Amplification of 16S rDNA reveals important fish mislabeling in Madrid restaurants

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ABSTRACT

Food fraud encompasses economic fraud and can produce health problems for
consumers, as well as conservation problems for the involved species. Nevertheless,
few studies tested for mislabeling in restaurants. In this study, we tested for mislabeling
of fish species in restaurants. We sampled 77 fish dishes from 53 different restaurants
located in 9 different districts of Madrid, Spain. A short fragment of the 16S rDNA was
employed for DNA amplification leading to species or genera identification. Results
showed that 7 species or genera and almost 30% of the samples were mislabeled.
Mislabeling was present in 37% of the sampled restaurants and in 71% of the sampled
districts. Mislabeling was common and it was not correlated with a districts' economic
status (i.e. with the official average square-meter price of apartments). The analyses also
showed that some species were more prone to be mislabeled than others.

Keywords:

25 16S rDNA; genetic identification; food control; fraud; mislabeling; species

26 authentication.

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1. Introduction

Food fraud can have important consequences for consumers (e. g. Spink & Moyer, 2011). The most obvious consequences for consumers are of economic nature. Intentional adulteration of food usually provides the vendor with a financial advantage, what is known as ‘Economic Motivated Adulteration (EMA) of food’ (Everstine, Spink, & Kennedy, 2013). Another consequence is the inadvertent consumption of species, which can produce serious health problems (Triantafyllidis, et al., 2010), and that may even lead to intoxication (e.g. with TTX; Giusti, et al., 2018). Last but not least, food fraud can also produce important problems for species conservation (e.g. Ward, Holmes, & Last, 2008), since it includes protected species, endangered species and species with capture quota. In these cases, food fraud generally masks the illegal exploitation (Horreo, Machado-Schiaffino, & Garcia-Vazquez, 2017; Pramod, Nakamura, Pitcher, & Delagran, 2014). Food fraud must be avoided, and its detection and the knowledge about how, where, and when food fraud occurs, is the first step to control it.

Seafood is extremely important in the European Union (EU). For example in 2015, the EU seafood supply (domestic production and import) reached more than 14.5 million tons, and households spent 54.8 billion Euros on seafood from fisheries and aquacultures (EUMOFA, 2017). Food fraud usually occurs due to financial incentives (von der Heyden, Barendse, Seebregts, & Matthee, 2010), and it can be detected using cost-effective, DNA based analytical methods (Tinacci et al. 2018). Fish fraud can happen during catching, at the wholesaler, during processing (Muñoz-Colmenero, Blanco, Arias, Martinez, & Garcia-Vazquez, 2016), in end-user markets (Muñoz-Colmenero, et al., 2015) or in restaurants. In restaurants, the percentage of mislabeling is suggested to be higher than in supermarkets and retailers (Bérnard-Capelle, et al.,

2015), but few studies exist so far and specific studies are required in order to confirm these suggestions (Pardo, Jiménez, & Pérez-Villarreal, 2016, Pardo, et al., 2018). In order to detect fish fraud, the fish species indicated on the product label (bought in a shop/supermarket/supplier/restaurant) can be compared with the fish species revealed by genetic analyses (e.g. Muñoz-Colmenero, et al, 2016). Fish can be mislabeled along the entire supply chain, i.e. when indicating where it was caught (in which geographic area, in the sea, or in a fish farm), during the acquisition of intermediate buyers, during processing, up to the place where the end-user buys it. This makes the origin of mislabeling difficult to track. For example, a restaurant may unintentionally purchase a wrongly labeled species, it may unintentionally use the wrong fish for preparing a meal, or it may intentionally change the species' name to increase his benefits (Kappel & Schröder, 2016), thereby defrauding the restaurant's guests. The detection of mislabeling is very important in order to alert and act against no-ethic and illegal behavior but unfortunately, as commented above, studies investigating mislabeling in restaurants are scarce.

Madrid is the capital of Spain (Europe) and Spain is the country with the highest household expenditure for fisheries and aquaculture in the European Union (EUMOFA, 2017). Three million inhabitants live in the center of the city and Madrid is Europe's 2nd biggest city. Madrid is one of the world's most touristic capitals, with more than 5.5 million tourists visiting Madrid per year (International, 2017). The hostelry is very important and the city center consists of 2,862 restaurants and 3,307 bars (Servilab, 2010) and to our knowledge, no studies assessed the prevalence of food fraud in this city except in grouper (Asensio, 2008). In this study, we tested for mislabeled food and measured the prevalence of mislabeling in fish by comparing whether species and genera indicated on a label coincided with the fish species unraveled by genetic

methods. Anonymous clients took tissue samples from fish meals in several restaurants located in different districts/areas in the city of Madrid and species identification was done employing DNA amplification and BLAST analysis. Results will provide evidence for the existence or absence of fish mislabeling in restaurants of one of the biggest cities of Europe.

2. Materials and Methods

2.1 Sampling

A total of 77 different fish samples (Table 1) were taken from meals served in 53 different restaurants located in 9 different districts of the city of Madrid: Arganzuela, Carabanchel, Centro, Chamartín, Chamberí, Latina, Moncloa, Salamanca and Tetuán. The average apartment price (Euro per square meter) of each of these districts was employed as an indicator of the district's economic level. Average apartment prices were obtained from the Madrid city council (<http://www.madrid.es>; accession date: 18 July 2018).

Employed fish samples included 17 different species or genera, depending on menu label. The number of samples per restaurant ranged between 1 and 7. Anonymous people acted as clients in those restaurants during the years 2017 and 2018 and sampled tissue from served dishes, including fresh fish, tataki, tartar, carpaccio, ceviche, sashimi, and coated and/or fried fish. The restaurant, the fish name appearing in the menu and the sampling date were annotated. Samples were put into Eppendorf tubes and stored frozen until laboratory analyses.

2.2 Genetic analyses

Genomic DNA was extracted from the samples using DNeasy Blood & Tissue Kit (Qiagen; Verlo, Netherlands). A primer pair amplifying a short fragment (75-125 basepairs, approximately) of the 16S rDNA was employed for DNA amplification. This gene has been demonstrated to allow for reliable species authentication, even in highly degraded samples (Horreo, et al., 2012; Muñoz-Colmenero, Martinez, Roca, & Garcia-Vazquez, 2016), and in canned pet food (Armani, et al., 2015). Given that the real processing of the samples (from the supplier until it appears on the dish) collected for this study is unknown, and given that the effective processing may not coincide with what is written on the menus, we used this fragment for species identification. The amplification of this fragment assures that the used genetic tools are highly sensitive, and that they will detect species or traces of species present in dishes with a high probability. Consequently, primers 16S-HF and 16S-HR were used. PCR conditions included a total volume of 25 µL containing 1µL DNA, 12.5 µL DreamTaq Master Mix Polimerase (Thermo Fisher Scientific; Beverly, USA), 1 µL of each primer (10uM) and 9.5 µL of water. PCR cycles included initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 45s with an annealing at 49°C for 60s and an extension at 72°C for 60s, and a final extension at 72°C for 10 min. PCR products were then purified and finally run on an ABI 3100 sequencer (Applied Biosystems; Foster city, USA). DNA sequences were viewed and edited with the BioEdit alignment editor software (Hall, 1999).

2.3 Species identification

The European Regulation (EU) 1379/2013 establishes which information needs to be declared when selling seafood at the retailer or mass caterer. Each EU Member State has to draft an official list with the trade names (including scientific, local or regional

names). These names are the names that need be officially used as product labels in the entire country. The Spanish Ministry of Agriculture, Food and Environment published this list on April 10th, 2014 (BOE-A-2014-3865). The names on this list were employed in this work in order to determine the scientific name that corresponds to each of the menu labels (Table 1). All menu labels detected in this study existed on the official list published by the Spanish Government, only with the exception of “pez mantequilla” (samples S18 and S19), which did not appear in the legislation valid during the samplings (BOE-A-2014-3865). Consequently such samples were deleted from the analyses.

Sequence comparison for species identification was done with the BLAST utility (<https://www.ncbi.nlm.nih.gov/BLAST>) of the GenBank public database. To this end, we first corroborated that the 16S rDNA sequence of the species indicated on the menu label was present in Genbank (<https://www.ncbi.nlm.nih.gov/genbank>). Thereafter, BLAST was used to find the most similar sequence. Successful identification existed if the sequence amplified from the collected tissue exhibited a similarity of 100% with a sequence present in GenBank, following Armani, et al., (2015). If the most similar sequence rendered by BLAST corresponded to a species/genera that did not coincide with the species/genera mentioned on the menu, we classified the sample as being mislabeled. In these cases, an additional BLAST comparison was done with the DNA sequence of the sample vs. the DNA sequence of the taxa mentioned on the menu to test for the robustness of our classification.

2.4 Economic level of Madrid districts

We tested whether there was a correlation between the average apartment price and the percentage of mislabeling. Only districts with two or more samples were included in the Spearman's rank correlation.

2. Results and discussion

All fish samples were successfully amplified. Fragment sizes ranged between 76 and 122 base pairs, which was enough to identify mislabeling on the species/genera level mentioned on the restaurant's menu (Table 1), demonstrating the usefulness of the employed primers even in cooked and processed food. In total, 32 samples exhibited a similarity of 100% with the species mentioned on the menu, and mislabeling appeared in 28.12% of these samples and in 37.5% of the restaurants. Moreover, using Kappel and Schröder (2016) 2% cut-off threshold (i.e. a sequence identity of $\geq 98\%$), mislabeling appeared in 36% of the samples. The here detected proportions of mislabeling do not differ among used thresholds ($\chi^2 = 0.62$, $P = 0.43$) and they are similar to the percentage recently found in Metro Vancouver, Canada (29%; Hu, Huang, Hanner, Levin, & Lu, 2018). Moreover, it is within the range found in European mass caterings (Pardo, et al., 2018). The high proportion of mislabeling demonstrates that a more effective control and/or management is required in order to avoid mislabeling and fraud. This is especially important, since among other reasons mislabeling exists for economic issues, and since it can produce health and conservation problems (Everstine, et al., 2013; Pramod, et al., 2014; Triantafyllidis, et al., 2010).

The detected amount of mislabeling is conservative for several reasons. First, we sampled all kind of fish, although one would predict that fraud and thus mislabeling would be most prevalent in expensive species that are difficult to get, e.g. in *Epinephelus marginatus*. Previous studies detected a much higher percentage of

mislabeling, namely 47.1% in the expensive grouper (*Epinephelus marginatus*; Asensio, 2008), 43% of mislabeling in hake in Spain (Muñoz-Colmenero, Blanco, et al., 2016), and up to 50% of mislabeling of hake in German restaurants (Kappel & Schröder, 2016). Second, in this study we sampled restaurants only once and only a few plates per restaurant (average number of plates sampled per restaurant = 1.42 ± 0.13 standard error), what reduces the chance of detecting mislabeled species. All these facts suggest that the percentage of mislabeled fish species/genera reported in the present study are conservative and likely underestimate the actual level of mislabeling and fraud.

7 species/genera names appearing on the menu were mislabeled (which corresponds to 43.75% of the sampled menu labels; Fig. 1), and these names correspond to the following species: *Stizostedion* spp., *Scophthalmus maximus*, *Merluccius merluccius*, *Lepidorhombus boscii*, *Galeorhinus galeus*, *Epinephelus marginatus*, and *Gadus* spp. (see BOE-A-2014-3865). Interestingly, some of the species/genera names appearing on the menu were consistently wrong and were thus fraudulent in all sampled cases. The two restaurants in which we supposedly consumed *Epinephelus marginatus*, actually sold two different fish species. In contrast, in other species the name on the menu consistently coincided with the real species, e.g. in *Micromesistius poutassou* (three samples from three different restaurants were correctly labeled). This suggest that some species are more prone to fraud than others, and that species subjected to fraud were substituted by several different species. This information is important in order to unravel the source of mislabeling and fraud and for developing management actions in relation to seafood fraud. If all restaurants sell a given species under a wrong name, it is more likely that mislabeling and fraud happen before the restaurants buy the fish, while

strong differences in the substitution among restaurants point to restaurant-specific tactics.

In addition, mislabeling appeared in the majority (71.43%) of the sampled districts of Madrid, namely in: Salamanca, Chamartín, Tetuán, Centro and Latina. In only one district, Carabanchel, mislabeling was absent, but it had only one sample. The detected percentage of mislabeling was not correlated with the apartment prices of the district where the sampled restaurant was located ($n = 6$, $r_s = -0.77$, $p = 0.10$). This result suggests that mislabeling is widely extended and independent of the economic level of the district in which the restaurants are located. However, a more extensive sampling is required to corroborate this result.

3. Conclusions

In conclusion, mislabeling of fish sold in restaurants in Madrid is important since it concerned 28.12% of the sampled fish dishes. It is more common in some fish species than in others and it is present in the majority of the districts of the city independently of their economic level, pointing to fraud at different levels of the sales hierarchy (restaurant and wholesaler).

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Figure captions

Fig. 1.- Proportion of Fish mislabeling (% , black bars) with respect to the menu labels detected in the present study (Y-axis, number of samples within brackets).

311 **Table 1.-** Fish and shark samples (S) obtained from restaurants (abbreviation: Rest) of eight different districts of Madrid, Spain. The table shows
312 the Spanish (Menu name) and scientific name (Menu taxa) of the fish taxa mentioned on the Menu, the species identified by BLAST
313 (<https://www.ncbi.nlm.nih.gov/BLAST>), and the similarity between the amplified and the most similar sequence existing in GeneBank (Sim, in
314 %). The presence of mislabeling was annotated with *Yes* in column (Mislab), and *No* indicated that menu name and BLAST species coincided.
315 Only 100% similarities were taken into account for results and analyses, and in mislabeled samples, similarity of the sample with the fish taxa
316 mentioned on the Menu (in %) is shown between brackets in column “Sim”.

District	Rest	S	Menu name	Menu taxa	BLAST	Sim	Mislab
Arganzuela	R45	S67	Corvina en escabeche	<i>Argyrosomus regius</i>	<i>Thunnus sp.</i> , <i>Katsuwonus pelamis</i>	99	-
Carabanchel	R2	S3	Atún	<i>Thunnus spp.</i>	<i>Thunnus sp.</i> , <i>Katsuwonus pelamis</i>	99	-
Carabanchel	R2	S4	Bacaladillas	<i>Micromesistius poutassou</i>	<i>Micromesistius poutassou</i> , <i>M. australis</i>	100	No
Centro	R15	S25	Carpaccio de Atún	<i>Thunnus</i> , <i>Katsuwonus pelamis</i>	<i>Gadus morhua</i> , <i>G. macrocephalus</i>	99	-
Centro	R16	S26	Tartar de atún	<i>Thunnus spp.</i>	<i>Salmo salar</i>	99	-
Centro	R19	S29	Merluza	<i>Merluccius merluccius</i>	<i>Merluccius paradoxus</i>	100	No
Centro	R20	S30	Mero	<i>Epinephelus marginatus</i>	<i>Argyrosomus japonicus</i> , <i>A. amoyensis</i>	98	-
Centro	R21	S31	Bacalao	<i>Gadus spp.</i>	<i>Gadus morhua</i> , <i>G. macrocephalus</i>	100	No
Centro	R23	S34	Pincho de bacalao	<i>Gadus spp.</i>	<i>Gadus morhua</i> , <i>G. macrocephalus</i>	98	-
Centro	R23	S35	Pincho de salmón	<i>Salmo salar</i>	<i>Salmo salar</i>	100	No
Centro	R24	S36	Salmón	<i>Salmo salar</i>	<i>Salmo salar</i>	98	-
Centro	R24	S37	Merluza	<i>Merluccius merluccius</i>	<i>Merluccius merluccius</i>	99	-
Centro	R31	S45	Salmón	<i>Salmo salar</i>	<i>Salmo salar</i>	98	-

Centro	R32	S46	Bacalao	<i>Gadus spp.</i>	<i>Gadus morhua, G. macrocephalus</i>	99	-
Centro	R36	S52	Salmón	<i>Salmo salar</i>	<i>Salmo salar</i>	99	-
Centro	R41	S61	Lubina	<i>Dicentrarchus labrax</i>	<i>Dicentrarchus labrax</i>	100	No
Centro	R48	S71	Bacalao	<i>Gadus spp.</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	100 (86)	Yes
Centro	R49	S72	Pescadilla	<i>Merluccius spp.</i>	<i>Merluccius merluccius</i>	100	No
Centro	R50	S73	Merluza	<i>Merluccius merluccius</i>	<i>Merluccius merluccius</i>	99	-
Centro	R53	S76	Salmón	<i>Salmo salar</i>	<i>Salmo salar</i>	100	No
Centro	R53	S77	Mero	<i>Epinephelus marginatus</i>	<i>Lates niloticus</i>	100 (75)	Yes
Chamartín	R4	S6	Cazón	<i>Galeorhinus galeus</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	100 (93)	Yes
Chamartín	R8	S10	Salmón	<i>Salmo salar</i>	<i>Oncorhynchus masou, O. tshawytscha, O. keta</i>	99	-
Chamartín	R8	S11	Mero	<i>Epinephelus marginatus</i>	<i>Pollachius virens</i>	100 (97)	Yes
Chamartín	R8	S12	Cazón	<i>Galeorhinus galeus</i>	<i>Prionace glauca</i>	98	-
Chamartín	R8	S13	Atún	<i>Thunnus spp.</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	99	-
Chamartín	R8	S14	Bacalao	<i>Gadus spp.</i>	<i>Gadus morhua, G. macrocephalus</i>	100	No
Chamartín	R8	S15	Salmón	<i>Salmo salar</i>	<i>Oncorhynchus keta</i>	99	-
Chamartín	R8	S16	Pescadilla	<i>Merluccius spp.</i>	<i>Merluccius merluccius</i>	99	-
Chamartín	R14	S24	Merluza	<i>Merluccius merluccius</i>	<i>Merluccius merluccius</i>	99	-
Chamartín	R38	S57	Tosta de sardina ahumada	<i>Sardina pilchardus</i>	<i>Sardina pilchardus</i>	100	No
Chamartín	R38	S58	Ceviche de atún	<i>Thunnus spp.</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	100	No
Chamberí	R10	S18	Sashimi de pez mantequilla	-	<i>Lepidocybium flavobrunneum</i>	99	-
Chamberí	R18	S28	Dados de Salmon	<i>Salmo salar</i>	<i>Salmo salar</i>	99	-
Chamberí	R29	S43	Dorada	<i>Sparus aurata</i>	<i>Sparus aurata</i>	100	No
Chamberí	R33	S47	Tartar de atún	<i>Thunnus spp.</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	99	-
Chamberí	R42	S62	Bacalao	<i>Gadus spp.</i>	<i>Gadus morhua, G. macrocephalus</i>	99	-
Chamberí	R43	S63	Bacalao	<i>Gadus spp.</i>	<i>Gadus morhua, G. macrocephalus</i>	100	No

Chamberí	R46	S68	Salmón	<i>Salmo salar</i>	<i>Salmo salar</i>	99	-
Chamberí	R46	S69	Atún	<i>Thunnus spp.</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	100	No
Chamberí	R47	S70	Nem de Bonito	<i>Sarda sarda</i>	<i>Merluccius merluccius</i>	99	-
Chamberí	R51	S74	Cazón	<i>Galeorhinus galeus</i>	<i>Prionace glauca</i>	98	-
Chamberí	R7	S9	Perca en ceviche	<i>Stizostedion spp.</i>	<i>Lates niloticus</i>	98	-
Chamberí	R9	S17	Merluza	<i>Merluccius merluccius</i>	<i>Merluccius merluccius</i>	99	-
Latina	R39	S59	Dorada	<i>Sparus aurata</i>	<i>Sparus aurata</i>	100	No
Latina	R40	S60	Ceviche de perca	<i>Stizostedion spp.</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	100 (75)	Yes
Moncloa	R6	S8	Salmón	<i>Salmo salar</i>	<i>Oncorhynchus keta</i>	99	-
Salamanca	R1	S1	Atún	<i>Thunnus spp.</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	98	-
Salamanca	R1	S2	Bacalao	<i>Gadus spp.</i>	<i>Xiphias gladius</i>	100 (81)	Yes
Salamanca	R5	S7	Dorada	<i>Sparus aurata</i>	<i>Sparus aurata</i>	98	-
Salamanca	R12	S21	Gallo	<i>Lepidorhombus boscii</i>	<i>Lepidorhombus whiffiagonis</i>	100 (93)	Yes
Salamanca	R13	S22	Ensalada de ventresca	<i>Thunnus spp., Katsuwonus pelamis</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	98	-
Salamanca	R13	S23	Cazón	<i>Galeorhinus galeus</i>	<i>Prionace glauca</i>	98	-
Salamanca	R25	S38	Rodaballo	<i>Scophthalmus maximus</i>	<i>Scophthalmus maximus</i>	99	-
Salamanca	R26	S39	Atún	<i>Thunnus spp.</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	98	-
Salamanca	R26	S40	Ventresca	<i>Thunnus spp., Katsuwonus pelamis</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	99	-
Salamanca	R28	S42	Pescadilla	<i>Merluccius spp.</i>	<i>Merluccius capensis</i>	99	-
Salamanca	R30	S44	Tartar de atún	<i>Thunnus spp.</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	100	No
Salamanca	R34	S48	Tataki de atún	<i>Thunnus spp.</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	98	-
Salamanca	R34	S49	Ceviche de corvina	<i>Argyrosomus regius</i>	<i>Argyrosomus japonicus, A. amoyensis</i>	98	-
Salamanca	R35	S50	Tataki de atún	<i>Thunnus spp.</i>	<i>Thunnus sp., Katsuwonus pelamis</i>	100	No
Salamanca	R35	S51	Sashimi de salmón	<i>Salmo salar</i>	<i>Salmo salar</i>	99	-

Salamanca	R37	S53	Limanda	<i>Limanda spp.</i>	<i>Limanda aspera</i>	100	No
Salamanca	R37	S54	Bacaladillas	<i>Micromesistius poutassou</i>	<i>Micromesistius poutassou</i> , <i>M. australis</i>	100	No
Salamanca	R37	S55	Salmón	<i>Salmo salar</i>	<i>Oncorhynchus masou</i> , <i>O. tshawytscha</i> , <i>O. keta</i>	99	-
Salamanca	R37	S56	Pescadilla	<i>Merluccius spp.</i>	<i>Merluccius merluccius</i>	100	No
Salamanca	R44	S64	Boquerones	<i>Engraulis spp.</i>	<i>Engraulis encrasicolus</i> , <i>E. eurystole</i>	100	No
Salamanca	R44	S65	Bacaladillas	<i>Micromesistius poutassou</i>	<i>Micromesistius poutassou</i> , <i>M. australis</i>	100	No
Salamanca	R44	S66	Rodaballo	<i>Scophthalmus maximus</i>	<i>Pleuronectes punctatissimus</i>	100 (78)	Yes
Salamanca	R52	S75	Lomos de Merluza	<i>Merluccius merluccius</i>	<i>Merluccius paradoxus</i>	99	-
Tetuán	R3	S5	Sargo	<i>Diplodus sargus</i>	<i>Diplodus sargus</i>	99	-
Tetuán	R11	S19	Pez mantequilla	-	<i>Ruvettus pretiosus</i>	100	-
Tetuán	R11	S20	Merluza	<i>Merluccius merluccius</i>	<i>Merluccius merluccius</i>	99	-
Tetuán	R17	S27	Merluza	<i>Merluccius merluccius</i>	<i>Thunnus sp.</i> , <i>Katsuwonus pelamis</i>	100 (96)	Yes
Tetuán	R22	S32	Salmón	<i>Salmo salar</i>	<i>Salmo salar</i>	100	No
Tetuán	R22	S33	Bacalao	<i>Gadus spp.</i>	<i>Gadus morhua</i> , <i>G. macrocephalus</i>	100	No
Tetuán	R27	S41	Emperador	<i>Xiphias gladius</i>	<i>Dicentrarchus labrax</i>	98	-