Authentication of ready-to-eat anchovy products sold on the Italian market by BLAST analysis of a highly informative cytochrome b gene fragment

Alice Giusti[1*¶], Lara Tinacci[1¶], Carmen G. Sotelo[2], Pier Luigi Acutis[3], Nicola Ielasi[1], Andrea Armani[1]

[1]*FishLab, Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, 56124, Pisa (Italy);*

[2] *Instituto de Investigaciones Marinas (IIM-CSIC), Rúa Eduardo Cabello 6, 36208 Vigo, Spain*

[3]*Experimental Institute of Zooprophylaxis Piedmont, Liguria and Aosta Valley, 10154 Turin, Italy;*

¶ These authors equally contributed to this work

*corresponding author:

Postal address: FishLab, Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, 56124, Pisa (Italy)

Tel: +390502210207; Fax: +390502210213

Email: alice.giusti183@outlook.it

Abstract

In this study, 111 ready-to-eat anchovy products were collected on the Italian market. The products were molecularly identified through a BLAST analysis of a highly informative *cytb* fragment amplified by a newly designed primer pair for the genus *Engraulis spp.* and the mislabelling rate was assessed. In addition, the labels were analysed in the light of the current EU law. Despite only one mislabelling case was observed (mislabelling rate 0.9%), which involved the substitution of the European anchovy (*Engraulis encrasicolus*) with the low-valuable Peruvian/Chilean anchoveta (*Engraulis ringens*), the molecular technique developed in this study was proved as suitable tool for detecting species in processed anchovy products. It could be therefore applied to carry out more extensive EU survey aimed at evaluating the mislabelling rate of such products, still poorly covered by a targeted and clear legislation system.

Keywords

Anchovy; *Engraulis spp.*; anchoveta; species identification; *cytb*; mislabelling

1. Introduction

Consumer protection throughout the single market is one of the EU policy benchmarks and the obligation they are appropriately informed about the food they consume has been fixed as key point by the General Food Law (Regulation (EC) No 178/2002). In this respect, the labelling of foodstuff represents a basic tool for guaranteeing traceability throughout the production and distribution chain, from the raw material supplier to the end-consumer. The Regulation (EU) No 1169/2011 on the provision of food information to consumers has been precisely issued to achieve a high level of health protection for consumers and to guarantee their right to information. Furthermore, such provisions consider the well-known food fraud phenomena that over the years have undermined consumer confidence and damaged the whole EU food supply chain and contribute to tackle this problem (European Parliament Resolution 2013/2091).

Food fraud is committed when food is illegally placed on the market with the intention of deceiving the customer, usually for financial gain (FAO, 2018). Intentional mislabelling and species substitution in seafood products, which generally occur when low-value or less-desirable fish species are swapped for more expensive varieties, are among the most reported kind of frauds at international level. Such practices are favoured by the fact that seafood chain is often long and complex, involving many food-business operators and an extremely wide range of species (i.e. 1200 different species being marketed in Europe). In addition to financially damage consumers, frauds may constitute an important global threat to sustainable fisheries as encouraging activities of Illegal, Unreported and Unregulated (IUU) fishing (Helyar et al., 2014; Petrossian, 2015). Health issues may even occur if poisonous species accidentally enter in the seafood chain (Giusti et al., 2016).

Available literature reported several mislabelling cases involving different kinds of products marketed within the EU in the last decade (Garcia-Vazquez et al., 2010; Pardo, Jiménez, & Pérez-Villarreal, 2016; Sotelo et al., 2018), even though an apparent reduction was observed during the last years. This trend was mainly attributed to the recent efforts in EU legislation that have played a pivotal role in shaping a more transparent market (Mariani et al., 2015). In this respect, in 2013

specific dispositions on seafood products labelling were provided by the Regulation (EU) No 1379/2013 on the common organisation of the markets in fishery and aquaculture products. Mandatory consumer information should include (a) the commercial designation of the species and its scientific name, (b) the production method ("caught", "caught in freshwater" or "farmed"), (c) the area where the product was caught or farmed, and the category of fishing gear used in capture of fisheries, (d) whether the product has been defrosted and (e) the date of minimum durability, where appropriate. However, as highlighted by D'Amico, Armani, Gianfaldoni, & Guidi (2016), the exclusion of some kinds of prepared and processed products from the application of thise Regulation ~~(EU) No 1379/2013~~ represents a significative shortcoming. Basically, these products only fall under the field of application of the Regulation (EU) No 1169/2011, which factually excludes the mandatory information above listed for seafood products. For this reason, with the Resolution No 2016/2532, the European Parliament encouraged the Member States, in the context of voluntary labelling, to state all available information that enables the consumer to make an informed choice (European Parliament Resolution No 2016/2532).

Semi-preserved anchovies are traditionally consumed within EU. Spain and Italy, the second and the fourth anchovy world producers respectively, are even the major EU consumers, covering alone the 71% of the total EU consumption (EUMOFA, 2018). In Italy, anchovies are mainly consumed in form of ready-to-eat products, i.e. salted, marinated or in oil. Except for salted products, that fall within the scope of Regulation (EU) No 1379/2013, tracing back the anchovy species used in marinated or in oil products may prove tricky since, as detailed above, no information on the scientific name and the catching area should be mandatory reported on the label of such products, except in form of voluntary claims.

Recently, Velasco, Aldrey, Pérez-Martín, & Sotelo (2016), which applied DNA-based methods for assessing the labelling accuracy of Spanish semi-preserved anchovies' products, highlighted a mislabelling rate higher than 15% and reported the Argentine anchovy (*Engraulis anchoita*) as the most substituted species. *Cytb* gene has been proved to be able to differentiate species belonging to

the Engraulidae family (Santaclara et al., 2006; Velasco et al., 2016). In particular, it was ~~*Cytb* was chosen as molecular marker as~~ reported as more suitable to discriminate some *Engraulis spp.* respect to the cytochrome oxidase I (COI) gene (Jérôme et al., 2008).

In this study, a PCR primer pair was designed for amplifying a highly informative fragment, proved as polymorphic among *Engraulis spp.,* from the mitochondrial cytochrome b (*cytb*) gene of processed anchovies. Then, a BLAST analysis was performed for molecularly identifying ready-to-eat anchovy's products sold on the Italian market. In addition, the labelling accuracy of the products was assessed. This study was proposed both as a survey for assessing which anchovy species are mainly used for manufacturing commercial products sold within the Italian market and as a useful tool for properly detecting mislabelling accidents involving this seafood category.

2. Materials and Methods

2.1 Sampling

A total of 111 ready-to-eat products (RTEs) made of anchovies, belonging to three different categories "salted" (n= 30), "marinated" (n=31) and "in oil" (n=50), were sampled in Tuscany (Northern Italy), at different points of sale of large-scale retail distribution (Table 1). A convenience, non-probabilistic sampling was conducted, structured to include a proportional number of products per type, according to the market supply and the brands variety.

2.2 Label analysis

Firstly, as already performed by Velasco, Aldrey, Pérez-Martín, & Sotelo (2016), the label accuracy was evaluated in the light of the mandatory information required by Regulation (EU) No 1169/2011. In detail, for each product, the presence of the commercial denomination, the ingredient list, the net and drained weight, the conservation instructions, the best before date, the company name or code and the batch number was assessed. Only for salted products, the presence of mandatory information required by Regulation (EU) No 1379/2013 (the commercial designation of the species and its scientific name, the production method, the area where the product was caught, and the category of used fishing gear and the date of minimum durability) was also verified. For marinated

and in oil RTEs voluntary claims on the species scientific name and on the catching area were considered when reported.

2.2 Molecular analysis

2.3.1 Total DNA extraction and evaluation. Total DNA extraction was performed starting from ~100 mg of tissue following the protocol described by Armani et al. (2014). The quality and quantity of the DNA from each sample were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, US). One microgram of DNA was electrophoresed on 1% agarose gel Gelly-PhorLE (Euroclone, Wetherby, UK), stained with GelRed™Nucleid Acid Gel Stain (Biotium, Hayward, CA, USA), and visualized via ultraviolet transillumination. DNA fragment size was estimated by comparison with the standard marker SharpMass™50-DNA ladder and Sharp-Mass™1-DNA ladder (Euroclone S.p.A-Life Sciences Division, Pavia, Italy). Each DNA sample was stored at -20°C until further analysis.

2.3.2 PCR Primer pair design. DNA sequences of the complete mitochondrial cytochrome b (*cytb*) gene belonging to the *Engraulis spp.* reported as valid on the official finfishes' database (www.fishbase.org) were retrieved from NIH genetic sequence database GenBank® (https://www.ncbi.nlm.nih.gov/genbank/). In detail, at least twenty sequences from the species *E. australis*, *E. encrasicolus* and *E. japonicus* and only the ~~unique~~ available sequences from *E. albidus* (n=1), *E. anchoita* (n=2), *E. eurystole* (n=1), *E. mordax* (n=3) and *E. ringens* (n=1) were retrieved. When possible, co-specific sequences belonging to specimens from different geographical areas were retrieved, given the reported intra-specific heterogeneity of *Engraulis spp.* (Jérôme et al., 2008), and especially the considerable number of haplotypes within both *E. encrasicolus* and *E. japonicus* (Magoulas, Castilho, Caetano, Marcato, & Patarnello, 2006; Yu et al., 2005). No sequences were available for *E. capensis* that, although reported as valid species on Fishbase (www.fishbase.org), was instead considered as unaccepted by the World Register of Marine Species (www.marinespecies.org) and classified as a synonym of *E. encrasicolus.* All the retrieved sequences were aligned in with Clustal W in BioEdit version 7.0.9 (Hall, 1999). The primer pair ANCH-531_F

157 (5'-GTTCTTYGCCTTCCACTTCYT-3') and ANCH-1059_R (5'-
158 YACTTGRCCAATAATAATGAATGG-3') was designed to amplify a 484 bp fragment according
159 to the parameters proposed by Giusti et al. (2016), and especially a) considering the level of DNA
160 degradation observed by electrophoresis (section 2.3.1), b) avoiding primer mismatches in critical
161 position, c) taking into consideration the inter-species variability of the selected DNA fragment.
162 *2.3.3 DNA amplification and sequencing.* DNA samples obtained from all RTEs were amplified
163 with the following PCR protocol: 20 µl reaction volume containing 2 µl of a 10X buffer
164 (BiotechRabbit GmbH, Berlin, Germany), 100 mM of each dNTP (Euroclone Spa, Milano), 200 nM
165 of forward primer, 200 nM of reverse primer, 1.0 U PerfectTaq DNA Polymerase (BiotechRabbit
166 GmbH, Berlin, Germany), 100 ng of DNA and DNase free water (Euroclone Spa, Milano). The
167 following cycling program was applied: denaturation at 95 °C for 3 min; 40 cycles at 95 °C for 30 s,
168 55°C for 30 s, and 72°C for 30 s; final extension at 72°C for 7 min. Five microliters of each PCR
169 products were checked by gel electrophoresis on a 2% agarose gel. The amplification of fragments
170 of the expected length was assessed by making a comparison with the standard marker SharpMass™
171 50-DNA ladder (Euroclone Spa, Milano) and the concentration of PCR products by making a
172 comparison with the intensity of the bands of the DNA ladder. A concentration of 10 ng/ml was used
173 as a threshold to destine the samples to the following sequencing phase. All the PCR products were
174 purified with EuroSAP PCR Enzymatic Clean-up kit (EuroClone Spa, Milano) and sequenced by the
175 Experimental Institute of Zooprophylaxis of Piedmont, Liguria and Aosta Valley (Turin, Italy).
176 The molecular analysis was carried out avoiding contaminations.
177 *2.3.4 BLAST analysis: species identification and mislabelling assessment.* The obtained sequences
178 were analysed using Clustal W in Bio Edit version 7.0.9 (Hall, 1999). Fine adjustments were manually
179 made after visual inspection. All the sequences were used to run a BLAST analysis on GenBank
180 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A top match with a sequence similarity of at least 99%
181 associated to 100% query coverage was used to designate potential species identification. Outcomes
182 from this phase were compared to data obtained from RTEs labelling analysis. The mislabelling rate

in relation to the scientific denomination was calculated according to the product category: on (a) salted products declaring the species scientific name (as mandatory information) (b) marinated/in oil products voluntary reporting the species scientific name and (c) marinated/in oil products not reporting the species scientific name but voluntary reporting geographical catching areas that could be unequivocally associated to a unique (or few) species.

3. Results and Discussion

3.1 Labelling compliance with EU legislation and voluntary claims assessment

Given the higher brands diversity and the stronger presence at Italian retail level, certainly related to a major consumers' demand, in oil RTEs were the most representative of the sampling (N=50).

3.1.1 Labelling compliances with Regulation (EU) No 1169/2011. 100% of the collected products were compliant with the disposition provided by the cited r~~R~~egulation ~~(EU) No 1169/2011~~, as all the mandatory information were reported on the label and no differences were observed among the distinct categories of products. In all the RTEs, the name of the product was associated to the commercial designation "anchovies" ("acciughe" in Italian), sometimes along with the wording "Mediterranean" (5/111), "Adriatic" (9/111), "Sicilian" (5/111), "Spanish" (5/111), "Cantabrian" (1/111) or "Chilean" (2/111), attributed regardless to the product category (Table 1). Of the whole, the absolute labels compliance with Regulation (EU) No 1169/2011 observed in this study was in accordance with that from Spanish anchovy products analysed by Velasco, Aldrey, Pérez-Martín, & Sotelo (2016), confirming that such law is well obeyed by EU FBOs.

3.1.2 Labelling compliances with Regulation (EU) No 1379/2013. The label analysis, uniquely performed on the salted RTEs, showed that the mandatory information on the commercial designation and the scientific name of the species, as well as the product's minimum durability, were correctly provided by 100% of the samples. ~~The Article 35 of the considered Regulation especially disposes~~As ~~that~~ the mandatory information on commercial designation and related scientific name should match with those reported on official lists drew up and published by each Member State (Article 35)~~;~~ ~~therefore,~~ the term "anchovy" ("acciuga" in Italian) should be uniquely related to the species *E.*

encrasicolus for products sold on the Italian market according to the list of Ministerial Decree No 19105 of September the 22th, 2017. In this respect, as *E. encrasicolus* was the species declared in all the salted RTEs, an actual compliance with the law was observed (Table 1). The catching area was present in all the products, but in 13 out of 30 samples (43.3%) the name in writing of the FAO sub-area or division, mandatorily introduced by the cited rRegulation (EU) No 1379/2013, lacked (Table 1). Five samples (from RTE-22 to RTE-26) indicated two distinct catching areas (FAO 37 and FAO 34) for the same product. Anyway, in both the declared areas, *E. encrasicolus* is the only present species (www.fishbase.org). Finally, for 23.3% of the samples (7/30) no information on both the production method and the fishing gear were provided. Such latter products belonged to the same brands that also reported the incorrect catching area in the label. So, 13 out of 30 salted RTEs (43.3%) was overall not fully compliant with the disposition of Regulation (EU) No 1379/2013.

3.1.3 Voluntary claims. For the marinated and in oil RTEs, voluntary claims on the species scientific name and/or on the catching area were reported in more than half of the samples (58%). This percentage is associated to the operate of EU FBOs are responding well to the European Parliament Resolution No 2016/2532 that encouraged the Member States, in the context of voluntary labelling, to state all available information that enables the consumer to make an informed choice. In detail, the scientific name was provided in 44.4% (36/81) of the samples, including 66% (33/50) of in oil products and only 9.7% of marinated products (3/31), and the catching area was provided in 38.3% of the samples (31/81) including 38% (19/50) of in oil products and 38.7% (12/31) of marinated products. *E. encrasicolus* was declared in 94.4% (34/36) of the product reporting the species name, while only 2 in oil RTEs (RTE-102 and RTE-103) reported the presence of *E. ringens* (Table 1).

Of the remained 45 samples not declaring the species, 10 however reported a catching area possibly related to both *E. encrasicolus* and *E. albidus* (FAO area 27 and FAO area 37) while 1 sample (RTE-90) reported a catching area clearly related to *E. encrasicolus* (FAO area 27) (Fig. 1). However, it should be noted that the taxonomic status of *E. albidus*, described from the Mediterranean

in 2004 (Borsa, Collet, & Durand, 2004) and currently considered as accepted in the World Register of Marine Species (www.marinespecies.org), is questioned by some scientists, specifically suggesting that this may be an eco-morph of *E. encrasicolus* (http://www.iucnredlist.org/details/18124888/0). Such samples were anyway considered as composed by *E. encrasicolus*/*E. albidus*, or only *E. encrasicolus* in the case of RTE-90.

3.2 Molecular analysis

3.2.1 Evaluation of total DNA fragmentation and primer design. The total DNA extracted from the RTEs showed a certain degree of fragmentation, with an electrophoretic pattern hardly visible above 500 bp. Such pattern is typically associated to degraded DNA from processed products packed as cans, tins, jars or tubes having experienced different level of processing (smoking, salting etc.) and also containing multiple additives, preservatives and flavours that may affect the quantity and quality of the DNA (Shokralla, Hellberg, Handy, King, & Hajibabaei, 2015). In this respect, the analysis of processed products has been generally performed by the means of molecular techniques focused on relatively short DNA fragments as genetic marker (Armani et al., 2015a; Meusnier et al., 2008; Shokralla, Hellberg, Handy, King, & Hajibabaei, 2015). Such approach was even proposed for applying next generation meta-barcoding technologies on highly processed complex food matrices (Giusti, Armani, & Sotelo, 2017).

For the RTEs analysis, the primer pair ANCH-531_F/ANCH-1059_R was thus designed for amplifying a DNA fragment shorter than 500 bp especially avoiding mismatches within the first three bases near the 3′ end since mismatches in this position may affect PCR more dramatically than mismatches located internally or at 5′ end (Armani et al., 2016) (Fig. 2). Of course, the high inter-species variability of the molecular marker is a basic prerequisite for ensuring the analysis success. In this respect, ten years ago, Jérôme et al. (2008) already tested the three mitochondrial genes (*16S*, *COI*, and *cytb*) for their suitability to differentiate and authenticate *Engraulidae* species. Even though it was proved that these markers can equally be used, *cytb* appeared slightly more variable between *E. japonicus* and *E. encrasicolus*, strongly distinguished with the maximum bootstrap value of 100%.

This is of great importance considering that in a previous study aimed at identifying fish species in ethnic food (Armani et al. 2015b) a low discrimination power of the *COI* gene between *E. japonicus* and *E. encrasicolus* was highlighted. In addition, *Cytb* was proved as suitable marker for distinguishing anchovy species even in the study of Santaclara, Cabado, & Vieites (2006) and, more recently, in the study of Velasco, Aldrey, Pérez-Martín, & Sotelo (2016), even though not all the currently valid eight species belonging to *Engraulis* genus were tested.

In this study, the 484 bp *cytb* fragment amplified by our primer pair showed an *Engraulis spp* inter-species variability that was ~~among all the *Engraulis spp*~~. comparable, and in many cases ~~even~~ higher, to that of the complete mitochondrial *cytb* sequence (Fig. 3)~~, especially regarding~~ for the species most used for manufacturing semi-preserved products (*E. encrasicolus*, *E. anchoita*, and *E. ringens*). In particular, the fragment shows a higher variability respect to the complete gene also in discriminating the species *E. encrasicolus* and *E. japonicus* that were reported as the most phylogenetically closer by Jerome et al., 2008. In addition, the design of primers pairs specifically intended to some species could even increase the amplification rate in processed products.

3.2.2. Species identity assessment. All the analysed products were successfully extracted obtaining a total DNA of good quality. All the samples produced at least one amplicon suitable for sequencing and one readable sequence. On the whole, most of the RTEs (102/111, 91.9%) were molecularly identified as *E. encrasicolus*, with ID values of 99% (35.3% of the cases) or 99-100% (64.7% of the cases), regardless the catching area (where it was provided) (Table 1). This difference between ID values may further prove the actual presence of a number of haplotypes within this species already showed by Magoulas, Castilho, Caetano, Marcato, & Patarnello (2006). The remaining 9 RTEs (all in oil products) were instead undoubtedly identified as *E. ringens* with ID value 99-100% (Table 1).

3.2.3 RTEs mislabelling rate. In this study, criteria for assessing RTEs mislabelling were related to the eventual discrepancy between the species declared on the product label and that actually recovered through the molecular analysis. This eventuality could in fact be related to the voluntary species substitution phenomenon that often occurs where low-value or less-desirable fish species are

Commented [AG1]: Commento di Carmen:

From my point of view you have obtained very homogenus identification results, in our case we had higher number of species coming from SAmerica. I tend not to use Blast as single identification tool. My suggestion is to compare with results with genetic distance approach

287 swapped for more expensive varieties (FAO, 2018). Mislabelling rate was calculated on 77 RTEs,
288 including all the salted RTEs (N=30), 12 marinated RTEs and 35 in oil RTEs, according to the
289 parameters described in section 2.5. The analysis revealed only one case of mislabelling (mislabelling
290 rate 0.9%), involving 1 in oil product (RTE-91) voluntarily labelled as "*E. encrasicolus*"/FAO 37.2.1,
291 that was instead proved *E. ringens.* This case can be fully considered as an intentional species
292 substitution, as the species *E. ringens* typically inhabits southeast Pacific Ocean (Fig. 1), and it could
293 not have been therefore erroneously by-caught with the con-generic species.

294 It was instead not possible to evaluate the mislabelling rate in the remaining marinated/in oil RTEs.
295 Differently from other categories of preserved products, such as tuna and bonito whose market
296 standards were opportunely fixed (Council Regulation (EEC) No 1536/92), labelling of ready-to-eat
297 anchovies currently presents objective legislative gaps. In fact, no dispositions on which species
298 should be standardly used in marinated or in oil products labelled as "anchovy/anchovies" were laid
299 down at European level yet. Some Member States currently refer to national legislation, such as in
300 the case of Spain, where the semi-preserved products labelled as "achovy" ("anchoa") must be made
301 only with *E. encrasicolus* (Spanish Royal Decree, 1984). Even the Italian legislation reports a Royal
302 Decree of 1927 stating that all canned products labelled as "anchovy" should be made with *E.*
303 *encrasicolus* (Italian Royal Decree, 1927). However, this old disposition is poorly accurate with
304 regard to the type of products included in the application field and, given the market developments
305 and growth since then, it may be actually considered as anachronistic. Except for that decree, any
306 related disposition has been provided. Not even the international Codex standards may be adopted,
307 as only referring to all commercial species of fish belonging to the family Engraulidae that have been
308 salted, boiled and dried and not covering products which have undergone an enzymatic maturation in
309 brine (CODEX STAN 236-2003). Actually, some information can be uniquely extrapolated from EU
310 codes from combined nomenclature, where the definition "prepared and preserved anchovy" covers
311 all the *Engraulis spp*. and even other anchovy species (*Anchovia macrolepidota*, *Lycengraulis*
312 *grossidena*) (EUMOFA, 2018). However, as already mentioned, this condition is not supported by a

313 concrete legislation system. This limit unavoidably leads to concerns such as the effective higher
314 possibility that cases of replacement with low-value species occur, as well as the concrete
315 impossibility for consumers to make informed choices on the products they purchase.

316 **3.3 Anchovy products in the context of the EU market: local species exploitation and**
317 **entrance of non-indigenous species**

318 *E. encrasicolus*, or European anchovy is a pelagic species distributed in the Eastern North and
319 Central Atlantic, Mediterranean, Black and Azov seas, as well as the Coast of West Africa to Angola
320 (EUMOFA, 2018) (Fig. 1). Given its abundance in such areas, it is the mainly caught, processed and
321 consumed species in EU (data also confirmed by the outcomes from this study). A report by the EU
322 Market Observatory for Fisheries and Aquaculture products (EUMOFA), specifically dedicated to
323 the anchovy market and providing data of the year 2015, states that 38% of anchovies consumed in
324 form of processed ready-to-eat products in Italy (38% of the total anchovy consumption) belong to
325 small processing industry relying on locally caught species flanked by large scale production
326 (EUMOFA, 2018). In both cases, *E. encrasicolus*, as inhabiting European waters, is mainly used.
327 However, large scale production even relies on extra-EU imports, and involving other *Engraulis spp.*
328 such as anchoveta (*E. ringens*), Argentine anchovy (*E. anchoita*) and Japanese anchovy (*E.*
329 *japonicus*). *E. ringens*, the other species found in the products analysed in this study is a pelagic fish
330 in the south-eastern Pacific Ocean, regularly caught by purse seiner vessels all along the Peruvian
331 coast including the north of Chile (Fig. 1). It represents the most caught fish in the world history in
332 terms of volume (FAO, 2016) and the Peruvian northern-central stock currently supports the single
333 most important mono-specific fishery in the world (cedepesca.net). It should be however noted that
334 the vast majority of the catch does not go for human consumption but is reduced to fishmeal and fish
335 oil and exported, primarily for aquaculture and animal feed (FAO, 2016). However, efforts in
336 encouraging the local anchovy value by addressing the Peruvian processing industry to manufacture
337 products for human consumption have been made in the last years. For instance, in July 2012,
338 "Compañía Americana de Conservas", the major Peruvian society of fishery industry, signed a

framework collaboration agreement with CeDePesca for the implementation of a fishery improvement project targeting the portion of the fishery fishing for direct human consumption (http://cedepesca.net/promes/small-pelagics/peruvian-anchovy-direct-human-consumption/). Other examples of private and public initiatives were reported in a recent report by *The Marine Ingredients Organization* (IFFO, 2017). However, data from that report showed a still low amount of exported product volume. This aspect may explain the low presence of this products within EU market and therefore the low percentage of *E. ringens* RTEs respect to *E. encrasicolus* RTEs in this study.

Conclusions

This study confirms how the use of simple and cost-effective DNA-based analytical techniques, allow to properly identify fish species and thus support traceability in the seafood supply chain. It also furnishes encouraging results showing a very low mislabelling rate of ready-to-eat anchovy products. This is particularly interesting considering these products are characterized by a high price on the Italian market and could thus represent an optimum target to perpetrate voluntary frauds. Overall, this survey confirms previous studies reporting a reduction of misdescription incidents in the EU highlighting how the market can positively respond to policies intended to regulate the seafood sector.

References

1. Armani, A., Tinacci, L., Xiong, X., Titarenko, E., Guidi, G., & Castigliego, L. (2014). Development of a simple and cost-effective bead-milling method for DNA extraction from fish muscles. *Food Analytical Methods*, *7*(4), 946-955.
2. Armani, A., Guardone, L., Castigliego, L., D'Amico, P., Messina, A., Malandra, R., Gianfaldoni, D., & Guidi, A. (2015a). 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.
3. Armani, A., Guardone, L., La Castellana, R., Gianfaldoni, D., Guidi, A., & Castigliego, L. (2015b). DNA barcoding reveals commercial and health issues in ethnic seafood sold on the Italian market. *Food Control*, 55, 206-214.
4. Armani, A., Giusti, A., Guardone, L., Castigliego, L., Gianfaldoni, D., & Guidi, A. (2016). Universal primers used for species identification of foodstuff of animal origin: Effects of oligonucleotide tails on PCR amplification and sequencing performance. *Food Analytical Methods*, *9*(5), 1199-1209.
5. Borsa, P., Collet, A., & Durand, J. D. (2004). Nuclear-DNA markers confirm the presence of two anchovy species in the Mediterranean. *Comptes rendus biologies*, *327*(12), 1113-1123.
6. CODEX STAN 236-2003. Standard for boiled dried salted anchovies. Available at: http://www.fao.org/fao-who-codexalimentarius/sh-proxy/it/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCODEX%2BSTAN%2B236-2003%252FCXS_236e.pdf

7. Council Regulation (EEC) No 1536/92 of 9 June 1992 laying down common marketing standards for preserved tuna and bonito. *Official Journal of European Communities*, L163, 1-4.
8. D'Amico, P., Armani, A., Gianfaldoni, D., & Guidi, A. (2016). New provisions for the labelling of fishery and aquaculture products: Difficulties in the implementation of Regulation (EU) n. 1379/2013. *Marine Policy*, *71*, 147-156.
9. EUMOFA, (2018). Case study: processed anchovies in Italy. Price structure in the supply chain. *European Market Observatory for Fisheries and Aquaculture Products*, February 2018. Available at: http://www.eumofa.eu/documents/20178/111808/Price+structure+-+Anchovy+in+Italy.pdf/347b0feb-a221-40e6-ad9f-84c21fb2dbc1?version=1.8
10. European Parliament Resolution 2013/2091(INI) of 14 January 2014 on the food crisis, fraud in the food chain and the control thereof. Available at: http://www.europarl.europa.eu/sides/getDoc.do?pubRef=-//EP//NONSGML+TA+P7-TA-2014-0011+0+DOC+PDF+V0//EN
11. European Parliament Resolution No 2016/2532 (RSP) of 12 May 2016 on traceability of fishery and aquaculture products in restaurants and retail. Available at: http://www.europarl.europa.eu/sides/getDoc.do?pubRef=-//EP//NONSGML+TA+P8-TA-2016-0222+0+DOC+PDF+V0//EN
12. FAO (2016). The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 pp. Available at: http://www.fao.org/3/a-i5555e.pdf.
13. FAO (2018). Overview of food fraud in the fisheries sector. Fisheries and Aquaculture Circular No. 1165. Rome, Italy.
14. Garcia-Vazquez, E., Perez, J., Martinez, J. L., Pardinas, A. F., Lopez, B., Karaiskou, N., ...& Triantafyllidis, A. (2010). High level of mislabeling in Spanish and Greek hake markets suggests the fraudulent introduction of African species. *Journal of Agricultural and Food Chemistry*, *59*(2), 475-480.
15. Giusti, A., Castigliego, L., Rubino, R., Gianfaldoni, D., Guidi, A., & Armani, A (2016). A Conventional Multiplex PCR Assay for the Detection of Toxic Gemfish Species (*Ruvettus pretiosus* and *Lepidocybium flavobrunneum*): a simple method to combat health frauds. *Journal of Agricultural and Food Chemistry*, *64*(4), 960-968.
16. Giusti, A., Armani, A., & Sotelo, C. G. (2017). Advances in the analysis of complex food matrices: Species identification in surimi-based products using Next Generation Sequencing technologies. *PloS one*, *12*(10), e0185586.
17. 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.
18. Helyar, S. J., Lloyd, H. A. D., de Bruyn, M., Leake, J., Bennett, N., & Carvalho, G. R. (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.
19. IFFO (2017). Peruvian anchovy: why feed not food? *The Marine Ingredients Organization.* Available at: http://www.iffo.net/system/files/Peru%20Anchovy%20-%20Feed%20not%20Food%20factsheet_110917.pdf
20. Italian Royal Decree (1927) No 1548 of July 7th. Norme per la fabbricazione, l'importazione ed il commercio dei prodotti alimentari, della pesca conservati in recipienti.
21. Jérôme, M., Martinsohn, J. T., Ortega, D., Carreau, P., Verrez-Bagnis, V., & Mouchel, O. (2008). Toward fish and seafood traceability: anchovy species determination in fish products by molecular markers and support through a public domain database. *Journal of Agricultural and Food Chemistry*, *56*(10), 3460-3469.
22. Kimura M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*, 111-120.
23. Magoulas, A., Castilho, R., Caetano, S., Marcato, S., & Patarnello, T. (2006) Mitochondrial DNA reveals a mosaic pattern of phylogeographical structure in Atlantic and Mediterranean populations of anchovy (*Engraulis encrasicolus*). *Molecular phylogenetics and evolution*, *39*(3), 734-746.
24. Mariani, S., Griffiths, A. M., Velasco, A., Kappel, K., Jérôme, M., Perez-Martin, R. I., & Boufana, B. (2015). Low mislabeling rates indicate marked improvements in European seafood market operations. *Frontiers in Ecology and the Environment*, *13*(10), 536-540.
25. Meusnier, I., Singer, G. A., Landry, J. F., Hickey, D. A., Hebert, P. D., & Hajibabaei, M. (2008) A universal DNA mini-barcode for biodiversity analysis. *BMC genomics*, *9*(1), 214.
26. Ministerial Decree No 19105 of September the 22th, 2017. Denominazione in lingua italiana delle specie ittiche di interesse commerciale. *Gazzetta Ufficiale* No. 266 of October, the 14th, 2017.
27. Pardo, M. Á., Jiménez, E., & Pérez-Villarreal, B. (2016). Misdescription incidents in seafood sector. *Food Control*, *62*, 277-283.

28. Petrossian, G. A. (2015). Preventing illegal, unreported and unregulated (IUU) fishing: A situational approach. *Biological Conservation*, *189*, 39-48.
29. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Official Journal of the European Communities* L31, 1-24.
30. Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. *Official Journal of the European Union* L304, 18-63.
31. Regulation (EU) No 1379/2013 of the European Parliament and of the Council of 11 December 2013 on the common organisation of the markets in fishery and aquaculture products, amending Council Regulations (EC) No 1184/2006 and (EC) No 1224/2009 and repealing Council Regulation (EC) No 104/2000. *Official Journal of the European Union* L354, 1-21.
32. Santaclara, F. J., Cabado, A. G., & Vieites, J. M. (2006). Development of a method for genetic identification of four species of anchovies: E. *encrasicolus*, *E. anchoita*, *E. ringens* and *E. japonicus*. *European Food Research and Technology*, *223*(5), 609-614.
33. Shokralla, S., Hellberg, R. S., Handy, S. M., King, I., & Hajibabaei, M. (2015). A DNA mini-barcoding system for authentication of processed fish products. *Scientific reports*, *5*, 15894.
34. Sotelo, C. G., Velasco, A., Perez-Martin, R. I., Kappel, K., Schröder, U., Verrez-Bagnis, V., ...& Griffiths, A. (2018) Tuna labels matter in Europe: Mislabelling rates in different tuna products. *PloS one*, *13*(5), e0196641.
35. Spanish Royal Decree (1984) of August 28th on the technical-health regulations establishments and fishery and aquaculture products for human consumption.
36. Tamura K., Stecher G., Peterson D., Filipski A., & Kumar S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular biology and evolution*, *30*, 2725-2729.
37. Velasco, A., Aldrey, A., Pérez-Martín, R. I., & Sotelo, C. G. (2016). Assessment of the labelling accuracy of spanish semipreserved anchovies products by FINS (Forensically Informative Nucleotide Sequencing). *Heliyon*, *2*(6), e00124.
38. Yu, Z. N., Kong, X. Y., Guo, T. H., Jiang, Y. Y., Zhuang, Z. M., & Jin, X. S. (2005). Mitochondrial DNA sequence variation of Japanese anchovy *Engraulis japonicus* from the Yellow Sea and East China Sea. *Fisheries Science*, *71*(2), 299-307.

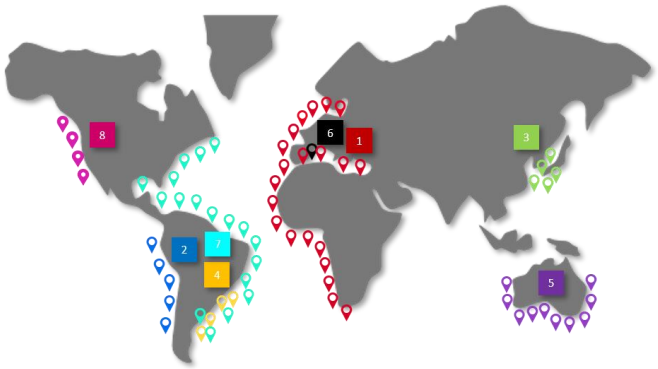
Figure captions

Fig. 1: Native distribution map for *Engraulis* species (source: www.fishbase.org). 1: *E. encrasicolus;* (European anchovy); 2: *E. ringens* (Anchoveta); 3: *E. japonicus* (Japanese anchovy); 4: *E. anchoita* (Argentine anchovy); 5: *E. australis* (Australian anchovy); 6: *E. albidus* (White anchovy); 7: *E. eurystole* (Silver anchovy); 8: *E. mordax* (Californian anchovy).

Fig. 2: Alignment between the primer pair ANCH-531_F/ANCH-1059_R projected in this study and the cytb sequences retrieved from GenBank; mismatches were highlighted in grey.

Fig. 3: Inter-species variability analyses conducted in MEGA 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) using the Kimura 2-parameter model (Kimura, 1980) on **a)** 484 bp fragment of this study and **b)** complete cytb gene. The analysis involved the following nucleotide sequences randomly selected among those used for primer projecting: *E. albidus* (MG958167); *E. anchoita* (JQ012416); *E. australis* (KJ007734); *E. encrasicolus* (JQ716614); *E. eurystole* (JQ012427); *E. japonicus* (KJ007662); *E. mordax* (JQ012421); *E. ringens* (JQ012426).

Fig. 1:



479

480 **Fig. 2:**

ANCH-531_F		ANCH-1059_R (reverse complement)	
GTTCTTYGCCTTCCACTTCYT		CCATTCATTATTATTGGYCAAGTR	
**********	*E. encrasicolus*	**********	*E. encrasicolus*
**********	*E. japonicus*	**********	*E. japonicus*
**********	*E. albidus*	**********	*E. albidus*
C**********	*E. anchoita*	**C**********	*E. anchoita*
**********	*E. australis*	**********	*E. australis*
A**********	*E. mordax*	**G***G*C**********	*E. mordax*
C**********	*E. ringens*	**C**********	*E. ringens*

481

482 **Fig. 3**

a)

	1	2	3	4	5	6	7	8
1. E. albidus								
2. E. anchoita	0.22063							
3. E. australis	0.05440	0.24473						
4. E. encrasicolus	0.03193	0.23852	0.05659					
5. E. eurystole	0.01255	0.22063	0.06369	0.04080				
6. E. japonicus	0.05915	0.24510	0.02755	0.05211	0.06382			
7. E. mordax	0.20146	0.24433	0.19542	0.20701	0.20146	0.19856		
8. E. ringens	0.23271	0.12723	0.22935	0.22034	0.23271	0.22063	0.24433	

b)

	1	2	3	4	5	6	7	8
1. E. albidus								
2. E. anchoita	0.22272							
3. E. australis	0.03665	0.23407						
4. E. encrasicolus	0.02646	0.22679	0.03971					
5. E. eurystole	0.01064	0.22132	0.03975	0.02746				
6. E. japonicus	0.04290	0.23713	0.02249	0.04182	0.04394			
7. E. mordax	0.18741	0.21489	0.18609	0.18465	0.18085	0.18359		
8. E. ringens	0.21561	0.09386	0.21149	0.20862	0.21423	0.21026	0.21752	

483