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(Article begins on next page)

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DNA barcoding as a tool for detecting mislabeling on incoming fishery products from third countries: an official survey conducted at the Border Inspection Post of Livorno-Pisa (Italy)

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27 **Abstract**

28 The importation of fishery products into the European Union (EU) is constantly rising. The aim 29 of this study was to conduct a survey on labeling non-compliances on fishery products imported 30 from non-EU/extra-European countries, in collaboration with the veterinary staff of the Italian 31 Ministry of Health Border Inspection Post of Livorno-Pisa (BIP)- The correspondence between the 32 products' identity and the scientific denominations reported on the accompanying certificates was 33 checked using the DNA barcoding method. Overall, 277 products belonging to different categories 34 (fish, cephalopods, crustaceans, bivalves, amphibian) were submitted to analysis for species 35 identification. The comparison of the molecular results with the scientific names declared on 36 accompanying documents highlighted that 22.5% (95%CI 17.8-28.0) of the analyzed products were 37 mislabeled. The highest percentage was observed on cephalopod based products (43.8%, 95% CI 38 32.3 – 55.9), followed by crustaceans (17.0%, 95% CI 9.2-29.2) and fish (14.0%, 95% CI 8.7-21.9). 39 A higher rate of mislabeling was found in products imported from China, Vietnam and Thailand. 40 This study is the first survey on mislabeling in products sampled at BIPs in Italy. The results 41 highlight the need of implementing analytical checks, based on DNA analysis, on incoming fishery 42 products.

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46 **Keywords**: Seafood products, Border Inspection Post, fraud, mislabeling, DNA barcoding, 47 official controls.

48

1. Introduction

Global fish production has grown steadily in the last five decades and currently seafood is the most traded food commodity in the world (Asche *et al*., 2015). In 2022, according to FAO, the world seafood production is expected to rise to 181 million tons, of which at least 42% will come from aquaculture (FAO, 2014). World *per capita* fish consumption increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 (FAO, 2014) and to meet the domestic demand many Countries worldwide must necessarily import a growing share of seafood from abroad. Currently, the major fish exporting countries are in Asia, where fish production (both from catch and aquaculture) has grown dramatically in the last twenty years, accounting now for about 70% of the global production (FAO, 2014). In the European Union (EU) seafood is largely imported from Eastern countries, especially China and Vietnam. These countries annually export to the Community market 5.3 million tons (9% of the total volume of EU seafood imports from non-EU countries) and 2.9 million tons (5%) of fish, respectively (European Market Observatory for Fisheries and Aquaculture Products, 2015). Asian countries, in particular Thailand, India, China and Vietnam, are also responsible for most of the seafood imported into Italy, followed by African countries such as Tunisia and Morocco and by North America (Italian Ministry of Health, 2015).

The complexity of the trade flows that characterize the fishery sector makes it difficult to trace back seafood origin (Sterling and Chiasson, 2014). Seafood often covers very long distances, changing hands several times among various intermediaries (brokers, wholesalers, processors and retailers) and this can favor the loss of traceability information along the chain as well as encourage frauds and commercialization of Illegal, Unreported, and Unregulated (IUU) fishing products (Miller and Sumalia, 2014; Sterling and Chiasson, 2014; Pramod *et al*., 2014). At a global level, seafood is among the foodstuffs most prone to illegal practices since it represents the second food product (after oil) and the first among foods of animal origin, most affected by frauds (Spink and Moyer, 2011; Johnson, 2014). Therefore, it is imperative that accurate and stringent checks are carried out by official authorities at border posts on incoming foodstuffs.

75 Veterinary border checks are key pillars for preventing the introduction of possible health risks
76 and non-compliant goods into a country and ensuring incoming foodstuffs meet the specific import
77 and transit conditions (Hinrich *et al.*, 2010). In the early 1990's, the EU provided for the
78 establishment in all major Community ports, airports and land borders of veterinary offices called
79 Border Inspection Posts (BIPs) (Hinrich *et al.*, 2010; Department for Environment, Food & Rural
80 Affairs of UK, 2013). According to Council Directive 97/78/EC and Commission Regulation (EC)
81 n. 136/2004 all food of animal originated from an extra-EU country (third country) have to pass
82 through a BIP (which must be authorized to receive that specific category of animal foodstuffs).
83 Currently, in the EU there are 222 veterinary BIPs. However, the list of the approved BIPs, which is
84 laid down in the Commission Decision 2009/821/EC and its amendments, is frequently updated.
85 (Directive 1997/78/CE; Commission Decision 2009/821/EC; Italian Ministry of Health, 2015).
86 Animal foodstuffs covered by the border checks regime are reported in Commission Decision
87 2007/275/EC.

88 Veterinary border controls are a series of documentary, identity and physical checks carried out
89 on each imported consignment or on samples, depending on several factors, such as type and
90 characteristics of consignment, exporting country, exporter reputation, history of non-compliance
91 and latest advice from the European Commission (Hinrich *et al.*, 2010; Department for
92 Environment, Food & Rural Affairs of UK, 2013; European Commission, 2013). All products of
93 animal origin must be pre-notified to the BIP and presented with the correct documentation,
94 including the health certificate issued by the competent authority in the third Country (as required
95 by Commission Regulation (EC) No 2074/2005 and Commission Implementing Regulation (EU)
96 No 1012/2012). Moreover, for fishery products, covered by the Fish Labelling Regulations (Art. 35
97 of the Regulation (EU) n. 1379/2013), accompanying documents must also report the commercial
98 designation and the scientific name of the fish species, the production method, the catch area and
99 the fishing gears used (D'Amico *et al.*, 2016). Moreover, starting from 31st December 2009, also a

100 validated catch certificate, required by the IUU Regulation (Council Regulation (EC) n.
101 1224/2009), has to be presented to the receiving BIP.

102 While documentary and identity checks are performed on all consignments, a physical check is
103 conducted only on a percentage of them. The frequency of physical checks is established by the
104 Commission Decision 94/360/EC (recently amended by Commission Decision 2006/590/EC).
105 According to FAO, there is a general rule of 1-5 percent random sampling at EU BIPs (Ababouch *et*
106 *al*., 2005) but such percentage can increase where serious infringement, such as presence of
107 unauthorized substance or exceeding of a maximum residue limit, are revealed (European
108 Commission, 2013). Physical checks may include sampling the product to detect pathogens or
109 illegal contaminants (veterinary drugs residues or heavy metals) or even physical tests, such as
110 cutting and cooking, sensory testing, control of temperature, weight and wrapping materials
111 (Hinrich *et al*., 2010; Department for Environment, Food & Rural Affairs of UK, 2013).

112 The BIP of Livorno-Pisa (port) is one of the Italian BIPs with the highest volume of traffic,
113 along with that of Genoa (port), Fiumicino (airport) and Malpensa (airport) (Italian Ministry of
114 Health, 2015). In 2015, according to the most recent data of the Italian Ministry of Health, 7383
115 consignments passed through the BIP of Leghorn-Pisa (port) and 78% (5767) of these were fishery
116 products (Fig. 1SM) (Italian Ministry of Health, 2015).

117 Analytical methods based on DNA may be a useful tool to support physical checks, especially
118 for processed fishery products, in order to deter operators from falsely labelling catches and prevent
119 frauds for species substitution. Despite the widespread use of these techniques for research purposes
120 (Cawthorn *et al*., 2015; Pardo *et al*., 2016; Vandamme *et al*., 2016) in assessing the identity of
121 products along the fishery supply chain, the use of this analysis for regulatory forensic programs is
122 still limited (Carvalho *et al*., 2015; Chang *et al*., 2016). However, this could be particularly
123 important in the light of the data provided by the EU Food Fraud Network (FFN) (European
124 Commission, 2015a). The FFN Activity Report 2015 has showed that the highest number of alleged
125 violations concerned fish and fish products and among the most common fraudulent activity (on all

126 food products), there were those related to labelling (36%) and illegal exports (18%) (European
127 Commission, 2015a).

128 The aim of this work was to conduct an analysis based on DNA barcoding to investigate labeling
129 non conformities on fishery products imported from third countries and entering the European
130 Union through the BIP of Livorno-Pisa. In particular, the analysis was conducted to verify the
131 scientific denominations declared on the accompanying documents. The correlation of the products
132 found most at risk of fraud for species substitution with their countries of origin will allow to better
133 address future checks.

134 2. Materials and methods

135 *2.1 Sample collection and tissue sampling*

136 A total of 277 fishery products were collected at the port of Livorno-Pisa BIP between April
137 2015 and June 2016 (Table 1SM-7SM). In particular, the 277 products collected consisted of fish
138 (129, of which 107 frozen, 3 salted or smoked and 19 canned fish, cephalopods (64), crustaceans
139 (53), a mix of cephalopods and crustaceans (6, mainly ready to cook skewers) and bivalves (20)
140 (Table 2). The remaining 5 were diverse products: 1 packet of frog legs, 1 packet of ready to eat
141 sushi, 1 loaf of fish skin and 2 products made of fish eggs. The products showed a wide range of
142 presentations. As regards fish, all the frozen products were unprocessed. Only 11 products were
143 composed of whole specimens, the remaining were filleted (74), beheaded fish (13) and fish slices
144 (12). Among the processed products the 2 smoked fish were whole herring specimens and the only
145 salted product was a fillet of cod. On the contrary, cephalopod products consisted in unprocessed
146 whole specimens (31), mixed rings and arms (14), rings (7), mantle slices (9) and arms (3).
147 Crustacean products consisted of peeled tails (30), whole specimens (9), not peeled tails (5),
148 crustacean meat (3) and claws, legs or half body (6). In addition, as mentioned, 6 products were a
149 mix of cephalopods and crustaceans: these were skewers of shelled shrimp tails and mantle slices
150 (4) or shelled shrimp tails mixed with cephalopods arms and rings. For what concerns bivalves, 12
151 products were not shelled while 8 were shelled.

152 The collected products were brought to the FishLab, Department of Veterinary Science,
153 University of Pisa, where they were visually inspected, registered by an internal code and
154 photographed. Tissue samples were collected and stored at -20 °C until further analysis. For those
155 products which, on the basis of the available information and of the visual inspection, appeared to
156 be composed only of a single seafood species, a variable number of tissue samples were taken, in
157 relation to the number of units in the product. In particular, in the case of products made of a
158 maximum of 4 units, a tissue sample was taken from each of them; in case of 5-10 units 3 samples
159 were taken and in case of more than 10 units 5 samples were taken. In both cases the samples were
160 randomly chosen. In the case of products made of a mix of different species, at least one sample per
161 species was taken. From these 277 products, 1010 tissue samples were obtained: 387 from fish
162 tissue, 310 from cephalopods, 214 from crustaceans, 94 from bivalves and 5 from amphibian.

163 ***2.2 Molecular analysis***

164 *2.2.1 DNA extraction and evaluation of DNA quality and concentration.* Total DNA extraction
165 was performed from all samples starting from 100 mg of tissue as described by Armani *et al*.,
166 (2014). The DNA quality and quantity was determined with a NanoDrop ND-1000
167 spectrophotometer (NanoDrop Technologies, Wilmington, DE, US). In the case of samples which
168 showed low amplification performances (see section 3.2), a total DNA run was performed: one
169 thousand nanograms of the total DNA extracted from the samples was electrophoresed on 1%
170 agarose gel GellyPhorLE (Euroclone,Wetherby, UK), stained with GelRed™ Nucleic Acid Gel
171 Stain (Biotium, Hayward, CA, USA), and visualized on a ultraviolet transilluminator (UVP,
172 Benchtop Variable Transilluminator, Cambridge, UK). DNA fragments' size was estimated by
173 comparison with the standard marker SharpMass™50-DNA ladder and Sharp- Mass™1-DNA
174 ladder (Euroclone S.p.A-Life Sciences Division, Pavia, Italy).

175 *2.2.2. DNA amplification and sequencing*. Different primer pairs for the amplification of
176 mitochondrial and nuclear genes were chosen according to the product category (fish, molluscs,
177 crustaceans, amphibian) and, in the above mentioned cases, the level of DNA degradation.

178 Briefly, three primer pairs were used for the amplification of a long fragment of the
179 mitochondrial *COI* gene (Handy *et al*., 2011; Mikkelsen *et al*., 2007; Folmer *et al*., 1994) and one
180 for a short fragment of the same gene (Armani *et al*., 2015a); two primer pairs were used for the
181 amplification of the mitochondrial gene *16S rRNA*, targeting a long (Palumbi, 1996) or a short
182 (Armani *et al*., 2015b) fragment and one pair for the amplification of the nuclear gene PEPCK
183 encoding the enzime phosphoenolpyruvate carboxykinase (Tsang *et al*., 2008). Details of the
184 primers' sequences, references and PCR conditions are reported in Table 1. Five µL of each PCR
185 product were checked by electrophoresis on a 1.8% agarose gel and the presence of expected
186 amplicons was assessed by a comparison with the standard marker SharpMass™50-DNA ladder.
187 Amplicons were purified and sequenced by High-Throughput Genomics Centre (Washington,
188 USA). The results of the amplification and sequencing of the samples belonging to different product
189 categories have been evaluated and discussed separately.

190 *2.2.3. Sequences analysis and comparison with the databases.* The obtained sequences were
191 analyzed with Clustal W in Bio Edit version 7.0.9. (Hall, 1999). Fine adjustments were manually
192 made after visual checking. All the sequences were submitted to a BLAST analysis on GenBank
193 and analyzed using the Identification System (IDs) on BOLD (Species Level Barcode Records). A
194 match with a sequence similarity of at least 98% was used to designate potential species
195 identification for the *COI* gene (Barbuto *et al*., 2010). For what concerns the *16S rRNA*, a specific
196 identification was attributed only for identity values of 99-100% (Armani *et al*., 2015b), due to the
197 lower interspecific variability of this *locus*. The same cut-off was used for the PEPCK gene.

198 Since the sequences obtained in this study were not derived from voucher samples or expert-
199 identified fish specimens, these sequences were not submitted to any international online database.

200 *2.2.3. Statistical analysis.* The χ^2 test was performed for proportion comparison between the
201 product categories. In particular, fish (frozen, salted, smoked and canned), cephalopods, crustaceans
202 and bivalves categories were compared. Results were considered significant when if $p<0,05$. For
203 each proportion a 95% Confidence Interval (95% CI), with alfa=5% was calculated using Wilson's

204 method (www.openepi.com) and reported on the presented chart (Fig. 1). After the overall
205 significance was assessed k-1 χ^2 were performed in order to better assess the difference, where k
206 indicates number of groups: for the analysis 4 groups were considered, therefore 3 comparisons
207 were made (cephalopods vs crustaceans; cephalopods vs frozen fish; cephalopods vs bivalves).

208 **2.3 Comparison between the molecular results and the scientific name reported on the**
209 **health certificate**

210 The results of the molecular identification obtained after submitting the obtained sequences to
211 the databases were compared with the scientific name of the species declared on the health
212 certificate accompanying the products, in order to highlight cases of species substitution.

213 **3. Results and discussion**

214 **3.1 Molecular analysis: DNA extraction, evaluation, amplification and sequencing**

215 All the samples were extracted obtaining DNA of good quality and yield. Of the total 277
216 products collected, at least one readable sequence was obtained for all unprocessed, salted or
217 smoked fish products (100%), for 15 canned fish (79%), for 59 cephalopods (92.2%), for 41
218 crustaceans (77.4%), for 12 bivalves (60%) and for 5 products composed of a mix of cephalopods
219 and crustaceans (83.3%). The remaining 5 diverse products were all successfully sequenced.

220 *3.1.1 Fish.* Out of the total extracted DNA samples from frozen unprocessed fish products (311),
221 304 were successfully amplified (97.7%) and 288 gave a readable sequence (92.6%) targeting the
222 *COI* gene. This category showed the highest sequencing rate. In particular, 247 long *COI* fragments
223 and 41 short *COI* fragments were obtained. The results are reported in Table 2 and in Table 1SM.

224 All the DNA samples extracted from smoked and salted products (5) were successfully amplified
225 and sequenced targeting the *COI* gene, obtaining, 2 full *COI* barcodes and 3 mini *COI* barcodes
226 (Table 2 and Table 1SM).

227 The *COI* gene was not amplifiable from canned products. Thus, a short fragment of the *16S*
228 *rRNA* was targeted. Out of the total extracted DNA samples (62), 48 were successfully amplified
229 (77.4%) and 45 gave a readable sequence (72.5%) (Table 2 and Table 2SM).

230 *3.1.2 Cephalopods.* Of the total extracted DNA samples (278), 232 were successfully amplified
231 (83.4%) and 223 gave a readable sequence (80.2%). For those samples that failed amplification of
232 the *COI* gene using Mikkelsen *et al*., (2006) or Folmer *et al*., (1994) primers, the alternative target
233 *16S rRNA* was amplified by using the primer pair proposed by Palumbi, (1996) (Table 1). Overall,
234 180 long fragments of the *COI* gene and 43 long fragments of the *16S rRNA* gene were obtained
235 (Table 2 and Table 3SM).

236 *3.1.3 Crustaceans.* Regarding crustaceans, 181 DNA samples were extracted, 152 were
237 successfully amplified (83.9%) and 142 gave a readable sequence (78.4%). The gene encoding for
238 PEPCK was chosen as the first target, obtaining 129 sequences. If products were not amplifiable
239 with this approach the *COI* was targeted using the primers of Folmer *et al*., (1994) and 13 additional
240 sequences were obtained (Table 2 and Table 4SM).

241 *3.1.4 Mixed products made of cephalopods and crustaceans (skewers).* Thirty DNA samples
242 were extracted from cephalopod tissue and 30 from crustacean tissue . Twenty-six samples of DNA
243 samples extracted from cephalopods were successfully amplified (86,7%) and 22 sequences were
244 obtained (73.3%). For these samples the primers of Folmer *et al*., (1994) were used for the
245 amplification of the *COI* gene, obtaining 18 sequences. Only for one product which failed
246 amplification of the *COI* gene the *16S rRNA* gene was targeted and 4 long sequences were obtained.
247 For what concerns crustacean tissues, 19 amplicons (63,3%) and 14 readable sequences were
248 obtained targeting the PEPCK gene(Table 2 and Table 5SM).

249 *3.1.5 Bivalves.* Only 53 (56.4%) of the bivalves' DNA samples (94) were successfully amplified,
250 giving 50 readable sequence (53.2%). Thirty-two *COI* sequences were obtained using the primers
251 designed by Mikkelsen *et al*., (2006) and Folmer *et al*., (1994). For those samples that failed
252 amplification the *16S rRNA* fragment of Palumbi, (1996) was targeted and 18 additional sequences
253 were obtained (Table 2 and Table 6SM).

254 *3.1.6 Diverse products.* Only a short *COI* gene fragment was successfully amplified from fish
255 eggs, while a long *COI* gene fragmentwas successfully amplified from all the 3 samples of fish skin

256 and from 4 out of 5 frog tissue samples. As for the ready to eat sushi product, 4 long fragments of
257 the *COI* gene were obtained from the fish and the cephalopod samples, while the 3 crustacean
258 samples were successfully amplified targeting the PEPCK gene.

259 **3.2 Comparison of molecular results with the scientific name reported on the health**
260 **certificates: assessing the mislabelling rate**

261 On the basis of the comparison between the molecular results and the scientific denomination
262 reported on the accompanying documents, results were classified in different categories. A first
263 distinction was made between molecular results that allowed an identification to species level or
264 not. When the result allowed specific identification, two possibilities occurred: i) the identified
265 species matched the species declared on the label or ii) the identified species did not match the label
266 declaration. On the other side, when the result did not allow specific identification, other
267 possibilities occurred: i) the molecular result matched the declared genus, ii) the molecular result
268 match the declared family, iii) the molecular result, although not specific, allowed to highlight the
269 presence of mislabelling, iv) the molecular results and the declared information were not
270 comparable. This latter case is generally due to the absence of reference sequences in the databases.
271 The results are described according to the different categories in the following sections and shown
272 in details in the corresponding SM tables. They are also summarized in Table 2.

273 *3.2.1 Fish.* Of the 288 sequences obtained from frozen fish samples, 142 (49.3%) allowed
274 identification to species level. Of these, 125 (43.4%) matched with label declaration, while 17
275 (5.9%) did not. Of the other 146 sequences (50.7%) for which identification to species level was not
276 possible, 104 (36.1%) and 21 (7.3%) matched the declared genus or family, respectively, while in
277 7.3% of the cases (21 sequences) a mislabelling was observed (Table 2 and Table 1SM). A
278 difference was observed in the discriminatory performance of the full and the mini *COI* barcode:
279 while the full *COI* barcode allowed specific identification for 55.1% of the sequences, the mini *COI*
280 barcode was discriminant only for 14.6% of them. However, in almost 50% of the cases the mini
281 barcode still allowed identification to the genus level.

282 Overall, mislabelling was identified in 15 (14%) different products made of unprocessed frozen
283 fish (Fig. 1). Details on the mislabelling are reported in Table 3 and described in section 3.3.
284 For what concerns smoked products, 2 full DNA barcodes and 2 mini DNA barcodes were
285 identified to species level and matched with label declaration (*Clupea harengus*), while 1 mini
286 DNA barcode obtained from the salted cod only allowed assignment to the family Gadidae.
287 Regarding canned products, 18 (40%) sequences, belonging to the 5 sardine products, were
288 identified to species level and the retrieved species matched the label information. The remaining
289 products, made of tuna and mackerel, were not identified at the species level This is likely due to
290 the fact that the *16S rRNA* gene does not represent the elective marker for species discrimination
291 within the Scombridae family. Other molecular markers have been proposed to get a precise species
292 discrimination within the genus *Thunnus* such as cytochrome b (cytb) and nuclear First Internal
293 Transcribed Spacer for rDNA (ITS-1) (Santaclara *et al*., 2015). However, sometimes two or three of
294 these markers need to be targeted in the same analysis (Vinas & Tudela, 2009). Considering the
295 high number of primers pairs and of samples involved in the project, we decided not to further
296 investigate these canned products in the present work and to exclude them from the mislabelling
297 rate. However, it is worth to highlight that *16S rRNA* amplicons were the only obtained from highly
298 degraded DNA extracted from canned products. Therefore, the mislabelling rate was not evaluated
299 for salted and canned fish products.
300 *3.2.2 Cephalopods.* Of the 223 sequences obtained from cephalopods, 201 (90.1%) allowed
301 identification to species level. Of these, 122 (54.7%) matched with label declaration, while 79
302 (35.4%) did not. Of the remaining 22 sequences (9.9%) for which identification to species level was
303 not possible, 7 (3.1%) and 3 (1.3%) matched the declared genus or family, respectively, while in in
304 5.4% of the cases (12 sequences) a mislabelling was observed (Table 2 and Table 3SM). Thus, in
305 this category mislabelling was identified totally in 28 products (43.8%) (Fig. 1).
306 *3.2.3 Crustaceans.* Of the 142 sequences obtained from crustaceans, 82 (57.7%) allowed
307 identification to species level. Of these, 73 (51.4%) matched with label declaration, while 9 (6.3%)

308 did not. The other 60 sequences (42.3%) for which identification to species level was not possible
309 gave the following results: 19 (13.4%) and 18 (12.7%) matched the declared genus or family,
310 respectively; 12 (8.5%) showed a mislabel and in 11 cases (7.7%) the match was not verifiable
311 (Table 2 and Table 4SM). Overall, in this category mislabelling was identified in 9 products (17%)
312 (Fig. 1).

313 *3.2.4 Mixed products made of cephalopods and crustaceans.* From the mixed products 36
314 sequences were obtained, of which 29 (80.5%) allowed specific identification. Of these, 18 (50%)
315 corresponded with the certificates' declarations, while 11 (30.6%) showed a substitution. Of the
316 remaining 7 sequences (19.4%), for which the identification at the species level was not possible, 4
317 (11.1%) agreed with the documents regarding the family, while 3 (8.3%) showed a mislabelling
318 (Table 2 and Table 5SM). Thus, a mislabelling was found in 4 different products (66% of the
319 products) (Fig. 1). In particular, in two products the species of the class Cephalopoda were
320 substituted, in one the crustacean species and in another one both.

321 *3.3.5 Bivalves.* A total of 50 sequences were obtained from bivalves. Of these, 41 (82%%)
322 retrieved a specific identification: 36 (72%) matched with label declaration, while 5 (10%) were
323 mislabelled. Of the other 9 sequences (18%) for which specific identification was not achieved, 6
324 (12%) and 3 (6%) matched the declared genus or family, respectively (Table 2 and Table 6SM).
325 The 5 non corresponding sequences belonged to one single product (5% of the total number of
326 products) that was declared *Meretrix lyrata* and was identified as *Gafrarium divaricatum*.

327 *3.3.6 Diverse products.* The 2 products made of fish eggs were identified to species level and the
328 retrieved species corresponded to the declared ones (*Zeus faber* and *Thunnus albacares*). The same
329 occurred for the product consisting of frozen frog legs that were identified as *Hoplobatrachus*
330 *rugolosus*, matching the label declaration. For what concerns the fish skin loaf, declared to be
331 *Oreochromis niloticus*, the obtained sequences only allowed identification to the genus level
332 (*Oreochromis* spp.), due to similarity of the *COI* gene in congeneric species. Finally, for what
333 concerns the ready-to-eat sushi product, the sequences derived from the fish and crustacean samples

334 matched the species declared in the label (*Salmo salar* and *Litopenaeus vannamei*), while those
335 retrieved from cephalopod samples showed the substitution of *Uroteuthis chinensis* with *U.*
336 *duvaucelii*.

337 **3.4 Analysis of the cases of mislabelling**

338 The mislabelling rates found in the different categories were: 14.0% (95% CI 8.7-21.9) of the
339 frozen fish products, 43.8% (95% CI 32.3-55.9) for cephalopod based products, 17% (95% CI 9.2-
340 29.2) for products made of crustaceans, 66.7% (95% CI 30.0-90.3) for products composed of a mix
341 of cephalopods and crustaceans, and 5% (95% CI 0.9-23.6) for bivalves (Fig. 1). No cases of
342 mislabelling were observed in smoked fish and in the diverse products (frog legs, fish skin and
343 eggs), except for the cephalopod sample in the ready-to-eat sushi. Results shows that the percentage
344 of mislabeled products in the cephalopod category was greater than in the other categories (χ^2=
345 31,42 $p<0,01$) , whereas the difference in proportion of mislabeled products between crustaceans,
346 bivalves and frozen fish was not statistically significant (χ^2=2,11 p=0,35).

347 The average value of mislabelling calculated on the total number of the analysed products was
348 22.5%.(95% CI 17.8-28) Interestingly, this value confirms the results of a recent report published
349 by Oceana, in which the results of more than 200 studies on mislabelling conducted in 55 globally
350 distributed countries were analysed
351 (http://usa.oceana.org/sites/default/files/global_fraud_report_final_low-res.pdf). In fact, from the
352 results of the report, issued from the analysis of more than 25,000 samples of fishery products, it
353 was found that problems related to the replacement of species affected one in five samples.

354 Mislabelling cases have been categorized and separately discussed in the following sections. The
355 results are also collected in Table 4.

356 *3.4.1 Substitutions between species belonging to the same genus.* In the cases of congeneric
357 species presenting high morphological similarities, overlapping distribution areas and shared
358 habitats, their erroneous identification can be the direct result of an unexperienced or not properly
359 formed operator. In fact, the increase of the variety of fish species fished and traded globally,

360 challenges morphological identification by operators (Rehbein, 2008; Armani *et al.*, 2015a). This
361 might have happened when the substitutive and the declared species presented a similar commercial
362 value. Rather than to intentional frauds, these cases may be related to an insufficient preparation of
363 the personnel.

364 Concerning fishes, the likely unintentional mislabelling cases highlighted in this study involve 5
365 different genera. In fact, considering also the *post mortem* partial or total loss of livery colour, the
366 morphological characters distinguishing *Psettodes belcheri* and *Psettodes bennetti*, *Epinephelus*
367 *areolatus* and *Epinephelus bleekeri*, *Merluccius paradoxus* and *Merluccius capensis*, *Mustelus*
368 *mustelus* and *Mustelus punctulatus*, *Synaptura cadenati* and *Synaptura lusitanica*, may not be easily
369 appreciable (Govindaraju and Jayasankar, 2004; see also the specific pages on
370 http://www.fishbase.org/). The poor training of operators in discriminating between related species
371 may be confirmed by the fact that some products for which mislabelling was found (*Psettodes* spp.,
372 *Mustelus* spp. and *Synaptura* spp.) originated from developing countries, such as Mauritania and
373 Senegal

374 As regards the 2 cases of intra-genus substitutions involving *Epinephelus* spp., only 1 out of the
375 3 examined samples did not correspond to the declared species. Considering the partial substitution
376 and the small size of the fillets and thus of the fished specimens (often young specimens are very
377 similar among related species, Govindaraju and Jayasankar, 2004), it is possible to speculate that
378 the presence of a different species may be due to the casual presence of a small number of
379 specimens in the lot due to the by-catch. In relation to the small size of the fillets, we need to
380 emphasize that fishing juvenile stages, other than constituting a further element of difficulty in the
381 identification of species, it can be considered one of the causes of depletion of fish stocks globally
382 (Froese, 2004).

383 For what concerns *Seriola dumerilii* and *Seriola quinqueradiata*, although they are
384 morphologically similar, *S. dumerilii* is worldwide distributed and generally wild caught, *S.*
385 *quinqueradiata* is only found in the Asiatic region, where it is also intensively farmed. Due to this

386 fact, its presence on Asiatic markets is constant and this has led to a decrease in its commercial
387 value (http://www.fao.org/fishery/culturedspecies/Seriola_quinqueradiata/en). Thus, in this case it
388 is plausible to hypothesize an intentional economically motivated adulteration.

389 Twenty-one cases of substitution among species of the same genus were found for cephalopods,
390 involving 4 genera: *Loliolus* spp., *Sepia* spp., *Sepiella* spp. and *Uroteuthis* spp.. Almost all these
391 non-conformities were found in products imported from Asian countries. As for fish species, it is
392 plausible to hypothesize that most of these mislabelling may be due to the presence of similar
393 species in the same fishing grounds. This can be the case of the substitution of *Loliolus japonica*
394 with *L. beka*, of *Sepiella japonica* with *Sepiella inermis*, of *Sepia officinalis* with *Sepia hierreda*, or
395 of the several substitutions (of part or all the samples of the product) found between *Uroteuthis*
396 *chinensis*, *Uroteuthis edulis* and *Uroteuthis duvaucelii*. For what concerns the genus *Sepia*, on the
397 contrary, considering that *Sepia aculeata* and *Sepia pharaonis* present different morphological
398 characteristics that may be appreciated even by non-experts
399 (http://www.sealifebase.org/Photos/ThumbnailsSummary.php?ID=57882;
400 http://www.sealifebase.org/Photos/ThumbnailsSummary.php?ID=57301), it is possible to
401 hypothesize that the substitution is unintentional and it is likely due to limits in labelling rules
402 awareness, since both species have a high commercial value. This is confirmed by the fact that both
403 species were found alternatively substituted.

404 As regards crustaceans, the only substitution among species of the same genus was found for
405 *Metapenaeus* spp., where the declared species *Metapenaeus monoceros* was substituted with the
406 species *Metapenaeus affinis*. These species share similar anatomical characters and geographical
407 distribution, so also in this case the mislabelling may be considered unintentional.

408 *3.4.2 Substitutions between species belonging to the same family.* Also in this case some species
409 substitutions may be caused by the inexperience of operators in distinguishing related species.

410 As regards fishes, this could be the case of the substitution of *Lepidotrigla microptera* with
411 *Chelidonichthys* spp.. Notwithstanding the morphological similarities between the species of these

412 genera, the high frequency and the recurrence of this substitution (total substitution in 6 products
413 from China), highlights again the existence of traceability and label issues in fishery products in
414 China (Xiong *et al*., 2016a, b, c).

415 On the countrary, the substitution of *Limanda aspera* with *Hippoglossoides* spp., may be
416 considered intentional due to their morphological differences and to the fact that while *L. aspera*
417 has a high commercial value, the two species belonging to the genera *Hippoglossoides* are of scarce
418 economic interest. Similarly, another possible example of intentional fraud may be represented by
419 the substitution of *Theragra chalcogramma* (pollack d'Alaska) with *Boreogadus saida*, considered
420 of low commercial value (http://www.fao.org/fishery/species/2233/en).

421 As regards cephalopods, probable accidental substitutions due to limits in labelling awareness
422 may have occurred in the case of *Cistopus indicus* substituted with *Amphioctopus* spp., or of
423 *Uroteuthis duvaucelii* with *Heterololigo bleekeri*. A different story may be hypothesized for the
424 products for which the declared species was *Octopus membranaceus*. In fact, its substitution with
425 *Amphioctopus fangsiao* may be explained considering that the stocks of the former species are
426 depleted and it is very rarely fished (FAO, 2016), while *A. fangsiao* is not included in the Italian
427 official list of seafood denominations. Therefore, selling a little known species under the name of a
428 highly commercial and depleted species may consent economic advantages. The same hypothesis
429 applies to the products in which *O. membranaceus* was substituted with *Cistopus* spp.

430 Also for crustaceans, in addition to unintentional or accidental mislabelling, some of them may
431 have been perpetrated with the aim of commercializing little known species with more common
432 ones. This might be the case of *Metanephrops thompsoni* substituted with species of the genus
433 *Nephropsis* spp., of *Metapenaeus affinis* substituted with *Metapenaeopsis* spp., or of *Litopenaeus*
434 *vannamei* substituted with *Parapenaeopsis* spp..

435 As for bivalves, the only mislabelling encountered was between *Meretrix lyrata* and *Gafrarium*
436 *divaricatum*. Considering the morphological differences of the two species, this substitution is
437 likely to be voluntary.

438 Particular attention must be given to the 6 products containing a mixture of cephalopods and
439 crustaceans, since mislabelling was found in 4 of them. Although the majority of the substitutions
440 may be unintentional, the case of product PIF265 is particularly interesting, since in this product all
441 the 3 species of cephalopods declared (*U. duvaucelii*, *S. pharaonis*, *O. membranaceus*) were found
442 to be substituted (*U. edulis, S. aculeata, Cistopus* spp.). In addition, the only species of crustacean
443 declared was also mislabelled (*Metapenaeus dobsoni* substituted with *Parapenaeopsis cornuta*).
444 Although, except for the substitution involving *O. membranaceus,* these intra-genus and intra-
445 family replacements can be considered accidental due to morphological similarities, they highlight
446 strong limits in species identification and product traceability.

447 *3.4.3 Substitutions between species belonging to the same order*

448 The only substitution occurring between species belonging to the same order, but to different
449 families, regarded *Arnoglossus kessleri* which was replaced with *Citharus linguatula.* This
450 substitution is likely due to shortfalls in the traceability legislative framework. .

451 **3.5 Relationship between countries of origin and mislabelling**

452 The majority of the products analysed in this study came from Asia (75.39%), followed by
453 Africa (18.46%), reflecting the high number of exports to the EU by developing countries in those
454 geographic areas (Smith *et al*., 2010) and confirming the data of the Italian Ministry of Health
455 (2015). In Fig. 2 the declared countries of origin for each category of product are shown. In
456 particular, 31.5% of collected samples had a Chinese origin. This high rate is not surprising
457 considering that China is one of the main producers of fishery products (FAO, 2014). As regards
458 mislabelling cases, the third countries most frequently involved were China, Vietnam and Thailand,
459 which were also among the main importers by number of products (Table 4). A recent survey aimed
460 at analyzing the Chinese legislative framework in the seafood compart highlighted the lack of a
461 mandatory legislation on seafood traceability and of an official naming system (Xiong *et al*.,
462 2016a). Moreover, molecular analysis conducted on Chinese products highlighted an impressive

463 rate of mislabeling and substitution with toxic or endangered species (Xiong *et al*., 2016b; Xiong *et*
464 *al*., 2016c).

465 In 2016, of the total number of notifications transmitted through the Rapid Alert System for
466 Food and Feed (RASFF), about 18% of these were related to seafood and in particular fishery
467 products (63.5%), bivalve mollusks (16.5%), crustaceans (12.8%) and cephalopods (7.2%) (RASFF
468 Portal, 2016). Among the third countries with the highest number of notifications there are those of
469 Asian (mainly Thailand, Vietnam and China) and African (Ghana and Senegal) origin (RASFF
470 Portal, 2016). The data concerning Asian countries in particular are perfectly in line with the issues
471 observed in the present study. However, of the total RASFF notifications concerning seafood, only
472 a very low percentage (0.15%) was due to labeling problems (absent/incomplete/incorrect) in 2016.
473 The percentage varied from 0.01% to 0.18% between 2010 and 2015 (Table 5).

474 These percentages do not represent an accurate estimate of mislabeling cases involving fisheries
475 products at EU border level. This is because RASFF data include mislabeling cases detected not
476 only at BIPs but also in intra-Community trade and at local level (within each Member State)
477 (RASFF, 2015). Moreover, it must not be underestimated that at the BIPs, physical and laboratory
478 checks are not carried out on each consignments and therefore the mislabeling cases detected at
479 BIPs do not rely on molecular analysis but just on documentary checks (Hinrich *et al*., 2010;
480 Department for Environment, Food & Rural Affairs of UK). Considering that document checks
481 mainly focus on the verification of the approval number of the establishment of origin, product
482 description, batch numbers and production dates, only the cases of broken labels, discrepancies
483 between label and accompanying documents and fraudulent trademarks, descriptions or stamps can
484 be revealed during border controls (European Commission, 2013). Therefore, other types of fraud
485 that need specific analysis, such as fish substitution, are not usually detected. Border controls on
486 fishery products are limited not only at European level, but also in the United States were is
487 estimated that less than 2% of incoming seafood is inspected specifically for fraud (Warner *et al*.,
488 2013). Therefore, it is likely that the data on mislabeling given by the RASFF are underestimated.

489 In this regard, data emerging from the coordinated testing program on fish species substitution,
490 organized by the European Commission (after horsemeat scandal) and based on analysis of
491 molecular identification, are more indicative (European Commission, 2015b). In 2015, during
492 official controls, 27 Member States and 2 European Free Trade Association (EFTA) Member States
493 collected 3906 samples of fish (predominantly white fish species) at different stages of the food
494 chain, including BIPs (European Commission, 2015b). The results showed that 6% of unprocessed
495 fish samples and 5% of processed ones were mislabeled. As it regards specifically the samples
496 taken at the BIPs, 7% of the total (135) resulted mislabeled with regard to the species declared on
497 the label (European Commission, 2015b). A higher mislabeling rate (14% for unprocessed fish
498 products, 11.6% for all fish products) was found in the present study. Many similar labelling issues
499 were found concerning species belonging to cod, haddock, grouper and flat fish
500 (https://ec.europa.eu/food/sites/food/files/safety/docs/official-controls_food-
501 fraud_fish_test_substitution_table3.pdf). However, in our study cephalopods, which were not
502 included in the EU study, were found to be the product most at risk for mislabeling.

503 **4. Conclusions**

504 While confirming the third countries characterized by the highest number of notification
505 (RASFF Portal, 2016) as those at highest risk of frauds for species substitution, discrepancies
506 between the available data concerning mislabelling at the EU level (RASFF Portal, 2016; European
507 Commission, 2015b) and the results of the present study were highlighted. Moreover, our data show
508 that, in addition to white fish, other categories of products, such as those made of cephalopods or of
509 a mix of cephalopods and crustaceans, are at high risk of mislabelling. Therefore, the
510 implementation of appropriate sampling plan (on the basis of the product category and of the third
511 country) together with the application of analytical methods (DNA barcoding) for the official
512 control of incoming fishery products is needed.

513

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516

517

518 **Figure captions**

519

520 **Fig. 1**. Mislabeling rates in the different categories of analyzed products. Error bars indicate the
521 95% Confidence intervals (CI). Salted/smoked products, mixed products made of cephalopods and
522 crustaceans and the diverse products were excluded due to their low number. Canned products were
523 excluded since mislabeling rates were not assessed due to technical problems. The continuous
524 horizontal line indicates the average mislabeling rate and the upper and lower discontinuous lines
525 indicate the 95% CI.

526

527 **Fig. 2**. Geographical origin of the products in relation to the different categories.

528

529

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