 2% bivalves (Figure 2).
163 Among the fish category, samples declared as cod and hake were the most frequently conferred to
164 our lab. Particularly, the Atlantic cod represented 85.7% (48/56) of the Gadiformes analyzed. Among
165 these products, 3 were salted (2/3 labelled as "*baccalà*"). Tunas was the second most sampled group
166 (23%, 42/182) and consisted almost entirely of Yellowfin Tuna and few Bigeye tuna products.
167 Groupers, leaded by Goldblotch grouper, and flatfish, mainly represented by European plaice and
168 Spiny turbot, were the third and fourth fish most collected groups. Less frequent self-checking
169 sampling were addressed to goatfish, salmon, trout and clupeids, and sporadically to a variety of
170 different species (redfish, gurnards, Nile perch, John dory, Amberjack, sharks and porgies) (Table 1;
171 Figure 2). About mollusks, the cephalopod products were constituted by longfin and shortfin squid,
172 octopus and cuttlefish. Finally, the 4 bivalves consisted of Chilean mussel, Blue mussel and one
173 veined clam product (Table 1).
174 ***3.2 DNA extraction and amplification***. The spectrophotometric analysis confirmed high yield and
175 quality (A260/A280 and A260/A230 ratio >2.0) for all the samples analyzed except for the total DNA
176 obtained from bivalves for which low A260/230 ratio (<1.5) was observed. For all the DNA samples
177 the elective target was successfully obtained at the first amplification, or eventually amplified using
178 one supportive molecular target, providing a final amplification and sequencing rate of 100%.
179 ***3.3. DNA Sequencing and post sequencing analysis***. The results of the post sequencing analysis
180 are shown in Table 1. In detail, 96.2% (175/182) of the samples were unambiguously identified at
181 species level by means of the elective target alone (92.4%, 168/182) or through the application of a
182 multi-target approach (3.8% 7/182). The remaining 3.8% of the products (2 redfish, and 2 pharaoh

cuttlefish products, 2 Chilean and 1 blue mussel) was only identified at genus level, despite the use of 2 targets (*COI* and *16S rRNA*). Overall, 33 (30 fish and 3 cephalopod products) out of the 182 products analyzed (18.1%) were found to be non-compliant to the commercial designation and scientific denomination provided by the suppliers. The highest non-compliance rate was highlighted in the grouper group (58.8% -10/17)in. flatfish (18.7% -3/16) and the cod/hake (16.1% -9/56)the highlighted (Figure 2). Tthe replacement of Atlantic cod withsubstitution of haddock (*Melanogrammus aeglefinus*) as Atlantic cod was the most frequently accounted. Notably, despite the high number of tuna products sampled in the study , oFor tuna products, only two non-compliances, represented by the substitution (represented by the replacement of *Thunnus albacares* with *Thunnus obesus* or vice versa, were highlighted (Table 1; Figure 3 1). Occasional substitution cases observed in less frequently sampled fish groups and mollusk category are detailed in Table 1.

As regard groupers, the majority of non-compliances were addressed to their substitution with Monrovia doctorfish (*Acanthurus monroviae*) and Goatbeard brotula (*Brotula multibarbata*); moreover, few cases of intra-genus substitution were found. Among the cod/hake (order Gadiformes), the non-compliances were limited to substitution within the same family or genus. The substitution of haddock (*Melanogrammus aeglefinus*) as Atlantic cod was the most frequently accounted. Nevertheless, *Gadus morhua* itself was verified as substituting Pacific cod (*Gadus macrocephalus*) in 1 salted product. About flatfish, 3 products conferred as Spottail spiny turbot (*Psettodes belcheri*) were identified as Spiny turbot (*P. bennetti*). Among the less frequently sampled fish categories, not compliances were highlighted for: West African goatfish (*Pseudupeneus prayensis*); Atlantic salmon (*Salmo salar*), Sardine (*Sardina pilchardus*), Greater Amberjack (*Seriola dumerilii*) Angolan dentex (*Dentex angolensis*) and John Dory (*Zeus faber*) (Table 1). Among cephalopods,1 European flying squid product was verified belonging to lesser flying squid (*Todaropsis eblanae*). The two "Pharaoh cuttlefish" were both identified as belonging to different genus or species (Table 1).

4. Discussions

208 Up to a few decades ago, food companies used to select suppliers on the basis of their cost and
209 location. As international government and industry put a stronger emphasis on food safety and
210 quality, evaluating the right supplier has become a critical business decision. Companies are required
211 to check the overall food safety status of their suppliers and highlight eventual significant compliance
212 or quality system failures that could affect the production of top-quality foods
213 (https://webapps.traqtion.com/traqtionportal/servlet/PDFServlet?filename=Costco_Food_Safety_Ex
214 pectations.pdf; Barach, 2016). Although the suppliers' selection process is complex and onerous,
215 especially for small companies, it may help to increase the brand reputation and drive the customers'
216 demand (Beil, 2009). To cope with the several cases of fraud, food companies have begun to adopt
217 the Vulnerability Assessment and Critical Control Points (VACCP) system, specifically aimed to
218 prevent or detect illicit practices (Cavin et al., 2016; Spink et al., 2016). The VACCP system aims at
219 identifying and monitoring "vulnerability areas" to detect, control and prevent fraudulent practices
220 that could damage their business (Cavin et al., 2016; Spink et al., 2016). It follows that when a raw
221 material is at risk of adulteration or replacement, the company must implement adequate verification
222 systems to minimize the risk (Manning & Soon, 2016). One of the most efficient and effective
223 measures to verify the authenticity of the products relies on specific laboratory analyses.
224 The species identification protocol developed in our lab was specifically thought to be applied to
225 both fresh and processed products belonging to different seafood categories, taking into consideration
226 the supplier needs in terms of procedure reliability, cost and time sustainability. Therefore,
227 considering that the specific features of each sample bring differences at various protocol steps
228 (primers coverage, PCR amplification efficiency, level of DNA degradation availability of reference
229 sequences on the official database), a decision tree was developed to follow the most appropriate
230 analytical pathway. The FLB *COI* barcode was selected as first choice and, for maximizing the
231 amplification efficiency, the primer pairs were selected according to the seafood category (fish or
232 mollusks). In the case of *Thunnus* spp., for which the *COI* gene doesn't exhibit sufficient variability
233 within species (Wong and Hanner, 2008; Lowenstein *et al.,* 2009, the analysis relied on the *cytb* gene.

In addition, the *cytb* and the *16S rRNA* were also used as supportive markers of the *COI* if the identity values did not reach 98%. The preliminary evaluation of DNA integrity in case of processed products and the application of two mini-barcode protocols for which the species resolution efficiency had already been proven (Armani et al., 2015) were considered in the decision tree to overcome issue related to DNA degradation. Moreover, the possibility to use a 5′-exonuclease deficient Taq polymerase, specifically verified for amplification performance in presence of several inhibitors (Schrader et al., 2012), represents an option to improve the PCR reaction efficiency in presence of contaminated DNA (as for example those extracted from for mollusk bivalves). All the samples were amplified using the amplification protocol specifically selected according to our decision tree.

About the post sequencing analysis, 92.4% of the FBOs' samples were effectively identified by means of the elective target alone (*COI* or cytb for the tuna products). The introduction of a supporting target to the *COI* gene analysis at the end of the decisional chart offered a decisive improvement of the species disambiguation for a further 3.8% of the analyzed samples and allowed to reach a final overall identification rate of 96.4%. (Table 1) Major limits to the successful species identification were recollected to the low resolution of the molecular target or to the absence of reference sequences.

Particularly, the lack of reference sequences prevented the species discrimination in cephalopod products and confirmed the noticeable need, particularly within Sepiidae family, of databases updating (Wen et al., 2017). Nevertheless, the molecular results were generally found informative enough to state the sample's non-compliance to the label information.

The overall non-compliances rate (18.1%), calculated over the two-year survey, was consistent with the mislabelling percentage highlighted at retail level in the US (Warner et al., 2013) (18%) and in South Africa (Cawthorn et al., 2015) (19%) in the same period. Interestingly, within the cephalopod category, a relative high non-compliance rate was highlighted (18.7%) considering the low number of products analysed. This data suggests that the enlargement of the sampling could result in a rise of the non-compliance and mislabelling rates within the category, as evidenced in Guardone et al. (2017). As already assessed in previous studies (Warner et al. 2013; Di Pinto et al., 2013) most of the

non-compliances (61.3% 19/31) were found in samples belonging to the Order Gadiformes (Gadidae and Merluccidae) and to the family Serranidae (sub family Epinephelinae). As in the study of Mariani et al., (2015) only 2 non-compliances were found among tuna samples. The non-compliances were recollected to deliberate substitution for economic gain purposes or lack of operators' training to species identification (Cawthorn et al., 2015). Significant deliberate substitution cases were highlighted in Atlantic cod, often replaced by haddock (*Melanogrammus aeglefinus*), and grouper, frequently substituted by low or no commercial value species such as Brotola or Doctorfish (*B. multibarbata* and *A. monroviae*) and 1 Atlantic salmon which was identified as Chum Salmon (*Oncorhynchus keta*). All the findings confirmed the need of an effective monitoring of the species identity for these products to prevent economic speculation to the detriment of both consumers and suppliers involved in the fisheries chain (Stawitz et al., 2016). Conversely, the lack of the operators' training plausibly posed the basis of unintentional species substitution in hakes, porgies, amberjack, turbots, clupeids and flying squid products. In these cases, the misidentification generally involved two species belonging to the same genus or family and the substitution is favoured by the species' similar morphological features, habitat overlapping or incidental bycatch phenomena (Table 1). Unintentional substitutions, although generally less relevant in term of economic loss, poses serious concerns about fishery transparency and sustainability since they may involve species below precautionary levels, such as *M. paradoxus* (Helyar *et al.*, 2014).

5. Conclusions

The implementation of a seafood traceability system is becoming a major issue for the different FBO's mainly due to the highly articulate flow from catch to customer purchase which is further complicated by the introduction of outsourcing to third-party countries of raw commodities processing before the reselling to the country of origin or on the international trade market (Costa Leal et al., 2015). In this context, the regular use of standard and cost-effective DNA based analytical methods may represent a great opportunity to improve the self-check programs within the internal traceability system, to achieve customers' satisfaction and protect the company's interests. Thus, the

study confirmed the usefulness of our optimized protocol for seafood identification to support traceability systems.

References

1. Armani A, Guardone L, Castigliego L, D'Amico P, Messina A, Malandra R, Guidi A, 2015b. DNA and Mini-DNA barcoding for the identification of Porgies species (family Sparidae) of commercial interest on the international market. Food Control, 50, 589-596.

2. Armani A, Guardone L, La Castellana R, Gianfaldoni D, Guidi A, Castigliego L, 2015a. DNA barcoding reveals commercial and health issues in ethnic seafood sold on the Italian market. Food Control, 55, 206-214

3. Armani A, Tinacci L, Lorenzetti R, Benvenuti A, Susini F, Gasperetti L, Ricci E, Guarducci M, Guidi A, 2017. Is raw better? A multiple DNA barcoding approach (full and mini) based on mitochondrial and nuclear markers reveals low rates of misdescription in sushi products sold on the Italian market. Food Control, 79, 126-133.

4. Armani A, Tinacci L, Xiong, X., Titarenko E, Guidi A, Castigliego L, 2014. Development of a simple and cost-effective bead-milling method for DNA extraction from fish muscles. Food anal. methods, 4(7), 946-955.

5. Aung MM, Chang YS, 2014. Traceability in a food supply chain: Safety and quality perspectives. Food control, 39, 172-184.

6. Barach JT, 2016. FSMA and Food Safety Systems: Understanding and Implementing the Rules. John Wiley & Sons. Ltd. First Edition. 2017

7. Beil D, 2009. Supplier Selection. Available from: http://www-personal.umich.edu/~dbeil/Supplier_Selection_Beil-EORMS.pdf

8. Cavin C, Cottenet G, Blancpain C, Bessaire T, Frank N, Zbinden P, 2016. Food Adulteration: From Vulnerability Assessment to New Analytical Solutions. CHIMIA International Journal for Chemistry, 70(5), 329-333.

Field Code Changed

311 9. Cawthorn DM, Duncan J, Kastern C, Francis J, Hoffman LC, 2015. Fish species substitution
312 and misnaming in South Africa: an economic, safety and sustainability conundrum revisited. Food
313 chemistry, 185, 165-181.
314 10. D'Amico P, Armani A, Gianfaldoni D, Guidi, A, 2016. New provisions for the labelling of
315 fishery and aquaculture products: Difficulties in the implementation of Regulation (EU) n.
316 1379/2013. Marine Policy, 71, 147-156.
317 11. Di Pinto A, Di Pinto P, Terio V, Bozzo G, Bonerba E, Ceci E, Tantillo, G, 2013. DNA
318 barcoding for detecting market substitution in salted cod fillets and battered cod chunks. Food
319 Chemistry, 141(3), 1757-1762
320 12. European Parliament and Council of the European Union, 2013. Regulation (EU) N.
321 1379/2013 of the Parliament and Council of the European Union of 11 December 2013 on the
322 common organisation of the markets in fishery and aquaculture products, amending Council
323 Regulations (EC) No 1184/2006 and (EC) No 1224/2009 and repealing Council Regulation (EC) No
324 104/2000 Regulation of the European Parliament and of the Council of 29 April 2004 laying down
325 specific hygiene rules for on the hygiene of foodstuffs. In: Official Journal, L 354/1, 28/12/2013.
326 13. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R, 1994. DNA primers for amplification of
327 mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular
328 Marine Biology and Biotechnology, 3, 294-299
329 14. Griffiths AM, Sotelo CG, Mendes R, Pérez-Martín, R I, Schröder U, Shorten, M, Silva HA,
330 Verrez-Bagnis V, Mariani S, 2014. Current methods for seafood authenticity testing in Europe: Is
331 there a need for harmonisation? Food Control, 45, 95-100
332 15. Guardone L, Tinacci L, Costanzo F, Azzarelli D, D'Amico P, Tasselli G, Magni A, Guidi A,
333 Nucera D, Armani A, 2017. DNA barcoding as a tool for detecting mislabeling of fishery products
334 imported from third countries: An official survey conducted at the Border Inspection Post of Livorno-
335 Pisa (Italy). Food Control, 80, 204-216.

16. Handy SM, Deeds JR, Ivanova NV, Hebert PD, Hanner RH, Ormos A, 2011. A single-laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. Journal of AOAC Int., 94(1), 201-210

17. Helyar, SJ, Lloyd HAD, De Bruyn M, Leake J, Bennett N, Carvalho GR, 2014. Fish product mislabelling: failings of traceability in the production chain and implications for illegal, unreported and unregulated (IUU) fishing. PloS one, 9(6), e98691.

18. Kocher TD, Thomas WK, Meyer A., Edwards, S. V., Pääbo, S., Villablanca, F. X., & Wilson, A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences, 86(16), 6196-6200.

19. Leal, M. C., Pimentel, T., Ricardo, F., Rosa, R., & Calado, R. (2015). Seafood traceability: current needs, available tools, and biotechnological challenges for origin certification. Trends in biotechnology, 33(6), 331-336.

20. Lowenstein, J. H., Amato, G., & Kolokotronis, S. O. (2009). The real maccoyii: identifying tuna sushi with DNA barcodes–contrasting characteristic attributes and genetic distances. PLoS One, 4(11), e7866.

21. Mariani, S., Griffiths, A. M., Velasco, A., Kappel, K., Jérôme, M., Perez-Martin, R. I., Schröder, U., Verrez-Bagnis, V., Silva, H.,Vandamme, S. G., Boufana, B., Mendes R., Shorten, M., Smith, C., Hankard, E., Hook, S. A., Weymer, A. S., Gunning, D., Sotelo C. G. (2015). Low mislabeling rates indicate marked improvements in European seafood market operations. *Frontiers in Ecology and the Environment*, *13*(10), 536-540.

22. Palumbi SR,1996. Nucleic acids II: The polymerase chain reaction. Molecular Systematics, 2(1), 205e247.

23. Schrader, C., Schielke, A., Ellerbroek, L., & Johne, R. (2012). PCR inhibitors–occurrence, properties and removal. Journal of applied microbiology, 113(5), 1014-1026.

24. Spink, J., Moyer, D.C., Speier-Pero, C. (2016). Introducing the Food Fraud Initial Screening model (FFIS). Food Control, 69, 306-314.

25. SSAFE (2016). Food fraud vulnerability assessment. Think like a criminal to fight food fraud. Disponibile su http://www.pwc.com/gx/en/food-supply-integrity-services/publications/food-fraud-vulnerability-assessment.pdf.

26. Stawitz, C. C., Siple, M. C., Munsch, S. H., Lee, Q., & Derby, S. A. F. S. (2016). Financial and Ecological Implications of Global Seafood Mislabeling. Conservation Letters

27. Stringer, C. et al. (2011) Shifting post production patterns: exploring changes in New Zealand's seafood processing industry. N. Z. Geog. 67, 161–173

28. Tamm EE, Schiller L, Hanner RH, (2016). Seafood Traceability and Consumer Choice. In Naaum M, Hanner RH. Seafood authenticity and Traceability a DNA based method. a DNA based perspective Elsevier Academic Press, UK,2016

29. Warner K, Timme W, Lowell B, Hirshfield M. (2013). Oceana study reveals seafood fraud nationwide. Available at http:// oceana.org/ reports/ oceana-study-reveals-seafood- fraud-nationwide

30. Wen J, Tinacci L, Acutis PL, Riina MV, Xu Y, Zeng L, Ying X, Chen Z, Guardone L, Chen D, Sun Y, Zhao J, Guidi A, Armani A (2017). An insight into the Chinese traditional seafood market: species characterization of cephalopod products by DNA barcoding and phylogenetic analysis using COI and 16SrRNA genes. Food Control, 82, 333-342.

Figures and Table Captions

Figure 1: Decision tree analysis developed by FishLab and used in this study to select the protocol of analysis

Figure 2: Circle chart of the samples conferred by FBOs as result of their periodic self-checking activity grouped per seafood category.

Figure 3: Distance tree inferred using the Neighbor-Joining method for the analysis of *COI* target sequences obtained from vouchered sequences of *Thunnus* sp, *Auxis thazard*, *Auxis rochei*, *Katsuwonus pelamis*, *Sarda sarda*, *Sarda orientalis*, *Euthynnus alletteratus*, *Euthynnus affinis*. The distance analysis was computed using the kimura 2-parameters model involving a total of 61

388 reference sequences. Bootstrap values (BP) > 70% obtained from 1000 replicates are shown below
389 the branches. The analysis was performed MEGA 6.06. The sequences related to the two commercial
390 samples are highlighted with (●).
391 **Table 1**: Molecular identification and compliance of the information provided by the suppliers. The
392 cases of interfamilial substitution have been highlighted in grey. Cases of substitution that mainly
393 determined economic loss to the detriment of the suppliers are reported in bold. Data about the
394 commercial interest were collected from FishBase (http://www.fishbase.us/search.php) and Sealife
395 (http://www.sealifebase.org/) databases