Elsevier Editorial System(tm) for Food Control

Manuscript Draft

Manuscript Number:

Title: Species identification in mussels (Mytilus spp.): a case study on products sold on the Italian market underlines issues and suggests strategies to face a not still solved problem

Article Type: Short Communication

Keywords: Mytilus chilensis, Mytilus galloprovincialis, Choromytilus chorus, seafood labelling, traceability, species identification, DNA-based methods

Corresponding Author: Dr. Andrea Armani,

Corresponding Author's Institution: University of Pisa

First Author: Alice Giusti

Order of Authors: Alice Giusti; Federica Tosi; Lara Tinacci; Lisa Guardone; Giuseppe Arcangeli; Andrea Armani

Abstract: Based on a case study concerning mussel products suspected to be mislabelled, this work wants to highlight the difficulties encountered in their molecular identification and propose new strategies for solving these issues. In November 2019, the FishLab (Department of Veterinary Sciences) was consulted by a wholesaler for identifying products labelled as "Chilean mussels" (Mytilus chilensis). The batch had been molecularly identified first as M. chilensis by an external private lab and, subsequently, as Choromytilus chorus following a second analysis entrusted to another external lab by the customer company. In this work, the samples could only be identified as Mytilus spp by sequencing the mtDNA COI gene. The amplification of the Polyphenolic Adhesive Protein (PAP) gene, a nuclear marker reported as more informative for mussel allowed to suppose the presence of M. chilensis and M. galloprovincialis based on the length of the obtained fragment. In fact, both the species, which are reported as inhabiting Chilean waters, present the same 123 bp amplicon. The low sequences quality obtained for this short fragment, however, did not allow a discrimination of the aforesaid species as this is based on a single mutation point. Results highlighted that the mtDNA COI gene does not allow the identification possibly due the presence in the genetic databases of erroneous sequences from misidentified specimens. In addition, the mtDNA in inheritance Mytilus spp. is unusual, and male and female mtDNA molecules are present in different tissue of male exemplars. The PAP discrimination power is reduced by the high similarity of the informative fragment between some species. In this case, improving the sequencing efficiency, such as applying protocols with oligonucleotide tails and high-fidelity Taq polymerase should be considered. In conclusion, issues in the approach one species-one name, currently adopted by the Italian legislator for mussel species were also underlined.

Suggested Reviewers: Domenico Meloni
Department of Veterinary Medicine, University of Sassari

dmeloni@uniss.it
He wrote the paper entitled: A case-study on compliance to the EU new requirements for the labelling of fisheries and aquaculture products reveals difficulties in implementing Regulation (EU) n.1379/2013 in some large-scale retail stores in Sardinia (Italy)

Pier Luigi Acutis
Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle d'Aosta
PierLuigi.Acutis@izsto.it
He is an expert in this field

Nelson Colihueque
Departamento de Ciencias Biológicas y Biodiversidad, Universidad de Los Lagos, Osorno, Chile
ncolih@ulagos.cl
He works on the same topic and produced the following article: Authentication of frozen Chilean blue mussel (Mytilus chilensis) commercialized in the town of Osorno, southern Chile, using PCR-RFLP analysis

Research Data Related to this Submission

There are no linked research data sets for this submission. The following reason is given:
Data will be made available on request

Species identification in mussels (*Mytilus* spp.): a case study on products sold on the Italian market underlines issues and suggests strategies to face a not still solved problem

Giusti A.[a], Tosi F.[b], Tinacci L.[a], Guardone L.[a], Arcangeli G.[b], Armani A.[a*]

[a]*FishLab, Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, 56124, Pisa (Italy)*

[b]*National Reference Center for Fish, Molluscs and Crustacean Diseases, Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Università 10, 35020 Legnaro (PD)*

***corresponding author**:

Postal address: FishLab, Department of Veterinary Sciences, University of Pisa, Viale delle Piagge 2, 56124, Pisa (Italy)

Tel: +390502210207; Fax: +390502210213

Email: andrea.armani@unipi.it

Abstract

Based on a case study concerning mussel products suspected to be mislabelled, this work wants to highlight the difficulties encountered in their molecular identification and propose new strategies for solving these issues. In November 2019, the FishLab (Department of Veterinary Sciences) was consulted by a wholesaler for identifying products labelled as "Chilean mussels" (*Mytilus chilensis*). The batch had been molecularly identified first as *M. chilensis* by an external private lab and, subsequently, as *Choromytilus chorus* following a second analysis entrusted to another external lab by the customer company. In this work, the samples could only be identified as *Mytilus* spp by sequencing the mtDNA *COI* gene. The amplification of the Polyphenolic Adhesive Protein (PAP) gene, a nuclear marker reported as more informative for mussel allowed to suppose the presence of *M. chilensis* and *M. galloprovincialis* based on the length of the obtained fragment. In fact, both the species, which are reported as inhabiting Chilean waters, present the same 123 bp amplicon. The low sequences quality obtained for this short fragment, however, did not allow a discrimination of the aforesaid species as this is based on a single mutation point. Results highlighted that the mtDNA *COI* gene does not allow the identification possibly due the presence in the genetic databases of erroneous sequences from misidentified specimens. In addition, the mtDNA in inheritance *Mytilus* spp. is unusual, and male and female mtDNA molecules are present in different tissue of male exemplars. The PAP discrimination power is reduced by the high similarity of the informative fragment between some species. In this case, improving the sequencing efficiency, such as applying protocols with oligonucleotide tails and high-fidelity Taq polymerase should be considered. In conclusion, issues in the approach one species-one name, currently adopted by the Italian legislator for mussel species were also underlined.

Keywords:

Mytilus chilensis, Mytilus galloprovincialis, Choromytilus chorus, seafood labelling, traceability, species identification, DNA-based methods

1. Introduction

Seafood traceability, sustainability and consumers' right to an informed purchase represent three of the main inspiring principles of the European Common Fisheries Policy (CFP) (Tinacci, Giusti, Guardone, Luisi, & Armani, 2019). The Common Organization of the Markets in Fishery and Aquaculture Products, an integral part of the CFP, is based on the Regulation (EU) No 1379/2013. The Article 35, in particular, fixes the mandatory information to be declared on seafood at retail or at the mass caterer, which include the commercial designation of the species and the associated scientific name, as reported on official lists of seafood trade names drawn up and updated by each Member State (Regulation EU No 1379/2013). In the same Regulation DNA-based methods are proposed as valid tools to support the traceability of seafood products in order to deter operators from falsely labelling practices. Intentional and involuntary mislabelling and species substitution are in fact reported as by far the most frequent fraud incidents in seafood products at international level, mainly favoured by the complexity of the seafood supply chain, involving many food-business operators and an extremely wide range of species which are often not sold as whole but prepared and processed (Tinacci et al., 2018; Donlan & Luque, 2019). In addition, difficulties in the correct implementation of Regulation (EU) No 1379/2013 at Italian level have been reported (D'Amico, Armani, Gianfaldoni, & Guidi, 2016; Esposito & Meloni, 2017).

To date, numerous diagnostic techniques relying on DNA-based methods have been developed for the identification of seafood species in a variety of product types. Even though the DNA barcoding of a ~655 bp region of the mitochondrial cytochrome c-oxidase I (*COI*) gene (Hebert, Cywinska, Ball, & deWaard, 2003) is among the most applied method for seafood species identification, other mitochondrial molecular markers as well as methods not based on sequencing are used. A recent survey within several EU accredited laboratories, highlighted in fact a significant diversity of approaches and a substantial need of standardization of molecular analysis (Griffiths et al., 2014). In addition, while mitochondrial markers have been proved as efficient for the identification of almost all the fish taxa, their utilization for recognizing other seafood is debated. In

this scenario it is of utmost importance that the analytical process is based on a preliminary systematic decision-making approach, aimed at evaluating the most appropriate lab pathways in the light of the features of the samples to be analysed (Tinacci et al., 2018).

Difficulties in unambiguously differentiating mussel species of the genus *Mytilus* (Mytilidae family, Bivalvia order), were already highlighted in the 1990s (Toro, Ojeda, Vergara, Castro, & Alcapan, 2005). *Mytilus* spp. exhibits a typical anti-tropical distribution with five species occurring in the Northern Hemisphere (*M. trossulus*, *M. edulis*, *M. galloprovincialis*, *M. californianus* and *M. coruscus*) and three in the Southern Hemisphere (*M. chilensis*, *M. galloprovincialis* and *M. platensis*) (Gaitán-Espitia, Quintero-Galvis, Mesas, & D'Elía, 2016). Species within this taxon are morphologically similar and difficult to distinguish, replacement of native species by an invasive taxon often occurs and individuals from different *Mytilus* spp. can hybridize in areas where their populations coexist (Zbawicka, Trucco, & Wenne, 2018).

Mussel consumption is traditional in Italy, which, together with Spain and France, contributed to 78% of the EU total consumption in 2016 (EUMOFA, 2018). More than 90% of the national production takes place in Emilia-Romagna, Veneto, Apulia, Friuli-Venezia-Giulia, Sardinia and Liguria and it is mainly addressed to the Mediterranean mussel (*M. galloprovincialis*). This species, which are mainly sold as fresh to the Italian territory, are however not enough to meet the national consumption demand, imposing imports from other countries. Italy imports of mussels, mainly from Spain and Chile, reached 73.066 tonnes in 2017. In fact, mussels cover about 3/4 of the Spanish aquaculture and Spain is by far the main EU producer and exporter of *M. galloprovincialis* (EUMOFA, 2018). At the international level, Chile has recently become the world's second largest producer and exporter of farmed mussels after China (Avendaño, Cantillánez, & González, 2017; FAO, 2018). Its production is mainly based on the native blue mussel (*M. chilensis*) (Larraín, Zbawicka, Araneda, Gardner, & Wenne, 2018), although other species are also farmed, such as *M. edulis*, *Aulacomya ater* and *Choromytilus chorus* (Avendaño et al., 2017).

104 All the above-mentioned species can therefore be found in products of Chilean origin and cases
105 of species substitution have been reported. Colihueque, Espinoza, & Parraguez (2020) recently
106 highlighted a 50% mislabelling rate in products labelled as *M. chilensis* in which the cholga mussel
107 (*A. ater*) was instead found. Harris, Rosado, & Xavier (2016) detected one clear mislabelling case
108 in a product sold on the Portuguese market as *M. chilensis* and identified as *C. chorus*. However,
109 mislabelling data could be underestimated probably due to the issues in molecular identification of
110 this taxon. In general, for all the invertebrate categories, there are insufficient data to produce useful
111 estimates on mislabelling rate (Donlan & Luque, 2019). In this respect, proper approaches should
112 be implemented in the analysis of this kind of seafood.
113 In November 2019, the FishLab (Department of Veterinary Sciences) was consulted by a local
114 wholesaler for identifying the mussel species in batches/a batch of pre-cooked products labelled as
115 "Chilean mussels" (*M. chilensis*). The batch had been molecularly identified first as *M. chilensis* by
116 an external private lab and, subsequently, as *C. chorus* following a second analysis entrusted to
117 another external lab during the self-monitoring procedure of the customer company. According to
118 the Italian official list of seafood (Ministerial Decree n. 19105 of September the 22nd, 2017) that
119 only includes *M. galloprovincialis, M. edulis, M. chilensis, C. chorus* does not possess an official
120 trade name.
121 Starting from the aforesaid case study, this work wants to highlight the difficulties encountered
122 in species identification in mussels' products. In addition, the limitations still present in the DNA-
123 based methods and molecular marker until now proposed were also discussed. Finally, issues in the
124 approach one species-one name, currently adopted by the Italian legislator for species closely
125 related by a phylogenetic point and in which the hybridization process could occur, such as mussels,
126 were also underlined.

127 **2. Materials and Methods**

128 ***2.1 Samples acceptance and documents analysis***

One plastic bag made of some shelled mussels randomly sampled from the batch of pre-cooked products under examination (RI-19) (Fig. 1a) was received by the FishLab, together with five identical pre-packaged products (PC-1; PC-2; PC-3; PC-4; PC-5) (1 kg each) made of pre-cooked in-shell mussels equally labelled as Chilean mussels – *M. chilensis* (Fig. 1b). All the samples were photographed and registered with an internal code. The reports of the analysis previously conducted on the same batch of samples in private laboratories were also received and analysed. It was found that two different molecular markers (both mitochondrial) had been used by the two laboratories involved: the species *M. chilensis* had been detected by using the *16S ribosomal RNA (16S rRNA)* gene and the species *C. chorus* by using the *COI* gene.

2.2. Molecular identification of the samples

Ten specimens from the batch RI-19 were randomly selected and codified with a progressive number (from RI-19.1 to RI-19.10). For each of the five pre-packaged products 5 specimens were randomly selected and codified with a progressive number (e.g. from PC-1.1 to PC-1.5). Total DNA was extracted with the lab standard method (Armani, Catigliego, Tinacci, Gianfaldoni, & Guidi, 2011). The standard *COI* gene barcode fragment was amplified using the primer pair LCO1490 and HCO2198 (Folmer, Black, Hoeh, Lutz, & Vrijenhoek, 1994). In addition, also the polyphenolic adhesive protein gene (PAP) was amplified with the primer pair Me15m-F and Me16m-R (Satto, Gastaldelli, Tosi, Zentilin, Turolla & Arcangeli, 2017). All the samples from which an amplicon of the expected length was obtained were purified with the kit EUROSAP® (Euroclone SPA, Milano) and sent to an external lab for standard Sanger sequencing. The obtained sequences were edited with the software Geneious R7 (Kearse et al., 2012) and analysed using the Basic Local Alignment Search Tool (BLAST) on GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For the *COI* sequences, also the Identification System (IDs) on BOLD (Species Level Barcode Records) (http://www.boldsystems.org/index.php/IDS_OpenIdEngine) was used. A top match with a sequence similarity of at least 98% was used to designate potential species identification when using the *COI* gene. For the PAP gene, an identity value of 100% was instead required, since the

155 estimates of inter-species divergence between the sequences available on the online databases were
156 very low (0.029±0.04) (data not shown).

157 **3. Results and discussion**

158 ***3.1 Samples molecular identification***

159 *3.1.1 COI gene.* All the DNA samples from the batch RI-19, except for RI-19.2, and four out of
160 the five DNA samples extracted from each PC product were successfully amplified. Even though,
161 according to the literature, the mitochondrial genes are not suitable for the identification of the
162 species belonging to *Mytilus* spp. (Larraín et al., 2018), the *COI* gene was selected in order to
163 compare the results obtained from our analysis with those previously achieved by the second
164 external laboratory. Contrariwise, the *16SrRNA* (targeted by the first laboratory) was not considered
165 given its even lower inter-species variability degree already observed for these and other species
166 (authors' note). For the *COI* gene, twenty-four PCR products were successfully sequenced. Identity
167 values higher than 98% were obtained with sequences deposited as *M. chilensis*, *M. edulis* and *M.*
168 *galloprovincialis* for almost all the samples, except for 4, where the identification was not achieved
169 even at genus level (identity values lower than 98%) (Table 1). No significant similarity with *C.*
170 *chorus* was found proving that samples from the batch RI-19 were not attributable to this species.
171 However, an identification to species level was not achievable for any sample. This occurrence
172 supported the outcomes by Harris et al. (2016), in which *COI* was equally proved as insufficient to
173 distinguish among various European mussel species, and especially *M. edulis* and *M.*
174 *galloprovincialis* because of mitochondrial introgression occurring between them. In the same
175 study, *C. chorus* was instead identified (Harris et al., 2016), recognizing the *COI* efficiency in
176 mussel inter-genera discrimination, as also reported by Khaksar et al. (2015), in which *M. trossulus*
177 and *C. meridionalis* were successfully discriminated by this marker. The *COI* limit in
178 discriminating among *Mytilus* species may be attributed to several factors. Firstly, it should be
179 noted that the taxonomic uncertainty could be due to the eventual presence of wrongly deposited
180 sequences that affects the reliability of the identification process. In the work of Abbadi et al.,

(2015), for example, one fresh specimen morphologically characterized as *M. galloprovincialis*, was then molecularly attributed to *M. chilensis*.

Therefore, a preventive screening of the sequences available on public database, that is very time-consuming and was therefore not performed in this study du to time restrains, is therefore recommended (Giusti et al., 2019). In addition, mitochondrial DNA (mtDNA) in *Mytilus* spp. is unusual in that it displays doubly uniparental inheritance (DUI), contrary to common uniparental mtDNA inheritance in animals. Distinctly different male and female mtDNA molecules are inherited with females having exclusively female mtDNA and males having both types with the male mtDNA concentrated in the gonadal tissue (Breton, Beaupre, Stewart, Hoeh, & Blier, 2007). The complexity of mtDNA inheritance means that mtDNA markers have not generally been developed for the purposes of species identification (Śmietanka, Zbawicka, Wołowicz, & Wenne, 2004). *Mytilus* spp. hybrid zones exist in many places in the world and can extend over hundreds of kilometres (Braby & Somero, 2006). Especially the three species of the Northern hemisphere (*M. edulis*, *M. galloprovincialis* and *M. trossulus*) show varying levels of hybridisation wherever they occur sympatrically, and their distribution patterns and hybridisation have been intensively investigated (Michalek, Ventura, & Sanders, 2016). However, cases of hybridizations have been observed worldwide (Gaitán-Espitia et al., 2016) including in Chile, where hybrids of *M. chilensis* x *M. trossulus* and *M. chilensis* x *M. galloprovincialis* were detected (Toro et al., 2005; Larraín, Díaz, Lamas, Vargas, & Araneda, 2012).

3.1.2 PAP gene. Since nuclear DNA of hybrids and their backcrosses is carried from both ancestral species, the use of nuclear marker has been encouraged by the scientific community for detecting hybridization and introgression events (Michalczuk et al., 2014). Inoue, Waite, Matsuoka, Odo, & Harayama (1995) identified the nuclear PAP gene as an alternative efficient marker to discriminate among the species *M. edulis, M. galloprovincialis* and *M. trossulus* that are included in the "*Mytilus edulis* species group", also known as "*Mytilus edulis* complex" (Hilbish et al., 2000). Inoue et al. (1995) observed that the length of the fragment amplified from the PAP non-repetitive

region was specific to each species, allowing to visually identify the species without recourse to sequencing. Hybrids can also be detected by visualizing a double amplification band, each corresponding to the species involved in the hybridization phenomenon. More recently by testing other species belonging to the genus *Mytilus* (Santaclara et al., 2006; Fernández-Tajes et al., 2011; Satto et al. 2017) it has been shown that not all the *Mytilus* species can be discriminated on the basis of the PAP marker length. In fact, the fragments obtained from the species *M. coruscus* has the same length of that obtained for *M. californianus* (~200 bp) and the same happens for *M. galloprovincialis/M. chilensis* (~120 bp). While for *M. coruscus/M. californianus,* that do not share the same distribution area (Sealifebase.org), a co-presence in a same commercial product is improbable and the species identity can be therefore with some certainty deducted from the product origin, the situation concerning *M. chilensis* and *M. galloprovincialis* is more complex, given both the habitat sharing and the hybridization possibility. Despite the above-mentioned limits, the region proposed by Inoue et al. (1995) was additionally analysed in this study due its simplicity and low cost of execution. In fact, it only required the purchase of a couple of primers and the set-up of a standard PCR program of amplification. For all the samples, the amplicon length matched with that of *M. chilensis/M. galloprovincialis* (123 bp), with no observed double amplification band. The samples were therefore sequenced in order to identify which of the two species was involved; however, this approach was unsuccessful. In fact, based on the Phred quality score (Ewing et al. 1998) the sequences were not considered reliable. Even though repeated many times, the sequences quality was always too low to allow the proper characterization of the species.

3.2 Complexity of *Mytilus* spp. identification: limitations in analytical methods and final considerations on mussels labelling

3.2.1. Limitations in the analytical methods for Mytilus spp. identification. The presented case report highlights that, although the *mt*DNA has been almost set aside since unsuitable for hybrids identification, also the use of a nuclear DNA target is not exempt from troubles, due to its high similarity among species phylogenetically closer. As regards the species *M. chilensis* and *M.*

233 *galloprovincialis,* the ~120 bp PAP marker factually only differs by a single mutation point
234 (Santaclara et al., 2006; Fernández-Tajes et al., 2011; Westfall & Gardner, 2013). Given both this
235 scarce inter-species variability and the shortness of the target fragment, a method based on the
236 sequencing approach must achieve high-quality sequences. In this respect, the use of proper
237 measures for improving the Sanger sequencing efficiency, such as protocols with oligonucleotide
238 tails (Armani et al., 2016) and high-fidelity Taq polymerase can be considered. In fact, this
239 approach represents the gold standard for producing DNA barcodes (Abbati et al., 2017) and even
240 though next generation sequencing technologies, such as pyrosequencing, are available, these
241 cannot easily implement in all labs. Alternatively, a Polymerase Chain Reaction-Restriction
242 Fragment Length Polymorphism (PCR-RFLP) method that allowed the discrimination among the
243 above-mentioned species is available (Santaclara et al., 2006; Fernández-Tajes et al., 2011). The *M.*
244 *galloprovincialis* amplicon contains in fact a single restriction site (that correspond to the single
245 mutation point described above) resulting in fragments of 69 and 57 bp after digestion with the
246 specific restriction enzyme AciI, whereas *M. chilensis* has a point mutation that prevent the cut
247 (Santaclara et al,. 2006; Fernández-Tajes et al., 2011). To date, this analytical approach seems to be
248 the most suitable for this purpose. However, the application of this method should be supported by
249 the production of a sufficient number of reference sequences from vouchered identified specimens
250 to confirm the efficiency of the method (authors' note). Therefore, in the present study, the PCR-
251 RFLP approach was not considered because of time and costs constrains.
252 *3.2.2. Uncertainties in the taxonomical status of the genus Mytilus and issues in the attribution*
253 *of specie-specific commercial designation.* Currently, the one-species one name approach is
254 internationally advocated as the goal system for ensuring a fair and transparent trade (Lowell,
255 Mustain, Ortenzi, & Warner, 2015; Tinacci et al., 2018). At European level, this approach has been
256 implemented by the Regulation EU No 1379/2013 stating that each EU Member State is delegated
257 to the drafting and updating of official lists reporting the trade names accepted throughout the
258 country for the product commercial designation and the scientific denominations referring to the

scientific names reported in the FishBase information system and in the ASFIS database and, exclusively for crustaceans, molluscs, echinoderms and tunicates, Sealifebase and Worms databases. Factually, different trade names are currently attributed to each *Mytilus sp.* whose status is accepted; *M. galloprovincialis* is reported as Mediterranean mussel, *M. edulis* as blue mussel, *M. trossulus* as foolish mussel, *M. coruscus* as Far eastern mussel, *M. chilensis* as Chilean mussel, etc. (sealifebase.org) and the Member States, in most of cases, simply translated these trade designations in the country language. In fact, the utilisation of qualifying adjectives referring to the geographical origin, as in the Italian Official list, can improve the recognition of the product by the consumer (Tinacci et al., 2019). Although in the last decades there has been a significant increase in the taxonomic understanding of *Mytilus* spp., mostly prompted by the analysis of molecular evidence, the taxon distribution is still not completely resolved (Gaitán-Espitia et al., 2016). While this taxon has been studied extensively in the Northern Hemisphere, disagreements remain regarding the number and identity of the species that live in the Southern Hemisphere, especially in South America (Larrain et al. 2017). Some authors have in fact suggested that mussels in the Pacific coast of South America could correspond to a Southern Hemisphere lineage of *M. galloprovincialis* (Hilbish et al., 2000; Gérard, Bierne, Borsa, Chenuil, & Féral, 2008; Westfall & Gardner, 2010; Borsa, Rolland, & Daguin-Thiébaut, 2012; Westfall & Gardner, 2013; Oyarzún, Toro, Cañete, & Gardner, 2016). In particular, it has been assumed that *M. chilensis* is a Southern hemisphere divergent lineage of *M. galloprovincialis* from the Northern hemisphere which was accidentally introduced or deliberately transported for aquaculture practices (Hilbish et al., 2000; Gérard et al., 2008; Westfall & Gardner, 2010; Borsa et al., 2012; Westfall & Gardner, 2013; Oyarzún et al., 2016). Despite of this, the term *M. chilensis* has long been employed on food product labels (Oyarzún et al., 2016; Larraín et al., 2018) and the name is also used in aquaculture production statistics (FAO, 2018).

4 Conclusion

284 The proposed study confirmed the need of a continuous implementation of molecular methods
285 with a problem solving approach in order to overcome unavoidable limits of the standard analytical
286 procedures, such as the DNA barcoding technique currently used and validated to support official
287 and self-check activities to support an efficient traceability system of fishery products. The accurate
288 interpretation of the analytical results and the adequate choice of the methodological approach
289 assume a pivotal value for the issue of an adequate and objective technical opinion. The reliability
290 of the method is even more important if the results are to be used as acceptable evidence in a court
291 of law (Beltramo et al., 2017). In addition, the availability of a reliable analytical techniques able to
292 discriminate the different mussel species is also useful in the aquaculture sector. In fact, it would
293 avoid the introduction of exotic species in farms where these are absent (Council Regulation (EC)
294 No. 708/2007).
295 Outcomes of this study further support the need to re-considered the nomenclature of this taxon
296 also considering the possible increasing presence of hybrids specimens on both intra and extra
297 Community market. Harmonizing taxonomy in the context of aquaculture production, traceability,
298 labelling and trade of *Mytilus* products is more complex respect to other seafood products (Larraín
299 et al. 2017). Therefore, the approach one species one name should be less stringent for *Mytilus spp.*
300 taking for granted that the product origin is declared as imposed by the European Regulation No.
301 1379/2013.

302 **Figure captions**

303 **Figure 1**. Samples analysed in this study; A: samples from the batch of pre-cooked products
304 under examination; B: two of the samples within the five pre-packaged products made of pre-
305 cooked in-shell mussels.

306 **References**

307 1 Abbadi, M., Marciano, S., Tosi, F., De Battisti, C., Panzarin, V., Arcangeli, G., & Cattoli, G. (2017). Species
308 identification of bivalve molluscs by pyrosequencing. Journal of the Science of Food and Agriculture, *97*(2),
309 512-519. DOI 10.1002/jsfa.7754
310 2 Armani, A., Castigliego, L., Tinacci, L., Gianfaldoni, D., & Guidi, A. (2011). Molecular characterization of
311 icefish, (Salangidae family), using direct sequencing of mitochondrial cytochrome b gene. Food Control,
312 22(6), 888-895. https://doi.org/10.1016/j.foodcont.2010.11.020

313 3 Armani, A., Giusti, A., Guardone, L., Castigliego, L., Gianfaldoni, D., & Guidi, A. (2016). Universal primers
314 used for species identification of foodstuff of animal origin: Effects of oligonucleotide tails on PCR
315 amplification and sequencing performance. Food Analytical Methods, 9(5), 1199-1209.
316 https://doi.org/10.1007/s12161-015-0301-9
317 4 Avendaño, M., Cantillánez, M., & González, J. (2017). Evaluation of culture of the mussels Choromytilus
318 chorus and Aulacomya ater (Molina) in northern coasts of Chile. Aquaculture Research, 48(7), 3556-3567.
319 https://doi.org/10.1111/are.13181
320 5 Beltramo, C., Riina, M. V., Colussi, S., Campia, V., Maniaci, M. G., Biolatti, C., Trisorio S., Modesto P.,
321 Peletto S. & Acutis, P. L. (2017). Validation of a DNA biochip for species identification in food forensic
322 science. Food Control, 78, 366-373. http://dx.doi.org/10.1016/j.foodcont.2017.03.006
323 1 Borsa, P., Rolland, V., & Daguin-Thiébaut, C. (2012). Genetics and taxonomy of Chilean smooth-shelled
324 mussels, *Mytilus* spp. (Bivalvia: Mytilidae). Comptes rendus biologies, 335(1), 51-61.
325 https://doi.org/10.1016/j.crvi.2011.10.002
326 2 Braby, C. E., & Somero, G. N. (2006). Ecological gradients and relative abundance of native (Mytilus
327 trossulus) and invasive (Mytilus galloprovincialis) blue mussels in the California hybrid zone. Marine Biology,
328 148(6), 1249-1262. https://doi.org/10.1007/s00227-005-0177-0
329 3 Breton, S., Beaupre, H. D., Stewart, D. T., Hoeh, W. R., & Blier, P. U. (2007). The unusual system of doubly
330 uniparental inheritance of mtDNA: isn't one enough? Trends in Genetics, 23(9), 465-474.
331 https://doi.org/10.1016/j.tig.2007.05.011
332 4 Colihueque, N., Espinoza, R., & Parraguez, M. (2020). Authentication of frozen Chilean blue mussel (*Mytilus*
333 *chilensis*) commercialized in the town of Osorno, southern Chile, using PCR-RFLP analysis. Recent patents on
334 food, nutrition & agriculture. https://doi.org/10.2174/2212798410666181231154406
335 5 Council Regulation (EC) No 708/2007 of 11 June 2007 concerning use of alien and locally absent species in
336 aquaculture. OJ L 168, 1–17.
337 6 D'Amico, P., Armani, A., Gianfaldoni, D., & Guidi, A. (2016). New provisions for the labelling of fishery and
338 aquaculture products: Difficulties in the implementation of Regulation (EU) n. 1379/2013. Marine Policy, 71,
339 147-156.
340 7 Donlan, C. J., & Luque, G. M. (2019). Exploring the causes of seafood fraud: A meta-analysis on mislabeling
341 and price. Marine Policy, 100, 258-264.
342 8 Esposito, G., & Meloni, D. (2017). A case-study on compliance to the EU new requirements for the labelling
343 of fisheries and aquaculture products reveals difficulties in implementing Regulation (EU) n. 1379/2013 in
344 some large-scale retail stores in Sardinia (Italy). Regional Studies in Marine Science, 9, 56–61.
345 https://doi.org/10.1016/j.rsma.2016.11.007
346 9 EUMOFA (2018) THE EU FISH MARKET. 2018 Edition.
347 https://www.eumofa.eu/documents/20178/132648/EN_The+EU+fish+market+2018.pdf
348 10 Ewing, B., & Green, P. (1998). Base-calling of automated sequencer traces using phred. II. Error
349 probabilities. *Genome research*, *8*(3), 186-194. doi:10.1101/gr.8.3.186
350 11 FAO (2018) The State of World Fisheries and Aquaculture 2018.
351 http://www.fao.org/documents/card/en/c/I9540EN/
352 12 Fernández-Tajes, J., Longa, A., García-Gil, J., Chiu, Y. W., Huang, Y. S., Méndez, J., & Lee, R. S. (2011).
353 Alternative PCR–RFLP methods for mussel Mytilus species identification. European Food Research and
354 Technology, 233(5), 791. https://doi.org/10.1007/s00217-011-1574-x
355 13 Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of
356 mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology
357 and Biotechnology, 3, 294–299
358 14 Gaitán-Espitia, J. D., Quintero-Galvis, J. F., Mesas, A., & D'Elía, G. (2016). Mitogenomics of southern
359 hemisphere blue mussels (Bivalvia: Pteriomorphia): Insights into the evolutionary characteristics of the
360 Mytilus edulis complex. Scientific Reports, 6(1), 1-10. https://doi.org/10.1038/srep26853
361 15 Gérard, K., Bierne, N., Borsa, P., Chenuil, A., & Féral, J. P. (2008). Pleistocene separation of mitochondrial
362 lineages of Mytilus spp. mussels from Northern and Southern Hemispheres and strong genetic differentiation
363 among southern populations. Molecular Phylogenetics and Evolution, 49(1), 84-91.
364 https://doi.org/10.1016/j.ympev.2008.07.006
365 16 Giusti, A., Guarducci, M., Stern, N., Davidovich, N., Golani, D., & Armani, A. (2019). The importance of
366 distinguishing pufferfish species (Lagocephalus spp.) in the Mediterranean Sea for ensuring public health:
367 Evaluation of the genetic databases reliability in supporting species identification. Fisheries Research, 210, 14-
368 21. https://doi.org/10.1016/j.fishres.2018.10.003

17 Griffiths, A. M., Sotelo, C. G., Mendes, R., Pérez-Martín, R. I., Schröder, U., Shorten, M., Silva, H. A., Verrez-Bagnis, V., & Mariani, S. (2014). Current methods for seafood authenticity testing in Europe: Is there a need for harmonisation? Food Control, 45, 95-100. https://doi.org/10.1016/j.foodcont.2014.04.020
18 Harris, D. J., Rosado, D., & Xavier, R. (2016). DNA barcoding reveals extensive mislabeling in seafood sold in Portuguese supermarkets. Journal of aquatic food product technology, 25(8), 1375-1380. https://doi.org/10.1080/10498850.2015.1067267
19 Hebert, P. D., Cywinska, A., Ball, S. L., & Dewaard, J. R. (2003). Biological identifications through DNA barcodes. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270(1512), 313-321. https://doi.org/10.1098/rspb.2002.2218
20 Hilbish, T. J., Mullinax, A., Dolven, S. I., Meyer, A., Koehn, R. K., & Rawson, P. D. (2000). Origin of the antitropical distribution pattern in marine mussels (*Mytilus* spp.): routes and timing of transequatorial migration. Marine Biology, 136(1), 69-77. https://doi.org/10.1007/s002270050010
21 Inoue, K., Waite, J. H., Matsuoka, M., Odo, S., & Harayama, S. (1995). Interspecific variations in adhesive protein sequences of *Mytilus edulis, M. galloprovincialis*, and *M. trossulus*. The Biological Bulletin, 189(3), 370-375.
22 Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., ... & Thierer, T. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics, 28(12), 1647-1649. https://doi.org/10.1093/bioinformatics/bts199
23 Khaksar, R., Carlson, T., Schaffner, D. W., Ghorashi, M., Best, D., Jandhyala, S., ... & Amini, S. (2015). Unmasking seafood mislabeling in US markets: DNA barcoding as a unique technology for food authentication and quality control. Food Control, 56, 71-76. https://doi.org/10.1016/j.foodcont.2015.03.007
24 Larraín, M. A., Díaz, N. F., Lamas, C., Vargas, C., & Araneda, C. (2012). Genetic composition of Mytilus species in mussel populations from southern Chile. Latin American Journal of Aquatic Research, 40(4), 1077-1084.
25 Larraín, M. A., Zbawicka, M., Araneda, C., Gardner, J. P., & Wenne, R. (2018). Native and invasive taxa on the Pacific coast of South America: Impacts on aquaculture, traceability and biodiversity of blue mussels (*Mytilus* spp.). Evolutionary Applications, 11(3), 298-311. https://doi.org/10.1111/eva.12553
26 Lowell, B., Mustain, P., Ortenzi, K., & Warner, K. (2015). One name, one fish: why seafood names matter. Oceana, 1-12. Available at: https://usa.oceana.org/sites/default/files/onenameonefishreport.pdf Last access 18-03-20
27 Michalczuk, J., McDevitt, A. D., Mazgajski, T. D., Figarski, T., Ilieva, M., Bujoczek, M., ... & Kajtoch, Ł. (2014). Tests of multiple molecular markers for the identification of Great Spotted and Syrian Woodpeckers and their hybrids. Journal of Ornithology, 155(3), 591-600. https://doi.org/10.1007/s10336-014-1040-1
28 Michalek, K., Ventura, A., & Sanders, T. (2016). Mytilus hybridisation and impact on aquaculture: a minireview. Marine genomics, 27, 3-7. https://doi.org/10.1016/j.margen.2016.04.008
29 Ministerial Decree n. 19105. (September 22nd 2017). Italian Official Journal, Series G, 6–33, Year 158°, no. 266 of 14-11-2017
30 Oyarzún, P. A., Toro, J. E., Cañete, J. I., & Gardner, J. P. (2016). Bioinvasion threatens the genetic integrity of native diversity and a natural hybrid zone: smooth-shelled blue mussels (*Mytilus* spp.) in the Strait of Magellan. Biological Journal of the Linnean Society, 117(3), 574-585. https://doi.org/10.1111/bij.12687
31 Regulation (EU) No 1379/2013 of the European Parliament and of the Council of 11 December 2013 on the common organisation of the markets in fishery and aquaculture products, amending Council Regulations (EC) No 1184/2006 and (EC) No 1224/2009 and repealing Council Regulation (EC) No 104/2000. OJ L 354, 1–21.
32 Santaclara, F. J., Espiñeira, M., Cabado, A. G., Aldasoro, A., Gonzalez-Lavín, N., & Vieites, J. M. (2006). Development of a method for the genetic identification of mussel species belonging to Mytilus, Perna, Aulacomya, and other genera. Journal of Agricultural and Food Chemistry, 54(22), 8461-8470. https://doi.org/10.1021/jf061400u
33 Satto, S., Gastaldelli, M., Tosi, F.; Zentilin, A., Turolla, E., & Arcangeli, G. (2017). Analisi biomolecolare per l'identificazione di *Mytilus galloprovincialis*, *Mytilus edulis* e *Mytilus chilensis*. Conference Proceedings VI Convegno Nazionale Società Italiana di Ricerca Applicata alla Molluschicoltura - La sostenibilità ambientale, economica e sociale nella molluschicoltura italiana, 51-52.
34 Śmietanka, B., Zbawicka, M., Wołowicz, M., & Wenne, R. (2004). Mitochondrial DNA lineages in the European populations of mussels (*Mytilus* spp.). Marine Biology, 146(1), 79-92. https://doi.org/10.1007/s00227-004-1418-3
35 Tinacci, L., Guidi, A., Toto, A., Guardone, L., Giusti, A., D'Amico, P., & Armani, A. (2018). DNA barcoding for the verification of supplier's compliance in the seafood chain: How the lab can support companies in ensuring traceability. Italian Journal of Food Safety, 7(2). https://dx.doi.org/10.4081/ijfs.2018.6894

426 36 Tinacci, L., Giusti, A., Guardone, L., Luisi, E., & Armani, A. (2019). The new Italian official list of seafood
427 trade names (annex I of ministerial decree n. 19105 of September the 22nd, 2017): Strengths and weaknesses
428 in the framework of the current complex seafood scenario. Food Control, 96, 68-75.
429 https://doi.org/10.1016/j.foodcont.2018.09.002
430 37 Toro, J. E., Ojeda, J. A., Vergara, A. M., Castro, G. C., & Alcapan, A. C. (2005). Molecular characterization of
431 the Chilean blue mussel (*Mytilus chilensis* Hupe 1854) demonstrates evidence for the occurrence of *Mytilus*
432 *galloprovincialis* in southern Chile. Journal of Shellfish Research, 24(4), 1117-1121.
433 https://doi.org/10.2983/0730-8000(2005)24[1117:MCOTCB]2.0.CO;2
434 38 Westfall, K. M., & Gardner, J. P. (2010). Genetic diversity of Southern hemisphere blue mussels (Bivalvia:
435 Mytilidae) and the identification of non-indigenous taxa. Biological Journal of the Linnean Society, 101(4),
436 898-909. https://doi.org/10.1111/j.1095-8312.2010.01549.x
437 39 Westfall, K. M., & Gardner, J. P. (2013). Interlineage *Mytilus galloprovincialis* Lmk. 1819 hybridization
438 yields inconsistent genetic outcomes in the Southern hemisphere. Biological Invasions, 15(7), 1493-1506.
439 https://doi.org/10.1007/s10530-012-0385-8
440 40 Zbawicka, M., Trucco, M. I., & Wenne, R. (2018). Single nucleotide polymorphisms in native South American
441 Atlantic coast populations of smooth shelled mussels: hybridization with invasive European *Mytilus*
442 *galloprovincialis*. Genetics Selection Evolution, 50(1), 5. https://doi.org/10.1186/s12711-018-0376-z

Highlights

A case-study involving mussel products sold as “Chilean mussels” is reported.

Two DNA targets, mitochondrial (mt) and nuclear, were investigated

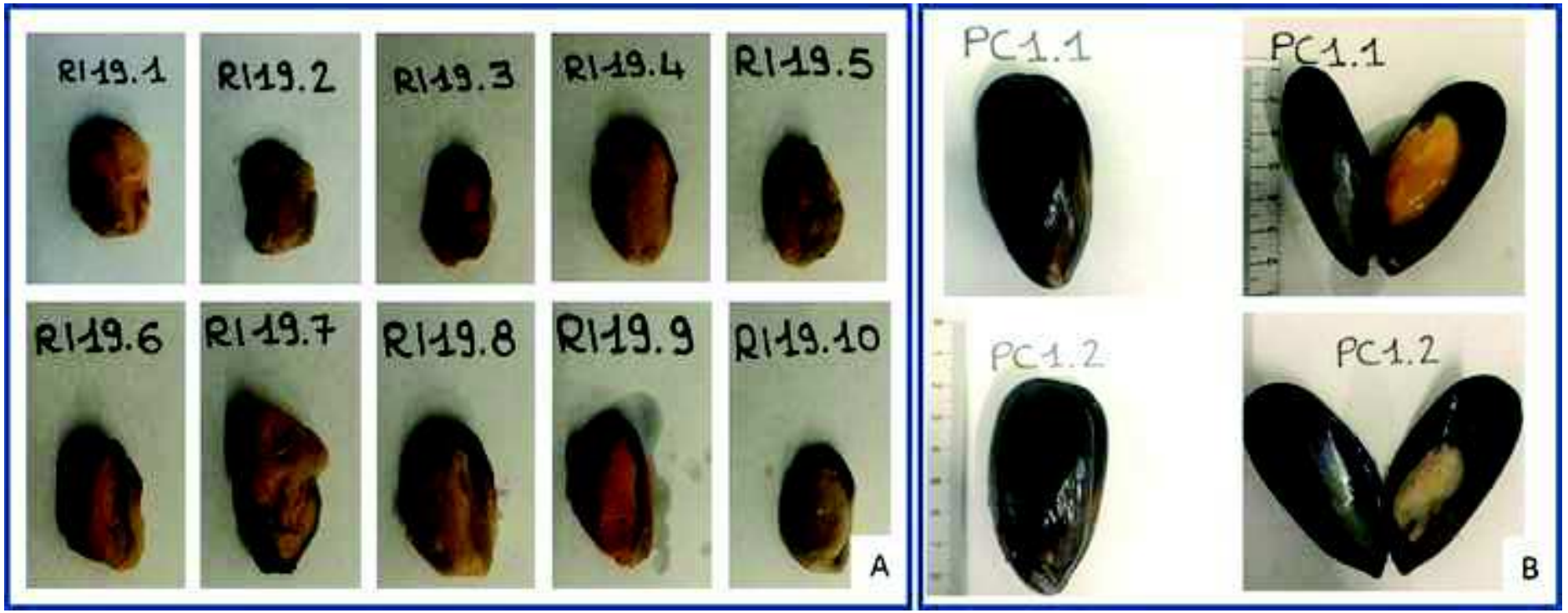
Mytilus spp. identification at species level is hindered by biological and technical issues

The approach one species-one name for *Mytilus* spp. labelling needs to be re-assessed

***Conflict of Interest Form**

Declarations of interest: none

Figure


R119.1
R119.2
R119.3
R119.4
R119.5
R119.6
R119.7
R119.8
R119.9
R119.10
A
PC1.1
PC1.1
PC1.2
PC1.2
B

Table

Sample code	Identity values	Molecular identification
RI 19.1	(1) *Mytilus chilensis* 99.55-98.67%; *Mytius edulis* 99.24-99.09%; *Mytilus galloprovincialis* 99.22-98.48%; *Mytilus trossulus* 98.95% (2) *Mytilus edulis* 99.87-92.71% *Mytilus chilensis* 92.63%	*Mytilus* sp.
RI 19.3	(1) *Mytilus edulis* 100-98.78%; *Mytilus chilensis* 99.69-98.78%; *Mytilus galloprovincialis* 99.36-97.85%; *Mytilus trossulus* 99.07% (2) *Mytilus edulis* 99.17-99%	*Mytilus* sp.
RI 19.4	(1) *Mytilus chilensis* 99.69-98.65%; *Mytilus edulis* 98.79-98.22%; *Mytilus galloprovincialis* 98.75-98.02% Mytilus trossulus 98.50% (2) *Mytilus edulis* 99.87-92.71% *Mytilus chilensis* 92.63%	*Mytilus* sp.
RI 19.5	(1) *Mytilus edulis* 100-98.78%; *Mytilus chilensis* 99.69-98.78%; *Mytilus galloprovincialis* 99.36-97.85%; *Mytilus trossulus* 99.07% (2) *Mytilus edulis* 92.83-92.67% *Mytilus chilensis* 93.11-92.95%	*Mytilus* sp.
RI 19.6	(1) *Mytilus chilensis* 98.43-97.96%; *Mytilus edulis* 98.12-97.96%; *Mytilus galloprovincialis* 98.27-98.11% (2) *Mytilus chilensis* 96.19-96.10%	*Mytilus* sp.
RI 19.7	(1) *Mytilus galloprovincialis* 100-97.47%; *Mytilus edulis* 100-97.89%; *Mytilus trossulus* 99.53-97.33%; *Mytilus chilensis* 99.53% (2) *Mytilus galloprovinialis* 99.76-99.17%	*Mytilus* sp.
RI 19.8	(1) *Mytilus chilensis* 99.54-98.92%; *Mytilus edulis* 99.22-98.92%; *Mytilus galloprovincialis* 99.38-99.22%; *Mytilus trossulus* 98.92% (2) *M. edulis* 98.92-98.81%	*Mytilus* sp.
RI 19.9	(1) *Mytilus chilensis* 99.85-98.81%; *Mytilus galloprovincialis* 99.38-98.63 %; *Mytilus edulis* 99.55-98.81%; *Mytilus trossulus* 99.10% (2) *Mytilus edulis* 98.67-98.32% *Mytilus galloprovincialis* 98.35%	*Mytilus* sp.

RI 19.10	(1) *Mytilus chilensis* 98.73-97.94%; *Mytilus galloprovincialis* 98.21-97.46 %; *Mytilus edulis* 99.18-97.94%; *Mytilus trossulus* 98.57% (2) *Mytilus edulis* 99.07% *Mytilus chilensis* 99.05%	*Mytilus* sp.
PC 1.1	(1) *Mytilus chilensis* 99.70-98.80% *Mytilus galloprovincialis* 99.38-97.46 % *Mytilus edulis* 99.39-98.80% *Mytilus trossulus* 99.13% (2) *Mytilus edulis* 99.07% *Mytilus chilensis* 99.05%	*Mytilus* sp.
PC 1.2	(1) *Mytilus chilensis* 99.69-98.65% *Mytilus galloprovincialis* 99.38-98.63%; *Mytilus edulis* 99.24-98.65%; *Mytilus trossulus* 98.95% (2) *Mytilus edulis* 99.06%	*Mytilus* sp.
PC 1.3	(1) *Mytilus chilensis* 99.53-98.37%; *Mytilus galloprovincialis* 99.23-98.47%; *Mytilus trossulus* 98.95% *Mytilus edulis* 98.94-98.37% (2) *Mytilus chilensis* 92.63-92.47%; *Mytilus edulis* 92.56-92.52%	*Mytilus* sp.
PC 1.5	(1) *Mytilus galloprovincialis* 100-96.97%; *Mytilus edulis* 99.53-96.97%; *Mytilus chilensis* 98.59% *Mytilus trossulus* 98.18-97.11% (2) *Mytilus galloprovincialis* 99.24-98.78%; *Mytilus edulis* 98-79%	*Mytilus* sp.
PC 2.1	(1) *Mytilus* sp. <93.32% (2) *Mytilus sp.* <89.30%	-
PC 2.2	(1) *Mytilus* sp. <93.30% (2) *Mytilus sp.* <89.35%	-
PC 3.1	(1) *Mytilus* sp. <93.30% (2) *Mytilus sp.* <89.35%	-
PC3.2	(1) *Mytilus chilensis* 99.85-98.96%; *Mytilus galloprovincialis* 99.53-98.78%; *Mytilus edulis* 99.55-81.90%; *Mytilus trossulus* 99.25% (2) *Mytilus edulis* 92.86-93.70%; *Mytilus chilensis* 92.62%	*Mytilus* sp.
PC 3.3	(1)	*Mytilus* sp.

	Mytilus chilensis 100-99.11%; *Mytilus galloprovincialis* 99.69-98.93%; *Mytilus edulis* 99.70-99.11%; *Mytilus trossulus* 99.40% (1 seq) (2) *Mytilus edulis* 93.33-93.18%; *Mytilus chilensis* 93.11%	
PC 4.3	(1) *Mytilus chilensis* 97.36-96.32%; *Mytilus galloprovincialis* 97.24-97.06%; *Mytilus edulis* 96.64-96.48%; *Mytilus trossulus* 96.48% (1seq) (2) *Mytilus chilensis* 97.47%; *Mytilus edulis* 97.90-97.55%	-
PC 4.5	(1) *Mytilus* sp. <92.36% (2) *Mytilus sp.* <85.78%	-
PC 5.1	(1) *Mytilus chilensis* 99.85-82.61%; *Mytilus galloprovincialis* 99.38-98.63%; *Mytilus edulis* 99.39-98.81%; *Mytilus trossulus* 99.10% (2) *Mytilus chilensis* 93.03-92.79%; *Mytilus edulis* 93.02-92.87%	*Mytilus* sp.
PC 5.2	(1) *Mytilus chilensis* 100-98.96%; *Mytilus galloprovincialis* 99.24-99.09%; *Mytilus edulis* 99.55-98.96%; *Mytilus trossulus* 99.25% (2) *Mytilus chilensis* 93.11%; *Mytilus edulis* 93.18-93.02%	*Mytilus* sp.
PC 5.3	(1) *Mytilus chilensis* 100-99.11%; *Mytilus galloprovincialis* 99.69-99.38%; *Mytilus edulis* 99.70-98.96%; *Mytilus trossulus* 99.40% (2) *Mytilus chilensis* 99.21%; *Mytilus edulis* 99.23% (2 seq)	*Mytilus* sp.

Table 1. Samples molecular identification using *COI* gene. The identity values was reported for the (1) Basic Local Alignment Search Tool (BLAST) on GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and for the (2) Identification System (IDs) on BOLD (Species Level Barcode Records) (http://www.boldsystems.org/index.php/IDS_OpenIdEngine)