Title:

The detection of Atlantic cod (*Gadus morhua*) using loop mediated isothermal amplification in conjunction with a simplified DNA extraction process.

Running title:

Gadus morhua LAMP assay

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Abstract

Atlantic cod (*Gadus morhua*) is a commercially important species of white fish, and one of three species legally identifiable as cod in the UK. Mislabelling of *G. morhua* does occur, as does the substitution of *G. morhua* for less expensive species. Sensitive molecular tests based on PCR have been developed for this species, but they have limitations, including the need for expensive thermal cycling equipment, and complex DNA extraction procedures. A loop mediated isothermal amplification (LAMP) assay was designed for the *G. morhua cytochrome b* gene, which was capable of detecting 0.1% w/w *G. morhua* in a homogenised raw fish mix. The LAMP assay was also able to detect *G. morhua* DNA when a rapid sample preparation was used, involving heating 100mg of fish in a 1ml aliquot of water and testing the supernatant, showing a higher tolerance of amplification inhibitors than a PCR assay. The LAMP assay did not generate a positive result when challenged with a range of non-target species, including *Gadus macrocephalus*, and *Gadus calchogrammus*, indicating a high level of specificity. Direct detection of a positive reaction using propidium iodide was also demonstrated.

1. Introduction

Atlantic cod (*Gadus morhua*), is a commercially important species of marine fish, and alongside Pacific cod (*Gadus macrocephalus*) and Greenland cod (*Gadus ogac*), constitute three species legally permitted to be labelled as cod in the UK (Fish Labelling (England) Regulations, 2010). Cod is the most heavily consumed whitefish in the UK, with cod products equating to 136,000 tonnes of whole fish, or 9% of the total world catch, sold each year (SEAFISH, 2013). During the last century, populations of *G. morhua* were greatly reduced primarily by overfishing and poor fisheries management, but also by disadvantageous climate effects (Mieszkowska *et al.*, 2009). The desire to protect the remaining *G. morhua* populations from catastrophic overfishing, and allow the recovery of Atlantic stocks lead to a concerted effort by EU fisheries authorities to conserve this resource, resulting in the instigation of the Total Allowable Catches (TAC) quotas, set in the Common Fisheries Policy. The instigation of these measures, alongside improved labelling regulations and traceability requirements has assisted in the protection of *G. morhua* stocks. Labelling schemes, such as the Marine Stewardship Council (MSC) ecolabel have been used to enable consumers to identify products that are produced from a certified sustainable source, and encourage the purchase of products from regulated sustainable fisheries (Kaiser & Edwards-Jones, 2006).

A significant danger to the sustainable management of *G. morhua* fish stocks is Illegal, Unreported and Unregulated (IUU) fishing, which can introduce mislabelled or untraceable fish products into the supply chain (Helyar *et al.*, 2014). The mislabelling of fish can be carried out by simple error or misinformation. It can also be carried out fraudulently, in order to misidentify a cheaper product as another more expensive one, for example the mislabelling of Vietnamese catfish (*Pangasius* spp.) as the more lucrative *G. morhua* (Miller et al., 2012). Deliberate mislabelling can be utilised to avoid fisheries regulations, as seen with the mislabelling of the highly regulated and vulnerable *G. morhua* as sustainably sourced *G. macrocephalus*, in the UK (Miller *et al.*, 2012). A recent Italian study found 10/65 salted cod, and 40/40 battered cod chunks were mislabelled non-cod fish (Di Pinto *et al.*, 2013). A study in Ireland tested 156 cod and haddock products from a variety of commercial retailers and restaurants, and found 25% to be mislabelled (Miller & Mariani, 2010), whilst a study of 95 cod products in the UK found a mislabelling rate of 7.4%, with substitute species including M*elanogrammus aeglefinus* and *Pangasius spp* (Miller *et al.*, 2012).

In order to detect mislabelled fish it is necessary to be able to determine the correct fish species present in the sample. When dealing with whole fish, this can be carried out via visual evaluation of

morphological traits, but this methodology is ill suited to dealing with highly processed or mixed samples, wherein the distinguishing features have been removed. Sensitive molecular testing, based on nucleic acid amplification tests (NAATs) such as PCR, has been applied for the determination of a wide range of fish species and food products, including caviar (Boscari *et al.*, 2014), grouper (Sumathi *et al.*, 2015), salmon (Herrero *et al.*, 2011) and European sole (Herrero *et al.*, 2012). Real-time PCR assays have also been developed for the authentication of *G. morhua*, targeting the genomic *Pan I* gene (Hird *et al.*, 2012) and cytochrome oxidase subunit 1 gene (CO1), located in the mitochondrial DNA (Herrero *et al.*, 2010). Mitochondrial DNA genes make attractive targets for speciation assays, as they are present in multiple copies in each cell and also differ significantly between species (Yang *et al.*, 2014). The cytochrome b gene (*cyt b*) has been used as a target for genetic barcoding in fish species (Ardura *et al.*, 2013), and for species identification in cod products using PCR-RFLP analysis (Akasaki *et al.*, 2006)

The use of PCR based testing from food or fish samples necessitates the isolation and purification of nucleic acid from the sample prior to the reactions, as certain compounds present in the samples may inhibit PCR (Rossen et al., 1992). A number of compounds found in common food ingredients have been demonstrated to inhibit PCR, including polyphenols and polysaccharides (Pinto *et al.*, 2007), and proteins and fats (Rossen *et al.*, 1992). Additionally, it has previously been shown that the heated lysates of seawater fish can inhibit PCR (Fach *et al.*, 2002). The requirement of pre-PCR DNA isolation is a significant drawback to the application of PCR for the speciation of fish in foodstuffs, as extraction methods can be expensive, time consuming, and require skilled technical staff (Vetrone et al., 2012). PCR also requires either time consuming post amplification analysis, such as agarose gel electrophoresis, or expensive real-time monitoring systems.

An alternative NAAT is loop-Mediated Isothermal Amplification (LAMP) a novel nucleic acid amplification method, designed to amplify target nucleic acid in a highly specific and rapid manner, under isothermal conditions (Notomi *et al.*, 2000). A strand displacement DNA polymerase is used in conjunction with a specially designed set of four primers (forward primer: F3, backward primer: B3, Forward inner primer: FIP, Backward inner primer: BIP), specific to a total of 6 distinct regions of the target DNA sequence. End point detection can be carried out via product separation using agarose gel electrophoresis, or visual detection via the addition of reporter dyes such as SYBR green (Chen et al., 2012) or propidium iodide (Hill et al., 2008). LAMP is known to have a higher tolerance to the presence of inhibitory compounds than PCR, due to the robust nature of the thermostable *bst* polymerase (Francois et al., 2011), enabling a simplified sample preparation processes.

This report details a *G. morhua* LAMP assay, specific to the mitochondrial cytochrome b gene (*cyt b*), and the application of this assay for the detection of *G. morhua* cooked and uncooked fish directly from heated fish lysates.

2. Materials and Methods

2.1 Samples

Samples of commercially available fish products were sourced from a supermarket in the UK (Table 1). All products were certified by the Marine Stewardship Council (MSC) ecolabel, requiring the display of the correct seafood species on the product packaging. After purchase, fresh fish products were stored at -20^{o}C to prevent spoilage and retain DNA integrity, whilst tinned products were kept unopened, at room temperature. The duration of freezing before use did not exceed 1 month. Prior to DNA extraction the samples were thawed, and washed in molecular grade water to remove any sauce or breadcrumb covering.

2.2. Commercial kit based DNA extraction

DNA extractions were carried out using a QIAmp DNA mini kit (QIAGEN Inc., USA), following the protocol for DNA extraction from tissue. The protocol utilised 25mg of tissue per extraction. DNA was eluted in a single 100µl volume of molecular grade water.

2.3. Simplified DNA extraction

Simplified DNA extractions were carried out by adding 100mg of fish to a 1ml aliquot of molecular grade water in an eppendorf tube. The end of a sterile microbiological inoculation loop was used to homogenise the sample by beating it against the tube walls for 10 seconds. The tubes were briefly centrifuged and the supernatant was removed as the template for the PCR or LAMP reactions.

2.4. Primer Design

LAMP primer sets were designed for the cytochrome B gene of *G. morhua*. A selection of available *cyt b* gene sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), with the following accession numbers; HM802896.1, KJ645860.1, KJ645857.1, KJ632817.1, KJ632815.1, KJ632814.1. Sequences were aligned using ClustalX (Larkin *et al.*, 2007), and primer binding sites were chosen to ensure coverage of all sequences. Alignments were also carried out between the *G. morhua cyt b* sequences, and *cyt b* sequences from the most genetically similar non-target organisms, *G. macrocephalus* (EU729384.1), *G. Ogac* (KC128864.1), and *Gadus chalcogrammus* (KC128867.1). Primer binding sites were chosen that would ensure sufficient mismatches between the non-target species (totalling between 6 and 8 mismatches per primer set), and particularly a mismatch at the 3' end of the B2 region of the BiP, and the 5' end of the B1c region of the BiP which have both been shown to prevent LAMP autocycling (Badolo *et al.*, 2012; Duan *et al.*, 2014). The

location of the primer binding sites on the *G. morhua cyt b* gene, and mismatches between the primer sequences and the most genetically similar non-target fish species are shown in Fig.1.

LAMP primers were designed using PrimerExplorer Version 4 (http://www.primerexplorer.jp/e/). The additional optional loop primers were not designed. Primer specificities were checked using BLAST (http://www.blast.ncbi.nlm.nih.gov/). LAMP primers were synthesised commercially (Eurofins, Germany). LAMP primer sequences are shown in table 2.

2.5. LAMP reactions

LAMP reactions were set up using reagents from a Mast Isoplex DNA amplification kit (Mast Group Ltd, UK; under license from Eiken Co., Ltd., Japan), using half volume reactions, as follows; 2.5µl five times LAMP reaction buffer, 0.5µl of 8U/µl *Bst* polymerase (New England Biolabs, USA), 0.5µl of primer mix containing 40pmol of FiP and BiP primers, and 5pmol of F3 and B3 primers, 6.5µl molecular grade water. Finally, 2.5µl of DNA sample or water was added to the reaction. Reactions were carried out in a heating block at 63^{o}C for 60 minutes. Reactions were terminated by heating to 80^{o}C for 1 minute in order to denature the *Bst* polymerase. The detection of LAMP reaction products was carried out via agarose gel electrophoresis (1.5% w/v), with UV transillumination. Endpoint direct detection was carried out via the addition of 1µl of 1mg/ml propidium iodide (Invitrogen, US), with UV transillumination applied to cause fluorescence.

2.6. PCR reactions

PCR reactions were set up as follows; 12.5µl RedTaq reaction mix (Sigma Aldrich, UK), 0.5pmol F3 primer, 0.5pmol B3 primer, 2.5µl DNA sample. Reactions were then made up to 25µl with molecular grade water. Reaction conditions were as follows; 94^{o}C for 5 minutes initial denaturation; 30 cycles of 94^{o}C for 30 seconds, 49^{o}C for 30 seconds and 72^{o}C for 30 seconds; 72^{o}C for 5 minutes final extension. Thermal cycling was carried out in an ABI 2720 instrument (Applied Biosystems, UK). The expected reaction product was an 185bp fragment. Reaction products were visualised using agarose gel electrophoresis (1.5% w/v), with UV transillumination.

2.7. Initial test of *G. morhua cyt b* LAMP reaction and detection methods

Nucleic acid was extracted from 100mg of uncooked *G. morhua*, *G. macrocephalus*, and *G. chalcogrammus* fillet, using the rapid extraction method. LAMP reactions were carried in duplicate using the extracted nucleic acid as a template. A reaction containing molecular grade water in place

of DNA was used as a negative control. Reaction products were visualised using agarose gel electrophoresis.

2.8. Direct detection of LAMP reaction positivity via end-point addition of propidium iodide

A positive LAMP reaction, containing *G. morhua* DNA extracted using the rapid method, and a negative reaction containing molecular grade water were carried out. Endpoint visual detection was carried out via the addition of propidium iodide (of 1µl of 1mg/ml stock), and images were taken of the visible fluorescence, both in the absence and presence of UV excitation, provided by a UV transilluminator.

2.9. Sensitivity of the *G. morhua cyt b* LAMP and PCR assays

A portion of uncooked *G. morhua* fillet was homogenised, and diluted in homogenised *Theragra chalcogramma* fillet, to produce a series of fish samples containing 100%, 10%, 1%, 0.1% and 0.01% w/w *G. morhua*. DNA extractions were carried out on each dilution using the rapid extraction method, and a commercially available spin column based kit. LAMP and PCR reactions were then carried out on each DNA sample produced by each method.

Specificity of the *G. morhua cyt b* PCR and LAMP assays

DNA was extracted from all available fish samples, using both extraction methods. PCR reactions were carried out using DNA from all samples, extracted using both methods. LAMP reactions were carried out using DNA extracted using the rapid extraction method.

3. Results and Discussion

3.1. Initial test of *G. morhua cyt b* LAMP reaction

The *G. morhua cyt b* LAMP reaction was successfully able to amplify *G. morhua* DNA, extracted from the *G. morhua* fillet using the rapid extraction method. The amplified product was visible after agarose gel electrophoresis, with the varying sized concatamers of amplified DNA appearing in the characteristic ladder-like pattern particular to the LAMP reaction (Fig.2.). Importantly, the reaction was not inhibited by compounds present in the crudely extracted DNA, which had not been processed to remove impurities.

The ability of LAMP to amplify target DNA from crude samples, without the need for elaborate DNA extraction methodologies, has been utilised with a number of different sample matrices, including urine (Edwards *et al.*, 2014), blood (Ebbinghaus *et al.*, 2012), egg white (Ohtsuka *et al.*, 2005) and supernatants from homogenised beef (Wang *et al.*, 2012). This is possible as the LAMP reaction has a greater tolerance to endogenous amplification inhibitors than PCR, due to the greater stability of the *bst* polymerase enzyme used to drive the reaction (Francois *et al.*, 2011). Direct amplification from heated fish lysate removes the need for complex, time consuming, and expensive extraction methodologies.

The *cyt b* LAMP assay did not produce amplified product from DNA extracted from *G. macrochepalus*, or *G. calchogrammus*, two of the most genetically similar species to *G. morhua*. This confirms the high level of specificity predicted with the intelligent choice of primer binding sites, with 8 mismatches present in the primer binding regions in the *cyt b* gene of these species (Fig.1.). Mismatches in primer regions that have been shown to prevent LAMP amplification (Badolo *et al.*, 2012; Duan *et al.*, 2014) were incorporated into the primer design to ensure amplification would not occur using the DNA of these organisms.

The ability of the assay to correctly detect *G. morhua* DNA, but not that of *G. macrocephalus* is significant, as mislabelling of *G. morhua* as the less endangered *G. macrocephalus* is one of the most commonly encountered mislabellings of *G. morhua* (Miller *et al.*, 2012). Mislabelling in such a way damages the ability of the consumer to make an ethically considered choice when purchasing cod, and increases the difficulty of maintaining traceability of the *G. morhua* fishery. The *cyt b* LAMP assay could be applied for the detection of such mislabelling, in addition to being used to detect substitution of relatively expensive *G. morhua* fish with less expensive species.

3.2. Direct detection of LAMP reaction positivity via end-point addition of propidium iodide

The visual detection of a positive reaction was enabled via the addition of propidium iodide after the reaction had been terminated (Fig.3.). The colour change was ambiguous in natural light, with no discernible difference between positive and negative reactions. Under UV light, however, the difference in fluorescence was readily visible, with the positive reaction producing a strong red fluorescence.

A range of indicators allowing visible detection of positive LAMP reactions via the detection of reaction by-products have been described, such as calcein and hydroxyl napthol blue, which produce a colour change in response to the generation of phosphate ions (Goto *et al.*, 2009; Tomita *et al.*, 2008). However, detection dyes such as these, which detect reaction by-products have been shown to reduce assay sensitivity when compared with the end-point addition of intercalating agents (Wastling *et al.*, 2010). The utilisation of this detection dye with the *G. morhua* LAMP assay enables sensitive molecular detection of *G. morhua* DNA without the requirement of an expensive and cumbersome real-time detection system, or lengthy post amplification processing.

3.3. Sensitivity of the *G. morhua* cyt b LAMP and PCR assay

The *G. morhua* LAMP assay, when combined with the rapid sample preparation protocol, was able to detect 1% of *G. morhua* flesh in 100mg of non- target fish (Fig.4.). This limit of detection of 1% w/w is the level of sensitivity suggested by the UK Food Standards Agency (FSA) for a meat speciation assay, and it is important that the LAMP assay can detect *G. morhua* material at this level in order to be of use as a detection method. This detection limit was equal to a PCR assay using the F3 and B3 primers from the LAMP reaction. Somewhat surprisingly, the PCR reaction was not inhibited by compounds present in the DNA extracted using the rapid method.

When paired with a commercially available DNA extraction kit, the LAMP assay was able to detect a 0.1% w/w concentration of the target fish species, from a 25mg total sample. The increase in sensitivity enabled by the commercial kit was expected for a number of reasons; the tissue lysis procedure in the kit fully lyses the cells, releasing the entire quotient of nucleic acids in the early stages of the extraction process. This is in contrast with the thermal lysis used in the rapid method, which does not completely lyse the tissue, leading to incomplete release of intracellular nucleic acids. The kit method also enables the elution of nucleic acid into a lower volume, concentrating the nucleic acid, and hence the starting copy number present in the downstream LAMP reaction.

This sensitivity of 0.1% target species in a binary meat mixture is consistent with other DNA amplification based methodologies for meat speciation, such as a pork specific PCR assay capable of detecting 0.1% w/w of target described in (Yusop *et al.*, 2012). A recently developed LAMP assay for the detection of ostrich meat could detect a minimum of 0.01% target meat (Abdulmawjood *et al.*, 2014).

Although the LAMP reaction offered no benefit in sensitivity when compared with PCR, the increased reaction speed and lack of a need for expensive thermal cycling equipment, combined with the ability to visually identify reaction positivity, give the LAMP assay a number of advantages over PCR.

3.4. Specificity of the *G. morhua cyt b* PCR and LAMP assays

In order to better determine the specificity of the *cyt b* LAMP reaction, the assay was challenged with DNA extracted from a range of commercially important fish species commonly encountered in foodstuffs. The *cyt b* PCR was also tested, with both the rapid and kit based extraction methods, in order to provide a comparison with the LAMP assay (Fig.5).

The *cyt b* PCR reaction was able to amplify the expected product from all three *G. morhua* samples when a commercial DNA extraction kit was used. There was no visible product produced when DNA from any of the non-target species were used, confirming the high level of primer specificity determined using BLAST.

When the rapid extraction method was employed, the PCR assay was still able to amplify a product, however when the smoked and breaded fish was tested the reaction products were significantly reduced, and smeared on the gel, making the determination of product size difficult. This could lead to an increased difficulty in interpreting the results of the assay, and is not optimal for a PCR test. The reduction in amplified product that occurred is likely due to the higher concentrations of compounds inhibitory to *taq* polymerase found in these more highly processed samples.

The smearing of the bands on the gel could potentially be caused by a high level of protein binding to the nucleic acid, inhibiting uniform migration of the DNA through the gel. This would be difficult to detect in a LAMP reaction, due to the nature of the products when visualised in this manner. Alternatively, it could be that a high ionic concentration in these particular samples is having a negative effect on PCR amplification specificity. This indicates that the PCR assay would benefit from a more thorough DNA purification step when dealing with more highly processed fish samples.

The LAMP assay was able to detect *G. morhua* DNA from all three *G. morhua* containing samples, including the smoked and breaded fillets. The ability to detect DNA from processed foods such as these, in the form of a heated lysate, is important as it shows that the assay is robust enough to handle any amplification inhibitors present in these samples that are carried over into the LAMP reactions.

4. Conclusion

Although LAMP assays have been developed for the detection of pork, chicken and bovine meats (Ahmed *et al.*, 2010), and recently ostrich meat (Abdulmawjood *et al.*, 2014), to our knowledge this is the first report of the application of LAMP for the determination of fish species. Molecular detection methods have been utilised for the speciation of *G. morhua* (Herrero *et al.*, 2010), and bluefin tuna (Lockley & Bardsley, 2000), amongst other fish species, but these have involved PCR assays, which require more complex and expensive equipment. The *G. morhua* LAMP assay, in conjunction with the rapid extraction method described and a visual detection dye, enables sensitive molecular testing for *G. morhua* DNA whilst only requiring the facility for pipetting and a waterbath to provide the necessary reaction temperature. This has the potential to allow for onsite surveying, enabling the testing of fish products or fresh fish at the point of processing, with the high sensitivity afforded by DNA amplification based methods. The limit of detection of the assay was found to be 1% w/w of target fish when combined with the rapid extraction method, and this could be improved to 0.1% by utilising a commercial spin column based kit. The *G. morhua* LAMP assay has the potential to be a useful tool for the identification of this fish species in whole or mixed samples, and can be applied for ensuring traceability and labelling, and for general fisheries control.

References

Abdulmawjood, A., Grabowski, N., Fohler, S., Kittler, S., Nagengast, H. & Klein, G. (2014). Development of Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid and Sensitive Identification of Ostrich Meat. *PLoS ONE* **9**, e100717.

Ahmed, M. U., Hasan, Q., Mosharraf Hossain, M., Saito, M. & Tamiya, E. (2010). Meat species identification based on the loop mediated isothermal amplification and electrochemical DNA sensor. *Food Control* **21**, 599-605.

Akasaki, T., Yanagimoto, T., Yamakami, K., Tomonaga, H. & Sato, S. (2006). Species Identification and PCR-RFLP Analysis of Cytochrome b Gene in Cod Fish (Order Gadiformes) Products. *Journal of Food Science* **71**, C190-C195.

Ardura, A., Planes, S. & Garcia-Vazquez, E. (2013). Applications of DNA barcoding to fish landings: authentication and diversity assessment. *ZooKeys*, 49-65.

Badolo, A., Okado, K., Guelbeogo, W., Aonuma, H., Bando, H., Fukumoto, S., Sagnon, N. F. & Kanuka, H. (2012). Development of an allele-specific, loop-mediated, isothermal amplification method (AS-LAMP) to detect the L1014F kdr-w mutation in Anopheles gambiae s. l. *Malaria Journal* **11**, 227.

Boscari, E., Barmintseva, A., Pujolar, J. M., Doukakis, P., Mugue, N. & Congiu, L. (2014). Species and hybrid identification of sturgeon caviar: a new molecular approach to detect illegal trade. *Molecular ecology resources* **14**, 489-498.

Di Pinto, A., Di Pinto, P., Terio, V., Bozzo, G., Bonerba, E., Ceci, E. & Tantillo, G. (2013). DNA barcoding for detecting market substitution in salted cod fillets and battered cod chunks. *Food chemistry* **141**, 1757-1762.

Duan, Y., Zhang, X., Ge, C., Wang, Y., Cao, J., Jia, X., Wang, J. & Zhou, M. (2014). Development and application of loop-mediated isothermal amplification for detection of the F167Y mutation of carbendazim-resistant isolates in Fusarium graminearum. *Sci Rep* **4**.

Ebbinghaus, P., von Samson-Himmelstjerna, G. & Krucken, J. (2012). Direct loop-mediated isothermal amplification from Plasmodium chabaudi infected blood samples: inability to discriminate genomic and cDNA sequences. *Experimental parasitology* **131**, 40-44.

Edwards, T., Burke, P. A., Smalley, H. B., Gillies, L. & Hobbs, G. (2014). Loop-mediated isothermal amplification test for detection of Neisseria gonorrhoeae in urine samples and tolerance of the assay to the presence of urea. *Journal of clinical microbiology* **52**, 2163-2165.

Fach, P., Perelle, S., Dilasser, F., Grout, J., Dargaignaratz, C., Botella, L., Gourreau, J.-M., Carlin, F., Popoff, M. R. & other authors (2002). Detection by PCR-Enzyme-Linked Immunosorbent Assay of Clostridium botulinum in Fish and Environmental Samples from a Coastal Area in Northern France. *Applied and environmental microbiology* **68**, 5870-5876.

Fish Labelling (England) Regulations (2010) Statutory instrument No. 420 FOOD, ENGLAND.

Francois, P., Tangomo, M., Hibbs, J., Bonetti, E. J., Boehme, C. C., Notomi, T., Perkins, M. D. & Schrenzel, J. (2011). Robustness of a loop-mediated isothermal amplification reaction for diagnostic applications. *FEMS immunology and medical microbiology* **62**, 41-48.

Goto, M., Honda, E., Ogura, A., Nomoto, A. & Hanaki, K. (2009). Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *BioTechniques* **46**, 167-172.

Helyar, S. J., Lloyd, H. a. D., de Bruyn, M., Leake, J., Bennett, N. & Carvalho, G. R. (2014). Fish Product Mislabelling: Failings of Traceability in the Production Chain and Implications for Illegal, Unreported and Unregulated (IUU) Fishing. *PLoS ONE* **9**, e98691.

Herrero, B., Vieites, J. M. & Espineira, M. (2011). Authentication of Atlantic salmon (Salmo salar) using real-time PCR. *Food chemistry* **127**, 1268-1272.

Herrero, B., Madrinan, M., Vieites, J. M. & Espineira, M. (2010). Authentication of Atlantic cod (Gadus morhua) using real time PCR. *J Agric Food Chem* **58**, 4794-4799.

Herrero, B., Lago, F. C., Vieites, J. M. & Espineira, M. (2012). Real-time PCR method applied to seafood products for authentication of European sole (Solea solea) and differentiation of common substitute species. *Food additives & contaminants Part A, Chemistry, analysis, control, exposure & risk assessment* **29**, 12-18.

Hird, H., Chisholm, J., Kaye, J., Colyer, A., Hold, G., Conyers, C., Núñez, J. & Macarthur, R. (2012). Development of real-time PCR assays for the detection of Atlantic cod (Gadus morhua), Atlantic salmon (Salmo salar) and European plaice (Pleuronectes platessa) in complex food samples. *Eur Food Res Technol* **234**, 127-136.

Kaiser, M. J. & Edwards-Jones, G. (2006). The role of ecolabeling in fisheries management and conservation. *Conservation biology : the journal of the Society for Conservation Biology* **20**, 392-398.

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007). Clustal W and Clustal X version 2.0. *Bioinformatics (Oxford, England)* **23**, 2947-2948.

Lockley, A. K. & Bardsley, R. G. (2000). Novel method for the discrimination of tuna (Thunnus thynnus) and bonito (Sarda sarda) DNA. *Journal of agricultural and food chemistry* **48**, 4463-4468.

Mieszkowska, N., Genner, M. J., Hawkins, S. J. & Sims, D. W. (2009). Chapter 3. Effects of climate change and commercial fishing on Atlantic cod Gadus morhua. *Advances in marine biology* **56**, 213-273.

Miller, D., Jessel, A. & Mariani, S. (2012). Seafood mislabelling: comparisons of two western European case studies assist in defining influencing factors, mechanisms and motives. *Fish and Fisheries* **13**, 345-358.

Miller, D. D. & Mariani, S. (2010). Smoke, mirrors, and mislabeled cod: poor transparency in the European seafood industry. *Frontiers in Ecology and the Environment* **8**, 517-521.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N. & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic acids research* **28**, E63.

Ohtsuka, K., Yanagawa, K., Takatori, K. & Hara-Kudo, Y. (2005). Detection of Salmonella enterica in naturally contaminated liquid eggs by loop-mediated isothermal amplification, and characterization of Salmonella isolates. *Applied and environmental microbiology* **71**, 6730-6735.

Pinto, A. D., Forte, V., Guastadisegni, M. C., Martino, C., Schena, F. P. & Tantillo, G. (2007). A comparison of DNA extraction methods for food analysis. *Food Control* **18**, 76-80.

Rossen, L., Norskov, P., Holmstrom, K. & Rasmussen, O. F. (1992). Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. *International journal of food microbiology* **17**, 37-45.

SEAFISH (2013). Responsible Sourcing Guide: Cod. **7**.

Sumathi, G., Jeyasekaran, G., Jeya Shakila, R., Sivaraman, B., Arunkumar, G., Manimaran, U. & Sukumar, D. (2015). Molecular identification of grouper species using PCR-RFLP technique. *Food Control* **51**, 300-306.

Tomita, N., Mori, Y., Kanda, H. & Notomi, T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nature protocols* **3**, 877-882.

Wang, F., Jiang, L. & Ge, B. (2012). Loop-Mediated Isothermal Amplification Assays for Detecting Shiga Toxin-Producing Escherichia coli in Ground Beef and Human Stools. *Journal of clinical microbiology* **50**, 91-97.

Wastling, S. L., Picozzi, K., Kakembo, A. S. L. & Welburn, S. C. (2010). LAMP for Human African Trypanosomiasis: A Comparative Study of Detection Formats. *PLoS Negl Trop Dis* **4**, e865.

Yang, L., Tan, Z., Wang, D., Xue, L., Guan, M.-x., Huang, T. & Li, R. (2014). Species identification through mitochondrial rRNA genetic analysis. *Sci Rep* **4**.

Yusop, M., Mustafa, S., Che Man, Y., Omar, A. & Mokhtar, N. (2012). Detection of Raw Pork Targeting Porcine-Specific Mitochondrial Cytochrome B Gene by Molecular Beacon Probe Real-Time Polymerase Chain Reaction. *Food Anal Methods* **5**, 422-429.

Tables

Table 1. Commercial fish products used in this study, with their state at purchase, and labelled species

Fish product	**state**	**Species (as labelled)**
Fresh Atlantic cod fillet	Uncooked	*Gadus morhua*
Smoked Atlantic cod fillet	Uncooked, smoked	*Gadus morhua*
Breaded Atlantic cod fillet	Uncooked, breaded	*Gadus morhua*
Fresh Pacific cod fillet	Uncooked	*Gadus macrocephalus*
Tinned herring	Cooked, tinned, tomato sauce	*Clupea herrengus*
Pollock fillet	Uncooked	*Gadus chalcogrammus*
Salmon fillet	Uncooked	*Oncorhynchus spp*
Haddock	Uncooked	*Melanogrammus aeglefinus*
Tinned sardines	Cooked, tinned, oil	*Sardina pilchardus*
Smoked herring	Cooked	*Clupea herrengus*

Table 2. Sequences and length of the *G. morhua cyt b* LAMP primers

Primer	Sequence	Length (bp)
G. morhua cyt b LAMP F3	CCTCAGACATCGAGACAG	18
G. morhua cyt b LAMP B3	AACCCCGATGTTTCATGT	18
G. morhua cyt b LAMP FiP (F1c + F2)	ATGTATATTCCGAATTAGTCAGCCG - CCTTCTCATCCGTAGTCCA	44
G. morhua cyt b LAMP BiP (B1c + B2)	ATGGTGCCTCTTTCTTTTTCATTTG - CTCTACAAAAAGATAGGAACCATA	49

Figures

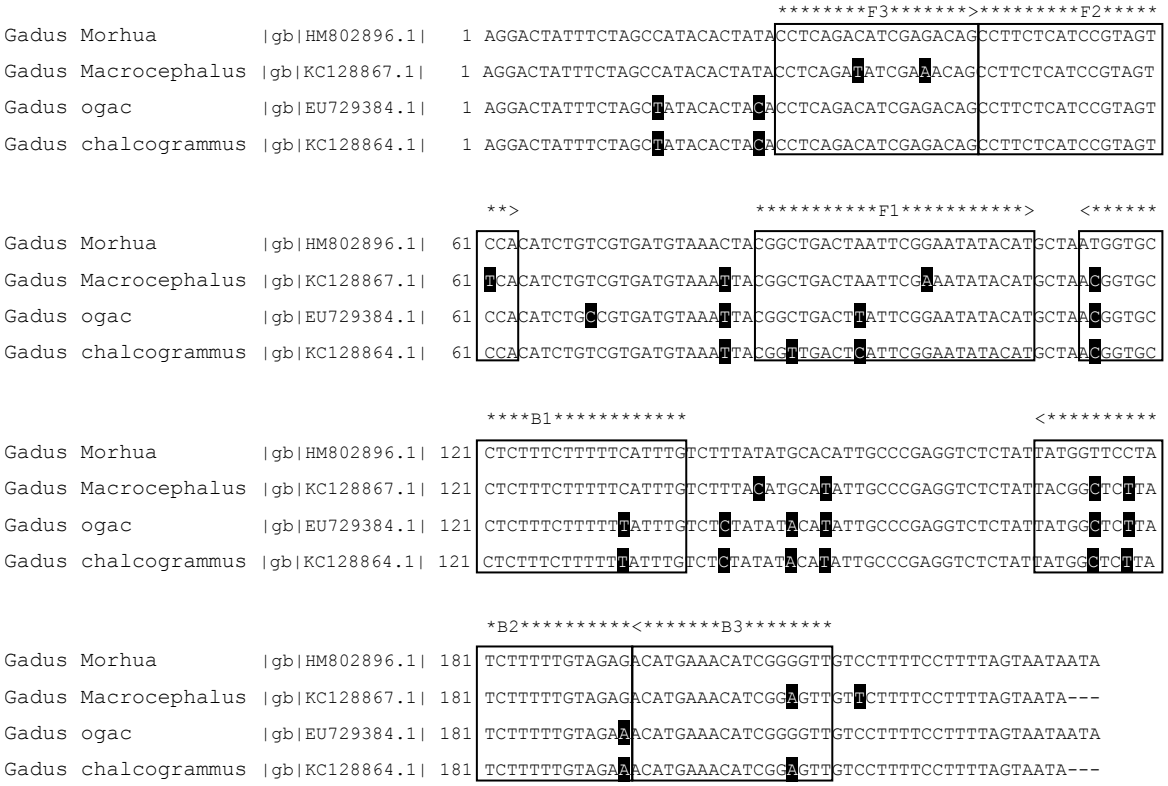


Fig. 1. Primer binding sites of the *G. morhua cyt b* LAMP primer set. Shaded bases indicate mismatches between the *cyt b* sequence and complementary primer sequences. The design of the primers includes mismatches in key positions, ensuring a high level of reaction specificity.

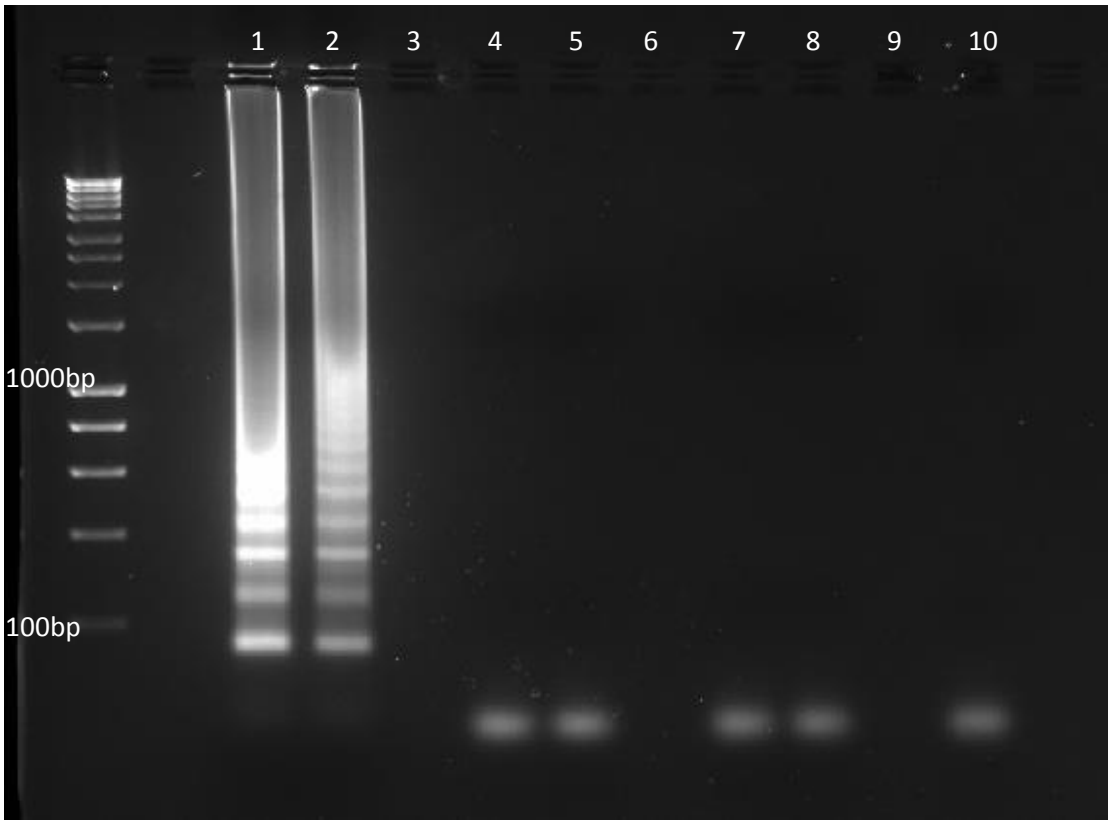


Fig.2. Agarose gel electrophoresis of *cyt b* LAMP reaction products. Reactions were carried out containing DNA extracted from *G. morhua* fillet (Lanes 1-2), *G. macrocephalus* fillet (L 4-5), and *G. chalcogrammus* fillet (L 7-8). A LAMP reaction containing molecular grade water in place of DNA was used as a negative control (L 10).

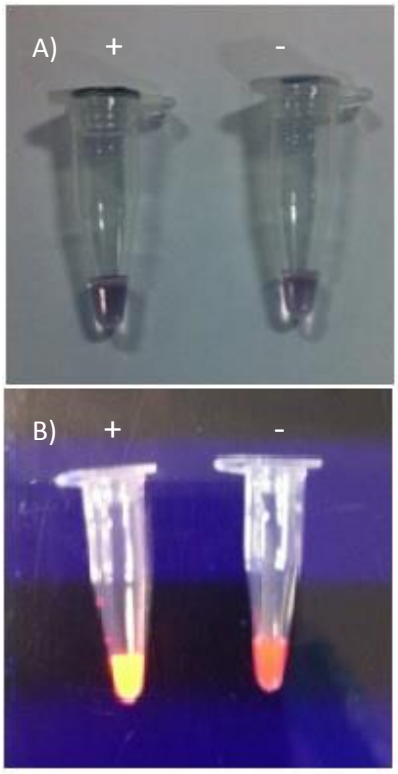


Fig.3. The post-reaction addition of propidium iodide (A) enabled visual determination of the reaction outcome via fluorescence under UV light (B).

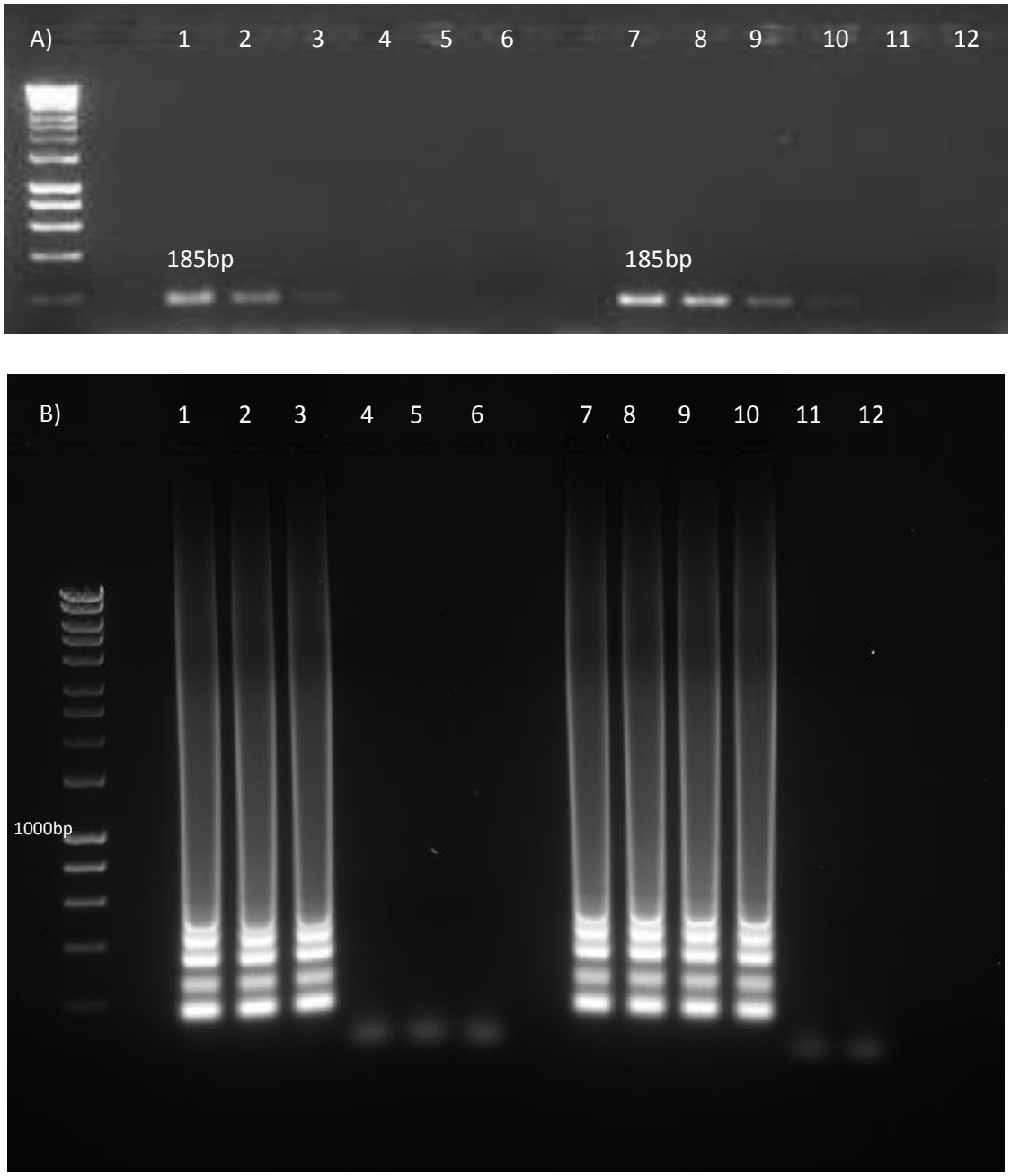
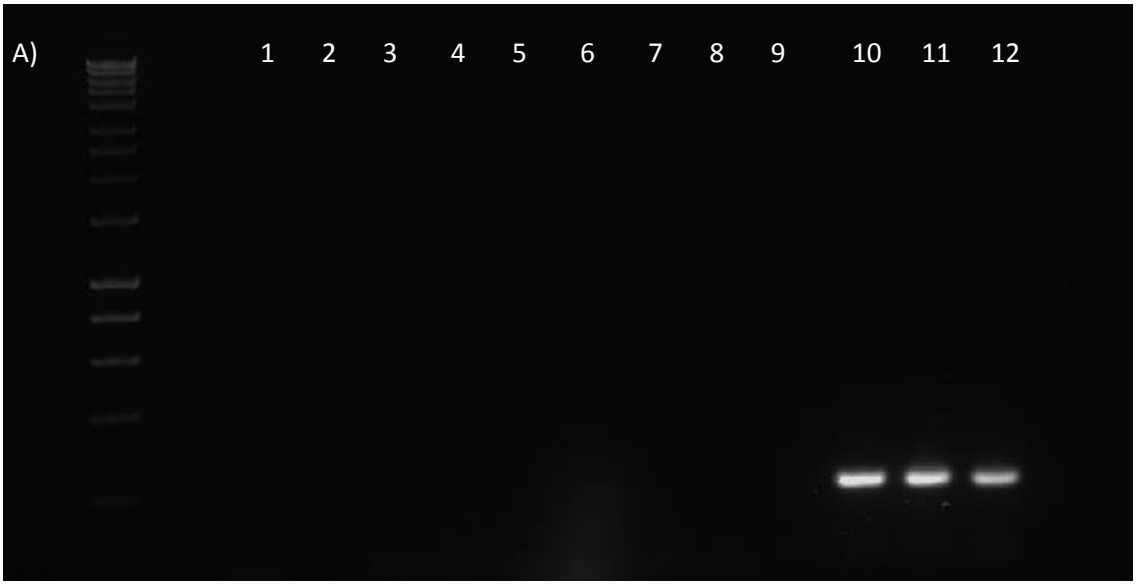


Fig.4. The sensitivity of the *cyt b* PCR (A) and LAMP (B) reactions for the detection of *G. morhua* DNA in a mixed sample. Reactions were carried out containing DNA extracted from mixes containing 100% (lanes 1 and 7), 10% (L 2 and 8), 1% (L 3 and 9), 0.1% (L 4 and 10), 0.01% (L 5 and 11)and 0% (L 6 and 12) w/w homogenised uncooked *G. morhua* fillet, diluted in homogenised *T. chalcogramma* fillet. Reactions were carried out using DNA extracted via the rapid method (L 1-6), and via a spin column based kit (L 7 - 12).


A)
1 2 3 4 5 6 7 8 9 10 11 12


B)
1 2 3 4 5 6 7 8 9 10 11 12

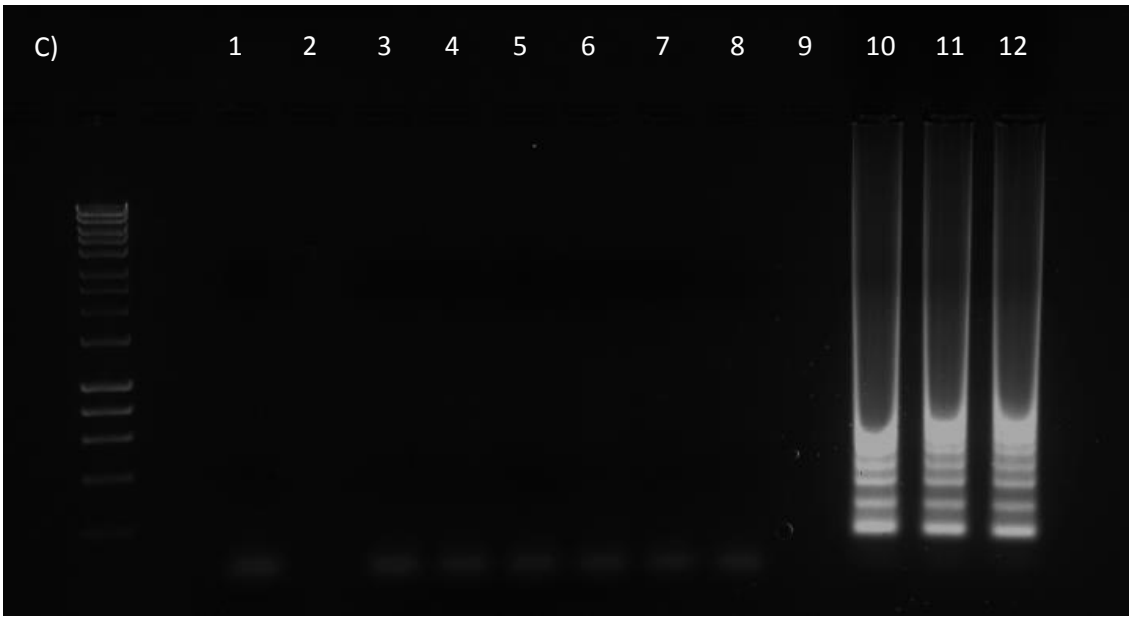

C)
1 2 3 4 5 6 7 8 9 10 11 12

Fig.5. The specificity of the *G. morhua cyt b* PCR (A, B) and LAMP (C) assays, when challenged with DNA extracted from a range of fish products using both the spin column kit (A) and rapid extraction methods (B, C). Reactions were carried out containing water (lane 1), and DNA extracted from *C. herrengus* (L 3), *G. chalcogrammus (L 4), Oncorhynchus spp* (L 5)*, M. aeglefinus* (L 6)*, S. pilchardus* (L 7)*, C. herrengus,* smoked (L 8), *G. morhua*, breaded (L 10), *G. morhua*, raw fillet (L 11), and G*. morhua*, smoked (L 12)