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Verification of authenticity: A rapid identification method for commercial scallop species through multiplex real-time PCR

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ABSTRACT

Seafood products are particularly vulnerable to food fraud and mislabelling, which may have negative implications on fisheries resources, economy, consumer health and trust. This is a considered problem for scallops, since these high-valued seafood products are usually sold without their morphologically characteristic shells. Scallop products differ in taste and value and usually species of the genus *Pecten* spp. are especially expensive in many European countries. The aim of the present study was the development of a multiplex TaqMan real-time PCR assay that allows a rapid and reliable authentication of the three commercially important species/genera *Pecten* spp. (usually King scallop *P. maximus*), Atlantic sea scallop *Placopecten magellanicus*, and Japanese scallop *Mizuhopecten yessoensis*. The design of primers and probes was based on mitochondrial *16S* rRNA gene amplifying fragments of 138–198 bp. Following the optimization of the multiplex real-time PCR assay, analyses on efficiency, limit of detection, specificity, robustness, and crosstalk were conducted for validation purpose. Average Cq values of 20 ng DNA obtained for *Pecten* spp. were 17.64 ± 0.89, for *P. magellanicus* 18.42 ± 0.83, and for *M. yessoensis* 17.08 ± 0.79. Non-target species produced either no fluorescence signal or the Cq differed significantly from those of target species ($p < 0.01$). Finally, the newly developed real-time PCR assay was tested on commercial samples from German supermarkets and fishmongers accompanied by simultaneous verification through Sanger sequencing, which revealed a high mislabelling rate of 48%, especially for products purchased at fishmongers. The results emphasize the need of a control method that allows the rapid analysis of sufficient sample quantities. The study is one of the first which presents a multiplex TaqMan real-time PCR approach for the authentication of commercially important seafood species.

1. Introduction

Mislabelling and food fraud is a recognized issue especially for seafood products due to their high diversity, often similar morphology, or even loss of morphological properties through processing (Verrez-Bagnis et al., 2018; Wisniewski & Buschulte, 2019). Incorrect labelling and adulteration of seafood products may have several negative implications such as deception of consumers, lowering of the product quality, contribution to illegal, unreported, and unregulated (IUU) fishing, or may even constitute a risk to the consumer's health (Agnew et al., 2009; Griffiths et al., 2014). In order to prevent food fraud and mislabelling, the European regulation EU No. 1379/2013 on Common Market Organisation was established which determines commercial and scientific designations with further information on labels of raw, thawed, unprocessed or slightly processed fishery products (European Comission, 2013).

Scallops, belonging to the diverse family Pectinidae, are among the top fifteen of the most consumed seafood products in Europe with 0.34 kg per capita in 2016 (EUMOFA, 2018). There are approximately 50 genera and over 250 species with a cosmopolitan distribution in this family, of which around 18 scallop species are of high commercial interest (Marin, Fujimoto, & Arai, 2015). Together with the roes, called "coral", the meat of the single adductor muscle is sold as high-prized seafood delicacy. On the European market and especially on the German market, the species King scallop *Pecten maximus* (Linnaeus, 1758), Great Atlantic scallop *Placopecten magellanicus* (Gmelin, 1791), and Japanese scallop *Mizuhopecten yessoensis* (Jay, 1857) are of major commercial importance. The global capture production for these species were 59,992 t, 20,6177 t, and 22,3216 t in 2016, respectively (FAO, 2020). A further species of *Pecten* spp., *P. jacobaeus* (St. Jame's scallop)

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has only a small contribution to European scallop landings, mainly from Mediterranean, Adriatic, and Aegean ports (Duncan, Brand, Strand, & Foucher, 2016).

Scallops are usually marketed fresh or frozen either in shells or as adductor muscle meats with or without the roe. Since scallops are often sold having the shells and morphological characteristics removed and they comprise rather expensive products, there is a high potential for mislabelling and food fraud. For the practical implementation of EU No. 1379/2013, each European country is required to draw up and publish an official list on commercial designations and scientific names. Pursuant to the Regulation on Fish Labelling (Federal Law Gazette, 2002/BGBl. I p. 3363), the German Federal Office for Agriculture and Food is competent for the compilation and management of a directory of commercial designations of seafood species. Accordingly, only species of *Pecten* spp. are allowed to be labelled as "Jakobsmuschel" (=King scallop) or "Pilgermuschel". Other members of the family Pectinidae can be sold under the designation "Kammmuschel"/scallop, with the extension of few allowed specifications such as "Japanische Kammmuschel"/Japanese scallop for *M. yessoensis* as an example. Mislabelling and adulteration of scallop species has already been described in several studies (Manthey-Karl, Lehmann, Ostermeyer, Rehbein, & Schröder, 2015; Näumann, Stumme, & Rehbein, 2012; Stephan et al., 2014).

Due to the absence of morphological characteristics in many scallop products, molecular methods are needed to examine the authenticity of the species. Several DNA-based methods have been presented for scallop species identification including mainly Sanger sequencing of mitochondrial fragments such as a *16S* rRNA gene and cytochrome oxidase subunit I (COI) (Feng, Li, Kong, & Zheng, 2011; Marin et al., 2015; Marín, Fujimoto, & Arai, 2013; Marin, Villegas-Llerena, Fujimoto, & Arai, 2017; Näumann et al., 2012; Wen et al., 2017). Additionally, a matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) was designed for the identification of scallop species (Stephan et al., 2014). Disadvantages of these methods are that Sanger sequencing is time-consuming and can take up several days if external sequencing services are needed and MALDI-TOF requires the generation of a database comprising various available spectra as reference for comparison.

An increasingly common method for species identification and for testing food authenticity is real-time PCR, including the use of dual labelled probes, which has some advantages due to its sensitivity, specificity, and it allows a rapid identification within a few hours since no post-PCR treatment is required (reviewed in Hellberg & Morrissey, 2011). Depending on the available channels of the real-time thermocycler, differently fluorescence-labelled species-specific probes can be combined simultaneously to a multiplex assay.

Despite their commercial importance, relatively few publications on scallop identification techniques are available. Therefore, the aim of the present study was the development of a rapid method that allows the identification of three market relevant scallop species/genera within a few hours. In the present study, three main commercial species/genera *Pecten* spp., *Placopecten magellanicus* and *Mizuhopecten yessoensis* were selected for the development of primers and probes. In addition, the newly designed multiplex real-time PCR method was validated on commercial samples in order to provide a general overview on the current status of correct labelling of scallop products on the German market.

Table 1
List of sequences of a *16S* rRNA gene fragment used for the design of the primers and probes.

Species	No. of sequences	No. of haplotypes	GenBank Accession numbers
Pecten maximus	42	34	AY650056.1-AY650084.1, KF982791.1, AJ972436.1, AJ972435.1, AJ571619.1, KC429258.1, KC250352.1, KC250353.1, JX624722.1, JQ611456.1, GU324150.1, EU3794524.1, FN667669.1, FN667668.1
Pecten jacobaeus	12	10	AJ245394.1, FN667671.1, FN667670.1, MF183948.1-MF183955.1
Pecten sulcicostatus	18	18	KP900974.1, KU754458.1-KU754475.1
Pecten albicans	10	2	KJ000188.1-KJ000195.1, JN896624.1, KP900974.1
Pecten fumatus	3	3	HM622690.1, JF339109.1, JF339110.1
Pecten novaezelandiae	6	2	JF339107.1, JF39108.1, AJ972445.1, AJ972446.1, EU379458.1, EU379459.1
Placopecten magellanicus	7	3	AJ972444.1 AJ972443.1 KX713249.1 FJ263647.1 EU379452.1 KC250354.1 DQ088274.1
Mizuhopecten yessoensis	24	18	KF577651.1-KF577663.1, JQ611477.1, FJ263649.1, DQ640893.1, DQ640894.1, AF362386.1, GU119977.1, GU119976.1, AB103394.1, NC_009081.1, HM630384.1
Argopecten irradians	9	3	KJ000142.1-KJ000148.1, AF526205.1, NC_012977.1
Azumapecten farreri	6	5	NC_012138.1, AF362385.1, GU119978.1-GU119980.1, HM622682.1
Mimachlamys nobilis	35	19	DQ873926.1-DQ873934.1, DQ640848.1-DQ640865.1, FJ415225.1, NC_011608.1, GU119963.1-GU119965.1, HM630532.1
Zygochlamys patagonica	6	2	HM630521.1, AJ972448.1, AJ972447.1, KY070310.1, KY070311.1, EU379466.1,
Amusium pleuronectes	33	23	HM630505.1, HM630501.1, HM630497.1, JF339128.1-JF339130.1, DQ640830.1-DQ640845.1, GU119962.1, AJ571616.1, KP900978.1, KC879126.1, KC879122.1, KC879118.1, EU379469.1, DQ873919.1, DQ873918.1, DQ873917.1
Aequipecten opercularis	13	4	AJ245397.1, AM494408.1-AM494413.1, JQ611439.1, EU379462.1, EU379463.1, JF808175.1, JF901824.1, KR078011.1
Mimachlamys varia	12	8	KT988340.1, JQ611446.1, EU379482.1, FN667674.1, AJ586481.1, AJ586480.1, AJ243575.1, AJ586476.1-AJ586479.1, HM630412.1,

2. Material and methods

2.1. Scallop samples and DNA extraction

A total of 56 scallop samples were analysed. Samples of target species and non-target species were either provided by German seafood traders and processors, by partners of the SEATRACES project (Instituto de Investigaciones Marinas (IIM-CSIC), Muséum National d'Histoire Naturelle (MNHN), Laboratoire SCL de Marseille (SCL)), and company Escal (Strasbourg, France), or samples were purchased at German supermarkets and fish mongers. Scallop samples were bought fresh, frozen, canned, or were provided in EtOH or RNAlater. After arrival at the Max Rubner-Institut (MRI), samples (except samples in EtOH and RNAlater) were frozen at −20 °C until further procedure. In addition, 27 reference samples of different taxa such as fish, crustaceans, other molluscs, and a shark from the MRI archive were used to test the specificity of the real-time assay (see Table 4).

For DNA extraction, 50–100 mg of sample tissue were prepared and isolated using the CTAB method as described in Rehbein (2005). An additional negative extraction control without sample material was performed within each extraction session. DNA concentrations were measured fluorometrically using the Hoechst test (Downs & Wilfinger, 1983) with calf thymus DNA as standard.

2.2. Species identification by Sanger sequencing

The species of all samples were determined by conventional PCR and Sanger sequencing of a 550–620 bp mitochondrial *16S* fragment as reference for scallop species identification with primers 16SAR (5′-CGCCTGTTTATCAAAAACAT) and 16SBR (5′-CCGGTCTGAACTCAGATCACGT) (Palumbi, 1991). The PCR analysis and species alignment was performed according to the official method BVL L 12.03/04–6 (Identification of scallop species through analysis of *16S* rRNA sequences), which is published by the §64 of the German Foods, Consumer Goods and Feedstuffs Code (LFGB) and has been validated by ring trials (BVL, 2020). PCR reactions were performed in volumes of 20 μL containing 10 μL HotStarTaq Plus Master Mix (Qiagen, Hilden, Germany), 10 pmol forward and reverse primer (Biomers, Ulm, Germany), each, and about 20 ng extracted DNA. PCR reactions included an initial denaturation at 94 °C for 5min, followed by 35 cycles of 95 °C for 60 s, 54 °C for 60 s, 72 °C for 60 s, and a final extension of 7 min at 72 °C. A negative control was added to each PCR run. PCR products were checked on a 2% (w/v) agarose gel. The 1:10 diluted post-PCR products and primers were sent for sequencing at LGC Genomics (Berlin, Germany). Sequenced forward and reverse strands were checked using Chromas Lite version 2.1 (Technelysium Pty Ltd, South Brisbane, Queensland, Australia). Primers were deleted from sequences and consensus sequences were assembled from both strands with MEGA10.0.5 (Kumar, Stecher, Li, Knyaz, & Tamura, 2018). For species identification, the obtained sequence data were compared with published GenBank data using the BLASTn algorithm (Altschul, Gish, Miller, Myers, & Lipman, 1990) and results were assessed as described in L 12.03/04–6.

2.3. Design of primers and probes for the real-time PCR

For the design of primers and probes, several sequences including *16S*, cytochrome-c-oxidase I (COI), and cytochrome *b* (*cytb*) for target species and closely related non-target species were retrieved from GenBank (www.ncbi.nlm.nih.gov/genbank) and BOLD (www.boldsystems.org) during October to December 2018. Concluding from these results, *16S* was chosen for the primer and probe design. Sequences of available scallop species matching the search "*16S*" in the NCBI taxonomy browser were downloaded and all sequences were aligned (Table 1) in MEGA10.0.5 (Kumar et al., 2018) to the target sequence sizes defined by the *16S* primer sequences (Palumbi, 1991). Alignments were additionally visualized in GeneDoc 2.7 (Nicholas & Nicholas, 1997).

For each target species, sequence regions were chosen that could potentially being used to differentiate the target species from other species. The suitability of primers and probes was tested on Primer 3plus (Untergasser et al., 2007), with a subsequent test for specificity and quality using PrimerBLAST (Ye et al., 2012). Additionally, a universal eukaryotic *18S* rDNA primer/probe system was selected to serve as internal amplification control: 18S Uni-F 5′-GTAATTTGCGCGCCTGCT -3′, 18S Uni-R 5′-GTTCGATTCCGGAGAGGGA-3′, 18S Uni-P Cy5-5-CCTTCCTTGGATGTGGTAGCCGTTTCTC- BBQ-650 (Zagon et al., 2017).

2.4. Real-time PCR and optimization

Preliminary tests of primers for functionality and specificity were conducted as singleplex reactions using only one primer pair for one target species/genus with SybrGreen (Biorad, Munich, Germany). For each target, two to three primer pairs were tested for their performance and specificity. Melting curve analyses were conducted to ensure the absence of non-specific amplification. The optimal temperatures for the primers were tested in a temperature gradient ranging from 56 to 68 °C. In the next step, the tests were repeated with addition of the probes. Four fluorescent markers that do not overlap in the wavelength spectrum were chosen and appropriate quenchers were selected according to the manufacturer's recommendations (Biomers, Ulm, Germany). Probes were labelled with the following fluorochromes: Pec FAM, Pmag Cy5, Myes Texas Red, and 18S Cy5-5 (Biomers, Ulm, Germany). The most specific primer/probe combinations, which produced preferably no or only a signal at a late real-time PCR cycle in non-target species, were chosen. In order to define optimal concentrations of primers and probes for the identification and the functionality in a multiplex assay, primers and probes were first tested in singleplex assays for each target species and later combined in a multiplex assay. Each primer/probe system was tested on the following conditions: 200, 300, 400 nM of primers, and 100, 200, 300 nM of probe. The combination that gave the lowest Cq (quantification cycle) value and the highest fluorescence was selected for the subsequent assays. In case there was no major difference in the Cq or the height of the signal, the lower concentration was chosen.

Resulting from these tests, primer and probe combinations for all three targets were combined to a multiplex assay. All PCR reactions were performed with a total volume of 20 μL including 20 ng DNA, 10 μL Sso Advanced Universal Probes Mastermix (2x) (Biorad, Munich, Germany), 200 nM forward and reverse primers, each, and 100 nM probe each. PCR reactions were performed in 96 Well 0.1 mL 8-Transformer Plate white and the respective Transformer Cap Strip Plate lids (Biozym, Hessisch Oldendorf, Germany) and run on an CFX-96 (Biorad, Munich, Germany) with the following 2-step thermal cycling protocol: 98 °C for 3 min followed by 39 cycles of 95 °C for 10 s and 62 °C for 30 s. All samples were analysed in duplicates and a non-template control was added to each PCR run.

2.5. LOD and standard curves (efficiency)

For validation of the multiplex PCR assay, tests were performed according to the MIQE guidelines (Broeders et al., 2014) and guidelines from the Federal Office for Consumer Protection and Food Safety (BVL, 2016).

The efficiencies (E) were determined using five tenfold serial dilutions of target DNA in the multiplex assay, separately for each of the three target species. Serial dilutions were tested with and without pig DNA as background DNA and the repeatability of the real-time PCR assay was investigated by analysing serial dilutions of target DNA from 20 to 0.002 ng without any background DNA in duplicates on three days. For comparability of Cq values, threshold values were defined and manually set on a certain value for each primer/probe set. The efficiency based on Cq values versus the log DNA amount of serial dilutions were used to calculate the efficiency according to formula $E = [10(-1/slope)-1]\times100$ (Bustin et al., 2009).

In order to determine the limit of detection (LOD), a serial dilution of seven tenfold dilutions with twelve replicates was compiled for each of the three primer/probe systems. First, a mix was created including the Sso Advanced Universal Probes Mastermix (2x), primers, and probes. DNA concentrations in the dilution series ranged from 20 to 0.00002 ng DNA per 20 μL reaction. The mix was then separated in seven tubes and DNA of the different dilutions was added. Each mix was vortexed well and distributed among a 96 Well 0.1 mL 8-Transformer Plate white (Biozym, Hessisch Oldendorf, Germany). Non-template controls of each dilution were added. The concentration generating a positive signal in eleven of the twelve measurements was defined as LOD.

2.6. Robustness

To determine the robustness of the real-time PCR assay, the testing scheme provided by the Guidelines for the single-laboratory validation

of qualitative real-time PCR methods (BVL, 2016) was adopted. The assay was tested with diverse deviations from the usual PCR conditions (Supplementary material). In brief, primer and probe concentrations were lowered for 30%, the mastermix volume was raised or lowered for 7%, and the annealing temperature was set 1 °C higher or lower than in the usual assay. Only one parameter was changed in each approach. In addition to the Sso Advanced Universal Probes Mastermix (2x), tests were performed using the QuantiNova multiplex PCR kit (Qiagen, Hilden, Germany).

In order to investigate whether the mixtures of target species DNA lead to shifts of the Cq values, DNA mixtures containing different contents were produced (50:50; 75:25, 90:10, 99:1, see Supplementary material).

2.7. Assay specificity and crosstalk

To test whether the designed primers and probes specifically hybridize only with the target species and whether there are no unexpected cross-reactions with other species, a theoretical and a practical test were carried out. The theoretical review of the primer/probe systems was done immediately after the primer design in the alignment. For this purpose, the potential primers and probes were blasted using the Primer-Blast program (see 2.3). All primer/probe systems were tested for specificity with 20 ng DNA isolated from a variety of seafood species. An additional test was conducted in order to analyse possible differences between frozen and fried products. Therefore, two individuals of each target species were picked and frozen tissue samples were taken. The scallops were then fried for approximately 3 min each side in a pan with oil and tissue samples were taken again. Real-time PCR tests were carried out according to the procedure described above.

Crosstalk, the occurrence of a fluorophore signal in a non-target filter (filter-bleed through) or a possible “cross-hybridization” of probes to a non-target sequence, was investigated by adding 20 ng DNA of two target species, each, in one 20 μL reaction. If there was no signal in the filter of the third primer/probe system, the absence of crosstalk was assumed.

2.8. Test of commercial samples

The acquired commercial samples were checked for completeness of the labelling according to the index of commercial designations for fisheries products published by the German Federal Office for Agriculture and Food. In order to verify results of the real-time PCR assay, samples were additionally sequenced using the above mentioned *16S* primers (conditions, see 2.2) and compared with available sequences in Genbank (Altschul et al., 1990). The assignment of the target species/genus using the newly developed real-time assay was based on the signal previously determined in the specificity test.

2.9. Data treatment

Average Cq values and standard deviations were determined for each target species tested. Cq values of the primer/probes assays Pec, Pmag, and Myes were individually tested for normality (Shapiro–Wilk’s W-test). In order to compare Cq values of target and non-target species for each primer/probe system, a Kruskal–Wallis rank sum test was applied. Two-tailed, unpaired Mann–Whitney *U* tests were performed to test for significant differences between the mastermixes in the robustness tests. Statistical tests were conducted in R (version 3.2.2), graphical visualisation in GraphPad Prism (version 5.01).

3. Results and discussion

Mislabelling and substitution of scallop species as a common phenomenon have already been described in previous studies, which are possibly related to economic benefits and the similar morphology of scallop products. However, correct labelling is important to comply with the European regulations and is essential as scallop species differ in value and marketability (Stephan et al., 2014). Therefore, the aim of the present study was the development and validation of a multiplex real-time PCR assay for the rapid identification of scallop species. The method allows food monitoring authorities a comprehensive monitoring of larger sample quantities within a short time period.

Table 2
Sequences, length, melting temperatures, and amplicon sizes of the newly designed primers and probes.

Name	Sequence	Bp	Tm [°C]	Amplicon size (bp)
Pec2-F	5′-GCAAATGCTTCCATGGGTAA-3′	20	60	154
Pec2-R	5′-CCCCAGCCAAAACTGCTAT-3′	19	61	
Pec2-P	FAM-AATTTAAGTTATTGGGAAGYTCCAAGGC-BMN-Q535	28	65	
Pmag1-F	5′-GCCTCCAACTGTCTCTAGGTTG-3′	22	60	198
Pmag1-R	5′-CCCAGCAAAAACCATTCACT-3′	20	60	
Pmag1-P	Cy5-CCCTGTTGGTGAAGGCTAGAGGG-BHQ-2	23	67	
Myes3-F	5′-TTGAAGGTCCCGGCTTTAT-3′	19	60	138
Myes3-R	5′-CACGATTTTCATGTTTTGTGG-3′	21	59	
Myes3-P	TexRed-TTGACGAGTTTTGGCTGGGGC-BBQ-650	21	68	

3.1. Design of the real-time PCR assay

In general, primers and probes should be designed to target DNA fragments with a high interspecific but low intraspecific variability in order to assure that they are species-specific on the one hand and bind to all individuals of one species on the other hand (Taboada, Sánchez, & Sotelo, 2017). For the real-time PCR assay development targeting scallop species, sequences of *16S* were chosen based on the fact that an official method for Sanger Sequencing of a mitochondrial *16S* fragment is available (BVL, 2020) and the number of available sequences of this fragment is highest in the NCBI GenBank database for target and non-target species of the Pectinidae. Due to the restrictions associated with the Nagoya Protocol, a development of primers and probes based on alternative fragments (e.g. nuclear DNA fragments) through generation of own sequences would have meant an enormous administrative and time-consuming expenditure as not only tissue of target species would have been required, but also of non-target species for specificity tests. That would not have been possible within a time-restricted research project. The sequences and amplicon lengths of the designed primer and probe sets are shown in Table 2. Fragment sizes are 154 bp, 198 bp, and 138 bp for the *Pecten* spp., *P. magellanicus*, and *M. yessoensis* assay, respectively. These partially large real-time PCR fragment sizes are due to an either high conservation of the fragment between or a large variability within species which limited the options to design specific primers and probes. In general, mitochondrial DNA is commonly used for species identification purposes and the development of real-time PCR assays (e.g. (Santaclara et al., 2015; Taboada et al., 2017; Velasco et al., 2013)). Mitochondrial DNA has some advantages for the use of species identification such as an absence of recombination due to usually maternal inheritance, rapid evolvement based on high mutation rates, and a large copy number per cell in muscle tissue. A specificity for some bivalves is doubly uniparental inheritance (DUI), in which mitochondrial DNA can be both maternally and paternally inherited (Doucet-Beaupré et al., 2010). However, strict maternal inheritance of mitochondrial DNA is found for Pectinids (Shumway & Parsons, 2016) and therefore DUI is not considered as an issue for species identification of scallops.

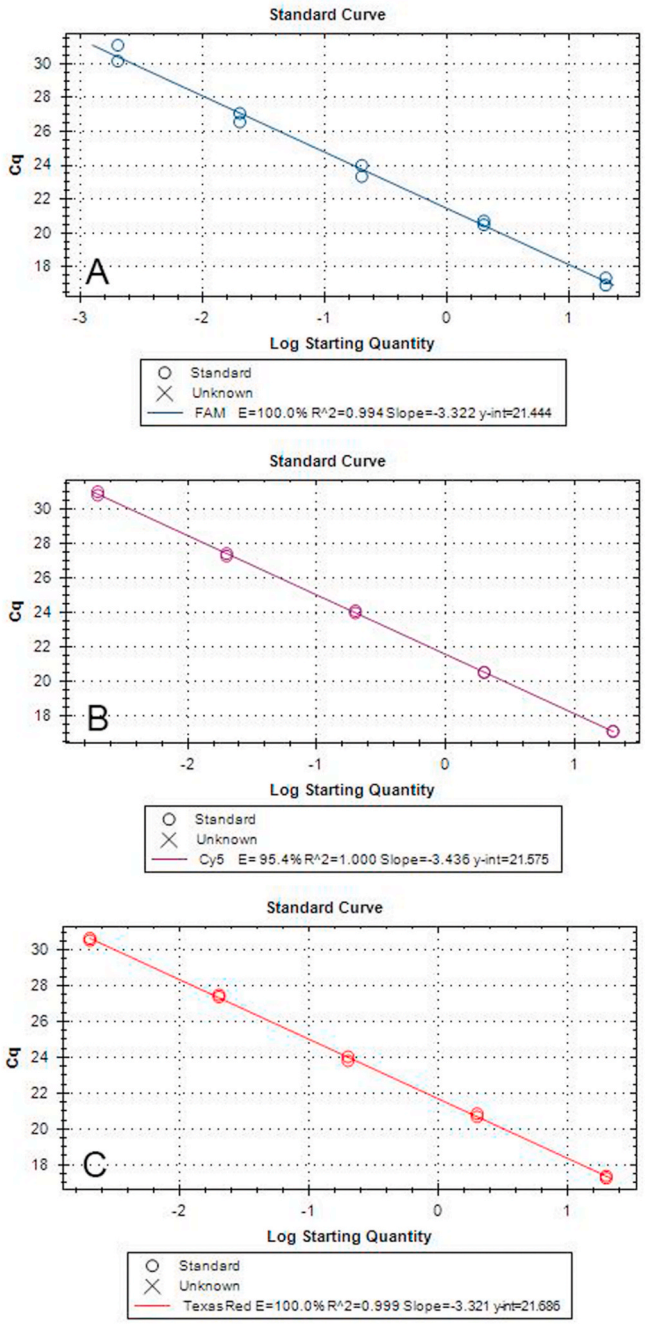

Fig. 1. Standard curves of efficiency tests on the three targets in the multiplex real-time PCR assay. Tenfold serial dilution series ranging from 20 to 0.002 ng DNA. A *Pecten* spp. B *Placopecten magellanicus* C *Mizuhopecten yessoensis*.

3.2. Efficiency and detection limit

Dilution series of template DNA were analysed for each target species to test the efficiency and limit of detection of the primers and probes of the multiplex assay (Fig. 1). For this purpose, serial dilutions of target DNA from 20 to 0.002 ng without any background DNA were prepared and tested over three days. The dilution series over this range were linear for all target species. Mean test results of *Pecten* spp. are an efficiency of E = 102.17 ± 1,90%, a coefficient of determination of R^2 = 0.997 ± 0.002, and a slope of −3.304 ± 0.064. For *P. magellanicus*, mean results are E = 96.67 ± 4,24%, R^2 = 0.999 ± 0.001, slope −3.407 ± 0.087, and for *M. yessoensis* efficiency, coefficient of determination, and slope are E = 100.37 ± 1,70%, R^2 = 1.000 ± 0.001, and slope −3.314 ± 0.033. The acceptable efficiency values for a multiplex assay encompass a range from 80 to 120% (Broeders et al., 2014). Therefore, our results are in an optimal range.

For the determination of the limit of detection (LOD), DNA concentrations in a dilution series from 20 to 0.00002 ng DNA in 20 µL reactions were analysed. Although the utilisation of background DNA is recommended in order to stabilize the system (BVL, 2016), tests using pig DNA as background DNA were unsuccessful and led to high shifts in the amplification curves. DNA in excess may presumably have an influence on the performance of the PCR. The LOD was defined as the lowest DNA concentration in which all twelve replicates display a positive result. Thus, the LOD was determined as 0.0002 ng DNA (Table 3).

3.3. Specificity

To analyse the specificity of the designed primers and probes, both theoretical and practical specificity were examined. An *in-silico* analysis of specificity was conducted immediately after the design of the primers and probes with existing sequences in the NCBI GenBank database, to test whether the designed primers and probes could theoretically result in non-specific amplicons in other non-target species. Results of the theoretical review of the primers and probes did not reveal any cross-reactions. However, the Pec primers and probes may anneal to several *Pecten* species such as *P. maximus, P. jacobaeus*, *P. sulcicostatus*, *P. novaezelandiae*, *P. fumatus*, *P. albicans*, and *P. keppelianus*. The design of specific primers and probes for commercial scallop species was difficult since the number of available sequences in the NCBI database and BOLD was low, and there was a high sequence homology, especially of *Pecten* spp.. As it is based on a *16S* rRNA gene PCR approach developed by Näumann et al., 2012, which enables the differentiation of genera and many species of Pectinidae, but *P. maximus* cannot be distinguished from its sister species *P. jacobaeus*, it was not possible to design a primer/probe system that only targets *P. maximus*. Although there are morphological differences between *P. maximus* und *P. jacobaeus*, their taxonomic relation is still not completely clarified (Canapa, Barucca, Marinelli, & Olmo, 2000; (Marín et al., 2015); Svåsand, Crosetti, García-Vázquez, & Verspoor, 2007; Wilding, Beaumont, & Latchford, 1999). Various gene markers revealed low genetic differentiation (Saavedra & Peña, 2004, 2005; Wilding et al., 1999). A recent

Table 3
Limit of detection results of the multiplex real-time assay. Results shown for each of the three primer/probe targets. Ø Cq = Average threshold cycle, SD = standard deviation, No. positive = number of positive signals from twelve replicates.

	Pec			Pmag			Myes		
Template	Ø Cq	SD	No. positive	Ø Cq	SD	No. positive	Ø Cq	SD	No. positive
20 ng	17.86	0.241	12/12	17.43	0.137	12/12	17.22	0.104	12/12
2 ng	20.99	0.187	12/12	21.01	0.102	12/12	20.54	0.143	12/12
0.2 ng	24.45	0.154	12/12	24.33	0.071	12/12	24.19	0.133	12/12
0.02 ng	27.73	0.313	12/12	27.61	0.123	12/12	27.60	0.171	12/12
0.002 ng	30.92	0.255	12/12	31.01	0.155	12/12	30.99	0.352	12/12
0.0002 ng	34.59	0.707	12/12	34.70	0.712	12/12	34.31	0.453	12/12
0.00002 ng	37.99	1.702	9/12	37.57	1.018	7/12	37.32	1.072	8/12

Table 4
Cq values of tested species obtained from 20 ng DNA in specificity and cross-reactivity tests. Target species are bold. N = number of samples tested. Negative results were set as Cq ≥ 40.

Taxon	Scientific name	N	Pec	Pmag	Myes	18s
Scallops	***Pecten* spp.**	17	**17.64 ± 0.89**	≥40	39.83 ± 0.37	16.01 ± 0.47
	Placopecten magellanicus	23	≥40	**18.42 ± 0.83**	39.66 ± 0.96	16.12 ± 0.33
	Mizuhopecten yessoensis	15	≥40	≥40	**17.08 ± 0.79**	15.39 ± 0.62
	Aequipecten opercularis	8	36.56 ± 2.96	38.39 ± 2.33	37.55 ± 1.65	16.61 ± 1.36
	Argopecten purpuratus	5	≥40	39.46 ± 1.21	39.46 ± 0.76	14.56 ± 1.58
	Azumapecten farreri	1	≥40	≥40	39.46	16.15
	Chlamys islandica	1	39.63	≥40	38.94	15.07
	Mimachlamys varia	2	33.33 ± 4.86	≥40	39.46 ± 0.76	15.82 ± 0.53
	Zygochlamys spp.	8	36.42 ± 3.23	39.79 ± 0.61	38.22 ± 2.86	17.12 ± 1.35
Other Bivalves	*Cerastoderma edule*	2	≥40	≥40	≥40	23.53 ± 0.23
	Ensis directus	2	≥40	≥40	39.60 ± 0.57	18.57 ± 1.8
	Glycymeris spp.	1	≥40	≥40	≥40	17.1
	Mercenaria mercenaria	1	≥40	≥40	35.43	17
	Mytilus edulis	1	≥40	≥40	≥40	18.11
	Ruditapes philippinarum	4	≥40	39.74 ± 0.74	≥40	17.85 ± 1.81
	Spisula solidissima	2	≥40	≥40	38.17 ± 2.59	19.66 ± 0.82
Cephalopods	*Doryteuthis gahi*	1	≥40	≥40	39.51	23.79
	Illex argentinus	1	≥40	≥40	≥40	17.34
	Sepia officinalis	1	≥40	38.90	32.65	18.41
	Uroteuthis chinensis	2	≥40	≥40	≥40	17.27 ± 1.02
Crustaceans	*Penaeus merguiensis*	1	≥40	≥40	≥40	15.7
	Liocarcinus holsatus	1	≥40	≥40	≥40	15.3
	Litopenaeus vannamei	2	≥40	≥40	≥40	15.32 ± 0.03
	Marsupenaeus japonicus	1	≥40	≥40	≥40	17.17
	Metapenaeus sp.	2	≥40	≥40	≥40	16.96 ± 0.36
	Nephrops norvegicus	1	≥40	≥40	≥40	15.71
	Mierspenaeopsis sculptilis	1	≥40	≥40	≥40	16.55
	Penaeus monodon	1	≥40	≥40	≥40	17.65
	Pleoticus muelleri	1	≥40	≥40	≥40	15.95
Fish	*Bolbometopon muricatum*	1	≥40	≥40	39.26	17.67
	Katsuwonus pelamis	1	≥40	≥40	39.34	19.96
	Lutjanus malabaricus	1	≥40	≥40	≥40	17.36
	Lutjanus sebae	1	≥40	≥40	≥40	16.02
	Pangasianodon hypophthalmus	1	≥40	≥40	≥40	16.35
	Scophthalmus maximus	2	≥40	≥40	≥40	16.45 ± 1.07
	Solea solea	2	≥40	≥40	≥40	15.26 ± 0.10
	Sparus aurata	1	≥40	≥40	≥40	16.46
	Thynnus albacares	2	≥40	≥40	≥40	17.26 ± 0.36
Sharks	*Squalus acanthias*	1	≥40	≥40	≥40	16.42

study used RAD sequencing to analyse the population structure of *P. maximus* and *P. jacobaeus* and found a divergence time estimate to be approximately 500,000 years ago, much less than previously expected (Vendrami et al., 2019). Low genetic differentiation was also found for other *Pecten* species. A comparison between mitogenomes of *P. maximus* and the Japanese baking scallop *P. albicans* displayed high nucleotide and amino acid identity, as well as the same gene order arrangement which is a special within Pectinidae (Marín et al., 2015). Based on these results, the authors suggested a recent speciation event. Regardless of the actual genetic differentiation of species within the *Pecten* genus, to our knowledge only *P. maximus* and *P. jacobaeus* are available on the German market. It was therefore not possible to carry out practical specificity tests of other *Pecten* species mentioned. This is not an obstacle from a legal perspective in Germany, because all *Pecten* spp. may be sold as "Jakobsmuschel" or "Pilgermuschel" (=King scallop). The same applies to other European countries such as Portugal, France and the UK, since there is also no distinction in the labelling of these *Pecten* species. Only in Spain a distinction is made between "Vieira o venera" (*P. maximus*) and "Concha de peregrino" (*P. jacobaeus*) (BOE, 2019).

Specificity was tested practically on a variety of different seafood species including various samples of the target and further scallop species. These included all scallop species available on the German market and reference samples of species from Spain and France. Additionally, other bivalves, cephalopods, crustaceans, fish, and a shark were tested (Table 4). The average Cq value for *Pecten* spp. was 17.64 ± 0.89, for *P. magellanicus* 18.42 ± 0.83, and for *M. yessoensis* 17.08 ± 0.79 using 20 ng DNA. To calculate the average Cq value, all undetected samples were assigned as Cq ≥ 40. Cq values for target species differed significantly from non-target species (Kruskal-Wallis rank sum test, $p < 0.01$). Although a signal occurred in the Pec primers and probes when testing *Aequiptecten opercularis* (ΔCq 18.92), *Mimachlamys varia* (ΔCq 15.69), and *Zygochlamys* spp. (ΔCq 18.78), the ΔCq values were sufficient to distinguish these species from *Pecten* spp.. This applies as well to the primers and probes for *P. magellanicus* and *M. yessoensis* ($p < 0.01$, see Table 4). Frying of scallops generating a degradation of the DNA did not lead to higher Cq values (Supplementary material). An *18S* fragment was deployed as internal amplification control to assure the functionality of the assay. The identification of all samples by real-time PCR was in line with the results of Sanger sequencing. A partly high variability of Cq values (Pec 15.41–18.67, Pmag 17.14–19.77, Myes, 16.30–19.46, DNA 20 ng) among samples of the same species may be explained by the use of mitochondrial DNA and consequently possibly different amounts of mitochondrial DNA depending on the tissue and species. Unfortunately, the fluorometrically determination of DNA concentration does not make a difference between nuclear and mitochondrial DNA because all double-stranded DNA is measured. Therefore, the 20 ng DNA inserted in the qPCR approach may consist of slightly varying mitochondrial DNA concentrations.

Additional crosstalk tests confirmed the absence of any "filter bleed-through" or a possible cross-hybridization of probes to a non-target sequence (Supplementary material). Thus, the primers and probes of the different targets do not lead to an unwanted signal in the multiplex approach.

3.4. Robustness and LODasym

To examine the robustness of the real-time multiplex PCR assay, tests with DNA isolates of the target species were separately performed in a multifactorial experimental design by varying the annealing temperature ±1 °C, the volume of the reaction mix (±7%), or by lowering the concentration of the target primers or probes (30% less). These tests were conducted by comparing two reaction mixes, the Sso Advanced Universal Probes Mastermix (2x) (Biorad, Munich) and QuantiNova multiplex PCR kit (Qiagen, Hilden). The tests show that Cq values were lower using the Sso Advanced Universal Probes Mastermix than those of the QuantiNova multiplex Mix for the Pmag and Myes primers and

Table 5
Analysis of commercial samples by using the newly developed multiplex real-time PCR assay and Sanger sequencing. Cq values are shown using 20 ng DNA. NA = not available.

Sample	Commercial name	Scientific name	Sequencing result	RT-PCR	Pec	Pmag	Myes	18S	Label correct	Shopping Type
1	Scallops	*Placopecten magellanicus*	*Mizuhopecten yessoensis*	+	40.00	40.00	16.30	15.82	No	Supermarket
2	Scallops (Mini Coquilles Saint-Jaques, small scallops)	*Zygochlamys patagonica, Chlamys nobilis, Chlamys opercularis, Pecten fumatus*	*Aequipecten opercularis*	–	34.42	40.00	36.82	16.79	Yes	Supermarket
3	Scallops (with roe)	*Argopecten purpuratus*	*Argopecten purpuratus*	–	40.00	40.00	40.00	13.64	Yes	Supermarket
4	Scallops (without roe)	*Placopecten magellanicus*	*Placopecten magellanicus*	+	40.00	17.31	40.00	16.37	Yes	Supermarket
5	Scallops (without roe)	*Placopecten magellanicus*	*Placopecten magellanicus*	+	40.00	17.80	40.00	16.43	Yes	Supermarket
15	King scallop (in wine sauce and breadcrumbs topping)	*Pecten maximus*	*Pecten* spp.	+	16.62	40.00	40.00	15.52	Yes	Supermarket
16	Atlantic sea scallop (fresh)	*Patinopecten yessoensis*	*Mizuhopecten yessoensis*	+	40.00	40.00	17.40	16.24	Yes	Supermarket
17	Scallops	*Mizuhopecten yessoensis*	*Mizuhopecten yessoensis*	+	40.00	40.00	16.66	14.94	Yes	Supermarket
18	Scallops	NA	*Mizuhopecten yessoensis*	+	40.00	40.00	17.47	15.21	Yes	Fishmonger
19	King scallop	NA	*Mizuhopecten yessoensis*	+	40.00	40.00	17.16	16.70	No	Fishmonger
20	King scallop	NA	*Mizuhopecten yessoensis*	+	40.00	40.00	16.83	14.92	No	Fishmonger
21	King scallop	NA	*Mizuhopecten yessoensis*	+	40.00	40.00	17.34	15.92	No	Fishmonger
22	King scallop	NA	*Mizuhopecten yessoensis*	+	40.00	40.00	16.74	15.22	No	Fishmonger
23	King scallop	NA	*Mizuhopecten yessoensis*	+	40.00	40.00	16.40	15.88	No	Fishmonger
41	Scallops	*Placopecten magellanicus*	*Placopecten magellanicus*	+	40.00	18.23	35.92	16.18	Yes	Supermarket
43	King scallop	NA	*Argopecten purpuratus*	–	40.00	37.29	35.17	14.68	No	Restaurant
44	Atlantic sea scallop	*Placopecten magellanicus*	*Placopecten magellanicus*	+	40.00	18.45	40.00	16.48	Yes	Supermarket
45	King scallop	*Pecten maximus*	*Pecten* spp.	+	17.28	40.00	40.00	15.63	Yes	Supermarket
46	Scallop (in delicate sauce)	NA	*Ruditapes phillipinarum*	+	40.00	40.00	40.00	16.12	No	Supermarket
47	King scallop (with roe)	*Chlamys opercularis*	*Aequipecten opercularis*	–	36.88	34.93	38.18	16.30	No	Supermarket
59	King scallop	*Pecten maximus*	*Placopecten magellanicus*	+	40.00	18.33	40.00	16.02	No	Supermarket
60	King scallop (à la Terrine “Breton")	*Amusium pleuronectes, Argopecten purpuratus, Chlamys albidus, Chlamys islandica, C. opercularis, P. maximus, P. magellanicus, Z. patagonica*	*Zygochlamys* spp.	–	33.52	38.28	38.27	16.43	No	Supermarket
61	King scallop	*Pecten maximus*	*Pecten* spp.	+	18.43	40.00	40.00	15.52	Yes	Supermarket
62	Scallops (without roe)	*Placopecten magellanicus*	*Placopecten magellanicus*	+	40.00	19.48	40.00	16.11	Yes	Supermarket
63	Atlantic sea scallop (raw, without roe, glazed, frozen)	*Placopecten magellanicus*	*Placopecten magellanicus*	+	40.00	19.10	38.85	15.86	Yes	Supermarket
64	Atlantic sea scallop	NA	*Placopecten magellanicus*	+	40.00	17.78	40.00	15.51	Yes	Supermarket
65	King scallop	*Pecten jacobaeus*	*Pecten* spp.	+	17.50	40.00	39.62	15.34	Yes	Fishmonger
66	King scallop	NA	*Mizuhopecten yessoensis*	+	40.00	40.00	17.44	15.04	No	Fishmonger
67	King scallop	*Placopecten* spp.	*Placopecten magellanicus*	+	40.00	18.96	40.00	16.29	No	Fishmonger
68	King scallop	NA	*Placopecten magellanicus*	+	40.00	21.28	40.00	15.39	No	Restaurant
69	King scallop	NA	*Mizuhopecten yessoensis*	+	40.00	40.00	16.57	15.37	No	Fishmonger
70	Scallop	*Placopecten magellanicus*	*Placopecten magellanicus*	+	40.00	17.37	40.00	15.80	Yes	Supermarket
71	King scallop	*Pecten maximus*	*Pecten* spp.	+	18.23	40.00	40.00	15.67	Yes	Supermarket

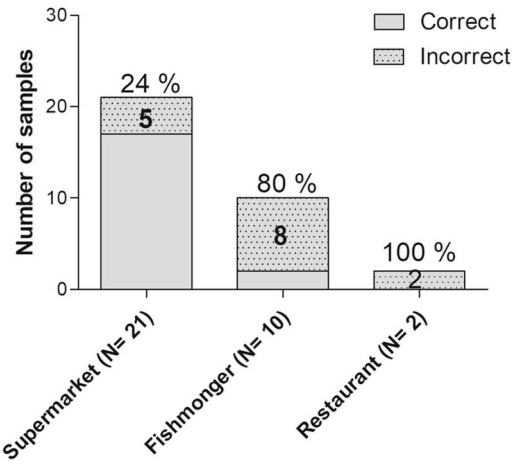

Fig. 2. Comparison mislabelling rate of samples from supermarkets and fish mongers. The number of incorrectly labelled samples are indicated in the grey bars. The mislabelling rate is shown above the bars. N = number of samples tested. Total N = 33.

probes (Mann-Whitney *U* test, Pmag; Myes: $p < 0.01$, Pec: $p > 0.05$). The mean Cq values using 0.2 ng DNA were 23.90 ± 0.24 for *Pecten* spp., 24.47 ± 0.37 for *P. magellanicus* and 23.88 ± 0.31 for *M. yessoensis* using the Sso Advanced Universal Probes Mastermix, while using the QuantiNova multiplex Mix, Cq values were 23.97 ± 0.28 for *Pecten* spp., 25.93 ± 0.45 for *P. magellanicus* and 25.97 ± 0.23 for *M. yessoensis* (Appendix A). Differences in Cq values by using distinct mastermixes may be explained by differential concentrations of PCR components in the mixes, especially for the use of a multiplex assay. However, these differences in Cq values were not substantial and did not influence the specificity of the real-time multiplex assay. Neither modifications in the temperature, nor in the primer or probe concentrations led to high variations in the Cq values using the same mastermix. Thus, the real-time multiplex PCR assay was found to be robust and both kits can be used.

The sensitivity in the presence of DNA from other target sequences for the multiplex PCR was validated by adding DNA of the remaining target sequences in excess (Broeders et al., 2014). The results show that the presence of DNA in excess of one target species leads to shifts in the Cq value of DNA from another target species with a lower concentration (Supplementary material). It was shown that Cq values could be shifted depending on which target DNA was used in excess and the degree of DNA in excess from one species. While mixtures of 1:1 and 1:2 did not lead to higher shifts in the Cq values, effects were observed in mixtures of 1:10. This was especially the case for the presence of DNA of *M. yessoensis* in excess. For example, the Cq values of 2 ng DNA of *Pecten* spp. in 20 ng of DNA from *M. yessoensis* (in a 20 μL reaction) had a mean of 30.10 ± 2.40, while in 20 ng of DNA from *P. magellanicus*, it was only 24.35 ± 2.67. Neither *Pecten* spp. nor *P. magellanicus* can be detected when *M. yessoensis* is in excess in a ratio of 0.2 ng in 20 ng DNA. In turn, 0.2 ng DNA of *M. yessoensis* is detectable in 20 ng of both, *Pecten* spp. and *P. magellanicus*. Thus, the presence of DNA from *M. yessoensis* seems to affect the detectability of the other target species. A possible explanation may be an influence due to an easier amplification of shorter amplicons since the amplicon length of Myes was shorter than the Pec and Pmag fragments. Therefore, it is recommended to preferentially test single scallop samples or only mixtures of up to three individuals.

3.5. Test and validation with commercial samples

In order to perform both, the validation of the designed multiplex assay and the assessment of the mislabelling rate of the scallop products, commercial samples were tested and compared with the results of Sanger sequencing. Results show an absolute agreement of the scallop real-time multiplex assay and the identification by sequencing and subsequent blast (Table 5).

Comparisons between the trade name and the scientific name were conducted to test whether they are in accordance with the European regulation EU No. 1379/2013 and the index of commercial designations for fishery products by the German Federal Office for Agriculture and Food. A label was considered as correct if the trade and scientific name on the label were in concordance and, in addition, agreed with the results of the real-time PCR/sequencing. In case the scientific name on the label was not provided, the trade name was compared to the real-time PCR/sequencing result. The results show that only 18 of 33 (52%) samples were correctly labelled (Table 5). In 12 (36%) samples, a scientific name was not provided. The mislabelling rate in supermarkets was relatively low (5 out of 21, 24%) in comparison to fishmongers (8 out of 10, 80%) and restaurants (2 out of 2, 100%, Fig. 2). Of the samples sold as "Jakobsmuschel" (*Pecten* spp.), 13 out of 18 samples (72%) were identified as a different species (Table 5). In many cases, scallop products sold as "Jakobsmuschel" were identified as *M. yessoensis*, especially in products purchased at fishmongers. Three samples from a restaurant, supermarket and fishmonger sold as "Jakobsmuschel" were identified as *P. magellanicus* instead. A frozen, packaged product from a supermarket was labelled as *P. magellanicus* was identified as *M. yessoensis*. One product sold as "Terrine Jakobsmuschel" already indicated to contain a variety of different species. Isolated scallop ingredients of this product were identified as *Zygochlamys* spp. by Sanger sequencing. A can labelled as "scallops" contained Manila clam *Ruditapes philippinarum*. Thus, mislabelling seems to be particularly common at fishmongers or in more processed products.

The results are in line with other studies on commercial samples from the German/Swiss market. Näumann et al., 2012 analysed 34 samples of commercial scallop products from Germany of which 15 were mislabelled, another 15 were correctly labelled, and 3 samples had either missing scientific names or the scientific name was not in concordance of the commercial name. Even higher mislabelling of scallop products were reported for samples collected from Switzerland (Stephan et al., 2014). In this study using a MALDI-TOF approach, 75% of scallop samples were labelled incorrectly as "Jakobsmuschel" (*Pecten* spp.), but were identified instead as *P. magellanicus*. In the present study, most of the incorrectly labelled products were *M. yessoensis*. This species is usually cheaper than *Pecten* spp. or *P. magellanicus*.

Summarizing the present and previous findings, the correct labelling of scallop products according to the legislative regulations still needs improvement. The high mislabelling rate shows the need of a method that allows a rapid identification of high sample quantities for the respective control.

4. Conclusion

The present study confirms the results of previous investigations identifying a relatively high mislabelling rate of scallop species which may have implications on the economy and consumer's trust. Therefore, methods to examine the labelling of these products are desirable that could facilitate regular inspections by food monitoring authorities. The newly designed multiplex TaqMan real-time PCR assay constitutes a rapid and cost-effective method with a high throughput of samples for the authentication of three commercially important scallop species and may contribute to a more effective monitoring.

CRediT authorship contribution statement

Regina Klapper: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Ute Schröder:** Conceptualization, Funding acquisition, Investigation, Methodology, Validation, Writing - review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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