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Assessment of the labelling accuracy of spanish semipreserved anchovies products by FINS (forensically informative nucleotide sequencing)

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Abstract

Anchovies have been traditionally captured and processed for human consumption for millennia. In the case of Spain, ripened and salted anchovies are a delicacy, which, in some cases, can reach high commercial values. Although there have been a number of studies presenting DNA methodologies for the identification of anchovies, this is one of the first studies investigating the level of mislabelling in this kind of products in Europe. Sixty-three commercial semipreserved anchovy products were collected in different types of food markets in four Spanish cities to check labelling accuracy. Species determination in these commercial products was performed by sequencing two different cyt-b mitochondrial DNA fragments. Results revealed mislabelling levels higher than 15%, what authors consider relatively high considering the importance of the product. The most frequent substitute species was the Argentine anchovy, *Engraulis anchoita*, which can be interpreted as an economic fraud.

Keywords: Food Science

1. Introduction

Anchovies are small pelagic fish belonging to the Engraulidae family, which play key roles in continental shelf food webs across the globe (FAO, 1988). As a result of their abundance, anchovies have been captured and consumed by humans for millennia, and of the 17 existing genera, *Engraulis*, *Anchoa*, and *Stolephorus*, are those with higher commercial relevance. In fact *Engraulis ringens* is one of the most exploited fish species in the world (FAO, 2014). The European anchovy (*Engraulis encrasicolus*) was the third species of the family in terms of catches in 2010 (529,615 t). *Engraulis encrasicolus* is a highly appreciated species in Europe, and particularly those caught along the northern Spanish coast, which are traded as “Anchoa del Cantabrico”, a denomination regularly employed in canned (“semipreserved”) products.

European legislation about labelling (EU 1169, 2011) lay down the rules about the information that would be provided to final consumers. In addition, the EU 1379/2013 establishes the information that must be reported in case of fishery and aquaculture products. In particular, commercial and scientific names of fish species should be exhibited in seafood products (live, fresh, chilled, frozen, dried, salted or in brine and smoked) at point of sale. This is an important tool helping consumers to identify the nature of the product they are about to buy. Starting from 2002 each European member state has published a list of approved commercial names that must be used for fishery products commercialized in its own territory. The commercial names “Anchoa” and “Anchoas” refer to a type of seafood product where the main ingredient is fish of the family *Engraulidae* (genus *Engraulis*) according to the Spanish approved list of commercial names for seafood products (BOE, 2015), which has been salted and matured for a certain period of time. Nevertheless, taking into account the specific Spanish legislation for semipreserved seafood (RD 1521, 1984), only products with *E. encrasicolus* should be labeled as “anchoa” (without “s”). This kind of product is a delicacy, which can reach commercial values in the Spanish markets from 90 up to 300 €/Kg, depending on the process employed (industrial versus artisanal), the species (*Engraulis encrasicolus* being the most prized of the genus) and the ingredients (type of oil used).

Fish Species identification relies in most cases on external morphological characters, however these are no longer recognizable in processed seafood, therefore several DNA based techniques have been developed to authenticate fish species in seafood products (Rasmussen Hellberg and Morrissey, 2011, Quinteiro et al., 1998; Hold et al., 2001; Griffiths et al., 2014); these have been also proven to be useful in the case of anchovy products (Bréchon et al., 2013; Jérôme et al., 2008; Santaclara et al., 2006; Sebastio et al., 2001).

Seafood mislabelling is a concern from different points of view, such as economic deception and loss of consumer confidence in the fishing industry; the use of cheaper fish species in products sold with the names of higher-value species remains the most common type of seafood mislabelling (Jacquet and Pauly, 2008) and this appears to be greater in processed products (Pardo et al., 2016; Everstine et al., 2013). Mislabelling can also hide illegal fishing practices which affect negatively the sustainability of a resource and damages the long term survival of an ancient human activity (Jacquet and Pauly, 2008). There might be also health implications since some substitute species might be toxic (Armani et al., 2015a; Cohen et al., 2009).

This study aims to evaluate the level of accuracy of the information provided to consumers in the case of processed anchovies in Spain: an important seafood product in the European market. We examine the information provided on the labels of this type of products in relation to its quality (species declared) and quantity (presence/absence of pieces of mandatory and voluntary information).

2. Material and methods

2.1. Samples

56 samples of authentic Engraulidae and Cupleidae species were obtained fresh, from local fish markets, and frozen from collaborating fish canneries (Table 1).

For all authors' knowledge in Spain there are about 130 brands for semipreserved anchovies, most of them are only available in particular cities or retailers, since anchovies market in Spain present a certain level of localism. The main commercial brands are covered in the sampling: the 11 most relevant Spanish commercial brands, present in all retailers sampled, and some of the city-specific and retail-specific brands. We analyzed 63 commercial samples, from a range of 42 brands, of commercial products of semipreserved anchovies, purchased in markets of four different Spanish cities, across three different geographical Spanish regions: Vigo, Santiago de Compostela (North West), Bilbao (North) and Madrid (Centre).

In all cities different locations of the city area were sampled, collecting products both in traditional markets, supermarkets and specialized stores. Samples were obtained in their original packaging and were immediately transported to the laboratory, where they were stored at $-20\text{ }^{\circ}\text{C}$ the same day of the purchase. Before opening the package, all products were photographed (Fig. 1) and label information was recorded (Table 2).

Table 1. Sequences of authentic species used as reference in the present study. “Specimens” column indicates each of the individuals used for performing the analysis. In the case of sequences obtained from a public database GenBank is indicated.

Keys	Common Name (Spanish)	Common name	Scientific name	Specimens	Origin	Provided by	Accession number
EENC	Anchoa	European anchovy	Engraulis encrasicolus	EENC1	Spain	Alfageme	KX062193
				EENC4	Spain	Alfageme	KX062194
				EENC6	Spain	Alfageme	KX062195
				EENC5	Spain	Alfageme	KX062196
				EENC3	Spain	Alfageme	KX062197
EJAP	Anchoa japonesa	Japanese anchovy	Engraulis japonicus	EJAP2	Japan	A. Takasuka	KX062166
				EJAP4	Japan	A. Takasuka	KX062167
				EJAP1	Japan	A. Takasuka	KX062168
				EJAP3	Japan	A. Takasuka	KX062169
				EJAP5	Japan	A. Takasuka	KX062170
EANC	Anchoita	Argentine anchovy	Engraulis anchoita	EANCH1	Argentina	Conxemar	KX062155
				EANCH10	Argentina	Conxemar	KX062156
				EANCH6	Argentina	Conxemar	KX062157
				EANCH8	Argentina	Conxemar	KX062158
				EANCH2	Argentina	Conxemar	KX062159
				EANCH3	Argentina	Conxemar	KX062160
				EANCH7	Argentina	Conxemar	KX062161
				EANCH4	Argentina	Conxemar	KX062162
				EANCH9	Argentina	Conxemar	KX062163
				EANCH5	Argentina	Conxemar	KX062164
				EANCHOITA1	Argentina	Conxemar	KX062165
ERIN	Anchoveta	Peruvian anchovy	Engraulis ringens	ERIN2	Peru	Conxemar	KX062198
				ERIN4	Peru	Conxemar	KX062199

(Continued)

Table 1. (Continued)

Keys	Common Name (Spanish)	Common name	Scientific name	Specimens	Origin	Provided by	Accession number
				ERIN3	Peru	Conxemar	KX062200
				ERIN1	Peru	Conxemar	KX062201
				ERIN5	Peru	Conxemar	KX062174
ANAS	Anchovetas indias, anchoveta blanca	Longnose anchovy	Anchoa nasus	ANAS1	Mexico	IIM	KX062149
				ANAS4	Mexico	IIM	KX062150
				ANAS6	Mexico	IIM	KX062151
				ANAS3	Mexico	IIM	KX062152
				ANAS5	Mexico	IIM	KX062153
				ANAS2	Mexico	IIM	KX062154
EMOR	Anchoa de California	Californian anchovy	Engraulis mordax	EMOR14GB		GenBank	JQ012350
				EMOR15GB		GenBank	FJ264393
				EMOR16GB		GenBank	FJ264392
				EMOR17GB		GenBank	AY923783
				EMOR18GB		GenBank	AY923782
SPIL	Sardina	European pilchard	Sardina pilchardus	SPIL10	Spain	IIM	KX062184
				SPIL13	Spain	IIM	KX062185
				SPIL12	Spain	IIM	KX062186
				SPIL14	Spain	IIM	KX062187
				SPIL11	Spain	IIM	KX062188
SAUR	Sardinelas	Round sardinella	Sardinella aurita	SAUR1	Senegal	IIM	KX062175
				SAUR2	Senegal	IIM	KX062176
				SAUR3	Spain	IIM	KX062177
				SAUR4	Spain	IIM	KX062178
				SAUR5	Spain	IIM	KX062179

(Continued)

Table 1. (Continued)

Keys	Common Name (Spanish)	Common name	Scientific name	Specimens	Origin	Provided by	Accession number
SSAG	Sardinopas	Southamerican pilchard	Sardinops sagax	SSAG1	Peru/Chile	IIM	KX062189
				SSAG4	Peru/Chile	IIM	KX062190
				SSAG3	Peru/Chile	IIM	KX062191
				SSAG2	Peru/Chile	IIM	KX062192
EMAC	Machete	Pacific Menhaden	Ethmidium maculatum	EMAC3	Peru/Chile	BIP Vigo	KX062171
				EMAC2	Peru/Chile	BIP Vigo	KX062172
				EMAC1	Peru/Chile	BIP Vigo	KX062173
SMAD	Sardinelas	Madeiran sardinella	Sardinella maderiensis	SMAD1	Senegal	IIM	KX062180
				SMAD2	Senegal	IIM	KX062181
				SMAD3	Senegal	IIM	KX062182
				SMAD4	Senegal	IIM	KX062183
CHAR	Arenque	Atlantic herring	Clupea harengus	CLUPEAHARENGUSGB		GenBank	EU492008



Fig. 1. Information collected for each commercial sample. Mandatory Information in anchovy labels (red marks) and not mandatory (yellow marks).

2.2. DNA extraction

Before DNA extraction, all commercial samples were desalted by soaking them in sterilized water for 3 to 4 hours at room temperature and rinsed afterwards with sterile water. DNA extraction from reference and commercial samples was carried out as previously described (Chapela et al., 2007) using Proteinase K (Thermo Fisher Scientific) digestion with Wizard DNA Clean-up System kit (Promega) for DNA isolation. DNA quality and concentration were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific). The resulting concentrations of DNA were in the range of 50–500 ng/μL. DNA extracts were kept at –20 °C until analysis.

2.3. DNA amplification

A 464 bp fragment of *cytb* gene was amplified by using the primers described by Burgener (1997) (H15149ad: GCICCTCARAATGAYATTTGTCCTCA and L14735: AAAAACCACCGTTGTTATTCAACTA). The thermal cycling protocol used was: 94 °C for 5 min, followed by 35 cycles of amplification (94 °C for 40 s, 55 °C for 80 s and 72 °C for 80 s) and a final extension step of 72 °C for 7 min. These PCR reactions were carried out in a GeneAmp PCR system 9700 Thermo cycler (Applied Biosystems), with a total reaction volume of 25 μL with 100 ng of DNA template, using PCR Ready-to-Go beads (GE Healthcare) with final concentrations of 1.5 mM of MgCl₂, 0.8 μM of each primer, 0.2 mM of dNTPs and 0.1 units of Taq polymerase.

A shorter cytochrome b fragment (100 bp) was also amplified with the same PCR mix using C-CB primers (Jérôme et al., 2003): C-CB284dF-AYGCNCA-CATTGGNCGRGG and C-CB425dR-CCTCAGAADGACATTTGBCCTC when

Table 2. Labelling information collected in anchovy products showing percentages of appearance.

INFORMATION	Mandatory	number of samples	%
Commercial denomination	Yes	63	100
Declared “anchoa”	Yes	28	44.44
Declared “anchoas”	Yes	35	55.56
Ingredients list	Yes	63	100
with olive oil (or Extra virgin olive oil)	Yes	39	61.9
with vegetable oil	Yes	15	23.81
with sunflower oil	Yes	8	12.7
with mixed oil	Yes	1	1.59
with % of ingredients	Yes	13	20.63
Net and drained weight	Yes	63	100
Conservation instructions	Yes	63	100
Best before date	Yes	63	100
Company name or code	Yes	63	100
Batch number	Yes	63	100
Country of origin (only mandatory for imported products)	Yes	3	100
Nutritional information	No	63	100
Reference to Cantabria*	No	16	25.4
Scientific name	No	23	36.51
Declared <i>E.encrasicolus</i>	No	11	17.46
Declared not <i>E.encrasicolus</i>	No	12	19.05
Artisanal elaboration	No	2	3.17
Elaborated from fresh fish	No	1	1.59
Product from Spain	No	2	3.17
Captured from April to June	No	3	4.76
No lactose	No	1	1.59
No gluten	No	3	4.76
Omega 3 source	No	3	4.76
Natural ingredients	No	1	1.59
Low salted	No	1	1.59
Consumer information telephone	No	5	7.94

*Processed in Cantabria, Processed in Santoña, From the Cantabrian Sea.

the 464 bp fragment amplification failed. In this case, the following thermal protocol was employed: 94 °C for 3 min, followed by 35 cycles of amplification (94 °C for 40 s, 55 °C for 40 s and 72 °C for 40 s) and a final extension step of 72 °C for 7 min.

PCR products were examined and recorded on a 2% agarose gel (Conda) using UV light with GelDOc XR (Biorad). In the case of PCR failure, DNA was run in agarose gels to check the DNA fragmentation status.

2.4. DNA Sequencing and data analysis

Enzymatic purification was applied to PCR products, by adding 3 μ l of illustra™ ExoStar™ 1-Step (GE Healthcare) and incubating at 37 °C for 15 min and 80 °C for 15 min. Two sequencing reactions were performed per PCR product, one with each primer of the set. Sequencing reactions were carried out with BigDye Terminator 1.1 (Applied Biosystems), following the manufacturer's instructions. After cleaning and drying, 15 μ l of Hi-Di Formamide (Thermo Fisher Scientific) were added to the sample tube, and Sanger sequencing carried out in an ABI PRISM 310 genetic analyzer (Applied Biosystems).

After automatic sequencing, forward and reverse sequences were edited with Chromas LITE (Technelysium) and aligned with Bioedit (Hall, 1999) to obtain the complete fragment. NCBI nucleotide database only allows to upload sequences longer than 200 bp, therefore sequences obtained with C-CB primers (100 bp) could not be submitted, and only the sequences obtained with the Burgener fragment have been assigned an accession number. (Table 3: [KJ563141](#) to [KJ563182](#), [KJ623921](#), [KJ645861](#) and [KJ645862](#)).

MEGA (Kumar et al., 2008) was used for a phylogenetic analysis. Genetic distance analysis was used to infer species from DNA sequences obtained from commercial samples using reference sequences obtained from Engraulidae and Cupleidae families (Table 1). The method Neighbor-Joining with Tamura-Nei model was used for the phylogeny reconstruction (Fig. 2). Each sequence was also matched in the Nucleotide BLAST (Basic Local Alignment Search Tool) of the NCBI (National Center of Biotechnology Information), by using the program Megablast with the default algorithm parameters.

103 sequences were used to build a Neighbor-Joining tree with Tamura Nei distances with the 465 bp fragment (44 from commercial samples, 53 from reference samples and 6 downloaded from GenBank) and for the 100 bp fragment Tree 76 sequences were used (14 from commercial samples, 56 from reference samples and 6 downloaded from GenBank).

3. Results and discussion

3.1. Species information in preserved anchovies

Commercial anchovy samples were analyzed using FINS (Forensically Informative Nucleotide Sequencing) (Bartlett and Davidson, 1992). This methodology is based on the analysis of DNA sequences, in this case a cytochrome b fragment of 464 bp

Table 3. Commercial anchovy samples used for the study showing the results of the analyses. Mislabelled samples appear in red.

Saple code	Label information	Date of sampling	City	Species declared	Fragment amplified	FINS result	BLAST result	% of match	GenBank ID	Correct/ mislabelled
AV1	Anchovies fillets in olive oil (37%)	02/05/2013	Vigo	Anchoas	464 bp	Engraulis anchoita	Engraulis anchoita	100%	KJ563154	correct
AV2	Anchovies fillets in olive oil. Anchoas	02/05/2013	Vigo	Anchoas	464 bp	Engraulis anchoita	Engraulis anchoita	100%	KJ563155	correct
AV3	Anchovies in olive oil	02/05/2013	Vigo	Anchoas	100 bp	Engraulis encrasicolus	Engraulis encrasicolus	100%		correct
AV4	Anchovies fillets in olive oil. Cantabrian Sea	02/05/2013	Vigo	Anchoas	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563156	correct
AV5	Anchovy fillets from the Cantabrian Sea in olive oil (30%) FAO 27. VIII. Engraulis encrasicolus	02/05/2013	Vigo	Anchoa	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563141	correct
AV6	Anchovy fillets in vegetable oil	02/05/2013	Vigo	Anchoa	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563157	correct
AV7	Anchovies fillets in sunflower oil, Engraulis anchoita	02/05/2013	Vigo	Engraulis anchoita	100 bp	Engraulis anchoita	Engraulis anchoita	100%		correct
AV8	Anchovy fillets in olive oil (40%)	02/05/2013	Vigo	Anchoa	100 bp	Engraulis anchoita	Engraulis anchoita	99%		mislabelled
AV9	Anchovies in vegetable oil, Engraulis ringens, FAO-087, P019-PAR-PRIS-PERÚ	02/05/2013	Vigo	Engraulis ringens	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563142	mislabelled
AV10	Anchovies fillets in vegetable oil, Engraulis ringens	02/05/2013	Vigo	Engraulis ringens	100 bp	Engraulis ringens	Engraulis ringens	95%		correct
AV11	Anchovies fillets in vegetable oil, Engraulis ringens	02/06/2013	Vigo	Engraulis ringens	100 bp	Engraulis ringens	Engraulis ringens	100%		correct
AV12	Anchovy fillets in olive oil	02/06/2013	Vigo	Anchoa	100 bp	Engraulis anchoita	Engraulis anchoita	100%		mislabelled
AV13	Anchovy fillets in olive oil. Engraulis encrasicolus	02/06/2013	Vigo	Engraulis encrasicolus	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563158	correct
AV14	Anchovy fillets in olive oil. Engraulis encrasicolus	02/06/2013	Vigo	Engraulis encrasicolus	100 bp	Engraulis encrasicolus	Engraulis encrasicolus	100%		correct

(Continued)

Table 3. (Continued)

Saple code	Label information	Date of sampling	City	Species declared	Fragment amplified	FINS result	BLAST re-sult	% of match	GenBank ID	Correct/ mislabelled
AV15	Anchovy fillets in sunflower oil	02/06/2013	Vigo	Anchoa	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	100%	KJ563159	correct
AV16	Anchovies from Santoña in olive oil. Anchovies from the Cantabrian Sea	02/08/2013	Vigo	Anchoas	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563160	correct
AV17	Anchovies in extra virgin olive oil. Cantabrian Sea	02/08/2013	Vigo	Anchoas	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563161	correct
AV18	Anchovies in extra virgin olive oil	02/08/2013	Vigo	Anchoas	100 bp	Engraulis encrasicolus	Engraulis encrasicolus	100%		correct
AV19	Anchovy fillets in olive oil	02/08/2013	Vigo	Anchoa	100 bp	Engraulis anchoita	Engraulis anchoita	98%		mislabelled
AV20	Anchovy fillets in olive oil. Engraulis encrasicolus, Mediterranean Sea (Morocco)	02/08/2013	Vigo	Engraulis encrasicolus	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563143	correct
AS1	Anchovies in olive oil (40%)	06/12/2013	Santiago de Compostela	Anchoas	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563162	correct
AS2	Anchovies fillets in vegetable oil, Engraulis ringens	06/12/2013	Santiago de Compostela	Engraulis ringens	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	98%	KJ563163	mislabelled
AS3	Anchovies fillets in vegetable oil, Engraulis ringens	06/12/2013	Santiago de Compostela	Engraulis ringens	100 bp	Engraulis ringens	Engraulis ringens	100%		correct
AS4	Anchovies fillets in sunflower oil	06/12/2013	Santiago de Compostela	Anchoas	100 bp	Engraulis ringens	Engraulis ringens	100%		correct
AS5	Anchovies fillets in vegetable oil, Engraulis ringens	06/12/2013	Santiago de Compostela	Engraulis ringens	100 bp	Engraulis ringens	Engraulis ringens	100%		correct
AS6	Anchovy fillets in virgin olive oil	06/12/2013	Santiago de Compostela	Anchoa	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563164	correct
AS7	Anchovies fillets in olive oil, from the Cantabrian Sea	06/12/2013	Santiago de Compostela	Anchoas	464 bp	Engraulis anchoita	Engraulis anchoita	99%	KJ563165	correct
AS8	Anchovies fillets in sunflower oil, Engraulis ringens	06/12/2013	Santiago de Compostela	Engraulis ringens	100 bp	Engraulis ringens	Engraulis ringens	100%		correct

(Continued)

Table 3. (Continued)

Saple code	Label information	Date of sampling	City	Species declared	Fragment amplified	FINS result	BLAST re-sult	% of match	GenBank ID	Correct/ mislabelled
AS9	Anchovies fillets in vegetable oil	06/12/2013	Santiago de Compostela	Anchoas	464 bp	Engraulis anchoita	Engraulis anchoita	100%	KJ563166	correct
AS10	Anchovies fillets in vegetable oil	06/12/2013	Santiago de Compostela	Anchoas	Not amplified					
AS11	Anchovies fillets in olive oil	06/12/2013	Santiago de Compostela	Anchoas	Not amplified					
AS12	Anchovy fillets in olive oil, <i>Engraulis encrasicolus</i>	06/12/2013	Santiago de Compostela	<i>Engraulis encrasicolus</i>	464 bp	<i>Engraulis encrasicolus</i>	<i>Engraulis encrasicolus</i>	99%	KJ563167	correct
AS13	Anchovy fillets in olive oil	06/12/2013	Santiago de Compostela	Anchoa	464 bp	<i>Engraulis encrasicolus</i>	<i>Engraulis encrasicolus</i>	99%	KJ623921	correct
AS14	Anchovy fillets in olive oil, Cantabrian Sea, <i>Engraulis encrasicolus</i>	06/12/2013	Santiago de Compostela	<i>Engraulis encrasicolus</i>	464 bp	<i>Engraulis encrasicolus</i>	<i>Engraulis encrasicolus</i>	100%	KJ563168	correct
AS15	Anchovies from the Cantabrian Sea in olive oil	06/12/2013	Santiago de Compostela	Anchoas	464 bp	<i>Engraulis encrasicolus</i>	<i>Engraulis encrasicolus</i>	99%	KJ563169	correct
AS16	Anchovy fillets in olive oil, <i>Engraulis encrasicolus</i>	06/12/2013	Santiago de Compostela	<i>Engraulis encrasicolus</i>	464 bp	<i>Engraulis encrasicolus</i>	<i>Engraulis encrasicolus</i>	99%	KJ563170	correct
AS17	Anchovy fillets in olive oil, made in Santoña	06/12/2013	Santiago de Compostela	Anchoa	464 bp	<i>Engraulis encrasicolus</i>	<i>Engraulis encrasicolus</i>	99%	KJ563171	correct
AM1	Anchovies fillets in oilve oil	26/06/2013	Madrid	anchoas	464 bp	<i>Engraulis anchoita</i>	<i>Engraulis anchoita</i>	100%	KJ563144	correct
AM2	Anchovy fillets in vegetable oil, made in Cantabria	26/06/2013	Madrid	anchoa	464 bp	<i>Engraulis encrasicolus</i>	<i>Engraulis encrasicolus</i>	99%	KJ563145	correct
AM3	Anchovy fillets from the Cantabrian in olive oil, <i>Engraulis encrasicolus</i>	26/06/2013	Madrid	<i>Engraulis encrasicolus</i>	464 bp	<i>Engraulis encrasicolus</i>	<i>Engraulis encrasicolus</i>	99%	KJ563146	correct
AM4	Anchovies in vegetable oil, <i>Engraulis</i> spp, product from Peru	26/06/2013	Madrid	<i>Engraulis</i> spp	Not amplified					
AM5	Anchovies fillets in sunflower oil, <i>Engraulis anchoita</i>	26/06/2013	Madrid	<i>Engraulis anchoita</i>	464 bp	<i>Engraulis anchoita</i>	<i>Engraulis anchoita</i>	99%	KJ563147	correct

(Continued)

Table 3. (Continued)

Saple code	Label information	Date of sampling	City	Species declared	Fragment amplified	FINS result	BLAST re-sult	% of match	GenBank ID	Correct/ mislabelled
AM6	Anchovy fillets in vegetable oil, Engraulis encrasicolus	26/06/2013	Madrid	Engraulis encrasicolus	464 bp	Engraulis encrasicollus	Engraulis encrasicollus	100%	KJ563148	correct
AM8	Anchovies in olive oil, low salt	26/06/2013	Madrid	anchoas	464 bp	Engraulis anchoita	Engraulis anchoita	99%	KJ563149	correct
AM9	Anchovy fillets in olive oil, from Santoña	26/06/2013	Madrid	anchoa	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563150	correct
AM10	Anchovies fillets in olive oil, from Santoña	26/06/2013	Madrid	anchoas	464 bp	Engraulis anchoita	Engraulis anchoita	100%	KJ563151	correct
AM11	Anchovy fillets in olive oil. Anchoa (Engraulis spp)	26/06/2013	Madrid	anchoa (Engraulis spp)	464 bp	Engraulis anchoita	Engraulis anchoita	100%	KJ563152	mislabelled
AM12	Anchovies fillets in vegetable oil	26/06/2013	Madrid	anchoas	464 bp	Engraulis ringens	Engraulis ringens	98%	KJ645862	correct
AM13	Anchovies fillets in olive oil	26/06/2013	Madrid	anchoas	464 bp	Engraulis japonicus	Engraulis japonicus	99%	KJ563153	correct
AB1	Anchovy fillets in sunflower oil	04/07/2013	Bilbao	anchoa	464 bp	Engraulis anchoita	Engraulis anchoita	98%	KJ563173	mislabelled
AB2	Anchovy fillets in olive oil	04/07/2013	Bilbao	anchoa	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	98%	KJ563174	correct
AB3	Filetes de anchoa del Cantábrico	04/07/2013	Bilbao	anchoa	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	100%	KJ563175	correct
AB4	Anchovy fillets in olive oil	04/07/2013	Bilbao	anchoa	464 bp	Engraulis anchoita	Engraulis anchoita	99%	KJ563176	mislabelled
AB5	Anchovy fillets from the Cantabrian Sea, Engraulis encrasicolus	04/07/2013	Bilbao	Engraulis encrasicolus	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563177	correct
AB6	Anchovies	04/07/2013	Bilbao	Engraulis anchoita	464 bp	Engraulis anchoita	Engraulis anchoita	99%	KJ563178	correct
AB7	Anchovies fillets in olive oil	04/07/2013	Bilbao	anchoas	100 bp	Engraulis ringens	Engraulis ringens	98%		correct

(Continued)

Table 3. (Continued)

Saple code	Label information	Date of sampling	City	Species declared	Fragment amplified	FINS result	BLAST result	% of match	GenBank ID	Correct/mislabelled
AB8	Anchovy fillets in olive oil	04/07/2013	Bilbao	anchoa	464 bp	Engraulis anchoita	Engraulis anchoita	99%	KJ563179	mislabelled
AB9	Anchovy fillets in olive oil	04/07/2013	Bilbao	anchoa	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563180	correct
AB10	Anchovy fillets in olive oil	04/07/2013	Bilbao	anchoa	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ563181	correct
AB11	Anchovy fillets in olive oil	04/07/2013	Bilbao	anchoa	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	95%	KJ563182	correct
AB12	Anchovies fillets in olive oil, <i>Engraulis encrasicolus</i>	04/07/2013	Bilbao	anchoas	Not amplified					
AB13	Anchovies fillets in olive oil	04/07/2013	Bilbao	anchoas	464 bp	Engraulis encrasicolus	Engraulis encrasicolus	99%	KJ645861	correct
AB14	Anchovies fillets in vegetable oil	04/07/2013	Bilbao	anchoas	Not amplified					

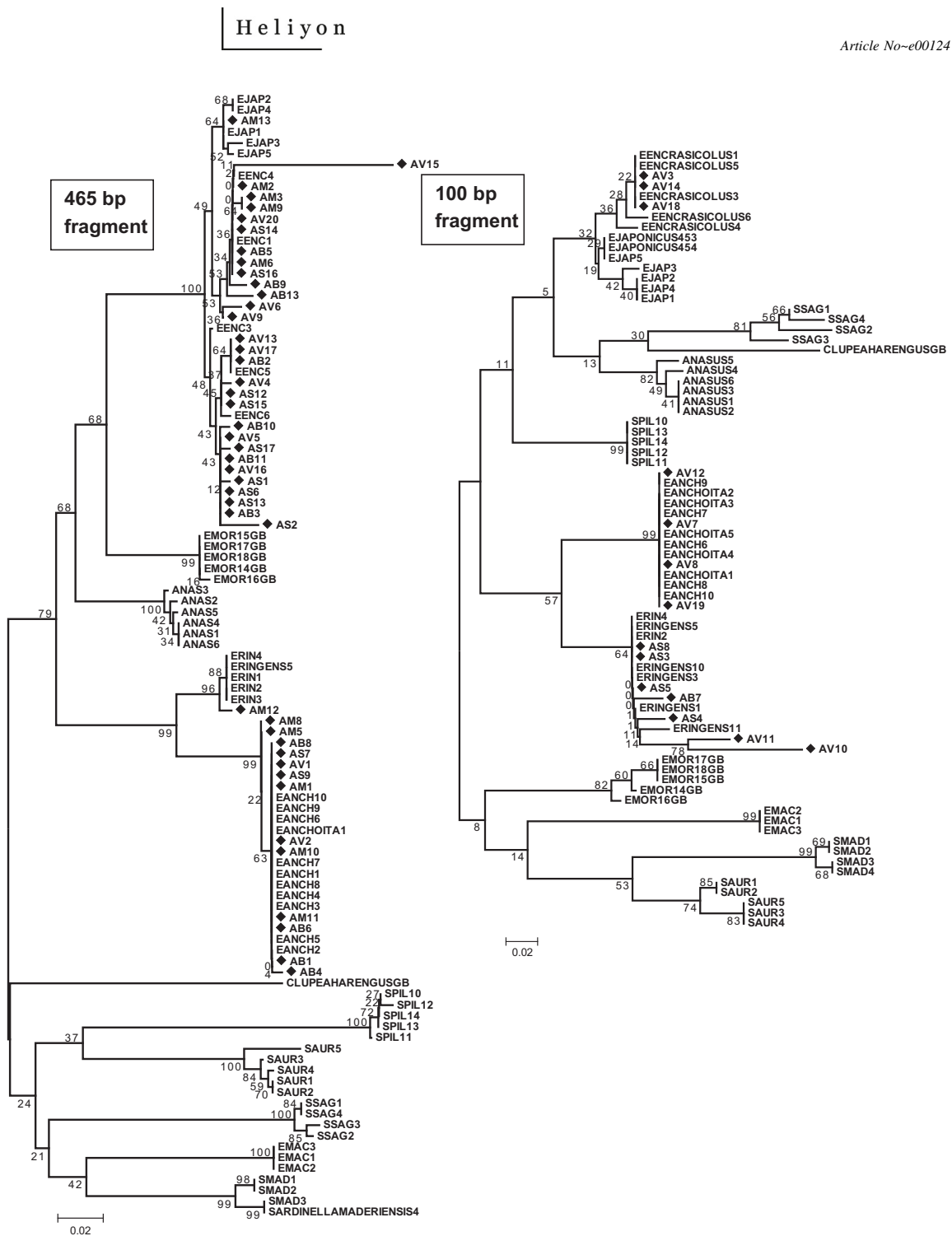


Fig. 2. Tamura-Nei trees with the 465 bp fragment (103 sequences) and 100 bp fragment (76 sequences) of cyt b. Commercial samples of this study are marked with ◆.

(Burgener fragment) and of 100 bp (C-CB fragment). These DNA markers were chosen because of their capability for differentiating among all anchovy species and, other possible substitute species, such as *Sardina pilchardus*, *Sardinops sagax*, *Sardinella aurita* or *Ethmidium maculatum* (Jérôme et al., 2003, Jérôme et al., 2008).

DNA quality was tested with NanoDrop, all DNA samples showed 269/280 ratios of 1.7 to 1.9. Although these ratios seemed acceptable for PCR, DNA fragmentation hindered the amplification of the 464 bp fragment in 19 cases, in which DNA quality had to be rechecked in agarose gels. In 14 of those cases it was necessary to use the shorter DNA fragment amplification target (C-CB, 100 bp), since DNA degradation did not allow the amplification of the 464 bp amplification, and in the other 5 samples DNA fragments sizes were below 100 bp hindering any DNA amplification. The total number of valid DNA sequences obtained was 58 out of 63 samples collected. Once DNA sequences were obtained, species were identified after determining their genetic distance with a set of reference sequences (Tamura Nei model, with 1000 replicates in the bootstrap test) and phylogenetic reconstruction using a Neighbor-Joining tree. Fig. 2 shows the obtained trees for the Burgener fragment (464 bp) and C-CB fragment (100 bp) with all 58 commercial samples together with reference samples listed in Table 1. The trees show the bootstrap support of each branch for the clades obtained. Table 4 shows the global result of the FINS analysis. Additionally, to support these results a homology search using BLASTn with Megablast algorithm and default parameters was carried out with the DNA sequences obtained from market samples. The results of both analyses are listed in Table 3 and it can be observed that coherent results between the two approaches were found in all cases.

Table 4. Commercial samples used in this study and FINS results.

	Number of samples	%
Total samples collected	63	
Successfully identified samples	58	92.06
Identified as <i>Engraulis encrasicolus</i>	32	50.79
Identified as other than <i>Engraulis encrasicolus</i>	26	41.27
Identified as <i>Engraulis anchoita</i>	17	26.98
Identified as <i>Engraulis ringens</i>	8	12.70
Identified as <i>Engraulis japonicus</i>	1	1.58
Correctly labelled	49	84.48
Mislabeled	9	15.52

3.2. Labelling analysis and species substitution

58 commercial samples were successfully identified. Approximately half of them were identified as *Engraulis encrasicolus*, and the other species found were *Engraulis anchoita*, *Engraulis ringens* and *Engraulis japonicus*. Following the mentioned Spanish legislation (BOE, 2015; RD 1521, 1984), products with *E. ringens*, *E. anchoita*, *E. japonicus* and *E. encrasicolus* under the commercial name “Anchoas” were considered correctly labeled, but products under the commercial name “Anchoa” containing other than *E. encrasicolus* were considered mislabelled. In the cases where the scientific name was present, we considered mislabeled samples with a wrong scientific name, even when the commercial name declared was the generic “anchoas”. A total of 9 samples were mislabeled regarding species, making a 15.52% of the total samples analyzed (Table 4). Regarding the commercial brands, 8 of them had mislabeled samples, resulting a 19.05% of the total brands (Table 5). The most frequent substitution found was *Engraulis anchoita*, labelled as *Engraulis encrasicolus* or anchoa. This substitution was found in 7 cases. *Engraulis anchoita*, is imported frozen from Peru and Argentina (MAGRAMA, 2013), most probably at a lower price. Therefore, this is an example of economic fraud. In 2 out of these 7 cases, the label showed a mention to Cantabrian region.

Another substitution found twice was *Engraulis encrasicolus* labelled as *Engraulis ringens*. This was quite unexpected, since the species *Engraulis encrasicolus*, the local species, is traditionally more appreciated by consumers and can also reach a higher price. One possible explanation is the low levels of biomass of the species *Engraulis encrasicolus*, which led the Authorities to close some fisheries on several years (ICES, 2010). This could represent an attempt to introduce over-quota catches by trading them as *Engraulis ringens*, whose distribution comprises the Eastern South Pacific, in the coasts of Peru and Chile (FAO, 2012). An accidental, genuine mistake is, of course, another probable reason.

The system adopted by Spanish legislation (BOE, 2015; RD 1521, 1984), that only allows to label as “Anchoa” products made of *E. encrasicolus* and “Anchoas” the other *Engraulis* species is very confusing and most consumers are not prepared for paying attention at that slight difference. The commercial name for preserved anchovies does not permit consumers to have an exact idea about the product they are buying. The European Commission recognizes also the commercial designation “anchoa” for *Engraulis encrasicolus* (http://mare.istc.cnr.it/site/engraulis_encrasicolus_cd.htm), but there is not such information for other *Engraulis* species. We therefore deem that legislation should be reviewed and adapted to the current situation of the processing industry (i.e. insufficient amounts of *E. encrasicolus* to meet the whole range of demand of anchovy products). It should be also adapted to the current needs of the market, as the number of anchovy species captured and

Table 5. Commercial brands tested in this study and FINS results.

Brand code	sample code	declared	FINS result
1	AV1	Anchoas	Engraulis anchoita
	AV5	Engraulis encrasicolus	Engraulis encrasicolus
2	AV2	Anchoas	Engraulis anchoita
3	AV3	Anchoas	Engraulis encrasicolus
4	AV4	Anchoas	Engraulis encrasicolus
5	AV6	Anchoa	Engraulis encrasicolus
	AS6	Anchoa	Engraulis encrasicolus
6	AV7	Engraulis anchoita	Engraulis anchoita
	AS8	Engraulis ringens	Engraulis ringens
	AM5	Engraulis anchoita	Engraulis anchoita
7	AV8	Anchoa	Engraulis anchoita
8	AV9	Engraulis ringens	Engraulis encrasicolus
	AM4	Engraulis spp	failed
9	AV10	Engraulis ringens	Engraulis ringens
	AS2	Engraulis ringens	Engraulis encrasicolus
	AS5	Engraulis ringens	Engraulis ringens
10	AV11	Engraulis ringens	Engraulis ringens
	AS3	Engraulis ringens	Engraulis ringens
11	AV12	Anchoa	Engraulis anchoita
	AM8	Anchoas	Engraulis anchoita
	AB8	Anchoa	Engraulis anchoita
12	AV13	Engraulis encrasicolus	Engraulis encrasicolus
13	AV14	Engraulis encrasicolus	Engraulis encrasicolus
14	AV15	Anchoa	Engraulis encrasicolus
	AS1	Anchoas	Engraulis encrasicolus
	AS4	Anchoas	Engraulis ringens
15	AV16	Anchoas	Engraulis encrasicolus
16	AV17	Anchoas	Engraulis encrasicolus
17	AV18	Anchoas	Engraulis encrasicolus
18	AV19	Anchoa	Engraulis anchoita
	AS13	Anchoa	Engraulis encrasicolus
19	AV20	Engraulis encrasicolus	Engraulis encrasicolus
20	AS16	Engraulis encrasicolus	Engraulis encrasicolus
21	AS7	Anchoas	Engraulis anchoita
	AS9	Anchoas	Engraulis anchoita
	AM1	Anchoas	Engraulis anchoita
22	AS10	Anchoas	failed

(Continued)

Table 5. (Continued)

Brand code	sample code	declared	FINS result
	AB7	Anchoas	Engraulis ringens
23	AS11	Anchoas	failed
	AM13	Anchoas	Engraulis japonicus
	AB1	Anchoa	Engraulis anchoita
24	AS12	Engraulis encrasicolus	Engraulis encrasicolus
25	AS14	Engraulis encrasicolus	Engraulis encrasicolus
26	AS15	Anchoas	Engraulis encrasicolus
27	AS17	Anchoa	Engraulis encrasicolus
	AM9	Anchoa	Engraulis encrasicolus
28	AM2	Anchoa	Engraulis encrasicolus
29	AM3	Engraulis encrasicolus	Engraulis encrasicolus
30	AM6	Engraulis encrasicolus	Engraulis encrasicolus
31	AM10	Anchoas	Engraulis anchoita
32	AM11	Anchoa	Engraulis anchoita
33	AM12	Anchoas	Engraulis ringens
34	AB2	Anchoa	Engraulis encrasicolus
35	AB3	Anchoa	Engraulis encrasicolus
	AB9	Anchoa	Engraulis encrasicolus
36	AB4	Anchoa	Engraulis anchoita
37	AB5	Engraulis encrasicolus	Engraulis encrasicolus
38	AB6	Anchoas	Engraulis anchoita
39	AB10	Anchoa	Engraulis encrasicolus
	AB11	Anchoa	Engraulis encrasicolus
40	AB12	Anchoas	failed
41	AB13	Anchoas	Engraulis encrasicolus
42	AB14	Anchoas	failed

traded has increased unceasingly since the last national specific legislation was issued (RD 1521, 1984).

Other works regarding seafood mislabelling have been reported all over the world focused in different species: rockfish (Logan et al., 2008), tuna from sushi in restaurants (Lowenstein et al., 2009) and red snapper (Marko et al., 2004) in the USA; cod (Miller and Mariani, 2010), hake (Machado-Schiaffino et al., 2008) and fish in pet food (Armani et al., 2015b) in Europe. All had rates of mislabelling higher than 20%. Despite our results reveal a lower level of mislabelling than the aforementioned, it could still be considered relatively high compared with other seafood products, such as gadoids (5,66%) in UK retailers (Helyar et al., 2014).

In this context, the relatively recent emergence on the market of the product named “Anchodina” (trademark registered by the company INKIELE S.L.), made with sardine (*Sardina pilchardus*) as raw material, should be mentioned. This product has an analogous elaboration process than semipreserved Anchovies, and it has also a very similar appearance, but uses a much cheaper raw material as fresh anchovy is 50% more expensive than fresh sardine (OPA-MAGRAMA, 2013). This eventually may lead to economic frauds if commercial names are not adequately used and regulated and consumers are consequently misled.

3.3. Labelling information in preserved anchovies

Typical labelling of this type of products is shown in Fig. 1. Most of the label is filled with mandatory information: commercial name, list of ingredients, company code, net and drained weights, conservation instructions and Best before Date, company name or code and batch number (not shown in Fig. 1). In imported products, the label should also reflect the country of origin (RD 1521, 1984; EU 1169, 2011). In this context, 3 samples were imported and showed the country of origin correctly in their labels: 2 from Peru and 1 from Morocco. All anchovy samples exhibited the complete mandatory information in their labels according to EU 1169, 2011 (Table 2).

Besides, labels sometimes include some non-mandatory additional information such as Nutritional Information (RD 930, 1992), or the absence of allergens, as it can also be seen in Table 2, the number of samples that present these pieces of non-mandatory information is variable: 100% of the samples gave nutritional information (which will be mandatory from December 2016) and 36.5% showed scientific names in the labels. Surprisingly, 25% of the samples indicate some link to a geographical location of the production (in this case Cantabria) but only 3% declared that the product was from Spain. Geographical location, such as “Cantabria”, is perceived by consumers as linked to high quality product for two reasons: species and process. Traditionally, the species *Engraulis encrasicolus* was seasonally captured by the artisanal fleet in the Cantabrian Sea, also the traditional preservation process was employed by the local industry, which led to great quality (MAGRAMA, 2013). Therefore, companies take profit of this cultural perception and rather indicate Cantabria than Spain in their labels.

However, the linkage to a geographical location or a particular type of process should be regulated and controlled, as in the case of quality labels for Protected Geographical Indication (PGI): in the case of Anchovies, such labelling authorization has not yet been approved, which forced the Spanish region of Cantabria to issue a regional normative to establish a quality label of Controlled Quality (CC) only for those manufactured in Cantabria with *Engraulis encrasicolus* (GAN 18, 2014).

In recent years, the decrease in the population of the Cantabrian *Engraulis encrasicolus* forced the limitation of captures (STECF, 2005) and even in some years the preventive closure of the fishery (EC 1116, 2006). The consequences of this limitation in raw material has resulted in the industry seeking other sources of anchovy, either from other close regions (Mediterranean) or even distant geographical locations (e.g. Asia, South America). Even provided that the product maintains its quality, consumers should still be informed on the species used, especially in the case of highly priced products. After this lockdown, anchovy stocks in the Gulf of Biscay have recovered, which led the authorities to allow a TAC (Total Allowable Catches) of 17,100 tons for Europe in 2013, 15,390 of which have been granted to Spain (EU 713, 2013).

In relation with the denomination of the product, in semipreserved anchovies, only the commercial names of the ingredients are mandatory (RD 1521, 1984). From that point of view, it was observed that all products exhibited the commercial name, and even a significant amount of the samples, 36.51%, provided extra-information by adding scientific names on their labels.

Labelling of Anchovies resulted ambiguous: up to three types of names were found in the market: “anchoas” (anchovies) (15.85% of the samples), “filetes de anchoa” (anchovy fillets) (44.4%) and “filetes de anchoas” (anchovies fillets) (39.7%); in addition, some brands added “of Cantabria”(23.8%). It is difficult for consumers to understand of the subtle differences among these commercial names. Besides, in some samples it was also possible to observe one name (“anchoa”) and the other (“anchoas”) in a different side of the package (23.8% of the samples).

4. Conclusions

In this work we have performed the first study to address the level of mislabelling of semipreserved anchovies in Spain, a European country: 15.52% of the analyzed samples were mislabelled, with the most frequent substitution being *Engraulis anchoita* sold as *E. encrasicolus*.

In light of our results, authors have also realized that there is some non-mandatory information presented in labels. Some of this extra information can be confusing since it does not follow clear rules, such a reference to a geographical location or the inclusion of scientific names. More specific regulation would be essential to harmonize when and how this information should be present in the labels.

Even more, authors consider that there is a need for more specific and updated pieces of legislation in Europe and particularly in Spain regarding the allowed commercial names for species in this type of products, in order to protect both consumers and fisheries.

Declarations

Author contribution statement

Amaya Velasco: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Anxela Aldrey: Performed the experiments.

Ricardo I. Pérez-Martín: Contributed reagents, materials, analysis tools or data.

Carmen G. Sotelo: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Competing interest statement

The authors declare no conflict of interest.

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Additional information

No additional information is available for this paper.

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