Food Control 130 (2021) 108257

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

SYBR-Green real-time PCR assay with melting curve analysis for the rapid identification of *Mytilus* species in food samples

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ARTICLE INFO

Keywords:
Mytilus
Species identification
Real-time PCR
Melting curve analysis
Labelling

ABSTRACT

Mytilus spp. mussels are one of the most commonly cultivated species in the European Union, being *M. galloprovincialis*, *M. edulis* and *M. chilensis* the main species marketed here. Therefore, the identification of this seafood species is critical for labelling, traceability and food safety purposes. In the present study, 174 samples have been employed for the development of an alternative fast, simple, reliable and cost-effective methodology for the identification of *Mytilus* spp. mussels commercialized in Europe. This real-time PCR assay with a SYBR Green post-PCR melting curve analysis targeting the polyphenolic adhesive protein gene developed was successfully applied to fresh, frozen and canned mussels, showing 100% of specificity, sensitivity and precision. Besides, results have demonstrated the applicability of the assay in different platforms without special software requirements. Therefore, the methodology described in this study is useful for the authentication of mussel samples and could be easily applied as a fast routine food control laboratory test.

1. Introduction

Mussels are widely distributed, and they are one of the most cultivated bivalves worldwide. Globally, 94% of the total production is supplied by aquaculture, which has shown an uptrend from 2007 to 2016. The European Union is one of the major contributors of mussels, being Spain its major producer with more than 200,000 tons per year (FAO, 2019b). European mussel farming relies mainly on two species: the Mediterranean mussel (*Mytilus galloprovincialis*), mostly cultivated in Spain, and the Blue mussel (*M. edulis*), mainly produced in the Netherlands and France (FAO, 2019b). Hybridization between these two species has been described in areas where their geographical origin overlaps (Beaumont, Turner, Wood, & Skibinski, 2004; Hilbish, Carson, Plante, Weaver, & Gilg, 2002; Michalek, Ventura, & Sanders, 2016). Besides, due to high consumer demand, *M. chilensis* is imported to Spain and other EU countries in significant volumes from Chile. Considering that Spain is one of the major EU consumers of mussels, any of the three aforementioned *Mytilus* species could be present in the Spanish market, mainly fresh, but also, can be found in processed, frozen and canned form.

Authentication of seafood products becomes a difficult issue when the external morphological characteristics are removed. Mussels are commercialized mostly without shells, hindering appearance-based species identification. European Union legislation (EC) No. 1379/2013 of the common organization of the markets in fishery and aquaculture products regarding traceability is very strict. In order to avoid possible labelling-related frauds, the species identification of seafood is becoming a topic of growing concern to assure traceability and fulfill related legislation (EU, 2013).

Molecular methods based on the amplification of a specific DNA marker by PCR is the most commonly employed technique for *Mytilus* species identification (Brooks & Farmen, 2013; Fernández-Tajes et al., 2011; Meistertzheim, Héritier, & Lejart, 2017; Santaclara et al., 2006; Zhang et al., 2019). Of all molecular markers reported to date, the Polyphenolic Adhesive Protein (PAP) gene is, by far, the most extensively used one for this purpose. This marker has been analyzed by using conventional PCR with the subsequent analysis of the amplified fragments by length polymorphism (Brooks & Farmen, 2013; Inoue, Waite, Matsuoka, Odo, & Harayama, 1995) or restriction fragment length polymorphism (RFLP) (Fernández-Tajes et al., 2011; Santaclara et al., 2006). To speed-up the time of the analysis, real-time amplification methods are carried out using TaqMan™ probes (Dias et al., 2008) or analyzing the melting curves by high resolution melting (HRM) (Jilberto, Araneda, & Larrain, 2017). Although these techniques help to reduce time considerably, they show some disadvantages such as an increase in the price of the analysis in the first case and the requirement

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https://doi.org/10.1016/j.foodcont.2021.108257
Received 8 February 2021; Received in revised form 14 May 2021; Accepted 15 May 2021
Available online 5 June 2021

of specific instrument and software in the second case. Conversely, SYBR-Green real-time PCR assay with melting curve analysis has been successfully applied to the identification of several species (Barcenas, Unruh, & Neven, 2005; Berry & Sarre, 2007; Castigliego, Armani, Tinacci, Gianfaldoni, & Guidi, 2015; Hsu et al., 2003; Winder et al., 2011; Yu, Chen, Zhang, & Yin, 2005). This method is based on the use of intercalating dyes rather than fluorescent probes, and it can be used in the simplest real-time cyclers without special software requirements unlike HRM analysis, therefore contributing to cost saving.

In this work, a real-time PCR assay with a SYBR Green post-PCR melting curve analysis was successfully developed for the fast, reliable, and cost-effective identification of the most important *Mytilus* species commercialized in Europe*: M. galloprovincialis*, *M. edulis* and *M. chilensis*, both in fresh samples and in highly processed canned products.

2. Materials and methods

2.1. Sample collection and DNA extraction

A total of 174 reference samples of mussels belonging to *Mytilus* genus were collected: 89 *M. galloprovincialis*, 26 *M. edulis*, 24 *M. chilensis*, 7 *M. trossulus* and 28 *M. galloprovincialis* x *M. edulis* hybrids. These reference samples were identified at species level following the PCR-RFLP Me15-16 *AciI* method as it is detailed below (Santaclara et al., 2006). Non-*Mytilus* bivalve species were selected as negative control: *Callista chione*, *Cerastorema edule*, *Crassostrea gigas*, *Ensis macha*, *Ostrea edulis*, *Perna viridis*, *Ruditapes decussatus*, *Tagelus dombeii* and *Venerupis philippinarum* (4 individuals from each species). In addition, 16 different commercial processed mussel products (pickled, in olive oil, cooked in sauce or spiced) were purchased from local markets in the Basque Country. Two individuals from each commercial product were analyzed independently.

DNA extraction was performed using Wizard® Genomic DNA Purification Kit (Promega), starting from 50 mg of tissue and following manufacturer's instructions. Prior to DNA extraction, excess oil was removed with paper tissue. Extracted DNA was eluted in sterile Milli Q water and quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). DNA was stored at −20 °C until use.

2.2. Mytilus species identification by PCR-RFLP Me15-16 AciI method

Species identification of the 174 reference mussel samples was firstly performed using the PCR-RFLP Me15-16 *AciI* method as described by Santaclara et al. (2006) to ensure correct identification. Briefly, the PCR was carried out in a 50 μl reaction mixture containing 20 ng DNA, 0.2 mM dNTPs, 2 mM $MgCl_2$, 1 μM forward and reverse primers and 1 U of Taq DNA Polymerase (Illustra™, GE Healthcare). PCR conditions were the following: a preheating step of 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 56 °C for 30 s, 72 °C for 30 s, followed by an extension step of 72 °C for 5 min. Amplified products were checked in 1.5% (w/v) agarose gel. For the differentiation of *M. galloprovincialis* and *M. chilensis*, amplicons were purified prior to enzymatic digestion with GFX PCR DNA and Gel Band Purification Kit (Illustra™, GE Healthcare) to eliminate dimers and primer excess. Digestion was carried out using 10 units of *AciI* enzyme (Thermo Fisher Scientific) and 5 μl of amplified DNA product. The mixture was incubated overnight at 37 °C in a final volume of 20 μl. Digested samples were loaded onto a DNA 1000 chip (Agilent technologies) and run in Bioanalyzer instrument (Agilent Technologies) for visualization.

2.3. Mytilus species identification by SYBR-Green real-time PCR with melting curve analysis

Real-time PCR amplification was performed on three different in-house thermal cyclers to evaluate possible differences among them: LightCycler® 480 Instrument II (Roche Molecular Systems, Inc Diagnostics), Step One™ (Applied Biosystems) and CFX96 Touch Deep-Well™ (BioRad). Furthermore, two PCR master mixes were compared: Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent technologies) and LightCycler® 480 SYBR Green I Master (Roche Molecular Systems, Inc Diagnostics).

The 10 μL reaction mixture contained 5 μL of master mix A (Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix; Agilent technologies) or master mix B (LightCycler® 480 SYBR Green I Master; Roche Molecular Systems, Inc Diagnostics), 20 ng of template DNA and 450 nM of each primer previously described by Dias et al., (2006). The reaction conditions were 3 min at 95 °C, followed by 45 cycles of 5 s at 95 °C and 60 °C for 10 s. The melting curves of the PCR products were monitored on the same instrument, from 65 °C to 95 °C with 0.1 °C increases, by continually recording the fluorescence produced by non-saturating dye molecules intercalated to double-stranded DNA. A negative control with no template DNA and the respective control-species were added. Melting curves were presented as a derivative plot showing the rate of change in fluorescent signal in relation to temperature.

2.4. Data analysis

In the real-time PCR analysis, melting curves were presented as a derivative plot showing the rate of change in fluorescent signal in relation to temperature. Each peak of the plot represented a melting temperature (T_m) specific for each *Mytilus* species. The T_m range for each species was calculated as the average of T_m (y) plus-minus two times its standard deviation ($T_m = y \pm 2SD$). The efficiency/performance of the real-time PCR assay with a SYBR® Green post-PCR melting curve analysis was evaluated using the following metrics: sensitivity, specificity, robustness, and precision. Sensitivity was calculated as the number of individuals in which the method correctly identified the species, divided by the total number of individuals sampled from that species. Specificity was calculated as the number of individuals that the method correctly excluded from the species, divided by the total number of individuals not belonging to that species. The robustness of the assay was evaluated by means of two different master mixes and three different thermal cyclers. The precision of the methodology, related to reproducibility and repeatability, was evaluated as the degree to which repeated measurements under unchanged conditions show the same results. The repeatability was calculated as the number of true positive results obtained after analyzing the same positive sample 10 times in the same laboratory by the same operator using the same equipment. The reproducibility was calculated as the number of true positives after analyzing the same positive sample 10 times by two different operators (ISO/20813, 2019).

3. Results and discussion

3.1. Development of the SYBR-Green real-time PCR with melting curve analysis

Real-time PCR with melting curve analysis with the universal set of primers (Dias et al., 2008) targeting the polyphenolic adhesive protein gene sequence (Inoue et al., 1995) has been successfully applied for the identification of the main important *Mytilus* species commercialized in Europe. The nuclear polyphenolic adhesive protein gene has been extensively used for *Mytilus* species identification by different PCR-based methods, including the visualization of PCR products by electrophoresis (Inoue et al., 1995), RFLP (Santaclara et al., 2006), TaqMan™ probes (Dias et al., 2008) and HRM (Jilberto et al., 2017). This monolocus marker has been shown to be a reliable and fast routine diagnostic marker, and it is the most common DNA marker for identifying *Mytilus* species. Certainly, using a monolocus approach has its limitations compared to multilocus approach; since this marker is not able to detect introgression patterns nor to diferenciate *M. chilensis* from

the Southern Hemisphere *M. galloprovincialis* from New Zealand and Australia. Despite these limitations, and considering that our aim is to rapidly identify *Mytilus* species in food samples commercialized in Europe, the use of a single locus is relatively straightforward (Araneda et al., 2021; Larrain, Gonzalez, Perez, & Araneda, 2019).

The real-time PCR developed in this study showed specific peaks in melting curves with unique T_m values for each species, while the hybrid (*M. galloprovincialis* x *M. edulis*) showed a double peak which correlated with the T_m of both species analyzed (Fig. 1). Representative T_m values were obtained for each species after analyzing 89 *M. galloprovincialis* individuals (78.85 °C ± 0.080); 26 *M. edulis* individuals (80.49 °C ± 0.12) 24 *M. chilensis* (78.35 °C ± 0.11) and 7 *M. trossulus* (79.17 °C ± 0.076). Sensitivity indicated that 100% of individuals (n = 174) were correctly identified applying the melting curve analysis, since none of the samples were incorrectly identified (Fig. 2) according to the results previously obtained using the conventional PCR-RFLP Me15/16 *AciI* method (Santaclara et al., 2006). Specificity was also successfully evaluated, since 36 samples, belonging to non-*Mytilus* bivalve species, were not significantly amplified (data not shown).

Real-time PCR with melting curve analysis is a useful tool for the rapid detection of PCR products which has been applied for the identification of microorganisms (Winder et al., 2011), mammalians (Berry & Sarre, 2007), fish (Castigliego et al., 2015), insects (Barcenas et al., 2005; Yu et al., 2005) and fungi (Hsu et al., 2003), among others organisms. We have developed a simple, fast, and cost-effective *Mytilus* species identification system based on the melting temperature ranges by real-time PCR. Conventional two steps PCR-based methods include the analysis of PCR products through the direct visualization of Me15/16 fragments by electrophoresis (Inoue et al., 1995) or the digestion of fragments by RFLP (Santaclara et al., 2006). Conversely, one step real-time PCR applications save time and, when compared to TaqMan™ probes (Dias et al., 2008), the use of a non-saturating intercalating SYBR® Green dye in combination with melting curve analysis is indeed cheaper than the addition of *Mytilus* specific fluorescent probes to the amplification reaction.

DNA melting curve analysis shares the fundamental principles with HRM analysis. HRM analysis is a relatively new application that has been successfully applied to the identification of *Mytilus* species (Jilberto et al., 2017). This technique is very powerful and precise, since it enables the discrimination of SNPs by using saturating binding dyes that monitor smaller melting differences (EvaGreen®, SensiFast™ etc …). However, HRM analysis requires special software to capture high density fluorescence data per °C, while melting curve analysis can be carried out with any basic real-time cycler (Herrmann, Durtschi, Wittwer, & Voelkerding, 2007). We have demonstrated that HRM is not required for effective discrimination of the two main *Mytilus* species cultivated in Europe, since the combination of GC content, length and sequence produce unique melting curves for each species (Fig. 1). Besides, we were also able to differentiate *M. galloprovincialis* from *M. chilensis* with only one SNP (G/T) (Jilberto et al., 2017) resulting two different melting curves for these species. The shortness of the amplified fragment (126 bp) enables a single substitution of one purine with a pyrimidine to result in a substantial effect on melting temperatures. However, despite the differences observed between *M. galloprovincialis* and *M. trossulus* melting peaks, their Tm values are too close to ensure a correct discrimination in processed food samples.

Robustness of the assay was tested with two different master mix A and B. Master mix A was more adequate than B for the resolving of the main *Mytilus* species, using the LightCycler® 480 platform, with a higher T_m variation range (as the difference between the higher and lower Tm obtained), 2.15 in the case of master mix A and 1.67 in the case of master mix B. (Table 1). Once master mix A was selected, different real-time cyclers platforms were evaluated using this master mix. Results show that the assay can be applied to different platforms with reliable results for the differentiation of the most commercially important mussel species; *M galloprovincialis*, *M. edulis and M. chilensis* (Table 2). Only one deviation was detected with the Step One™ thermal cycler (Agilent), in which *M. trossulus* was not entirely satisfactorily resolved. This constraint could be reduced by using a formulation recommended for each platform. As differences in Tm between some species can be low, positive controls using reference material of each of the target species should be included in each run to ensure a correct species identification. Consequently, the real-time assay developed can be easily implemented in different platforms with minimal variations such as the use of the more sensitive master mix formulation in the amplification, as previously was described in similar tests (Graziano, Gullì, & Marmiroli, 2017).

Finally, the precision of the real-time PCR assay was also evaluated. The repeatability was obtained after analyzing the same reference sample of *M. galloprovincialis* 10 times, in different runs. All samples were correctly assigned to this species. The reproducibility was eventually evaluated after analyzing the same sample 10 times in different runs by two different operators. Similarly, all samples were correctly identified as *M. galloprovincialis* with a 100% rate. Previous studies reported similar precision values of the analysis of DNA melting curves for *Mytilus* species identification (Jilberto et al., 2017).

3.2. Analysis of commercial canned mussel products

The main objective of this study was the final optimization of a

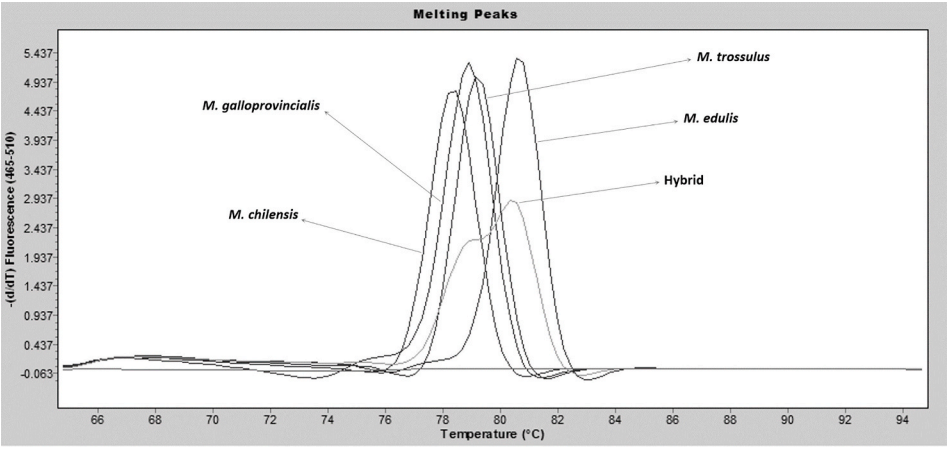

Fig. 1. Melting curves presented as a derivative plot showing the rate of change in fluorescent signal with respect to temperature. Each peak of the plot represents a melting temperature (Tm) specific for each *Mytilus* species. These assays were performed using master mix A and a LightCycler® 480 Instrument II (Roche Diagnostics). Hybrid refers to *M. galloprovincialis x M. edulis*.

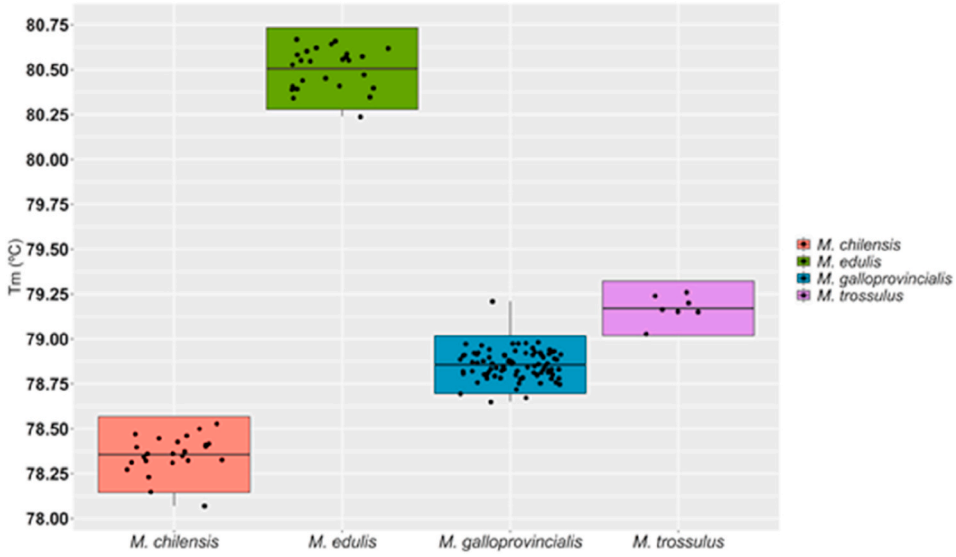

Fig. 2. Representative melting temperature (Tm) values obtained for each species (n = 146). *M.galloprovincialis* (n = 89): 78.85 °C ± 0.080 |*M. edulis* (n = 26): 80.49 °C ± 0.12 |*M. chilensis* (n = 24): 78.35 °C ± 0.13 |*M. trossolus* (n = 7): 79.24 °C ± 0.086. Tm range was calculated as the average of Tm plus-minus two timesits standard deviation (Tm ± 2*SD). These assays were performed using master mix A and a LightCycler® 480 Instrument II (Roche Diagnostics).

Table 1
Robustness of qPCR performed on purified *Mytilus* species DNA in the presence of two different master mixes.

Species	Master mix A		Master mix B	
	Mean	SD	Mean	SD
M. galloprovincialis	78.85	0.08	78.09	0.01
M. edulis	80.50	0.11	79.31	0.15
M. trossulus	79.17	0.07	78.01	0.03
M. chilensis	78.35	0.10	77.64	0.01

All reactions were performed using LightCycler® 480 Instrument II (Roche Diagnostics). Mean T_m are reported as mean of four replicates. SD: standard deviation. Variation ratio is the difference between the higher and lower Tm obtained.

Table 2
Robustness of qPCR performed on purified *Mytilus* species DNA in different thermal cyclers.

Species	LightCycler® 480		CFX96 Touch DeepWell™		Step One™	
	Mean	SD	Mean	SD	Mean	SD
M. galloprovincialis	78.86	0.08	77.86	0.07	78.20*	0.355
M. edulis	80.50	0.11	79.44	0.08	79.20	0.354
M. trossulus	79.24	0.07	78.18	0.02	78.20*	0.355
M. chilensis	78.36	0.10	77.38	0.04	76.94	0.005

All reactions were performed using master mix A. Mean T_m are reported as mean of four replicates. SD: standard deviation. * indicates unresolved species.

robust and precise identification assay for the correct identification of the main *Mytilus* species commercialized in Europe. This includes canned mussel products, which usually contain ingredients and additives acting as a source of inhibitors that can negatively affect the methodological effectivity. Moreover, the sterilization process in canning process produces a strong fragmentation of DNA into pieces of about 200 bp or even less (Chapela et al., 2007; Lopez & Pardo, 2005; Pardo & Pérez-Villareal, 2004). Therefore, it becomes mandatory to include this sort of samples to validate the performance of the real-time assay. The obtained PCR products have been demonstrated to be small enough for successful amplification of canned mussels (Dias et al., 2008; Jilberto et al., 2017; Santaclara et al., 2006). Only a few previous works have included canned commercial samples purchased in local supermarkets and stores in Spain (Fernández-Tajes et al., 2011; Santaclara et al., 2006). We have acquired 16 samples in local markets covering the main presentations commercialized in Spain, from different brands and sauces, including brine and oil among others (Table 3). All samples were successfully amplified by real-time PCR, and subsequently identified by DNA melting curves analysis. In all cases, the T_m values obtained were assigned to one species, *M. galloprovincialis*. These results confirm the applicability of our assay to identify the *Mytilus* species in canned mussel samples commercialized in different presentations and brands.

Table 3
qPCR test applied to 16 commercial canned mussel food samples.

Sample ID	Type of canned mussel	Mean	SD	*Mytilus* species assignation
C01	in brine	78.79	0.080	*M. galloprovincialis*
C02	in brine and olive oil (brand 1)	78.66	0.050	*M. galloprovincialis*
C03	in brine and olive oil (brand 2)	78.83	0.030	*M. galloprovincialis*
C04	in brine and olive oil (brand 3)	78.65	0.078	*M. galloprovincialis*
C05	in brine and olive oil (brand 4)	78.69	0.080	*M. galloprovincialis*
C06	with algae in brine and olive oil	78.68	0.055	*M. galloprovincialis*
C07	fried in brine and olive oil (brand 1)	78.66	0.081	*M. galloprovincialis*
C08	fried in brine and olive oil (brand 2)	78.71	0.050	*M. galloprovincialis*
C09	fried in brine and olive oil (brand 3)	78.70	0.026	*M. galloprovincialis*
C10	fried in "cabrales" cheese sauce	78.74	0.070	*M. galloprovincialis*
C11	fried in "albariño" wine sauce	78.74	0.010	*M. galloprovincialis*
C12	with garlic	78.76	0.061	*M. galloprovincialis*
C13	fried with olive oil	78.67	0.090	*M. galloprovincialis*
C14	with peeper	78.71	0.049	*M. galloprovincialis*
C15	in brine with "gallega" paprika sauce	78.68	0.071	*M. galloprovincialis*
C16	in salted water	78.68	0.066	*M. galloprovincialis*

All reactions were perform3ed using LightCycler® 480 Instrument II (Roche Diagnostics) using master mix A. Mean T_m are reported as mean of four replicates. SD: standard deviation.

European Union labelling law states that seafood products must be labelled with the complete scientific name of the species (i.e. genus and species, Latin binomial nomenclature)(EU, 2013). Attending to this regulation, all samples were correctly labelled, and identified as *M. galloprovincialis*, and therefore no deviation was detected in the Spanish market. However, Spain continues to be the main client for frozen mussel exports from Chile and China, which are directed as raw material towards the Spanish canning industry (FAO, 2019a). In this sense, we consider extremely important to develop authentication methodologies allowing geographical origin identification of commercialized mussels in any type of format to protect the local industry and the final consumer.

4. Conclusion

The real-time PCR with melting curve analysis targeting the polyphenolic adhesive protein gene here described has been successfully applied for the identification of the most relevant *Mytilus* species on the European market, *M. galloprovincialis*, *M. edulis* and *M. chilensis* with high specificity and sensitivity. Besides, the assay has proven to be able to successfully identify fresh, frozen, and canned mussel species in a laboratory with standard qPCR equipment without special software requirements. Therefore, the methodology described in this study is useful for the traceability of mussel samples and could be easily applied as a fast routine food control laboratory test.

In order to ensure transparency in seafood labelling and traceability (to fulfil European labelling regulation) and to increase consumers' confidence, there is a need to explore the potentials of DNA-based techniques not only for species identification, but also for tracing the geographical origin of mussels.

CRediT authorship contribution statement

Ane del Rio-Lavín: Methodology, Data curation, Formal analysis, Writing – original draft. **Elisa Jiménez:** Conceptualization, Writing – review & editing. **Miguel Ángel Pardo:** Conceptualization, Formal analysis, Supervision, Writing – original draft, Writing – review & editing.

Acknowledgments

This study was carried out thanks to the financial support of the SEA-TRACES (EAPA_87/2016) INTERREG Atlantic Area EU funded project. Ane del Rio-Lavín is the recipient of a PhD grant from the Education Department of the Basque Government. Samples were kindly provided by IIM-CSIC (Spain), Consello Regulador Mexillón de Galicia (Spain), Fepromodel (Spain) and Norwegian Institute for Water Research (NIVA). This manuscript is contribution number 1034 from the Food Research Division of AZTI.

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