Rapid species identification of fresh and processed scallops by multiplex PCR

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Abstract

Food control policies regarding to seafood label authenticity have become a global issue due to increased incidence of species substitution or mislabelling. Proper species-level identification in processed scallop products is hindered by the lack of morphological characters such as their valves. In order to identify four commercially important scallop species (*Argopecten purpuratus*, *A. irradians*, *Mizuhopecten yessoensis*, *Pecten albicans*) a species-specific multiplex PCR reaction is described herein. Novel reverse species-specific primers in combination with one universal forward primer designed to amplify a partial region of the mitochondrial 16S rRNA gene were assayed in fresh as well as in manufactured scallop samples. All PCR reactions showed a high specificity allowing an unambiguous species authentication.

Keywords Pectinidae • Scallop • Food control • 16S rRNA gene • Multiplex PCR

1. Introduction

Scallops are bivalve mollusks belonging to the family Pectinidae. They do not only play an important role in marine ecosystems but also have tremendous worldwide commercial importance. Scallop fisheries are distributed globally (Tracey and Lyle, 2011), being Japan the world largest scallop producer, with a total production of 565,600 tons in 2009 (FAO-Globefish, 2011). In Japan, *Mizuhopecten yessoensis* ("hotate-gai" or "Japanese scallop") is one of the most important fishery products (Sato et al., 2005) and is mainly cultured in Hokkaido prefecture. Another economically important scallop species in Japan is the baking scallop *Pecten albicans* ("itaya-gai"), which is less abundant but a substitute for the commonest *M. yessoensis*, especially in southern Japan (Wongso and Yamanaka, 1998). *M. yessoensis* naturally occurs also in the northern Korean Peninsula and the far east of Russia, and in 1982 was introduced into China to increase scallop cultivation (Hou et al., 2011). The Japanese scallop is now widely farmed in northern China, where it was accepted quickly due to its large size and high market value (He et al., 2012; Li, Xu, & Yu, 2007). The bay scallop *Argopecten irradians* is another commercially valuable species in China where it was introduced from America in 1982 (Zhang, He, Liu, Ma, & Li, 1986), and has become one of the most important aquaculture species (Li, Liu, Hu, Bao, & Zhang, 2007). The Peruvian scallop *A. purpuratus*, which is widely cultured in Peru and Chile, is a highly economically important aquaculture species and it has recently been introduced into China and hybridized with the bay scallop *A. irradians* (Wang et al., 2011). According to the Peruvian Association of Exporters (ADEX), during the year 2011, total exportation of *A. purpuratus* from Peru reached 10,409 m tons for a total value of

$ 137 million (U.S.) dollars. France, United States and Belgium were the main destinations. In 2011, the European market was dominated by France, with 36,700 tons of scallops imported, followed by Spain (9,800 tons) Belgium and Germany (3,500 tons each; FAO-Globefish, 2012).

Globalization of the seafood market and increasing tendencies of international seafood trade are making governments around the world to enforce strict policies and new regulations (e.g. the European Council Regulation (EC) No 104/2000) in order to ensure food safety and prevent frauds (Santaclara et al., 2006; Zhan et al., 2008). Indeed, identification of food species is now a main concern not only for government entities and companies but also for consumers due to economic, regulatory, health and religious reasons. In Japan, food labelling of processed food is regulated according to the Law on Standardization and Proper Labelling of Agricultural and Forestry Products (JAS Law), product name, production site, and country of origin are required, particularly for imported products (Namikoshi, Takashima, Iguchi, Yanagimoto, & Yamashita, 2011). Seafood mislabelling is a very common issue; for example examinations performed during a nine-year period (1988-1997) by the National Marine Fisheries Service's National Seafood Inspection Laboratory (NSIL) showed that 37% of fish and 13% of other seafood were mislabelled (Buck, 2010). Manufactured scallop products are mostly commercialized without shells or in a processed form, consequently hampering species identification. According to FAO (2011), mislabelling import products (i.e. labelling Japanese scallop as USA domestic product) has been used fraudulently to obtain higher prices or to replace the product due to lack of supply. Furthermore, substituted and/or

109 mislabelled seafood is considered misbranded by the FDA (Food and Drug
110 Administration) and is a violation of Federal law (FDA, 2011).

111 Advances in biomolecular methods have allowed the development of
112 effective procedures for the identification of species in processed food. However,
113 most of these procedures (e.g. FINS, PCR-RFLP, RAPD) require further secondary
114 analyses (sequencing, endonuclease digestion, fingerprinting, phylogenetic
115 analysis) resulting in extra time and cost. On the other hand, species-specific PCR
116 technique has been proven to be faster, cheaper and more useful to control food
117 authenticity and identify a large number of samples (Marshall, Johnstone, & Carr,
118 2007; Rodriguez et al., 2003; Wen, Hu, Zhang, & Fan, 2012). So far, nuclear
119 markers such as microsatellite, ribosomal subunits, internal transcribed spacers
120 (Fernández-Tajes, Freire, & Mendez, 2010; Lopez-Pinon, Insua, & Mendez, 2002;
121 Bendezu, Slater, & Carney, 2005, Santaclara et al., 2006; Zhan et al., 2008), as
122 well as mitochondrial markers such as COI, 16S rRNA, Cyt b genes (Bendezu et al.,
123 2005; Colombo, Trezzi, Bernardi, Cantoni, & Renon, 2004; Jen, Yen, Liao, &
124 Hwang, 2008; Marshall et al., 2007) have been used for the identification to
125 species level in bivalve molluscs. However, it is well known that mitochondrial
126 DNA is more abundant in cells than nuclear DNA. Moreover, intense heating with
127 high-pressure conditions applied in food canning and drying process cause
128 severe DNA degradation (Bellagamba, Moretti, Comincini, & Valfre, 2001; Pascoal,
129 Prado, Calo, Cepeda, & Velasquez, 2005). These facts make mitochondrial DNA an
130 easier and more effective target for PCR amplification, especially in case of
131 tissues from heavily processed products, on which highly sensitive diagnostic
132 procedures are required.

Consequently, we select a partial region of the mitochondrial 16S rRNA gene as a marker. Generally, the COI mitochondrial gene has been proposed as the more suitable marker for DNA barcoding among taxa (Hebert, Cywinska, Ball, & deWaard, 2003), due mainly to the existence of robust universal primers, and a relatively greater range of phylogenetic signal than other mitochondrial genes with an evolution rate three times greater than that of 12S or 16S rDNA (Feng, Li, & Zheng, 2011). However, it has been shown that the proposed universal primers for DNA barcoding are not applicable for species of the family Pectinidae (Feng et al., 2011). Moreover, it has been previously reported that mitochondrial 16S rRNA gene can be useful for the identification of bivalve species (Bendezu et al., 2005; Feng et al., 2011; Jen et al., 2008). In this study, we designed and evaluated the utility of novel species-specific oligonucleotides for the identification of four commercially important scallop species: *A. purpuratus*, *A. irradians*, *M. yessoensis* and *P. albicans*. In order to evaluate if processed food label is correct, the new primers were assayed in canned, frozen and boiled scallop products. The efficiency of the methodology and primers applied herein was increased by the application of multiplex PCR reaction designed to amplify one species-specific fragment of the mitochondrial 16S rRNA gene for each tested scallop species. Additionally, a longer specific fragment of the mitochondrial 12S rRNA gene was amplified using novel primers and used as common positive control in all multiplex PCR reactions.

2. Materials and methods

2.1. Materials

157 Fresh samples of *A. purpuratus* were collected in Independencia and
158 Sechura Bay (Peru). *M. yessoensis* specimens were sampled in Funka Bay
159 (Hokkaido, Japan) and *P. albicans* samples were collected in Shimoda Bay and
160 Oki Island (Shimane, Japan). Samples of *A. irradians* were supplied by Dr.
161 Linsheng Song (Institute of Oceanology, China). Approximately, 200 mg of
162 adductor muscle was dissected, preserved in 95% ethanol and stored at −20 °C.
163 In order to confirm the authenticity of the labelling of processed scallops, twelve
164 samples were analyzed: three samples of canned *A. irradians* (labelled as "itaya-
165 gai"), and three samples of canned *M. yessoensis* (labelled as "hotate-gai") were
166 purchased from a Japanese supermarket; three samples of boiled *A. irradians*
167 (labelled as "itaya-gai" and imported from China), and three samples of frozen *A.*
168 *purpuratus* (labelled as "concha de abanico") were purchased from a Peruvian
169 local market.
170
171 *2.2. DNA extraction*
172 From fresh samples, total genomic DNA was extracted using the standard
173 phenol-chloroform protocol, quantified and adjusted to a concentration of 100
174 ng/μl for PCR reactions. DNA from processed scallop samples was extracted
175 using the protocol described in Sokolov (2000) with minor modifications: briefly,
176 70 mg of tissue was soaked in distilled water for 10 minutes, transferred to a 1.5
177 ml microtube containing 400 μl of TNES buffer (10 mM Tris-HCl at pH7.4, 10 mM
178 EDTA at pH 8, 125 mM NaCl, 0.5% SDS and 4M urea) and 10 μl of proteinase K
179 (20mg/ml), mixed softly by vortexing and incubated for 2 h at 55 °C. Then, 75 ml
180 of 3M sodium acetate (pH 5.2) was added and mixed by tube inverting. All
181 samples were incubated on ice for 10 min and centrifuged at 13,500 rpm for 15

182 min. The supernatant was recovered and mixed with the same volume of
183 phenol/chloroform/isoamyl alcohol (25:24:1) by tube inverting and centrifuged
184 at 13,500 rpm for 15 min. The clear supernatant was recovered again mixed with
185 an equal volume of isopropanol, incubated for 10 min at room temperature, and
186 centrifuged at 13,500 rpm for 20 min. The pellet was washed twice with 70%
187 ethanol, dried and dissolved in 100 μl of TE buffer (10mM Tris-HCl, 1mM EDTA
188 at pH 8.0).
189
190 *2.3. Primer design for species-specific mitochondrial 16S rRNA gene fragment and*
191 *common mitochondrial12S rRNA gene fragment amplification*
192 The full mitochondrial 16S rRNA gene sequences for *M. yessoensis*
193 (accession number: FJ595959), *A. irradians* (accession number: DQ665851), and
194 *A. purpuratus* (accession number: HQ677600) were retrieved from GenBank
195 database. Whereas for *P. albicans*, we determined its complete mitochondrial 16S
196 rRNA gene sequence by "primer walking" strategy using the primer sets reported
197 by Marín, Fujimoto, and Arai (2012) designed for the determination of the same
198 gene in *A. purpuratus*, with two additional primers designed in this study: one
199 specific primer Palb16S 5'-CTAAGTATAGCTCTTCGGTTGATG-3' and one
200 degenerated primer ND1F 5'-TGTGCCGGAGCAGCttyccncgnta-3' (Table 1). PCR
201 products were sequenced using an ABI PRISM 3130XL Genetic Analyzer (Applied
202 Biosystems).
203 To design species-specific primers for each scallop species, all complete
204 mitochondrial 16S rRNA gene sequences were multi aligned using MEGA 5.05
205 software (Tamura et al., 2011). Genetic distances were calculated using the
206 Kimura-2 parameter method. Based on interspecific variations, one species-

207 specific reverse primer was designed for each scallop (Table 2) according to the
208 following criteria: a) all primers were at least 18 bp in length; b) at least three
209 nucleotides from the extreme 3' of the primer end had to match perfectly with
210 the complementary region of the target sequence; c) since the sterilization
211 process in manufactured bivalves can fragment the DNA to sizes ranging from
212 200 to 700 bp (Fernández-Tajes et al., 2010), all primers were designed to
213 amplify PCR products no larger than 700 bp (see Table 2). The forward primer
214 16S arl (Palumbi et al., 1991) was used as the common primer for all species in
215 all PCR reactions (Table 2). Additionally, the complete mitochondrial 12S rRNA
216 gene of *M. yessoensis* and *A. irradians* and a partial region of the same gene from
217 *A. purpuratus* were multi aligned and two "universal" pectinid primers
218 (12SpectU forward and reverse) were designed (Table 2) based on highly
219 conserved regions. The fragments amplified by these novel primers were used as
220 positive control in all multiplex PCR reactions.
221
222 *2.4 Multiplex PCR amplification*
223 Firstly, PCR amplification was standardized for each species-specific
224 primer separately. To check the specificity of the species-specific primers, they
225 were tested on both target and non-target species DNA. The optimum PCR
226 conditions were the following: 100 ng of template DNA, 0.2 mM each for dNTPs,
227 1X Ex *Taq* buffer (TaKaRa, Japan), 0.25 μM each for species-specific reverse and
228 common 16S arl forward primers, 0.05 μM each for 12SpectU forward and
229 reverse primers, and 0.5 U of Ex *Taq* polymerase (TaKaRa, Japan) in a total
230 volume of 20 μl. Thermocycling conditions were: initial denaturation for 5 min at
231 94 ˚C, followed by 30 cycles of denaturation for 15 s at 94 ˚C, annealing for 15 s

232 at 55 ˚C, and extension for 15 s at 72 ˚C, followed by a final extension for 7 min at
233 72 ˚C.

234 In order to set up a rapid and reliable species identification method from
235 commercial scallop samples, a multiplex PCR reaction was performed. The
236 common forward 16S arl primer was pooled together in a single reaction with
237 the three species-specific reverse primers for *A. irradians* (AirraR3 primer), *A.*
238 *purpuratus* (ApurpR3 primer), and *M. yessoensis* (MyessoR4 primer). The
239 positive control primers 12SpectU were used in all multiplex reactions. Different
240 combinations of primer concentrations were tested. The optimum primer
241 concentrations were: 0.5 μM for the common 16S arl forward primer, 0.25 μM
242 each for ApurpR3, AirraR3 and MyessoR4, and 0.1 μM each for 12SpectU forward
243 and reverse primers. Except for primer concentrations, PCR conditions were the
244 same as described above. All PCR products were electrophoresed for 35 min in a
245 1.5 % agarose gel, stained with ethidium bromide and visualized under UV light.
246

247 **3. Results and discussion**

248 After the alignment of the complete mitochondrial 16S rRNA gene from
249 the scallop species studied herein, the lowest sequence divergence was found
250 between *A. purpuratus* and *A. irradians* (14 %, 167 variable sites, data not
251 shown). Nevertheless, this divergence allowed us to design a specific primer also
252 for these species. In this study, species-specific primers in combination with one
253 universal primer designed to amplify a partial fragment of the mitochondrial 16S
254 rRNA gene were successfully assessed for a reliable identification at species level
255 of fresh and processed scallop food samples. In the respective target species the
256 pool consisting of the reverse species-specific primer in combination with the

257 forward common 16S arl primer and the universal 12SpectU forward and
258 reverse control primers, gave one species-specific product (the mitochondrial
259 16S rRNA fragment) and one higher size band (the mitochondrial 12S rRNA gene
260 fragment) amplified by the positive control primers (Fig. 1), as expected. Instead,
261 PCR reactions containing non-target species DNA amplified only the positive
262 control amplicon, as expected too.

263 The positive control fragment from mitochondrial 12S rRNA gene ranged
264 from 616 to 660 bp, depending on species, (Fig. 1 and 2). It showed whether a
265 failure in the amplification with species-specific primers in the absence of target
266 DNA was truly due to the primer species-specificity or to a failure in the overall
267 PCR reaction. Overall, the co-amplification of the positive control fragment was
268 very consistent in all multiplex reactions that included target and non-target
269 DNA.

270 No cross amplification (false positive) was observed when the species-
271 specific primers were assayed with non-target species DNA (Fig. 1). Different
272 PCR products were consistently amplified in fresh and processed samples of *A.*
273 *irradians* (272 bp), *A. purpuratus* (344 bp) and *M. yessoensis* (425 bp) as reported
274 in Fig. 2. All species-specific primers were successfully amplified in 29 fresh
275 samples of *A. purpuratus*, 30 samples of *M. yessoensis*, 16 samples of *A. irradians*,
276 and 9 samples of *P. albicans*, as well as in processed food (canned, boiled, frozen)
277 samples of *A. purpuratus*, *A. irradians* and *M. yessoensis*. As for *P. albicans*. the
278 complete nucleotide sequence of the mitochondrial 16S rRNA gene (1423 bp)
279 was deposited in GenBank/DDBJ/EMBL DNA databases under the accession
280 number JN896624. The species-specific primer designed for *P. abicans* (PalbiR2)
281 in combination with the universal 16S arl primer produced a 270 bp size

amplicon. By using our species-specific primers, all commercial food samples labelled as "concha de abanico" (*A. purpuratus*, frozen) and "hotategai" (*M. yessoensis*, canned) were found to contain the species declared in their labels. Regarding to the product labelled as "itayagai", three canned and three boiled samples (from Japan and China, respectively) were identified as *A. irradians* (bay scallop). The use of the same common name for different species is a frequent issue in marine processed food, especially when referred to products obtained from foreign species. In fact, the same vernacular name can be referred to different species or a single species may be diversely labelled depending on the region or country (Barbuto et al., 2010). For example, the baking scallop *P. albicans* is widely known as "itayagai" in Japan, but the bay scallop *A. irradians* is also known by the same vernacular name and sometimes referred as "America-itayagai" (Takahashi and Muroga, 2008). Our results thus suggested that Chinese bay scallop exported to Japan are sometimes labelled as "itayagai" due to unintended misconception, which may cause confusion among consumers. The use of similar names for different products introduced from one country to another where a market niche already exists makes it difficult to enforce rules, even if national food legislations indicate that the label must not mislead consumers (FAO-Globefish, 2012). However, it must be specified that a real misleading can be considered when there is a difference of quality traits among different species. Thus, some species can have the same commercial name if there are no reasons for giving them different names. Moreover, FDA generally recommends use of common name as the market name unless a common or usual name has been established by regulation or law.

This study highlights the importance of an accurate labelling of seafood products and the results demonstrated the specificity of the novel species-specific primers used and multiplex PCR technique for the forensic identification of scallop food products. This simple, inexpensive, timesaving, and powerful single step method can be reproduced easily by any basic molecular genetics laboratory. Progress in the area of authentication of traded seafood products requires the use of molecular tools to ensure a proper species identification, thus enhancing the application of effective food control regulations and consumer protection.

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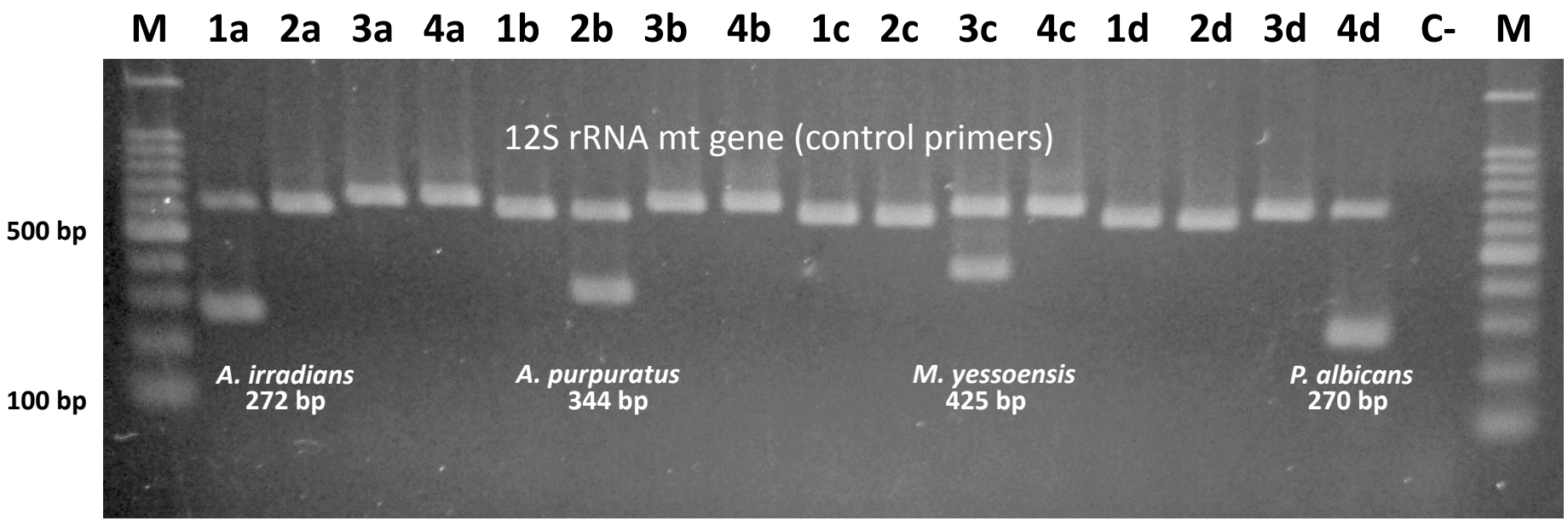
447

448 Fig 1. Agarose gel electrophoresis of PCR products amplified using species-specific
449 primers in fresh scallop samples. Lanes 1a to 4a: 16Sarl/AirraR3. Lanes 1b to 4b:
450 16Sarl/ApurpR3. Lanes 1c to 4c: 16Sarl/MyessoR4. Lanes 1d to 4d:
451 16Sarl/PalbiR2. Lanes 1a, 1b, 1c, 1d: *A. irradians* DNA template. Lanes 2a, 2b, 2c,
452 2d: *A. purpuratus* DNA template. Lanes 3a, 3b, 3c, 3d: *M. yessoensis* DNA template.
453 Lanes 4a, 4b, 4c, 4d: *P. albicans* DNA template. M: 100 bp ladder, C-: negative
454 control.

455

456 Fig 2. Agarose gel electrophoresis of multiplex PCR products amplified using
457 species-specific primers in fresh and processed scallop samples. All species-
458 specific primers were included in every single reaction. Lanes 1 to 4: *A. irradians*
459 DNA templates (lanes 1 and 2: fresh, lanes 3 and 4: boiled). Lanes 5 to 8: *A.*
460 *purpuratus* DNA templates (lanes 5 and 6: fresh, lanes 7 and 8: frozen). Lanes 9 to
461 12: *M. yessoensis* DNA templates (lanes 9 and 10: fresh, lanes 11 and 12: canned).
462 A partial fragment of the mt 12S rRNA gene (616-660 bp) was used in all
463 multiplex PCR reactions as a positive control. M: 100 bp ladder, C-: negative
464 control.

465


M 1a 2a 3a 4a 1b 2b 3b 4b 1c 2c 3c 4c 1d 2d 3d 4d C- M
12S rRNA mt gene (control primers)
500 bp
100 bp
A. irradians
272 bp
A. purpuratus
344 bp
M. yessoensis
425 bp
P. albicans
270 bp

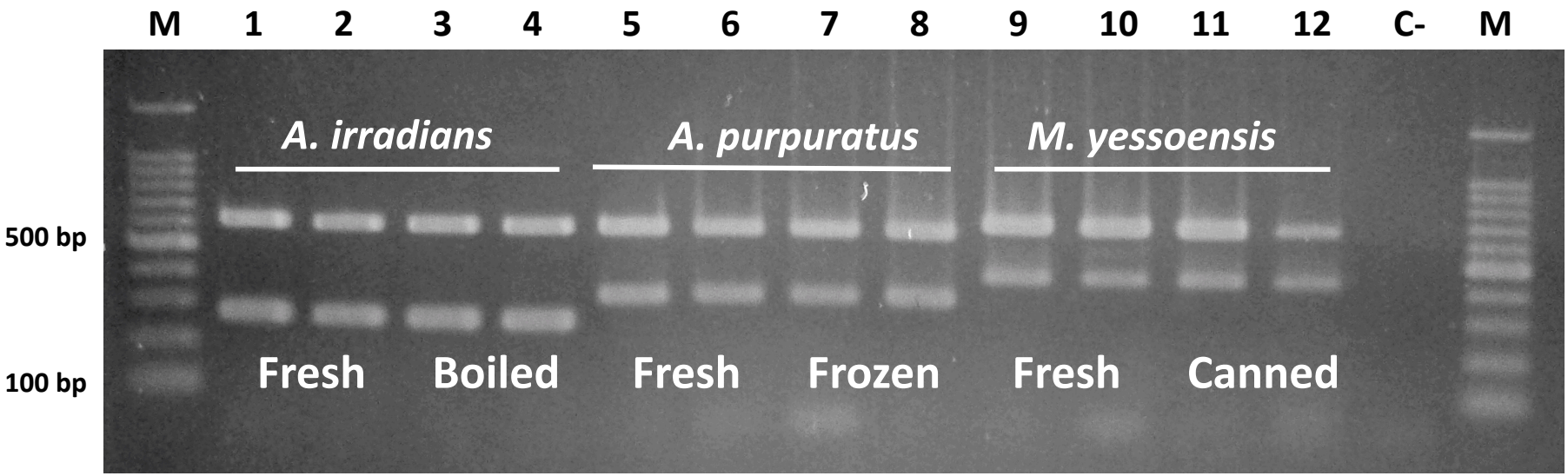

M
1
2
3
4
5
6
7
8
9
10
11
12
C-
M
A. irradians
A. purpuratus
M. yessoensis
500 bp
100 bp
Fresh
Boiled
Fresh
Frozen
Fresh
Canned

Table 1. Primers used to determine the complete mitochondrial 16S rRNA gene sequence in *P. albicans.*

Primer name	Direction	Sequence (5'-3')	Source
16S arl	Forward	CGCCTGTTTAACAAAAACAT	Palumbi et al. (1991)
16S R	Reverse	CCGRTYTGAACTCAGCTCACG	Puslednik and Serb (2008)
ND1F	Forward	TGTGCCGGAGCAGCttyccncgnta	This study
CO1AB	Reverse	GGTGCTGGGCAGCcayatnccngg	Marín et al. (2012)
Palb16S	Forward	CTAAGTATAGCTCTTCGGTTGATG	This study
16SCC	Forward	GCGTAATCCGTCTTGACAGT	Marín et al. (2012)

Table 2. Species-specific, common and universal primers used in multiplex PCR.

Species	Primer name	Sequence	Fragment size (bp)
A. irradians	AirraR3	5'-AAAGTAGCCTCCCACTATTCT-3'	272
A. purpuratus	ApurpR3	5'-GCTCACCAGCACTTAGAA-3'	344
M. yessoensis	MyessoR4	5'-CGTATCCGTTTAGTGATTA-3'	425
P. albicans	PalbiR2	5'-GCCTTGGAACTTCCCAATAACT-3'	270
Forward common	16S arl	5'-CGCCTGTTTAACAAAAACAT-3'	_
	12SpectUF forward	5'-AGTCCAACCAGGTGCCAGCA-3'	_
	12SpectUR reverse	5'-GAGAGCGACGGGCAGTTTGT-3'	_