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Seafood labelling compliance with European legislation and species identification by DNA barcoding: a first survey on the Bulgarian market

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Abstract

The present study aimed at assessing the labelling compliance of seafood products sold on the Bulgarian market in the light of the European legislation and at verifying their identity by DNA barcoding. A preliminary analysis of the official Bulgarian seafood denomination list was conducted. The labels of 97 seafood products collected from Bulgarian wholesalers were analysed to verify their compliance with the requirements of the Regulation (EU) n. 1379/2013. Then, the products were molecularly identified by DNA barcoding and the species substitution rate was calculated. The analysis of the official seafood denomination list highlighted the lack of national and international relevant market species. Moreover, 19.3% of the listed items were found referring to invalid scientific names. The main shortcomings found with the labels analysis were: the presence of commercial and scientific names not included within the official list (59.2% of the products), the lack of the scientific name (34.1%), the incomplete reference to the catching area (85.2%) and the absence of the fishing gear (55.2%). Finally, tThe DNA barcoding revealed a species substitution rate of 17.7%. The outcomes of this study underline the urgency to review and update the Bulgarian official seafood list. Even though the relatively low species substitution rate found in this study supports the reduction of unfair practices in the EU seafood chain, official controls aimed at verifying seafood labelling along the Bulgarian supply chain are still needed.

Keywords

Bulgarian market, seafood labelling, mislabelling, DNA barcoding, species identification

1. Introduction

World seafood consumption has strongly increased during the last fifty years, mainly due to the population growth and to the higher consumers' attention toward food sources of high nutritional value (Kearney, 2010; FAO, 2016). According to FAO data, world per capita apparent fish consumption increased from an average of 9.9 kg in the 1960s to 14.4 kg in the 1990s and to 19.7 kg in 2013, with preliminary estimates for 2014 and 2015 pointing towards further growth beyond 20 kg (FAO, 2016). This trend is particularly evident in Europe, where seafood consumption ~~had already overcome the threshold estimated for 2014~~ reached~~ing~~ an average value of 25.5 kg/capita (EUMOFA, 2017).

Despite the European increasing trend, a study of Vanhonacker, Pieniak & Verbeke (2013) found that consumers' habits and attitudes toward fish purchasing and consumption were different across the investigated EU countries (Portugal, UK, Sweden, Germany, Italy, Greece, Czech Republic and Romania). The authors highlighted few major barriers to seafood purchase, mainly represented by price, lack of traditional culinary habits, fish organoleptic characteristics and absence of decisive motivations or drivers to fish consume. As also shown by the European Market Observatory for Fishery and Aquaculture (EUMOFA), these factors constitute an actual ~~brake~~ hindrance to the household expenditure for fisheries and aquaculture products, especially in Central and Eastern countries such as Czech Republic, Slovenia, Poland, Romania and Bulgaria (EUMOFA 2017b).

As regards ~~Also in~~ Bulgaria in particular, seafood consumption is limited due to the aforesaid barriers against these kind of products (EUMOFA, 2017b). Currently, the Bulgarian consumers' choice is essentially addressed to local species (carp, sprat, trout) and to several wild and farmed fish species imported from other European or third countries, such as herring, Atlantic, Pacific and pink salmon, Alaska pollock, hake and mackerel (Todorov, 2017).

With the aim to further raise the awareness of consumers on seafood healthy characteristics, and promote the national products, studies have been conducted to highlight the content of polyunsaturated fatty acids (PUFA) of several Bulgarian fresh water and Black Sea species (carp, catfish, rainbow trout, sprat, turbot, garfish) (Merdzhanova, Ivanov, Dobreva & Makedonski, 2017). The authors confirmed Black Sea fish, particularly the marine species and rainbow trout among fresh water species, as a very good and balanced source of PUFA, which play a significant role for the prevention of cardiovascular diseases, atherosclerosis, obesity and diabetes. However, dDespite an informative campaign organized by the Ministry of Health since 2006 induced a slight increase in seafood consumption (Bulgaria Ministry of Health, 2006; EUMOFA, 2017b), the seafood market and the fishery sector still play a marginal role in the Bulgarian economy (Todorov, 2017). At present, the yearly domestic consumption is 5.2 kg/per capita, which rises to 8.8 kg/per capita if restaurant consumption is included (Todorov, 2017).

The fishery sector is well recognised as one of the most susceptible to fraudulent practices which, by the years, raised Governments awareness about the need of enhancing systems for the control of seafood supply chain traceability and labelling (Tamm, Schiller & Hanner, 2016). Seafood fraud incidents may encompass any illegal activity aimed at misrepresenting the products origin, composition and identity (Upton, 2015). Falsification and mislabelling practices represent the most frequently highlighted fraud incidents with direct impact on consumers trust and supply chain economy, potential harmful impacts on consumers' health and negative impact on seafood stocks preservation (Pardo, Jiménez & Pérez-Villarreal, 2016).

With respect to the labelling requirements in force at the European level, the Regulation (EU) No. 1379/2013 establishes the obligation to provide the consumer with the commercial and scientific name of the product, together with the geographical area, production method and fishing gear in the case of caught fish. Each Member State is required to draw up, publish and update a list of the commercial designations accepted in their territory for the sale of fishery products (D'Amico, Armani, Gianfaldoni & Guidi, 2016). In this respect Bulgaria, which has joined theis a member state of the European Union since the 1st January 2007, had published its own official list in 2006 (Ministry of Agriculture and Forestry. Decree n. 4 of 13.01.2006) collecting the main commercial species present at that time on the market. However, the list has not been updated since then.

Molecular analysis involving the use of DNA-based methods are ~~V~~valuable tools to verify species identity and enhance traceability along the supply chain~~ are those based on molecular analysis and especially involve the use of DNA-based methods~~, ~~which~~and are clearly mentioned in the Regulation (EU) n. 1379/2013 (foreword 23) as means to deter operators from falsely labelling catches. These methods are irreplaceable verification tools especially in the presence of filleted or processed seafood, in which the species are no longer recognizable through the visual examination of the whole specimen's morphology (Griffiths et al., 2014).

Among DNA-based methods, the analysis of a 655 bp fragment of the Cytochrome c Oxidase I (*COI*) has been proposed as a standard ~~tool~~ procedure for species identification, the so-called DNA barcoding (Hebert, Ratnasingham & de Waard, 2003). The technique has also been validated by the US Food and Drug Administration (FDA) (Handy et al., 2011). DNA barcoding has been recently successfully applied in several market surveys to verify the labelling compliance of commercial seafood goods (fish, molluscs and crustacean) collected at retail, both at International and European level (Galal- Khallaf, Ardura, Mohammed-Geba, Borrell, & Garcia-Vazquez, 2014; Khaksar et al., 2015; Cawthorn, Duncan, Kastern, Francis & Hoffman, 2015; Lamendin, Miller & Ward, 2015; Vandamme et al., 2016; Armani et al., 2017; Nagalakshmi, Annam, Venkateshwarlu, Pathakota, & Lakra, 2016; Nedunoori, Turanov, Kartavtsev, 2017, Günther, Raupach & Knebelsberger, 2017). Moreover, a mini DNA barcode of 139 bp was ~~proved~~ shown to be effective for the analysis of processed seafood (Armani et al., 2015).

Nevertheless, data on seafood labelling compliance on Central-Eastern and Eastern European seafood markets are still rarely reported. Among the countries overlooking the Black Sea, a survey has been conducted only in Romania (Popa et al., 2017). Data on seafood mislabelling in Bulgaria have been recently collected in a project of the European Commission in 2015. However, only white fish species were analysed (https://ec.europa.eu/food/safety/official_controls/food_fraud/fish_substitution_en).

The present study provides a more extensive survey on seafood products sold at retail level on the Bulgarian market. After a preliminary analysis of the Bulgarian official list of seafood commercial designations, this study aimed at: 1) assessing the labelling compliance of seafood products in the light of the European legislation and 2) verifying their identity by DNA barcoding.

2. Material and methods

2.1 Analysis of the Bulgarian official list

The official Bulgarian list of seafood commercial designations published by the Bulgarian Ministry of Agriculture and Forestry (Ministry of Agriculture and Forestry. Decree n. 4 of 13.01.2006) was analysed with the aim to describe the document's outline and assess the official designations provided as C~~c~~ombination I~~i~~tems (CI) of C~~c~~ommercial (CN) and S~~s~~cientific (SN) N~~n~~ames. Then, the congruency between the Bulgarian CN, the SN and the associated commercial name translated in English ~~has be~~wasen verified. Finally, the correctness and validity of the scientific name in the light of the FAO official Fishbase and Sealife databases (fishbase.org; sealifebase.org) ~~has been~~was assessed. In addition, a comparison among the total number of CI in the Bulgarian list and those provided by ~~-~~the last updated version of the lists ~~-~~of other 25 Member States (available at https://ec.europa.eu/fisheries/cfp/market/consumer-information/names_en) was performed. Moreover, the ratio among the total number of different CNs (the CNs that were repeated in the list were excluded) and the corresponding number of SNs reported as binomial nomenclature (SNs only referred to the genus were excluded), was calculated. As described in Xiong et al. (2016a), this ratio can be considered as an Index (Species Index, SI) that reflects the accuracy of the countries in managing the commercial designations~~nomenclatures~~.

2.2 Samples collection

A total of 97 seafood products (fish N=86 and cephalopod molluscs ~~(cephalopods)~~ N=11) were purchased from major wholesaler companies at Stara Zagora, Bulgaria, from March 2016 to June 2017. According to the definition provided by the Regulation (EC) 852/2004, they were classified as unprocessed (fresh and frozen, beheaded, cleaned, sliced filleted, trimmed) or processed (marinated, smoked and precooked) products. Each product was recorded with a progressive numerical code and all the labelling information (see section 2.3) were tabulated together with a short description of the product presentation at purchase (Table 1SM). 1-5 g of muscle tissue were collected, dehydrated by means ofunder 95% ethanol and sent to the FishLab (Department of Veterinary Sciences, University of Pisa) for the molecular analysis (see section 2.4 and 2.5).

2.3 Labelling Analysis

The products were classified according to the Tariff commercial categories reported in the Regulation (EU) 1379/2013 Annex I in order to define products falling into the scope of the Regulation and, therefore, to be included into the labelling analysis (D'Amico et al., 2016).

The labels or, in the case of non-prepacked products, the information collected at purchase on the billboards, were checked with respect to: I) the presence of the commercial designation and of the scientific name, for which the compliance with the official CI reported on the Bulgarian list was also verified; II) the country of origin and the catching or farming geographical area; III) the declaration of the gear category according to the designated terms listed on Annex III of the same Regulation.

2.4 Total DNA extraction, amplification and sequencing

Total DNA extraction for each sample was performed according to the salting out procedure proposed by Armani et al. (2014) starting from 50 mg of tissues for both fish and cephalopod tissues. Final DNA concentration and quality were evaluated with Nanodrop ND-1000 spectrophotometer according to the manufacturer guidelines and absorbance ratios A260/A280 > 2.0 and A260/A230 >1.8 were set as minimum values of nucleic acid purity.

The *COI* gene was selected as target for the analysis of both fish and cephalopod products. The amplification was set using two distinct primer pairs proposed by Handy et al., (2011) and Folmer, Black, Hoeh, Luttz &Vrijenhoek, (1994) for the amplification of a 655 bp fragment (Full Length Barcode, FLB) from fish and metazoan organisms, respectively. The PCR reactions were settled starting from 100 ng of total DNA in a final volume of 20 µl containing 2 µl of 10X Buffer

(BiotechRabbit GmbH, Berlin, Germany), 100 mM of each dNTP (Euroclone Spa, Milano), 250 nM of each primer, 25 ng/mL of BSA (New England BioLabs, Inc. USA), 1.0 U PerfectTaq DNA Polymerase (BiotechRabbit GmbH, Berlin, Germany), DNase free sterile water (Euroclone SPA, Milano). The PCR was performed as follows: initial denaturation at 95° C for 3 min; three steps 45 cycles at 95 °C for 30 sec, 53°C for 30 sec for Handy et al. (2011) or 47°C for 25 sec for Folmer et al. (1994) primer pair, 72 °C for 35 sec; final extension at 72 °C for 5 min.

Five µl of each PCR product were checked on a 1.8% agarose gel (GellyPhorLE, Euroclone, Milano) previously stained with GelRed™ Nucleid Acid Gel Stain (Biotium, Hayward, CA, USA). The presence of the expected amplicon and the final concentration were verified by comparison with the standard molecular marker SharpMass™50-DNA. A concentration ≥ 5 µg/µl of PCR product was set as a threshold value for the subsequent sequencing reaction, according to the sequencing laboratory operative procedures. In case of amplification failures, DNA samples were reamplified ~~by the application of~~ targeting a mini *COI* DNA barcode of 139bp (MDB) ~~protocol~~ as described in Armani et al., (2015). All the PCR products were purified with EuroSAP PCR Enzymatic Clean-up kit (EuroClone Spa, Milano) according to the manufacturer instructions and sent to the Laboratory of Ichthyopathology of the Lazio and Tuscany's Experimental Zooprophylactic Institute, Pisa (Italy), for sequencing.

2.5 Molecular identification by database comparison and calculation of species substitution rate

The raw forward and reverse sequences were manually checked and edited using Bioedit 7.0 software (Hall, 1999) and aligned with the software Clustal W included to obtain the final barcode. The final sequences were queried by Basic Local Analysis Search Tool (BLAST) and Identification System (ID's) (Ratnasingham & Hebert, 2007) against the reference sequences available on GenBank (http://www.ncbi.nlm.nih.gov) and BOLD (http://www.boldsystems.org/) databases, respectively. The highest similarities percentages obtained within the first 100 top match records by BLAST and ID's query were registered (Table 3 SM). An identity value ≥ 98% was set as cut-off for final species attribution (Barbuto et al., 2010). To verify the compliance between the molecular results and the labelled information the products were split in two subgroups: ~~–~~I) products presenting a species-specific SN for which the species substitution rate was calculated after the comparison of the molecular results with the scientific name declared; II) products offered for sale with the sole presence of commercial name or presenting genus SN, which were not included in the calculation of the substitution rate.~~.~~

3. Results and discussion

3.1 Descriptive analysis of the Bulgarian official list

The Ministerial Decree published on January 2006 contains the unique Bulgarian official list of accepted designations for seafood labelling. The CI listed are composed by CNs in Bulgarian and English language associated to SN referring to species or genus. The list includes 68 CI, divided into three sections corresponding to the main commercial seafood categories: fish N=59, (86.8% of the total CI), crustaceans N=5 (7.3%) and molluscs N=4 (5.9%). The comparison of the Bulgarian decree with the other 25 Member States official lists highlighted that the Bulgarian list includes the lowest number of CI, preceded by Latvia (N=152), Slovakia (N=154), Sweden (N=171) and Belgium (N=191)~~ (data not shown)~~. This finding is reasonably related to the fact that the Bulgarian list has never been updated since its first publication in 2006. Interestingly, the analysis of the list revealed~~s~~ the lack of a significant number of fish species reported as commercial leading products by EUMOFA~~,~~ (EUMOFA, 2017) and others relevant in the Bulgarian market (Todorov, 2017) (Table 1). ~~–~~In fact, no official CI are provided for several fish species of commercial interest ~~inhabiting~~ from the Black Sea, such as: Mediterranean sand smelt (*Atherina hepsetus*), Garfish (*Belone belone*), Round sardinella (*Sardinella aurita*), Black scorpionfish (*Scorpaena porcus*), Brill (*Scophthalmus rhombus*), Stargazer (*Uranoscopus scaber*), John Dory (*Zeus faber*). Finally, except for carp species (*Cyprinus carpio*, *Hypophthalmichthy~~i~~s molitrix*, *Ctenophary~~i~~ngodon idella*, *Aristichthys nobilis* (valid name *Hypophthalmichthys nobilis*) and *Carassius* sp.), the official list does not include any of the species leading the Bulgarian aquaculture sector (see section 3.2).

As for crustaceans and molluscs, the list includes a paltry number of species despite the consumers' demand and the products imports of these macro-categories have increased in the last years (Todorov, 2017). Particularly, only one or two species for each of the most relevant crustacean subgroups (shrimp, crab, lobster, crayfish) and, within mollusc macro-category, one mussel (*Mytilus galloprovincialis*), one scallop (*Pecten maximus*), one whelk (*Buccinum undatum*) and two cuttlefish species (*Sepia officinalis* and *Rossia macrosoma*) are mentioned in the list, with the total exclusion of clam, octopus and squid products. The remarkable absence of rapa whelk (*Rapana venosa*), ~~which represents~~ one of the specie most exploited along the Black Sea coasts, also emerged (Keskin et al., 2017).

About the congruency between CN, expressed both in Bulgarian and English, and the SN listed in the Decree, two notable incongruences were found, related to the terms Мерлуза and Морски език. The two CNs are associated to two and three distinct specie or genus SN referring to fish belonging to taxonomically unrelated families. ~~In particular, t~~The term Мерлуза, corresponding to the English term hake and congruently associated to the species SN *Merliccius merluccius* is also coupled with~~to~~ two taxonomically distant genus SN, *Lepidorhombus* sp. and *Brama* sp., including turbot and pomfrets species respectively. Similarly, the term Морски език, corresponding to sole, in addition to being correctly associated with the genus *Solea* sp., also refers to two scabbardfish species (*Lepidopus caudatus* and *Aphanopus carbo*). In both cases, since the first scientific name proposed is properly associated with the respective commercial name, the presence of the other scientific names might be attributable to errors during the document's drafting. This hypothesis is further supported by the fact that each SN is correctly associated to the relative CN in English language listed in the Decree.

Moreover, from the comparison of the SN reported in the list against the reference scientific name available on FAO Fishbase and Sealife databases (www.fishbase.org; www.sealifebase.org), the presence of obsolete names for 19.3% (12/62) of the species was highlighted (Table 2SM).

Finally, with respect to the association between CN and SN, 80.9% (55/68) of ~~-~~the CI reported in the list were made of one CN corresponding to only one SN, while in 14.7% (10/68) of the cases one CN corresponded to two or more SN and in 4.4% (3/ 68) of the combinations two CN correspond to the same SN. The SI calculated as described in section 2.2, was then introduced ~~as an objective index of~~to assess the list's accuracy. According to Xiong et al., (2016a), SI = 1 corresponds to the most accurate situation, as a single CN is associated to only one species. SI> 1 indicates the presence of a number of commercial names greater than the listed species and the consequent marketing of the same species with more than one trade name. In contrast, when SI <1 different species share the same commercial name. The ~~final~~ Bulgarian list SI was 1.04. Although this value suggests a good level of accuracy of the list, it is opportune to underline that in 13 CIs the CN was associated to a SN referred to an entire genus. If this is considered, the SI value would be lower. In particular, by adding all the species (N=229) belonging to the mentioned genera, a value of 0.22 is obtained, highlighting the list structure is still far from the "one-species-one-name" approach advocated at international level as a tool to guarantee a fair and transparent marketplace and to contrast IUU fishing and seafood fraud incidents (Oceana, 2014).

3.2 Samples collection: frequency and type of products

Overall, 66% (64/97) of the samples was made of unprocessed products (28.9% fresh and 37.1% frozen products, both consisting of whole or filleted/sliced seafood). The remaining 34% (33/97) consisted of different type of processed products of which: 18 smoked, 9 marinated and 6 breaded precooked products (Table 2; Table 1SM). The sampling frequency ~~thus~~ reflected the Bulgarian consumers' choice, generally oriented to unprocessed fresh or frozen products followed by molluscs and smoked/canned fish and crustaceans. Fish fillets and sliced fishery products, particularly, account for almost 60% of total fish EU and extra EU imports (Todorov, 2017).

As regards the species, 16.5% of the total products (16/97) derived from the national production and consisted of sprat (3/97) and four farmed products: grass carp (2/97), bighead carp (5/97), trout (4/97) and African sharptooth catfish (2/97) (Table 2, Table 1SM). ~~In particular, s~~Sprat is one of the main species of local interest accounting for around 74% of the Black Sea catches; grass carp, bighead carp and trout constitute the majority of national aquaculture production and African sharptooth is one of the species recently introduced together with Barramundi (*Lates calcarifer*), as result of the farming system diversification (Todorov, 2017). Nevertheless, most of the samples collected in the study (81.4%, 79/97) derived from both intra-EU (27/97) and extra-EU import (52/97). Spain, Portugal and Great Britain were recorded as the main supplier of intra-EU imported products, while most products of non-European origin were found to be imported, in order of frequency, from n, Norway, USA, China and Vietnam (Table 2). As reported in Table 2, the imported products mainly consisted of salmon (19.6%, 19/97), Alaska pollock (13.4%; 13/97), mackerel (9.3%; 9/97), herring (8.2%, 8/97) and squid (9.3%, 9/97), followed by garfish (4.1%; 4/97), blue hake (3.1%; 3/97), pangasius (3.1%; 3/97), and sprat (3.1%; 3/97). The sampling frequency agrees with the Bulgarian National Statistical Institute data, recorded in the two-year period 2015-2016, related both to the principal market suppliers and to the rates and types of products imported. These data reported an increasing demand for mid- to high-end species (such as salmon, squid and hake) over less valued national freshwater and marine species (Todorov, 2017). The high market availability and the increase in market demand of salmon, herring and mackerel, particularly rich in ω3 fatty acids, might be plausibly related to a greater attention of the Bulgarian consumer towards the healthy action of the intake of polyunsaturated fatty acids in the prevention of cardiovascular diseases and hypertension and in the modulation of immunological and inflammatory responses (Kris-Etherton, Harris & Appel, 2002; Gottrand, 2008; Merdzhanova, Stancheva & Makedonski, 2012; Merdzhanova et al., 2017).

3.3 Analysis of the labels

All the information on products' labels are reported in Table 1SM. Eighty-two out of the 97 products collected, consisting of 64 unprocessed and 18 smoked products, feall within the scope of the Regulation and were thus included in the label analysis (see section 2.3).

3.3.1 Commercial designation and scientific name. The commercial designation in Bulgarian language was present in all the analysed samples and it was accompanied by a designation in English language in 17% of the products (14/82), all unprocessed prepacked products. On the contrary, the lack of the scientific name was recorded in 28 samples, all unprocessed non-prepacked products, with a 34.1% (28/82) final non-compliance rate. This rate~~e percentage found~~ is consistent with the findings obtained by Esposito & Meloni (2017), in a similar study conducted in Sardinia (Italy) in 2017. The presence of the complete CI including both the commercial name (CN) and scientific name (SN) (of species or genus) was verified for 65.8% (54/82) of the products. Among these 54 products only 7 (13.0%) were compliant with the Ministerial Decree. Thirty-two products (59.2%) showed CN and SN not included in the list. Products for which no official CI was provided on the Ministerial Decree (Table 2 and section 3.2) are listed in Table 3~~(Table 3)~~.

As detailed in Table 4, in 11 products (20.4%) the SN declared, even though included in the official list, was associated to an incomplete or different CN with respect to the official item.

Finally, in 4 products (7.4%) the commercial name "Треска" reported on the label, ~~and~~ which was also present in the official list referring to *Gadus morhua*, was instead used in association with the SN *Theragra chalcogramma*, not included in the list.

Interestingly, all the marinated products and 4 out of the 6 breaded precooked products (1 fish and 3 cephalopod products), although not covered by the Regulation EU n.1379/2013, presented both commercial and scientific name on the label (Table 3).

3.3.2 Declaration of catching area and fishing gear. The products covered by the Regulation (EU) n.1379/2013 (N=82) were preliminarily split into two subgroups according to the production method reported on the label: 58 products were caught while 24 originated from aquaculture. The product's origin (as catching area or country) was declared in 93% (54/58) of the caught products and in 100% of the farmed products. About the caught products, despite the low percentage of labels without any reference to the catching area, in 85.2% (46/54) of the cases the origin was not properly declared, lacking the reference to the full name of the FAO area and subarea required by law (Table 1SM).

The fishing gears were declared only in the 44.8% (26/58) of the wild products. Interestingly, although not compulsory required, all the marinated products, together with the complete commercial name (see section 3.3.1), reported information about both the FAO area and the fishing gear used during the catching activities.

3.4 Total DNA extraction, amplification and sequencing

All 97 products analysed were successfully extracted, obtaining a total DNA of good quality, characterized by average absorbance ratios always higher than 1.90 for both the absorbance indices (A260 / A280 and A260 / A230).

All the samples produced at least one amplicon suitable for sequencing and one readable sequence, except for BM57, for which no PCR products were obtained neither using full-length barcode (FLB) nor the mini DNA barcode (MDB) protocol.

A total of 96 PCR products, 88 FLB and 8 MDB, were purified for sequencing analysis. All the MDB belonged to DNA samples extracted from smoked products (BM38, BM39, BM40, BM78, BM81, BM82, BM83, BM84). The FLB amplification failure in these products may be recollected to a partial DNA fragmentation and oxidation induced by the smoking process, although this procedure was shown not directly affecting the DNA integrity (Pollack et al., 2018).

All the PCR products were successfully sequenced. The final length of the FLB sequences ranged from 411 to 655 bp, with an average length of 539 bp (82.3% of the expected amplicon length), while all the MDB sequences reached the expected amplicon length (139 bp).

3.5 Post sequencing analysis: molecular species identification and assessment of species substitution rate

3.5.1 Molecular identification by database comparison. Overall, by the combination of BLAST and BOLD ID's results, 87 products out of 96 (91%) were unequivocally allocated to species level (Table 2SM). Subsequently, issues highlighted during the analysis of the sequences related to 4 products (BM4, BM26, BM45, BM61), showing a maximum identity value of 100-98% against numerous *Pangasianodon hypophthalmus* reference sequences and a 100% identity with two sequences belonging respectively to the species *Pangasius krempfi* and *Pangasius bocourti*, were resolved. The analysis of the distance tree provided on BOLD ID's system, by the application of a Neighbor-Joining clustering analysis (Saitou & Nei, 1987) on Kimura 2-parameters distance model (Kimura, 1980), highlighted the presence of well separated species clusters for the three species with the solely exception of the two aforementioned sequences, which were grouped within the *Pangasianodon~~P~~. hypophthalmus* branch. Therefore, these 4 products were confirmed ~~belonging to~~as *Pangasianodon~~.~~ hypophthalmus*. Thus, the final number of products identified at species level reached 91 (94.8%).

Three of the 5 remaining sequences, represented by 2 DNA samples belonging to tuna products (BM9, BM27) and 1 DNA sample from hake product (BM59), were finally allocated to *Thunnus* sp. and *Merluccius* sp. genera. Limits in the species discrimination, also highlighted in previous studies, were recollected to a reduced divergence rate of the *COI* target within the genus (Rasmussen & Morrissey, 2008; Kochzius et al., 2010). In this case, the substitution or the association of the *COI* gene with an alternative DNA target such as cytochrome b (*cytb*), mitochondrial Control Region or nuclear First Internal Transcribed Spacer for rDNA (*ITS-1*) could contribute to improve the efficiency of the DNA barcoding technique (Santaclara et al., 2015; Mitchell & Hellberg, 2016; Armani et al., 2017).

Finally, two DNA samples related to grass carp products were only confirmed belonging to Ciprinidae family. Failure of species and genus identification in this case was due to the obtaining of maximum identity values (100-99%) with deposited reference sequences from commercial inter-species hybrids commonly used in aquaculture and fishery stocking programs (He et al., 2013; Bartley, Rana & Immink, 2000).

3.*5.2 Assessment of Species substitution rate.* All the molecular results are reported in Table 3SM. Only 62 out of the 96 products, for which a readable sequence was obtained, were originally provided with a species-specific scientific name at purchase while the remaining 32 products had been offered for sale with the sole presence of a commercial name (N=29) or associated to a genus SN (N=3). Thus, the species substitution rate was calculated only accounting the molecular results related to the first products subset (N=62).

Overall, 11 products were found not correctly labelled with a final species substitution rate of 17.7% (11/62). The substitutions were equally distributed between unprocessed or processed smoked products (6/62; 9.7%) falling within the scope of the Regulation (EU) n. 1379/2013 and processed products (marinated and breaded precooked) (5/62; 8.0%) for which the SN declared on the label was voluntary introduced by the producers.

Three out of the 11 species substitution incidents, characterized by the replacement of *Salmo salar* with *Oncorhynchus mykiss* (2 cases) and *Gadus chalcogrammus* (previously *Theragra~~.~~ chalcogramma*) with *Pangasianodon hypophtalmus*, can be taken as examples of typical deliberate substitution for the operators' economic benefit, driven by the considerable price gap between the substituent and replaced species that outweighs the risk of detection (Table 5) as also observed by Helyar et al., 2014 in a survey on whitefish products sold on the UK market. Striped catfish (*Pangasianodon~~.~~ hypophtalmus*) in particular, which has been reported as substituent species since early 2000s together with the booming of the species aquaculture and its expansion to the global market (Rehbein, 2008), is still reported as substituent species of several medium-high price marine and farmed fish species such as grouper (*Epinephelus* sp.) Nile ~~P~~perch (*Lates niloticus*), r~~R~~ed ~~S~~snapper (*Lutjanus campechanus*), European plaice (*Pleuronectes platessa*), ~~and~~ halibut (*Hippoglossus hippoglossus*) and Greenland halibut (~~;~~ *Reinharditius hippoglossoides*), both in unprocessed and processed products purchased at retail level and restaurants (Galal-Khallaf et al., 2014; Tantillo et al., 2015; Nagalakshmi et al., 2016; Kappel & Shroder, 2016, https://ec.europa.eu/food/sites/food/files/safety/docs/official-controls_food-fraud_fish_test_substitution_table3.pdf). Similarly, the substitution of Atlantic salmon (~~*Salmo*~~ *Salmo~~.~~ salar*) with farmed exemplars belonging to *Oncorhyncus~~.~~ mykiss* or other species of the genus *Oncorhynchus* sp. has already been described (Zhang & Cai, 2007; Filonzi, Chiesa, Vaghi & Marzano, 2010; Cline, 2012) and recently remarked by a survey on salmon imported in the US (Warner et al., 2015).

Less relevant mislabelling incidents concerned: 1 frozen whole exemplar of mackerel (BM55), labelled as *Scomber japonicus* and confirmed as *Scomber scombrus*, 1 marinated product sold as Garfish (*Belone belone*), actually belonging to *Scomberesox saurus*, 1 marinated product sold as *Sardinella aurita* with the commercial designation "anchovy" (Аншоа) and verified belonging to *Sardina pilchardus* (Sardine), 1 product declared as *Theragra chalcogramma* (BM63) instead identified as *Gadus morhua* (Atlantic cod) (Table 3SM). The substitution with species of analogous or low commercial value and consumer's appeal was more plausibly to be recollected to the improper training of the operator to the species morphological identification than the voluntary replacement. This underlines the need to improve the operator's awareness on the pivotal informative role of the labelling tool according to the general objectives of the Regulation (EU) n. 1169/2011, aimed at ensuring consumer's informed choices and preventing consumer's misleading. However, the attempt to reallocate product belonging to illegal practices (IUU fishing) on the market cannot be completely excluded. Similar issues have in fact reported in recent survey conducted in EU (Helyar et al., 2014) and extra-EU market (Xiong et al., 2016b).

The last mislabelling incidents concerned 3 squid-based processed ready to cook products, labelled as argentine squid (*Illex argentinus*) and finally identified as the Humboldt squid (*Dosidicus gigas*). This finding is consistent with the study conducted by Wen et al. (2017), which reported all the processed squid products analysed as solely belonging to the Humboldt squid. Given the current similar availability of these two cephalopod species on the international market a plausible explanation to the systematic substitution of Argentine squid with Humboldt squid could rely on the current lower price and high post-processing yield of the latter species against to the substitute (Arkhipkin et al., 2015; Wen et al., 2017).

~~Interestingly, t~~The overall value of mislabelling rate found in the present study (17.7%) is slightly lower than the value of 20% recently reported by Oceana, as a result of the analysis of more than 200 studies on mislabelling conducted in 55 globally distributed countries (http://usa.oceana.org/publications/reports/deceptive-dishes-seafood-swaps-found-worldwide). —A similar value (22.5%) was also obtained in a recent study on 277 products imported into the EU from Third countries (Guardone et al., 2017). However, as reported above, in this study 34 out of 96 products (35.4%) weren't included in the analysis due to the absence of scientific name on the labels that hampered the assessing of the species substitution rate. This could plausibly result in an underestimation of the real value and the final substitution rate that may have in fact precisely matched with the percentage obtained from the aforesaid report. Our data seem to confirm the reduction of unfair practices in the EU territory as already observed by Mariani et al., (2015) that, in a survey conducted across 6 countries (France, Germany, Ireland, Portugal, Spain and UK), demonstrated an apparent reduction of seafood mislabelling in Europe. The positive correlation between a well-structured traceability legal framework and a low rate of species substitution also emerge from a study conducted in Tasmania where the labelling accuracy is central for the seafood market (Lamendin et al., 2015). However, in the aforesaid study, the absence of mislabelling was also considered as a consequence of a high consumer awareness in the marketplace and of the geographical location. Conversely, in countries outside Europe characterized by a different legal framework respect to the one implemented in the EU, species substitution rates are still high reaching, in some cases, impressive values (Xiong et al., 2016a).

4. Conclusions

The Bulgarian seafood market, although gradually growing, still represents a minor sector of the national economy. Currently, data on the effective application of the ~~current~~ European legislation on traceability and product labelling are limited in this country. The study highlighted numerous non-compliances with respect to the obligations imposed by European regulations on seafood labelling. These issues probably arise from the lack of update of the Bulgarian official list of commercial designations which presents objective criticalities as regards the number and validity of items provided. The species substitution rate highlighted, although falling within the average percentage observed at the international level, contributes to emphasize the need to improve systems for verifying the products' identity along the supply chain. In fact, the introduction of molecular techniques as both FBO self-control and official control analytical tools could prevent and reduce voluntary and involuntary mislabelling incidents.

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Tables

Table 1: List of the main commercial fish species consumed within Europe (EUMOFA, 2017) and not included in the Bulgaria list. Highlighted in bold are the species highly appreciated by the Bulgarian consumers (Todorov, 2017).

Commodity category*	Commercial name	Scientific name
Salmonids	**Atlantic salmon**	***Salmo salar***
	Chum salmon	*Oncorhynchus keta*
	Pink salmon	*Oncorhynchus gorbuscha*
	Coho salmon	*Oncorhynchus kisutch*
	Rainbow trout	***Oncorhynchus mykiss***
Tunas and Tuna like species	Yellowfin tuna	*Thunnus albacares*
	Blackfin tuna	*Thunnus atlanticus*
	Longtail tuna	*Thunnus tonggol*
	Suthern bluefin tuna	*Thunnus maccoyii*
	Pacific bluefin tuna	*Thunnus orientalis*
	Skipjack tuna	*Katsuwonus pelamis*
	Bullet tuna	*Auxis rochei*
	Frigate tuna	*Auxis thazard*
	Bonitos	*Sarda* sp.
	Swordfish	*Xiphias gladius*
Groundfish	Hake	*Merluccius* sp.
	Alaskan Pollock	***Gadus chalcogrammus***
Freshwater fish	**European eel**	***Anguilla Anguilla***
	Nile Perch	*Lates niloticus*
Flatfish	Atlantic halibut	*Hippoglossus hippoglossus*
	Greenland Halibut	*Reihnardtius hippoglossoides*
Other marine fish	Gilthead seabream	*Sparus aurata*
	Other seabreams	*Dentex sp., Pagrus sp., Pagellus sp.*
	European seabass	*Dicentrarcus labrax*

*The commodity groups reported are provided by the EUMOFA for the data management of the main commercial species for statistical purposes

Family	Product category	Product type

Table 2: Product categories and sampling frequency

	Bulgarian language	English translation	Unprocessed		processed		total
			N. of samples	Origin	N. of samples	Origin	
			FISH				
Salmonidae	Сьомга	Salmon	6	Extra-EU, Norway (6)	9	Extra-EU, Norway (9)	15
		Pacific salmon	4	Extra-EU, Alaska (4)	0	-	4
	Пъстърва	Trout	4	**Bulgaria (4)**	0	-	4
Gadidae	Морска треска	Alaska Pollock	12	Extra EU, NR (8) Extra EU, China (4)	1	Extra EU, NR (1)	13
	Треска	Cod	1	EU, Holland (1)	0	-	1
Merluccidae	Хоки	Blue grenadier, Blue hake	3	Extra-EU, USA (3)	0	-	3
Clupeidae	Шпроти/ Цаца	Sprat	6	**Bulgaria (3)** EU import, NR (3)	0	-	6
	Херинга/ Салака	Herring	2	EU-Estonia (1) EU-Latvia (1)	6	Extra EU, Norway (6)	8
	Аншоа	Anchovy	0		1	EU-Spain (1)	1
Scombridae	Скумрия	Mackerel	1	EU-NR (1)	8	EU, Great Britain (5) Extra EU, Faroe Islands (2) Extra EU, Norway (1)	9
	Риба тон/	Tuna	2	Extra EU, Vietnam (2)	0	-	2
Belonidae	Зарган	Garfish	1	EU, Spain (1)	3	Extra EU, NR (1) EU, Spain (2)	4
Ciprinidae	Толстолоб	Bighead Carp	5	**Bulgaria (5)**	0	-	5
	Амур	Grass Carp	2	**Bulgaria (2)**	0	-	2
	Мрена	Albanian barbel	1	EU, Greece (1)	0	-	1
Moronidae	Лаврак	European Seabass	2	EU, Greece (2)	0	-	2
Clariidae	Африкански сом	African shartooth catfish	2	**Bulgaria (2)**	0	-	2
Pangasiidae	Пангасиус	Pangasius (Striped catfish)	3	Extra-EU, Vietnam (3)	0	-	3
Nototheniidae	Нототения		1	EU, Great Britain (1)	0	-	1
			CEPHALOPODS				
Ommastrephidae/L oliginidae	Калмар	Squid	4	EU-Portugal (2) EU, Great Britain (2)	5	EU, Spain (3) NR (2)	9
Octopodidae	Октопод	Octopus	2	Extra EU, Morocco (2)	0	-	2
TOTAL			**64**		**33**		**97**

Table 3: List of unprocessed and processed products presenting both CN and SN declared on the label. In brackets the valid scientific name according to FAO fish base database (fishbase.org)

CN labelled on the product	English translation of the original CN	SN labelled on the product	Type of product	n. of products
Products covered by the labelling requirement of the Regulation (EU) n. 1379/2013				
Сьомга	Salmon	*Salmo salar*	Processed, Smoked	9
		Oncorhynchus gorbuscha	Unprocessed	4
Морска треска	Alaska Pollock	*Theragra chalcogramma* (*Gadus chalcogrammus*)	Unprocessed	8
Пъстърва	Trout	*Salmo gairdneri irideus* (*Oncorhynchus mykiss*)	Unprocessed	3
Хоки	Hake	*Merluccius* sp.	Unprocessed	3
Пангасиус	Pangasius	*Pangasianodon hypophthalmus*	Unprocessed	1
Риба тон	Yellofin tuna	*Thunnus albacares*	Unprocessed	1
Мрена	Barbel	*Barbus albanicus*	Unprocessed	1
Нототения	Notothenia	*Patagonotothen ramsay*	Unprocessed	1
Зарган	Garfish	*Belone belone*	Unprocessed	1
Products not covered by the requirement of the Regulation (EU) n. 1379/2013				
Морска треска	Alaska Pollock	*Theragra chalcogramma* (*Gadus chalcogrammus*)	Processed, preacooked breaded	1
Калмари	Squid	*Illex argentines*	Processed, preacooked breaded	3
Херинга	Herring	*Clupea harengus*	Processed marinated	3
Салака	Herring	*Clupea harengus*	Processed marinated	1
	Mackerel	*Scomber scombrus*	Processed marinated	2
Зарган	Garfish	*Belone belone*	Processed marinated	1
		Scomberesox saurus	Processed marinated	2

Table 4: Products for which an incomplete or different CN with respect to the official item was provided.

Bulgarian Decree		Labelling information		n. of products
Official SN	Official CN	SN	CN	
Scomber scombrus	Атлантическа с~~С~~кумрия	*Scomber scombrus*	Скумрия	8
Scomber japonicus	Японска с~~С~~кумрия	*Scomber japonicus*	Скумрия	1
Clupea harengus	Херинга	*Clupea harengus*	Салака	2

Table 5: List of products found mislabelled by the comparison of the molecular results with the labelled scientific name. The products not falling within the scope of Regulation EU n.1379/2013, for which species scientific name was voluntary declared on the label, are highlighted in bold.

Codes	Type of product	Scientific name Declared	Comm. interest*	N	SN molecularly verified	CN associated	Commercial interest*
BM42	Processed smoked fillet	*Salmo salar*	High	2	*Oncorhychus mykiss*	Rainbow trout	low
BM43	Processed smoked slice				*Oncorhychus kisutch*	Coho salmon	medium
BM45	Unprocessed frozen fillet	*Theragra. chalcogramma (G.chalcogrammus)*	High	1	*Pangasianodon. hypophthalmus*	Striped catfish	low
BM63	Unprocessed frozen fillet			1	*Gadus morhua*	Atlantic cod	high
BM48	**Processed marinated fillet**	***Belone belone***	**NR**	**1**	***Scomberesox saurus***	**Atlantic saury**	**low**
BM64	Unprocessed frozen beheaded	*Belone belone*	NR	1	*Scomberesox saurus*	Atlantic saury	low
BM47	**Processed marinated fillet**	***Sardinella aurita***	**High**	**1**	***Sardina pilchardus***	**Sardine**	**high**
BM55	Unprocessed frozen whole exemplar	*Scomber japonicus*	High	1	*Scomber scombrus*	Mackerel	high
BM92	**Breaded precooked slices**	***Illex argentinus***	**Medium-high**	**3**	***Dosidicus gigas***	**Humboldt squid**	**Medium-high**
BM93	**Breaded precooked slices**						
BM94	**Breaded precooked slices**						

*value estimates collected by species card directly accessible from fishbase.org and sealifebase.org. SN: Scientific name; CN: commercial name N: number of products; NR: Not Reported.

Highlights

- Label compliance with EU law of seafood products sold in Bulgaria was verified
- DNA barcoding was used to assess products' species identity and substitution rate
- Several shortcomings and incongruences were found with the labels analysis
- The DNA barcoding revealed a species substitution rate of 17.7%
- The Bulgarian official list of the seafood designations need updating

e-component
Click here to download e-component: Table 1SM.docx

e-component
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