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Novel decaplex PCR assay for simultaneous detection of scallop species with species-specific primers targeting highly variable 5' end of the 16S rRNA gene

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Abstract

Scallops (family Pectinidae) comprise species of high commercial value, supporting both commercial fisheries and mariculture activities. Accurate and reliable molecular methods for the species level identification are of outstanding utility for taxonomic and food authentication surveys. The mitochondrial 16S rRNA gene has been used to design species-specific primers for identification of different bivalve species. However, the low interspecific variability at the 3' end of this gene has limited its utility and only few scallop species have been assessed. In this study, we used the high variable 5' end of the 16S gene to develop a novel decaplex PCR assay that enabled a fast and accurate identification of 8 commercially important scallop species in a single PCR reaction. A total of 285 individuals including fresh and manufactured samples from 8 different processed presentations representing 11 different scallop species were collected from diverse locations around the world and successfully. Our assay accurately identified all the analyzed samples at the species level. Furthermore, to enhance the utility of our assay, the PCR product amplified by the family-specific primer set that was utilized as positive control was also used for the identification of unknown (non-target) scallop species by DNA sequencing analysis. In its present form, our multiplex PCR method can be of great utility for different types of studies involving scallop species and for research institutes and governmental agencies that regulate seafood authentication around the world.

Keywords • Pectinidae • Decaplex PCR • Aquaculture • 16S rRNA gene

Introduction

Scallops, members of the family Pectinidae, are among one of the most important marine groups of high commercial value, supporting both commercial fisheries and mariculture activities. There are about 350 extant scallop species globally distributed, from which 40 are commercially exploited but only 18 species account for the live weight global production (Seafish 2013). Among them, the most important wild capture scallop species are the Japanese scallop *Mizuhopecten yessoensis* and the Atlantic sea scallop *Placopecten magellanicus*. Both species are included in the list of FAO top 70 marine resources capture production by principal species (FAO 2014). The Japanese scallop occurs naturally in Japan, Korea and Russia, and has been introduced into China, France and Canada (FAO 2006; Guo 2009). On the other hand, the Atlantic sea scallop occurs in the northwestern Atlantic Ocean ranging from north shore of the Gulf of St. Lawrence in Canada to Cape Hatteras in US (Naidu & Robert 2006). During the year 2012 sea scallop commercial landings valued at $559 millions making this species among the highest value fishery in the US (National Marine Fisheries Service, US commercial landings data). Primary commercial markets are restaurants and retailers in the US and in the European Union, where they are available fresh, frozen and in many processed foods (Marine Stewardship Council MSC 2013). In Europe, the most commercially important scallop species is the king scallop *Pecten maximus*. This species is distributed along the Northeast Atlantic from Norway down to North Africa (Beaumont & Gjedrem 2007) and constitutes a major commercial resource in Great Britain, Ireland, France and Spain (Le Pennec, Paugam & Le Pennec 2003). *P. maximus* is sold

shucked and fresh to the European and domestic market. Scallop fisheries of *M. yessoensis* (Hokkaido, Japan), *P. magellanicus* (US and Canada) and *P. maximus* (UK) are three of the only five scallop fisheries that currently hold the sustainable certification labeling from the MSC (Marine Stewardship Council). In China, two native (*Chlamys farreri* and *Mimachlamys nobilis*), and two exotic species (*M. yessoensis* and *Argopecten irradians*) are the most important cultured scallops (Spencer 2008). Another species of high commercial value is the Peruvian scallop *A. purpuratus*. This species is widely exploited in Peru and Chile, where occur naturally. During the year 2013, Peruvian scallop exportations exceeded 14,000 tons valued at $161 millions, shipped to 42 countries around the world (PROMPERU 2014).

Processed scallop products frequently consist just of the adductor muscle, which prevents the identification to the species level using morphological characteristics. Even when scallops are commercialized as whole animals with shell, a proper identification to the species level may not be possible for the untrained eye, due to morphological similarity among species. Fraud is a serious concern among seafood industry. Food product misbranding usually implies whole or partial substitution of more expensive species by low value species or species with a potential food safety hazard (Handy, Deeds, Ivanova, Hebert, Hanner, Ormos, Weigt, Moore & Yanci 2011). Typically, reports on mislabeled food products have shown that high valued seafood are being substituted by cheaper species that not always belong to related groups. For instance, it has been detected that skate (a cartilaginous fish) wings were fraudulently mislabeled as scallops (Jacquet and Pauly 2008). In another case, mislabeled

scallop imports have been used fraudulently to obtain higher prices or to replace the product due to lack of supply in the US (FAO-Globefish 2011). Thus, high throughput assays that can enable the identification of different commercially important scallop species are needed for species conservation and consumer protection.

Over the last two decades, molecular methods have allowed the development of effective assays for species identification. A variety of DNA-based techniques including multiplex PCR have been developed and successfully applied for meat and seafood species identification (Marín, Fujimoto & Arai 2013; Ali, Razzak & Hamid 2014). Mitochondrial markers have proven to be an effective tool for species identification, especially when the quality and quantity of DNA is not the most favorable (e.g. museum specimens, processed food). The relatively abundant mitochondrial cell content makes it an effective target when working with tissues that have been through high temperatures and pressure (i.e. canned food), which cause severe DNA degradation. Among the several mitochondrial markers, the 16S rRNA gene has been successfully applied for the accurate identification of different bivalve species (e.g. Bendezu, Slater & Carney 2005; Jen, Yen, Liao & Hwang 2008; Feng, Li & Zheng 2011; Marín *et al*. 2013, 2015). Species-specific primers (hereafter referred to as "SSPs") combined with multiplex PCR is a fast, low-cost and widely used technique for species identification and food authentication (Wang & Guo 2008). However, despite its demonstrated utility, so far few studies have focused on this technique for the identification of scallop species, and only few species have been assessed (Colombo, Trezzi, Bernardi, Cantoni & Renon 2004; Bendezu *et al*. 2005;

Marshall, Johnstone & Carr 2007; Marín *et al*. 2013). Therefore, there is still a need for the development of a rapid multilplex PCR assay for the simultaneous identification of a broader range of scallop species, especially those of high commercial value, which are more prone to be subjected to species substitution resulting in commercial fraud.

The aim of this study was to develop an assay for the identification of some of the most economically important scallop species (fresh and processed samples) by a single-step multiplex PCR. For this purpose, we designed 8 SSPs and one *Pecten* genus specific primer (hereafter referred to as "GSP") based on the high diverged 5' end of the mitochondrial 16S rRNA gene, which in combination with a scallop family-specific primer set, allowed us to develop a novel decaplex PCR assay for fast and simultaneous identification of 8 scallops at species level (*M. yessoensis*, *A. irradians*, *A. purpuratus, C. farreri*, *P. magellanicus*, *M. nobilis, Bractechlamys vexillum,* and *Annachlamys macassarensis*) and one at genus level (*Pecten*). To our knowledge, this study represents the first report on the simultaneous identification of these 8 economically important scallop species by single step multiplex PCR.

Materials and methods

Ethics statement

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals in Hokkaido University, Japan. Field sampling required for this work included only invertebrate species, which are not endangered.

Sample collection and DNA isolation

A total of 285 individuals (243 fresh and 42 samples from 8 different processed presentations) from 11 different scallop species were collected from diverse locations around the world (Table 1). For the 9 target scallop species, from 14 to 65 samples were analyzed, except for *A. macassarensis*, for which only five samples were collected. When possible, different geographical locations were sampled, attempting to cover a broader genetic variation of each species. Scallop samples were taxonomically identified based on morphological characteristics and by DNA barcoding approach (Marín *et al*. 2015). In processed samples containing several fibers of adductor muscles (canned and simmered) or many full adductor muscles (dried, cooked, seasoned, boiled and smoked), three fibers or whole adductor muscles were randomly analyzed (Table 1). In frozen presentations, where one package contained limited number of full adductor muscles (i.e. from 5 in *A. purpuratus* to 10 in *P. magellanicus*), all adductor muscles where analyzed. Adductor muscles were excised and preserved in ethanol 95%. For all fresh samples, DNA isolation was performed using the standard phenol-chloroform protocol (Sambrook et al., 1989). DNA from manufactured samples was isolated following the protocol described in Sokolov (2000) with minor modifications by Marín *et al*. (2013). DNA amount and quality was determined using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, US).

Species-specific primer design based on the 5' end of the 16S rRNA gene and PCR amplification

Previously, we reported a high interspecific variability at the 5' end of the mitochondrial 16S rRNA gene, after the multialignment of the complete 16S rRNA genes of 15 scallop species (Marín *et al*. 2015). The complete 16S mitochondrial gene from all the scallop species analyzed herein were multialigned using MEGA 5.2 (Tamura, Peterson, Peterson, Stetcher, Nei & Kumar 2011). Accordingly, 8 reverse SSPs and one GSP were designed based on non-conserved regions at the 5' end of the 16S rRNA gene. A Pectinidae family-specific forward primer PECT16BCF (Marín *et al*. 2015) was used as the common forward primer for all PCR reactions performed in this study. This forward primer was also used in combination with a family-specific reverse primer as positive control. The family specificity of this primer set was previously validated by amplifying it in 14 different scallop species and by obtaining unspecific or no amplification at all in two close bivalve families namely Mytilidae and Ostreidae (Marín *et al*. 2015). To ensure a robust PCR amplification, all SSPs ranged from 18 to 24 nt in length, at least five nucleotides from the 3' end of the primer matched perfectly to its complementary template DNA region, and primers for processed scallop samples were designed to produce PCR products no larger than 500 bp. Lowest PCR product sizes (<200 bp) were given to species with heavily processed samples (e.g. canned). First, to test for individual primer specificity, each reverse SSPs was PCR amplified individually with the common forward primer using four "fresh" individuals of its target species DNA as template. Each SSP and the GSP were also tested against all non-target DNA templates (all "fresh" samples listed in Table 1 were tested) to check for cross-species reactions. The optimal annealing temperature was determined using a gradient Gene Atlas G02 gradient thermal cycler (ASTEC,

Fukuoka, Japan). The PCR master mix consisted of 50 ng of template DNA, 0.2 mM each dNTPs, 1X Ex *Taq* buffer (TaKaRa, Osaka, Japan), 0.5 μM each primer, and 0.5 U of Ex *Taq* polymerase (TaKaRa, Japan) in a total volume of 20 μL. Thermal cycling conditions were as following: initial denaturation for 5 min at 94 °C, followed by 27 cycles of denaturation for 15 s at 94 °C, annealing for 25 s at 57 °C, and extension for 15 s at 72 °C, followed by a final extension of 3 min at 72 °C. Finally, PCR products were electrophoresed and visualized on 1.5% agarose gel stained with ethidium bromide under UV light.

Decaplex PCR

In order to perform a rapid and cost-effective scallop species identification, a total of 11 primers (Table 2) were combined in a multiplex PCR reaction: 8 species-specific reverse primers and one genus-specific reverse primer, with one family-specific primer set Pect16BC forward and reverse (Marín *et al*. 2015). We expected to obtain two PCR products in each of the 9 target species: one species-specific band of different size length (amplified by each SSP and GSP and the forward family-specific common primer), and a positive control product (amplified by the family-specific primer set). The final optimized PCR master mix consisted of 50 ng of template DNA, 0.2 mM each dNTPs, 1X Ex *Taq* buffer (TaKaRa, Japan), 0.5 U of Ex *Taq* polymerase, 0.4 μM of common forward primer Pect16BCF and SSP BrVe846R, 0.05 μM of common reverse primer Pect16BCR, 0.35 μM of SSPs MiYe113R and MiNo565R (each), 0.3 μM of PeMa323R and AnMa918R (each), 0.25 μM of ArIr197R, and 0.20 μM of ArPu228R, ChFa376R, and PlMa496R (each), in a total volume of 20 μL. Thermal cycling conditions

were the same as described above. Furthermore, to confirm the reproducibility and specificity of our decaplex PCR assay, three additional TaKaRa PCR enzymes (Emerald Amp Master Premix, Sapphire Amp Fast PCR Master Mix, and HS Perfect Mix) were tested using 4 fresh and processed (when available) individuals per species, using the same decaplex primer concentrations described above and following manufacture's instructions. PCR products were visualized in a 1.5% agarose electrophoresis gel. Besides, in our experimental design, one additional scallop species, *Amusium pleuronectes*, was included. This DNA template was intended to amplify only the positive control band. The single PCR product obtained was further sequenced using an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, Hitachi, Tokyo, Japan). The species identification of the obtained DNA sequence was performed using the NCBI web Blast service.

RFLP assay for differentiation between *P. maximus* and *P. albicans*

Due to the low genetic distance between *P. maximus* and *P. albicans*, the GSP PeMa323R amplified a PCR product of similar size as in *P. albicans* (data not shown). Consequently, in order to further differentiate both species, we performed an assay based on RFLP (restriction fragment length polymorphism). We analyzed the complete 16S gene from both species (GenBank accession numbers KF982791 and JN896624) to find putative restriction enzyme sites located within the sequence flanked by the primers Pect16BCF and Pect16BCR. Our RFLP assay was validated using 14 and 19 individuals of *P. maximus* and *P. albicans* respectively (samples were collected from two localities for each

247 species). PCR reactions consisted of 50 ng of template DNA, 0.2 mM each dNTPs,
248 1X Ex *Taq* buffer (TaKaRa, Japan), 0.25 μM of the Pect16BCF and Pect16BCR
249 primers (each), and 0.5 U of Ex *Taq* polymerase (TaKaRa, Japan) in a total
250 volume of 20 μL. Thermal cycling conditions were as following: initial
251 denaturation for 5 min at 94 °C, followed by 30 cycles of denaturation for 15 s at
252 94 °C, annealing for 25 s at 60 °C, and extension for 30 s at 72 °C, followed by a
253 final extension of 5 min at 72 °C. Subsequently, 5 μL of PCR products were
254 digested using *Hae*III (Roche Diagnostics, Mannheim, Germany). Enzymatic
255 digestions were performed following manufacture's instructions in a final
256 volume of 20 μL and incubated at 37 °C overnight. Digested fragments were
257 visualized in a 1.5 % agarose gel under UV light.

258

259 **Results**

260 Primers specificity and decaplex PCR

261 All SSPs were designed to yield PCR products of different length for each species
262 in combination with the common forward primer. All SSP showed high
263 specificity, reflected in clear and unambiguous species identification by
264 visualization of amplicons of different sizes in agarose gel (see Table 1 for
265 species sample size information). Decaplex PCR amplification resulted in one
266 species-specific band for each species, ranging from 113 in *M. yessoensis* to 918
267 bp in *A. macassarensis*, plus the positive control product that ranged from 600 to
268 700 bp, depending on species (Fig. 1). The different length sizes of the positive
269 control products were due to the presence of indels and gaps found after the

multialignment of partial mitochondrial 16S rRNA gene fragments of the scallop species studied herein. Multiplex PCR amplifications showed consistent results for the other three different *Taq* DNA polymerase enzymes analyzed (data not shown). However, different total PCR amplification times were achieved by each *Taq* DNA polymerase: 40 min for HS Perfect Mix, 48 min for Sapphire Amp Fast PCR Master, and 65 min for Ex *Taq* and Emerald Amp Master Premix (the variation in amplification time is due to the different annealing and elongation times required in each different *Taq* polymerases). No cross species amplification was found, except for the primer ArPu228R that amplified a weak unspecific band of about 1.5 kb in fresh samples of *A. irradians*. This unspecific PCR product was not yielded in processed DNA samples of *A. irradians*.

Processed scallop samples authentication

Overall, 8 processed scallop presentations representing four different species were authenticated in this study. Even though some processed samples (i.e. smoked and canned) showed relatively low (Nanodrop 260/280 ratio: 1.4 to 1.5) to medium (Nanodrop 260/280 ratio: 1.6 to 1.7) DNA purity probably due to physical treatment and contamination by food additives, it was sufficient for PCR analysis. Thus a 100% success PCR amplification rate for all processed samples was obtained. After multiplex PCR amplification, we found that all products contained the species declared in their labels. However, only one presentation displayed the scientific name on its label, whereas in all the other samples only their common name was indicated (Table 1).

293

294 Alternative utility of the positive control amplicon

295 In order to enhance the effectiveness of our multiplex assay, we intentionally
296 included a Pectinidae family-specific primer set as the positive control. The aim
297 of this was to assure the amplification of any scallop species. In such a way, if an
298 unknown scallop species (different than the 9 species targeted herein) is
299 analyzed with our assay, at least a positive control PCR product will be obtained.
300 The unknown sample can be identified by further analysis (e.g. BLAST, DNA
301 barcoding). To demonstrate this, we included the DNA of one "unknown and non
302 target" species that in fact was extracted from one fresh individual of the Asian
303 moon scallop (*A. pleuronectes*, BLAST nucleotide sequence match 100% identity).
304 After multiplex PCR, the single positive amplicon was purified and sequenced.
305 The sequence was deposited in GenBank database under the accession number
306 KM502555. The submission of this novel sequence is a contribution to the DNA
307 barcoding of the family Pectinidae species list based on the 5' end of the 16S
308 rRNA gene (Marín *et al*. 2015).

309

310 *Hae*III RFLP assay for identification of *P. maximus* and *P. albicans*

311 In *P. maximus* and *P. albicans* the primers Pect16BCF and Pect16BCR amplified a
312 fragment of 654 and 655 bp respectively. After DNA sequence analysis, a unique
313 restriction site was identified only for *P. albicans* in the position 369 bp. Thus, a
314 digestion reaction by *Hae*III yielded two diagnostic bands of 286 and 369 bp in *P.*
315 *albicans*. The 654-bp amplicon of *P. maximus* remained uncut (Fig. 2), which

316 allowed a clear discrimination between these *Pecten* species by agarose gel
317 visualization. A total of 14 and 19 individuals of *P. maximus* and *P. albicans* were
318 successfully identified with this RFLP assay, respectively.

319

320 **Discussion**

321 There are different DNA based assays that have been successfully applied for
322 seafood species identification. Nonetheless, most of these procedures require
323 further secondary analyses (sequencing, endonuclease digestion, fingerprinting,
324 phylogenetic analysis) resulting in extra time and cost (Marín *et al*. 2013). In this
325 study, we selected the SSPs design and multiplex PCR amplification method
326 because it allows a simple, accurate, cost-effective and timesaving species
327 identification. The main drawback of this technique is that prior sequence
328 knowledge from the targeted species is required for primer design. This need
329 was fulfilled by a previous study on DNA barcoding based on the variability of
330 the 5' end of the 16S RNA gene in Pectinidae (Marín *et al*. 2015).

331 Mitochondrial DNA has been widely used as a source of molecular
332 markers for species identification. An accelerated evolutionary rate, reflected in
333 a significant amount of sequence divergence in closely related species, and lack
334 of introns make the mitochondrial DNA suitable for species identification (Yang,
335 Tan, Wang, Xue, Guan, Huang & Li 2014). The most commonly used
336 mitochondrial genes for species-level determination in seafood studies are the
337 COI, Cytb and 16S rRNA genes. Even though the COI gene is 2.5 times more
338 variable than the 16S rRNA gene (Nicolas, Schaeffer, Missoup, Kennis, Colyn,

339 Denys, Tatard, Cruaud & Laredo 2012), the mutations frequently found in
340 variable regions of the 16S gene, which correspond to loops in the ribosomal
341 RNA structure (Vences, Thomas, van der Meijden, Chiari & Vieites 2005),
342 enhance the utility of this gene for the development of SSPs for species
343 authentication assays by multiplex end-point PCR. Previous studies have
344 demonstrated the low efficiency of universal COI primers for different organisms,
345 such as marine molluscs (Layton, Martel & Hebert 2014) including scallops (Feng
346 *et al*. 2011). On the contrary, universal primers based on the 16S gene have been
347 successfully applied in organisms where universal COI primers have failed (e.g.
348 Vences *et al*. 2005). Another important reason to choose the 16S gene for the
349 design of SSPs was the presence of gaps in interspecific sequence alignments.
350 Gaps in DNA alignments are more commonly found in ribosomal genes than in
351 protein coding genes (Barr, Cook, Elder, Molongoski, Prasher & Robinson 2009).
352 To facilitate the amplification of only target species, the 3' end of some SSPs was
353 anchored in gap mismatches from no target species (SSPs position sites for each
354 species are available in the supplementary material). Noteworthily, almost all
355 reports on scallop species identification based on 16S gene relied on the
356 variability of the 3' end (e.g. Feng *et al*. 2011; Marín *et al*. 2013). This is probably
357 because of the existence of universal primer sets designed to amplify a partial
358 region of the 3' end, which have been widely used for researchers in
359 phylogenetic, forensic, DNA barcoding, population structure studies, etc (Marín
360 *et al*. 2015). However, the occurrence of large stretches with high degree of
361 sequence conservation within this region restricts the design of SSPs to a limited
362 number of species, especially when working with highly related or congeneric
363 species (e.g. Marín *et al*. 2013). On the other hand, the 5' end of the 16S gene in

the family Pectinidae is characterized for a higher interspecific molecular divergence, which allowed the clear identification of 14 scallop species by DNA barcoding approach (Marín *et al*. 2015). Furthermore, the low intraspecific variation found at the 5' end of the 16S gene (0 to 0.1%, Marín *et al*. 2015) makes this region an ideal target to design markers for scallop species identification.

To evaluate the performance of our multiplex PCR assay using different *Taq* DNA polymerase enzymes, three additional PCR kits were assayed. The same primer concentrations were used for all the PCR reactions, which resulted in consistent results allowing an unambiguous species identification. Each *Taq* polymerase enzymes achieved different PCR amplification times. HS Perfect Mix (TaKaRa) reached the shortest time (40 min). Nevertheless, we chose to show the amplification results only for Ex *Taq* polymerase (TaKaRa, Japan) because we consider this enzyme of standard use in more laboratories (among all the enzymes used in this study).

Except for the weak unspecific PCR product amplified by the primer ArPu228R by cross-reaction with non-target *A. irradians* template, all our SSPs showed only specific amplification when tested against non-target scallop species. Besides, ArPu228R did not amplify any processed samples of *A. irradians*. The unspecific amplicon size (about 1.5 kb) observed only in fresh samples of *A. irradians* was larger than all species-specific bands (maximum size was 918 bp in *A. macassarensis*). Thus, this result would not interfere with the ability to detect all target species. In this study, only five individuals of *A. macassarensis* were analyzed. Thus, it should be noted that more individuals of this species are required to further validate the AnMa918R primer. In this work, the scallops *M.*

388 *yessoensis* and *A. irradians* included heavily processed presentations (e.g.
389 canned). The small PCR amplicon sizes intentionally designed for these species
390 increased the chance of successful PCR amplification using its degraded target
391 DNA. However, in some canned samples, a weak positive control band was
392 obtained, due to the higher molecular size amplicon and degraded target DNA.
393 Thus, caution must be taken if canned presentations or heavily degraded target
394 DNA from other scallop species are analyzed with our assay, especially when
395 using the SSPs designed to amplify PCR products higher than 400 bp.

396 The restriction enzyme *Hae*III was successfully applied for the accurate
397 differentiation between *P. maximus* and *P. albicans*. Even though an additional
398 RFLP analysis was necessary for the discrimination between these two species,
399 this assay can be useful for routine taxonomic surveys. Nowadays, *P. albicans* is
400 commercialized only spontaneously in some Japanese local markets, and there is
401 neither exportation nor processing industry of this species. Whereas *P. maximus*
402 is commonly commercialized throughout Europe as fresh and processed food.
403 Thus, it is unlikely that those two scallop species occur in the same market. We
404 encourage the design of further assays for the discrimination among more *Pecten*
405 species such as *P. jacobeus* (Mediterranean Sea), *P. sulcicostatus* (South Africa), *P.*
406 *fumatus* (Australia) and *P. novaezelandiae* (New Zealand). All aforementioned
407 *Pecten* species have similar morphological appearance and represent
408 commercially important fisheries.

409 The present study provided some important advantages over previous
410 related studies. Previous reports on scallop species identification by SSPs
411 multiplex covered from 2 to 3 scallop species (Colombo *et al*. 2004; Bendezu *et al*.

2005; Marshall *et al*. 2007; Marín *et al*. 2013) that occur or are sold in the same geographic region, thus restricting the utility of those assays only to a limited group of researchers or institutes. In this study, by designing GSP and SSPs for a total of nine scallop species, which occur naturally or artificially in five continents and most of them are shipped internationally, the utility of our multiplex assay was outstandingly increased. Besides, our assay showed to be more advantageous not only because it addressed the deficit of SSPs of commercially important scallop species by developing a relatively higher number of SSPs, but also because of the secondary potential utility of the positive control reaction. Typically, multiplex PCR assays include a positive control as an indicator for any enzymatic failure during PCR reaction. Indeed, in our multiplex assay, the family-specific primer set used as positive control has an essential secondary function: it can be used as a "wildcard" reaction because it will amplify the DNA from every Pectinidae species. Therefore, our assay is not only useful for the identification of the 9 target species analyzed herein, but also for many more members of the Pectinidae family. If only the positive control PCR product from an unknown scallop species is obtained, it can be further sequenced and identified using BLAST or DNA barcoding. To demonstrate the utility of the positive control reaction contained in the multiplex PCR cocktail, we included the DNA of one more scallop species, *A. pleuronectes*, which was expected to produce one single band amplified only by the Pectinidae family-specific primer set. This PCR product was further sequenced and the "unknown" species was correctly identified by DNA barcoding approach (data not shown).

All scallop species identified in this study are of high commercial importance, and most of them are commercialized internationally. Consequently, in its present form, our multiplex PCR method is of great utility and could be easily used as a commercial kit by laboratories from different research institutes and governmental agencies that regulate seafood labeling around the world.

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559

560 **Figure captions**

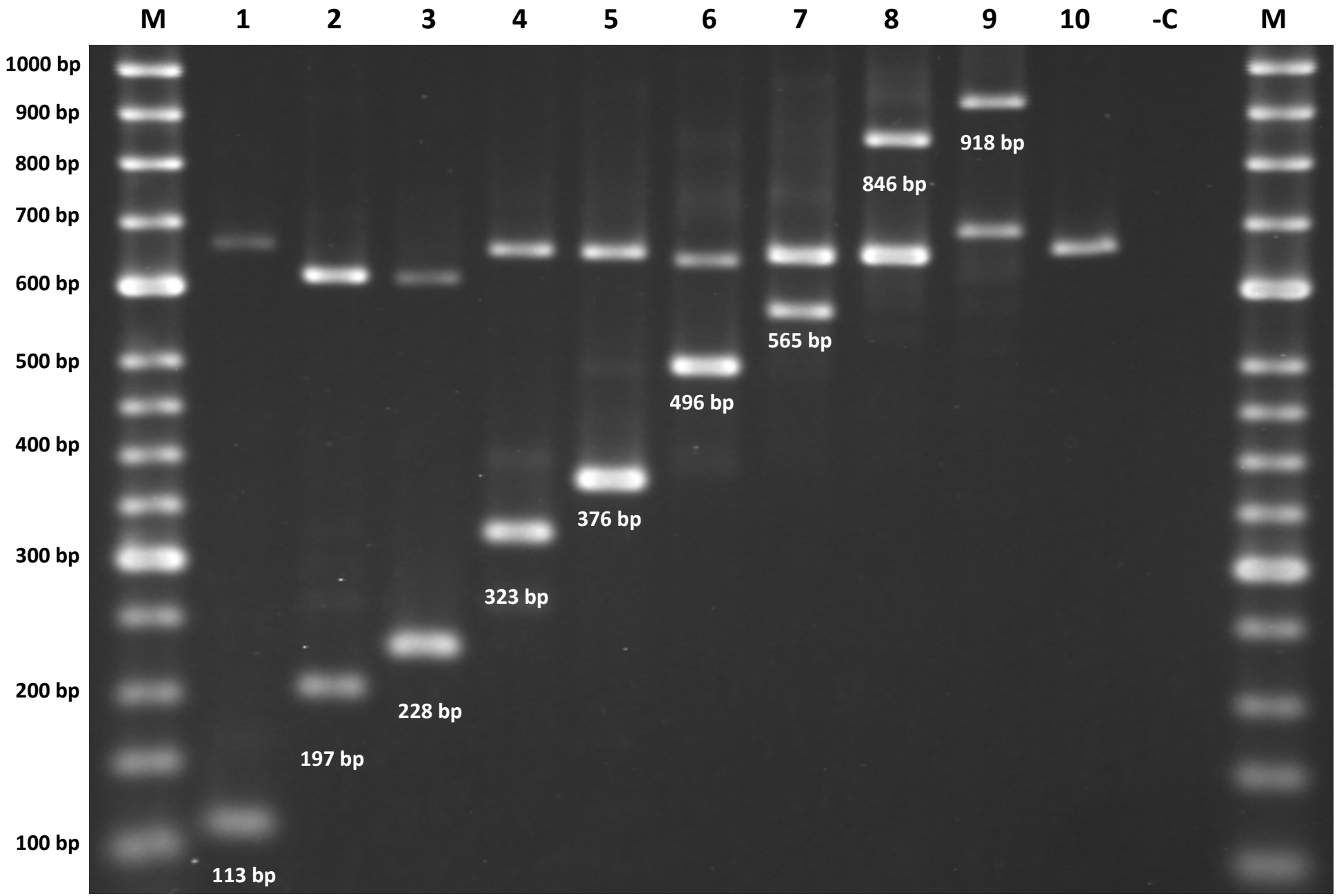
561 **Figure 1** Agarose gel electrophoresis of decaplex PCR using 8 species-specific
562 primers and one genus-specific primer in 9 scallop species. Each species
563 yielded a PCR product of different size (indicated in bp: base pairs) plus a
564 positive control band that ranged from 600 to 700 bp, depending on species.
565 M: 50 bp ladder, lane 1: *Mizuhopecten yessoensis* 113 bp, lane 2: *Argopecten*
566 *irradians* 197 bp, lane 3: *Argopecten purpuratus* 228 bp, lane 4: *Pecten*
567 *maximus* 323 bp, lane 5: *Chlamys farreri* 376 bp, lane 6: *Placopecten*
568 *magellanicus* 496 bp, lane 7: *Mimachlamys nobilis* 565 bp, lane 8:
569 *Bractechlamys vexillum* 846 bp, and lane 9: *Annachlamys macassarensis* 918
570 bp. Only a positive control band is observed in Lane 10: *Amusium*
571 *pleuronectes* (non-target species). –C: negative control

572 **Figure 2** Agarose gel electrophoresis of a RFLP (restriction fragment length
573 polymorphism) assay for the identification of *Pecten maximus* and *Pecten*
574 *albicans* using *Hae*III enzyme. A 654 base pair (bp) size amplicon in *P.*

575 *maximus* (lanes 1 to 5) amplified by the Pectinidae family-specific primer set
576 Pect16BC was uncut, whereas the 655 bp size amplicon amplified by the
577 same family-specific primers in *P. albicans* (lanes 6 to 10) resulted in two
578 diagnostic fragments of 286 and 369 bp. M: 100 bp ladder

579 **Supplementary material 1**

580 Alignment of a partial fragment of the 5' end of the 16S gene in 9 scallop
581 species showing their species-specific reverse primer site locations. Target
582 species names are highlighted in bold and primer sites for each target
583 species are highlighted in grey


M
1
2
3
4
5
6
7
8
9
10
-C
M
1000 bp
900 bp
800 bp
700 bp
600 bp
500 bp
400 bp
300 bp
200 bp
100 bp
113 bp
197 bp
228 bp
323 bp
376 bp
496 bp
565 bp
846 bp
918 bp

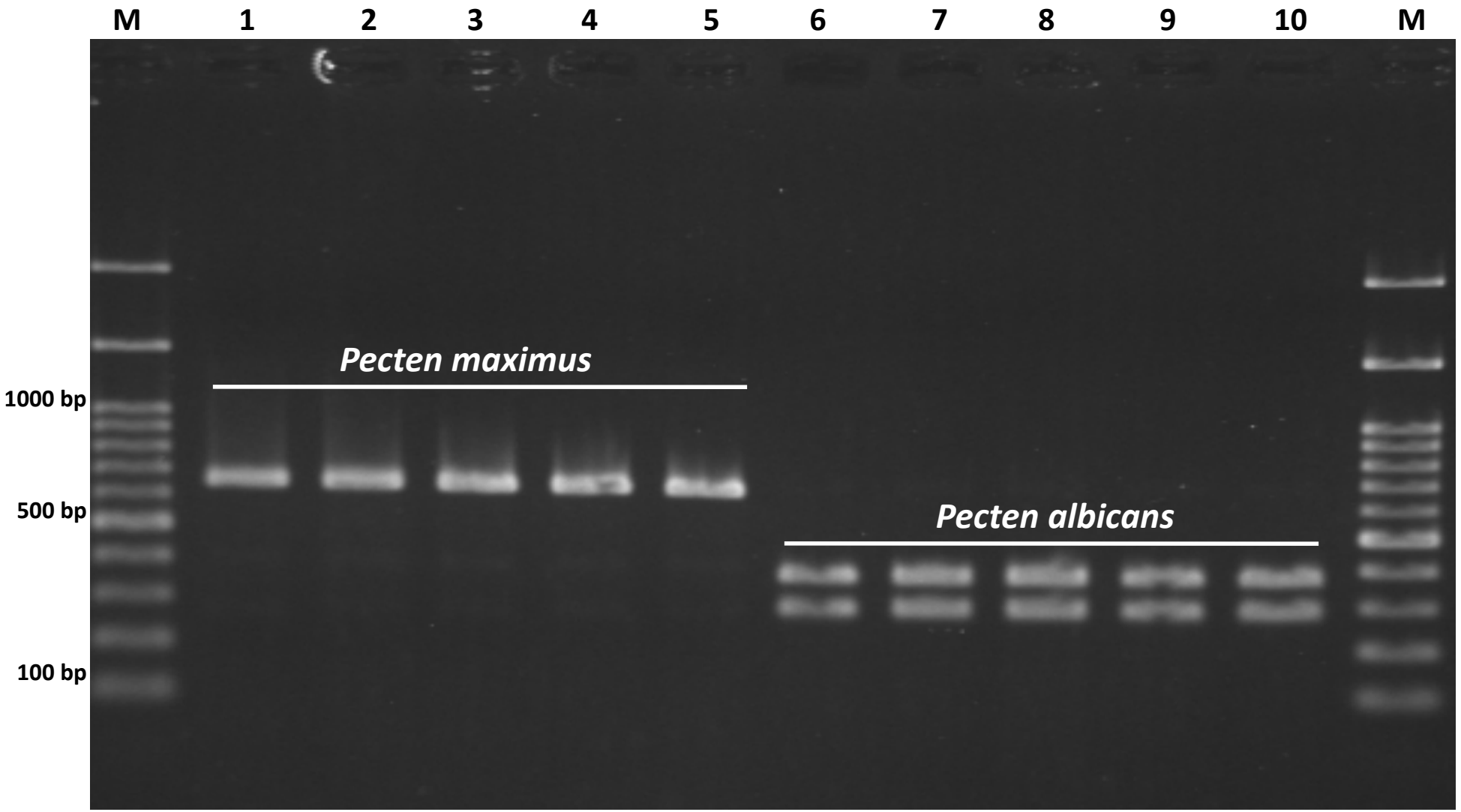

M
1
2
3
4
5
6
7
8
9
10
M
1000 bp
500 bp
100 bp
Pecten maximus
Pecten albicans

Table 1 Information of all scallop species collected from wild and local markets as fresh and processed food analyzed in this study

Species	Type	Origin/ Treatment	Sample size	Name on label (Japanese name)	Origin on label
Mizuhopecten yessoensis	Fresh	Mori, Japan	15		
		Muroran, Japan	15		
	Processed	Local market, Japan			
		- Dried	3	Japanese scallop (Hotategai)	Wakkanai (Japan)
		- Smoked and canned	3	Japanese scallop (Hotategai)	Japan
		- Seasoned	3	Japanese scallop (Hotategai)	Japan
		- Cooked and seasoned	3	Japanese scallop (Hotategai)	Japan
		- Simmered	3	Japanese scallop (Hotategai)	Japan
		- Canned	3	Japanese scallop (Hotategai)	Japan
Argopecten irradians	Fresh	Qingdao, China	16		
	Processed	Local market, Japan			
		- Boiled and frozen	3	Bay scallop (Itayagai)	China
		- Canned	3	Bay scallop (Itayagai)	
		- Simmered	3	Bay scallop (Itayagai)	
Argopecten purpuratus	Fresh	Sechura Bay, Peru	30		
		Independencia Bay, Peru	30		
	Processed	Local market, UK			
		- Frozen	5	Peruvian scallop	
Pecten maximus	Fresh	Rye Bay, UK	2		
		Local market, UK	2	King scallop	South Coast
		Local market, UK	10	King scallop-*Pecten maximus*	North East Atlantic
Chlamys farreri	Fresh	Hakodate, Japan	15		
Placopecten magellanicus	Fresh	Local market, Canada	5	East Coast scallops	
	Processed	Local market, UK			
		- Frozen	10	Wild Atlantic raw scallop	North West Atlantic
Mimachlamys nobilis	Fresh	Oki Islands, Japan	30		
Bractechlamys vexillum	Fresh	Sicogon Island, Philippines	18		
		Molocoboc Island, Philippines	30		
Annachlamys macassarensis	Fresh	Molocoboc Island, Philippines	5		
Pecten albicans	Fresh	Shimoda, Japan	2		
		Oki Islands, Japan	17		
Amusium pleuronectes	Fresh	Bangkok, Thailand	1		
TOTAL			285		

Table 2 List of primers used in the decaplex PCR assay based on the 5' end of the 16S gene in scallops

Species	Primer	Sequence 5'-3'	Amplicon length (bp)	Reference
All Pectinidae	Pect16BCF	CGTACCTTTTGCATCATGG	600-700	Marín *et al*. 2015
All Pectinidae	Pect16BCR	GCACGATTTACCGCGTCCGTTTA	600-700	Marín *et al*. 2015
Mizuhopecten yessoensis	MiYe113R	TCTCGTCTCTGGATGATAC	113	This study
Argopecten irradians	ArIr197R	CCAGCCTACTTGATGAAA	197	This study
Argopecten purpuratus	ArPu228R	GGCTCCCACCAAAAACTAAA	228	This study
Pecten maximus	PeMa323R	CCACTTCTTAGCCTTTCC	323	This study
Pecten albicans			324	
Chlamys farreri	ChFa376R	GCAATGAAGGTTAGTGTGAGGTGA	376	This study
Placopecten magellanicus	PlMa496R	CCCCTTACTAGCTTTTCTTCTA	496	This study
Mimachlamys nobilis	MiNo565R	GATAAACCAGGCGGTTCC	565	This study
Bractechlamys vexillum	BrVe846R	CAAAAGTCGACCCTACGG	846	This study
Annachlamys macassarensis	AnMa918R	ACAGCCCAGGAATCAGAT	918	This study

bp, base pairs

Novel decaplex PCR assay for simultaneous detection of scallop species: designing species-specific primers on highly variable 5' end of the 16S rRNA gene

Aquaculture Research

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	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125
M. yessoensis	T	G	G	T	G	T	T	G	T	A	**G**	**T**	**-**	**A**	**T**	**C**	**A**	**T**	**C**	**C**	**A**	**G**	**A**	**G**	**A**	**C**	**G**	**A**	G	A	A	G	A	T	A	G	T	G
A. irradians	T	G	G	C	G	T	A	A	T	A	A	-	-	G	C	T	A	T	C	T	T	T	A	G	A	T	G	G	A	A	A	A	A	T	A	G	T	G
A. purpuratus	T	G	G	C	G	T	A	G	A	A	G	-	-	G	C	T	A	T	C	T	T	T	A	G	A	T	G	G	A	A	G	A	A	T	A	G	T	G
P. maximus	T	G	G	C	G	T	T	A	T	A	T	T	T	G	C	T	A	T	C	T	G	G	A	G	A	T	G	G	G	G	A	G	A	T	A	G	T	G
C. farreri	T	G	G	C	G	T	T	G	A	A	T	T	-	G	C	T	A	T	C	C	A	G	A	G	A	T	G	G	G	G	A	G	G	T	A	G	C	G
P. magellanicus	T	A	A	C	G	T	T	T	T	A	T	G	-	G	T	T	A	T	C	C	A	A	A	G	A	T	A	G	G	G	G	G	A	T	A	G	T	G
M. nobilis	T	G	G	C	G	T	T	G	A	A	T	T	-	G	C	T	T	T	C	C	G	T	A	G	A	C	A	A	G	A	A	T	G	T	A	G	T	G
B. vexillum	T	G	G	C	G	T	T	G	A	A	G	A	-	G	C	T	A	T	C	C	G	A	A	G	A	T	A	G	G	G	A	G	A	T	A	G	T	G
A. macassarensis	T	G	G	C	G	T	T	G	G	A	T	A	-	G	C	T	A	T	C	C	G	G	A	G	A	C	G	G	A	G	A	G	A	T	A	G	T	G

	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224
M. yessoensis	T	A	G	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	T	-	T	T	T	T	G	G	G	G	C	G	G	G	T	T	C	T	A	C	G
A. irradians	T	A	G	G	**T**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**T**	**T**	**C**	**-**	**-**	**A**	**T**	**C**	**A**	**A**	**G**	**T**	**A**	**G**	**G**	**C**	**T**	**G**	**G**	G	G	G	A
A. purpuratus	T	A	G	G	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	C	C	-	-	A	C	C	T	A	G	-	A	A	G	C	T	A	A	T	G	G	G
P. Maximus	T	A	G	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	T	T	C	G	G	T	T	G	A	T	G	G	G	G	T	T	T	A	C	A	G	T
C. farreri	T	A	G	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	T	A	C	T	T	T	G	G	G	A	C	A	G	A	T	T	C	T	A	C	A	G
P. magellanicus	T	A	G	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	C	T	T	T	T	T	G	G	G	A	C	A	G	G	T	T	C	T	A	C	A	T
M. nobilis	T	A	G	C	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	C	A	T	T	T	T	G	G	G	A	C	G	G	A	T	T	C	T	A	C	G	G
B. vexillum	T	A	G	C	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	C	T	C	T	A	G	T	G	G	-	A	T	G	G	G	T	G	T	T	A	A	A	A
A. macassarensis	T	A	G	C	T	T	T	T	T	G	T	A	G	G	A	T	G	C	A	C	T	C	T	A	A	G	G	G	T	A	A	G	G	G	T	T	T	T	T	A	A	G

	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286
M. yessoensis	T	T	T	A	T	T	T	T	G	T	G	T	T	A	C	-	A	A	G	A	T	T	C	C	G	C	C	C	T	C	T	T	T	T	A	G	G	A	G	A	T	G	G
A. irradians	A	A	C	C	C	C	C	A	G	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	T	T	T	G	A	T	G	G	T	A	G	C	T	C	A	G
A. purpuratus	G	A	C	C	T	**T**	**T**	**A**	**G**	**T**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**T**	**T**	**T**	**T**	**G**	**G**	**T**	**G**	**G**	**G**	**A**	**G**	**C**	**C**	C	A	T
P. maximus	T	A	A	T	T	C	T	A	A	T	A	G	T	A	T	T	C	G	G	-	-	-	G	C	T	G	T	T	T	T	T	C	G	A	A	G	G	G	G	T	C	A	G
C. farreri	T	T	T	T	T	C	T	T	G	G	C	T	T	G	C	C	A	A	G	A	T	G	C	T	G	T	T	C	T	C	T	T	T	T	A	G	G	A	G	A	T	G	G
P. magellanicus	-	-	T	T	A	T	A	G	T	T	G	T	T	T	C	-	-	-	-	-	-	-	-	T	G	T	T	T	T	C	T	A	A	A	A	G	G	A	G	A	T	G	G
M. nobilis	A	A	-	-	-	-	-	-	G	T	G	T	C	T	T	T	A	G	G	A	T	T	G	T	-	C	A	G	T	C	T	T	T	T	G	G	G	A	G	A	T	G	G
B. vexillum	A	A	A	C	T	T	A	G	T	C	G	T	T	G	G	G	C	G	G	T	G	-	G	T	G	T	T	T	T	C	T	A	G	T	G	A	A	A	G	T	T	G	A
A. macassarensis	T	A	T	T	T	A	A	G	G	T	T	T	T	A	G	A	A	G	G	T	A	A	G	T	G	T	T	T	T	T	T	T	T	A	A	A	A	A	G	T	T	G	G

	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366
M. yessoensis	G	G	T	G	A	A	G	T	T	T	T	A	G	G	T	-	-	-	T	T	A	C	A	A	G	T	A	G	G	T	T	T	C	A	A	G	G	A
A. irradians	T	A	T	T	A	A	T	A	A	A	A	T	A	A	A	-	-	-	T	G	G	A	A	A	G	T	G	G	G	C	T	T	A	A	A	G	G	A
A. purpuratus	T	T	T	A	G	A	G	A	A	A	A	G	A	G	A	-	-	-	T	G	G	A	A	A	G	T	G	G	G	C	T	T	A	A	A	G	A	A
P. Maximus	A	A	T	T	A	A	G	A	A	T	**G**	**G**	**A**	**A**	**A**	**G**	**G**	**C**	**T**	**A**	**A**	**G**	**A**	**A**	**G**	**T**	**G**	**G**	G	C	T	T	G	A	A	G	T	A
C. farreri	G	G	T	G	A	A	G	T	C	T	T	A	G	G	T	-	-	-	T	T	G	G	A	A	G	T	A	G	G	T	T	T	T	A	A	G	G	A
P. magellanicus	G	G	T	G	G	A	G	T	G	T	T	G	G	G	T	-	-	-	T	A	G	T	A	A	G	T	G	G	G	C	T	T	G	A	A	G	A	A
M. nobilis	G	G	T	T	A	A	T	T	T	T	T	G	G	G	G	-	-	-	T	T	A	G	A	A	G	T	A	G	G	T	T	T	T	A	A	G	A	A
B. vexillum	G	T	C	G	A	A	G	T	G	G	T	G	A	G	C	-	-	-	A	A	G	G	A	A	G	T	G	G	G	C	T	T	A	A	A	G	G	A
A. macassarensis	G	G	C	G	A	A	G	C	T	A	A	A	G	A	A	-	-	-	T	T	T	G	A	A	G	T	G	G	G	C	T	T	G	A	A	G	G	A

	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419
M. yessoensis	G	T	G	G	G	T	T	A	T	A	C	C	A	C	A	T	A	T	G	G	G	C	C	T	T	T	C	T	T	T	A	A	-	-	A	A	T	T
A. irradians	G	T	G	G	G	T	T	A	A	T	C	C	T	C	A	A	-	T	A	T	T	T	T	T	T	A	T	-	-	T	A	G	T	G	A	-	T	C
A. purpuratus	G	T	G	G	G	T	T	A	A	T	C	C	T	C	A	G	-	T	A	T	T	T	C	T	A	G	T	-	-	T	A	A	T	G	A	-	T	C
P. maximus	G	T	G	G	G	T	T	A	A	A	C	C	A	C	A	A	A	T	G	A	G	C	C	T	T	G	T	A	-	T	A	A	T	A	A	A	T	T
C. farreri	G	T	G	G	G	T	T	**T**	**C**	**A**	**C**	**C**	**T**	**C**	**A**	**C**	**A**	**C**	**T**	**A**	**A**	**C**	**C**	**T**	**T**	**C**	**A**	**T**	**T**	**G**	**C**	A	-	-	A	A	T	A
P. magellanicus	G	T	G	G	G	T	T	A	T	A	C	C	T	C	A	A	T	C	A	G	G	C	T	C	T	T	G	A	-	-	T	G	T	A	A	A	T	A
M. nobilis	G	T	G	G	G	T	T	A	C	A	C	C	A	C	A	C	A	T	A	A	G	C	T	C	T	A	T	T	T	T	A	A	T	A	A	A	T	A
B. vexillum	G	T	G	G	G	T	T	T	G	T	C	C	T	C	A	C	A	A	G	T	G	C	T	C	T	T	T	T	-	-	C	A	T	A	A	A	T	A
A. macassarensis	G	T	G	G	G	T	T	A	T	A	C	C	T	C	A	A	G	G	G	T	T	T	T	T	A	G	C	A	G	G	C	A	T	A	A	A	T	A

	524	525	526	527	528	529	530	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563
M. yessoensis	T	T	T	G	-	C	T	G	C	G	G	G	G	T	A	A	A	A	A	A	T	A	A	A	A	A	T	G	T	A	A	G	A	G	T	G	C	G
A. irradians	G	T	T	-	A	A	T	T	C	G	G	G	A	A	A	T	T	A	G	A	C	G	T	A	A	A	A	C	A	A	A	-	-	-	-	T	A	A
A. purpuratus	A	T	T	T	G	G	T	T	C	A	G	G	A	A	A	T	T	A	A	A	C	G	C	T	A	A	G	T	G	A	A	-	-	-	-	C	G	A
P. Maximus	T	T	T	A	A	G	G	T	C	G	G	G	G	A	A	A	G	A	A	A	C	G	T	A	A	G	G	T	T	A	T	G	T	-	T	C	C	G
C. farreri	T	T	T	G	-	T	T	T	C	A	G	G	G	T	A	A	A	A	A	A	T	A	A	G	A	A	T	A	T	A	A	G	A	A	T	G	C	G
P. magellanicus	T	C	T	T	G	T	T	C	**T**	**A**	**G**	**A**	**A**	**G**	**A**	**A**	**A**	**A**	**G**	**C**	**T**	**A**	**G**	**T**	**A**	**A**	**G**	**G**	**G**	**G**	G	T	G	A	T	G	-	-
M. nobilis	T	T	T	A	-	T	T	A	C	A	A	G	G	A	A	A	A	A	A	A	T	G	T	T	A	A	G	G	T	A	A	G	-	-	T	A	A	T
B. vexillum	C	T	T	A	G	T	T	T	C	G	G	G	G	T	A	T	A	G	G	G	C	G	A	T	A	A	G	G	G	G	G	A	T	A	A	A	-	G
A. macassarensis	T	T	T	A	-	-	T	T	C	G	G	G	G	G	A	T	A	A	A	A	C	G	T	G	A	A	G	G	G	T	T	A	A	A	A	C	C	G

	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626
M. yessoensis	G	G	C	A	A	A	A	G	A	G	A	G	G	A	C	C	G	C	C	T	G	-	T	T	T	A	G	C	A	A	A	A	A	C	A	T	G	G
A. irradians	G	G	C	A	A	A	A	A	T	G	A	A	A	A	C	C	G	C	C	T	G	-	T	T	T	A	A	C	A	A	A	A	A	C	A	T	G	G
A. purpuratus	G	G	C	A	A	C	A	A	C	G	A	A	A	A	C	C	G	C	C	T	G	-	T	T	T	A	G	C	A	A	A	A	A	C	A	T	G	G
P. maximus	G	G	C	A	A	A	T	A	A	G	G	G	G	A	C	C	G	C	C	T	G	-	T	T	T	A	T	C	A	A	A	A	A	C	A	T	G	G
C. farreri	G	G	C	A	A	A	A	T	A	G	A	G	G	A	C	C	G	C	C	T	G	-	T	T	T	A	T	C	A	A	A	A	A	C	A	T	G	G
P. magellanicus	G	G	C	A	A	A	A	T	A	G	G	A	G	A	C	C	G	C	C	T	G	-	T	T	T	A	T	C	A	A	A	A	A	C	A	T	G	G
M. nobilis	G	G	C	A	A	A	A	G	A	G	**G**	**G**	**A**	**A**	**C**	**C**	**G**	**C**	**C**	**T**	**G**	**G**	**T**	**T**	**T**	**A**	**T**	**C**	A	A	A	A	A	C	A	T	G	T
B. vexillum	G	G	C	A	A	A	A	T	A	G	G	G	G	A	C	C	G	C	C	T	G	-	T	T	T	A	T	C	A	A	A	A	A	C	A	T	G	G
A. macassarensis	G	G	C	A	A	G	A	A	A	G	A	G	G	A	C	C	G	C	C	T	G	-	T	T	T	A	A	C	A	A	A	A	A	C	A	T	G	G

	897	898	899	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934
M. yessoensis	-	C	G	T	T	A	A	A	G	A	G	C	A	-	-	G	A	T	A	G	G	G	C	T	T	T	G	G	A	T	C	T	T	T	T	T	T	T
A. irradians	G	C	T	A	C	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	T	T	C	T	-	-	-	-	-	-	-	-
A. purpuratus	G	T	T	A	C	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	C	C	T	T	-	-	-	-	-	-	-	-
P. maximus	G	C	T	A	T	T	T	G	T	G	G	C	C	G	-	-	-	-	-	-	-	-	-	A	A	T	C	G	T	T	G	A	G	G	A	G	C	A
C. farreri	G	C	G	T	T	T	T	A	G	A	A	C	T	T	T	A	A	T	T	T	T	C	C	A	A	T	A	C	G	G	C	T	A	G	A	G	T	T
P. magellanicus	G	G	C	T	A	G	-	A	G	G	G	T	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	A	G	G	G	A	T	T	T	G	G
M. nobilis	T	T	T	T	T	T	T	T	G	A	G	T	T	-	-	-	-	-	-	-	-	-	-	-	-	T	G	T	A	G	A	T	G	T	A	A	G	T
B. vexillum	G	C	**C**	**C**	**G**	**T**	**-**	**A**	**G**	**G**	**G**	**T**	**C**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**G**	**A**	**C**	**T**	**T**	**T**	**T**	**G**	A	A
A. macassarensis	G	T	T	T	A	T	G	A	G	G	G	T	T	T	-	-	-	-	-	-	-	-	-	-	G	G	G	G	G	T	T	T	G	T	T	T	A	T

	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963
M. yessoensis	T	C	T	T	T	T	T	T	T	G	A	A	G	G	T	C	C	C	G	G	C	T	-	-	T	T	A	T	G	G	C	T	T	T	T	G	A	G
A. irradians	T	-	-	-	-	-	-	-	-	-	-	-	-	G	C	C	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. purpuratus	T	-	-	-	-	-	-	-	-	-	-	-	-	A	G	C	T	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. maximus	T	G	A	G	G	A	G	C	A	C	T	A	G	A	G	C	T	T	A	G	T	G	T	-	-	-	-	-	-	-	-	-	-	T	T	C	A	G
C. farreri	G	C	T	A	G	A	G	T	T	A	G	G	G	G	T	A	T	T	G	G	A	T	A	T	T	T	A	T	A	G	T	T	T	T	T	A	A	G
P. magellanicus	G	G	G	A	T	T	T	G	G	T	T	G	G	T	T	T	C	T	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	G
M. nobilis	G	A	T	G	T	A	A	G	T	T	G	A	G	A	T	C	T	T	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	G	A	G
B. vexillum	A	C	T	T	T	T	G	A	A	T	T	A	G	T	T	T	C	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	G
A. macassarensis	T	T	T	G	T	T	T	**A**	**T**	**C**	**T**	**G**	**A**	**T**	**T**	**C**	**C**	**T**	**G**	**G**	**G**	**C**	**T**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**G**	**T**	T	A	T	T	A	G