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High resolution melting analysis for identification of commercially-important *Mytilus* species

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ABSTRACT

Mytilus are edible mussels, including commercially-significant species such as *M. chilensis*, *M. galloprovincialis* and *M. edulis*. The scientific name of the species must be indicated on commercial products to satisfy labelling and traceability requirements. Species identification using morphological criteria is difficult due the plasticity of these characteristics and the absence of shells in processed products, and conventional PCR-based methods are laborious and time-intensive. As alternative, we propose high resolution melting (HRM) analysis as a simple tool to detect and identify SNP (single nucleotide polymorphisms) and length polymorphisms in *Mytilus* spp. We designed HRM-specific primers for the *Mytilus* genus to identify *M. chilensis*, *M. galloprovincialis, M. edulis* and their hybrids through clearly-distinguishable melting curves. HRM analysis showed high sensitivity (0.9639), specificity (1.0000) and precision (1.0000) compared to a conventional PCR-RFLP test. HRM is a fast and low cost method, being a reliable tool for species identification within the *Mytilus* genus.

1. Introduction

Marine mussels are one of the most cultivated and marketed bivalves. The *Mytilus* genus includes major commercial species such as *M. chilensis*, *M. edulis* and *M. galloprovincialis.* At 2014, these three species represent the 80% of the *Mytilus* genus mussels world catches (FAO., 2016) and they are widely distributed throughout the oceans and found in cold and temperate waters of both hemispheres (Gerard, Bierne, Borsa, Chenuil, & Feral, 2008). When *Mytilus* species coexist, they may interbreed and create hybrid zones (Hilbish, Carson, Plante, Weaver, & Gilg, 2002; Oyarzún, Toro, Cañete, & Gardner, 2016; Westfall & Gardner, 2013).

Mussels may be marketed with the shell (refrigerated) or without (frozen or canned). Morphological characteristics (shell color and size) are not highly informative for species identification within the *Mytilus* genus due their plasticity (Krapivka et al., 2007), being impossible to apply these criteria in products without shells (Rego et al., 2002). The situation is even more problematic for *Mytilus* species from hybrid zones. Accurate species identification is an important goal as it is the first of the three levels of traceability for seafood products (Ogden, 2008). Species identification is also a required component of labelling for fishery products exported to the European Union (EU, 2013). Therefore, rapid, low-cost techniques are needed.

Molecular methods can be used to address traceability requirements, using techniques such as PCR, quantitative real-time PCR, microarrays or HRM analysis targeting specific genes, molecular markers or length polymorphisms (Madesis, Ganopoulos, Sakaridis, Argiriou, & Tsaftaris, 2014; Pasqualone et al., 2016; Teletchea, Maudet, & Hanni, 2005). PCR-based molecular methods, such as AFLP (amplified fragment length polymorphism), RAPD (random amplified polymorphism) and microsatellite-based techniques, have been designed to target various genes for identification of specific species or even for distinguish among different groups within the same species (Groenenberg et al., 2011; Pasqualone, Lotti, & Blanco, 1999; Rego et al., 2002). The most common methods for species identification targets the polyphenolic adhesive protein gene. Inoue, Waite, Matsuoka, Odo, and Harayama (1995) identify *M. edulis*, *M. galloprovincialis, M. trossulus* and their hybrids using a length polymorphism revealed by PCR amplification with specific primers (Me15-16). Santaclara et al. (2006) applied the same primer pairs, adding a RFLP (restriction fragment length polymorphism) step involving digestion of the amplicons with *Aci*I endonuclease. This method (RFLP Me15-16 *Aci*I) clearly differentiated *M. chilensis* from the three above-

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mentioned species and their hybrids. Another RFLP-based method recognized *M. chilensis*, *M. californianus, M. coruscus, M. edulis, M. galloprovincialis* and *M. trossulus* with the primers Myti-F/R (Fernandez-Tajes et al., 2011). However, RFLP techniques are time-consuming due to the enzymatic reaction required as well as the two-step visualization of the results with gel electrophoresis.

High resolution melting (HRM) analysis allows for detection of single nucleotide polymorphisms (SNP) and length polymorphisms in small amplicons using primers specifically designed for this technique. HRM is an interesting high-throughput approach for food analysis (Druml & Cichna-Markl, 2014) since it involves real-time PCR with saturated DNA-binding dyes, high resolution instruments to detect variations in fluorescence and software capable of analyzing melting curves (Herrmann, Durtschi, Voelkerding, & Wittwer, 2006). SNP genotyping or length polymorphism detection is then performed by analyzing the various melting curves associated with each SNP allele and/or length polymorphism (Seipp, Herrmann, & Wittwer, 2010). HRM analysis does not require a gel electrophoresis step, making it faster, more efficient and less expensive than methods that rely on gel electrophoresis, such as RFLP, AFLP or PCR (Wittwer, 2009; Yang et al., 2014). HRM analysis has been used to detect food adulteration and to identify bacteria, plant and animal species (Iacumin et al., 2015; Jin, Li, Kong, Yu, & Zhong, 2014; Pasqualone et al., 2015; Vietina, Agrimonti, & Marmiroli, 2013).

This manuscript presents an alternative method for identification of three important commercial species within the *Mytilus* genus (*M. chilensis*, *M. edulis* and *M. galloprovincialis*) and their hybrids, based on HRM analysis of amplicons obtained from the polyphenolic adhesive protein gene.

2. Materials and methods

2.1. Mussel sample collection

A total of 471 individuals from the *Mytilus* genus (Supplementary Table 1) were analyzed: 323 from southern Chile, 60 from Canada, 21 from Mexico, 20 from New Zealand, which were collected along the coast. Samples from Spain (47 individuals) were bought alive in a costal local market. Non-*Mytilus* mussels species served as controls: *Aulacomya atra*, *Choromytilus chorus*, *Perumytilus purpuratus* and *Semimytilus algosus* (8 individuals from each species).

2.2. DNA extraction

DNA extraction was performed on 50–100 mg of tissue using the phenol-chloroform method, adapted for mussels (Larrain, Diaz, Lamas, Vargas, & Araneda, 2012). The DNA concentration of all individuals was estimated with a NanoDrop spectrophotometer (NanoDrop ND-2000, Thermo Scientific, Wilmington, DE, USA), and integrity was assessed using gel electrophoresis. All samples were diluted to a concentration of 20 ng/μL.

2.3. Species identification

Species identification was performed for all individuals using the RFLP Me15-16 *Aci*I method, identifying 281 individuals as *M. chilensis*, 96 as *M. galloprovincialis*, 52 as *M. edulis*, 10 as *M. trossulus*, 12 as *M. chilensis* × *M. edulis* hybrids, 19 as *M. chilensis* × *M. galloprovincialis* hybrids and 1 as a hybrid of *M. galloprovincialis* × *M. edulis*.

2.4. Primer design

Polyphenolic Adhesive Protein of Mussels (PAPM) Primers (PAPM-F: 5′-GGAACAAAGCATGGACCA-3′ and PAPM-R: 5′-GACAG CTTCTTTGCAAGTGG-3′) were designed targeting the sequence of the polyphenolic adhesive gene with the program Primer3 (Koressaar & Remm, 2007; Untergasser et al., 2012), taking into account the need for a small PCR product for the HRM method. We used the consensus sequence obtained by aligning the GenBank sequences for the nonrepetitive region of the polyphenolic adhesive protein gene of *M. chilensis* (GenBank ID DQ640609.1) and *M. galloprovincialis* (GenBank ID D63778.1). The specificity of the PAPM primers was confirmed using Primer-Blast (Ye et al., 2012). The absence of artifacts, primer-dimers and nonspecific PCR products was verified by agarose gel electrophoresis. To confirm the amplification of the interest zone in the polyphenolic adhesive protein gene, PAPM PCR products were sequenced in samples of *M. chilensis* (n = 4), *M. edulis* (n = 4), *M. galloprovincialis* (n = 4) and the hybrid *M. chilensis* × *M. galloprovincialis* (n = 2).

2.5. High resolution melting (HRM) analysis

HRM was performed in an Eco Real-Time PCR System 4.0 (Illumina®), which allowed the additional melting analysis step. The following conditions were used: 96 °C for 2 min followed by 35 cycles at 96 °C for 15 s and 59 °C for 1 min. The final melt curve analysis was performed from 55 °C to 95 °C with 0.2 °C increases. The reaction contained 0.1 μM of forward and reverse primers, 20 ng of genomic DNA and Fast EvaGreen® qPCR Master Mix (Biotium®) or SensiFast™ HRM Kit (Bioline®) in a final volume of 8 μL. Amplification curves, dissociation curves and post-HRM analysis of melt curves were performed using the ECO Studio software v5.0489 (Illumina®). To obtain the respective control-species curves, we used samples of each species (in triplicate) previously verified using RFLP Me15-16 *Aci*I. In all HRM analyses performed to identify species we used these control individuals. Also, a negative control without DNA was included in all runs.

2.6. Data analysis

To evaluate the performance of the HRM method, the results were compared with those of the RFLP Me15-16 *Aci*I method separately for each species. The following metrics were calculated:

2.6.1. Sensitivity (S)

Number of individuals in which the HRM method correctly identified the species, divided by the total number of individuals sampled from that species. Sensitivity reflects the test ability to correctly identify individuals of a given species (Altman & Bland, 1994).

2.6.2. Specificity (E)

Number of individuals that the HRM method correctly excluded from the species, divided by the total number of individuals who do not belong to that species. Specificity reflects the test ability to exclude individuals who do not belong to a given species (Loong, 2003).

2.6.3. Precision (P)

Also called the positive predictive value, reflects the proportion of individuals identified as belonging to a given species that truly belong to the species. Calculated as the number of individuals correctly identified with both methods, divided the total number of individuals identified by the RFLP Me15-16 *Aci*I method (Lever, Krzywinski, & Altman, 2016).