The variable 5' end of the 16S rRNA gene as a novel barcoding tool for scallops (Bivalvia, Pectinidae)

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Abstract

Scallops (Bivalvia, Pectinidae) are among the most valuable source of marine food. With about 350 extant species distributed worldwide and a total global production comprising 18 species, the development of proper species-level identification assays is imperative. DNA barcoding has proven to be a useful tool in species identification. A partial region at the 5' end of the mitochondrial cytochrome c oxidase subunit I (COI) gene, known as the ''Folmer region,'' was proposed as the most suitable DNA barcoding marker. However, Folmer primers have failed to amplify polymerase chain reaction (PCR) products in different organisms, including scallops. Searching for an alternative barcoding gene region, we analyzed the complete mitochondrial 16S rRNA gene in 15 scallop species. We found that the interspecific variation at the 5' end is twice as high as that at the 3' end. Based on that evidence, we designed a novel Pectinidae family-specific primer set, aiming to amplify a partial region at the 5' end of the 16S rRNA gene, and tested its suitability as a barcoding tool. A neighbor-joining analysis identified correctly 100 % of the scallop specimens analyzed, with high bootstrap support. Our new primers are well suited for DNA barcoding analysis and may contribute to scallop food industry surveys, as well as routine taxonomic surveys.

Introduction

Scallops (family Pectinidae) are ecologically and commercially important marine bivalves that occur throughout the world's oceans. There are about 350 scallop species with abundant fossil records, and total global production is comprised of 18 species [1]. During 2012, overall global production was estimated at 2.4 million t valued at approximately $4.3 billions [2]. The Japanese scallop *Mizuhopecten yessoensis* is among the most important wild scallop species. In 2011, total Japanese production of *M. yessoensis* was estimated at 303,000 t [3]. In 2013, the Japanese scallop fishery, managed by the Hokkaido Federation of Fisheries Cooperative Association in Japan gained certification for meeting the global standard for sustainable and well-managed fisheries set by the Marine Stewardship Council (MSC) (http://www.japanfs.org/en/news/archives/news_id034273.html). The global scallop market shows clear increasing trend. For instance, exportation of Peruvian scallop *Argopecten purpuratus* increased from 5,300 t during 2012 to 9,000 t in 2013, demonstrating a 68% increase (http://www.globefish.org/bivalves-june-2014.html). Mislabeled scallop import products have been used fraudulently to obtain higher prices or to replace the product due to lack of supply (http://www.globefish.org/bivalves-august-2011.html). Manufactured scallop products are mainly traded without shells, hampering species identification [4]. Thus, the development of proper species-level identification assays is imperative for the detection of mislabeled processed products and sustainable fishery management.

DNA barcoding is currently used in various fields including conservation, food science and cryptic species identification. Due to its high nucleotide variability and

66 relatively fast evolution rate, the COI gene is widely used for species identification [5].
67 Universal primers designed by Folmer et al. [6] are the most commonly used for
68 amplification of a partial fragment of the COI gene across multiple taxa. Folmer primers
69 have been chosen as consensus primers by the Consortium for the Barcode of Life [5].
70 However, in spite of the robustness of the Folmer primers, they have failed to amplify
71 PCR products in different marine organisms, including fish [7], crabs [8], echinoderms
72 and ascidiacea [9], decapods [10, 11], venus clams [12] and scallops [13]. Furthermore,
73 putative pseudogenes (i.e., nuclear mitochondrial DNA "numts") have been also
74 reported after PCR amplification with Folmer primers [8, 11]. Due to low COI universal
75 primer specificity reported in some animal groups and also because of the variable
76 mutation rate of COI gene among different groups, this marker may not be the most
77 appropriate for all taxa [14]. Consequently, the performance of alternative
78 mitochondrial barcoding markers, such as cytochrome b and 16S rRNA genes, has been
79 analyzed in different organisms [13, 15].

80 Universal primers designed by Palumbi et al. [16] to amplify a partial fragment of
81 the 3' end of the 16S rRNA gene have been extensively used in marine bivalves and
82 scallops, including phylogenetics [17-22], population genetics [23-25], forensics [4] and
83 barcoding studies [13]. However, in spite of the usefulness of Palumbi's primers, there
84 are some issues regarding the performance of broad-range universal primers that will
85 be discussed following our initial discussion. Our previous study about the population
86 genetic structure of *A. purpuratus* [26] was the first to describe a higher variation rate at
87 the 5' end of the 16S rRNA gene of the Pectinidae family, after multiple alignment of the
88 full gene sequences from 15 individuals using novel specific primers. Since then,
89 Pectinidae studies have not explored the utility of the "other side" of the 16S rRNA gene,

the 5' end. Taking our previous results into account, we analyzed and tested the potential of the 5' end region to act as a molecular marker and its utility as DNA barcoding tool in scallops. For this purpose, we developed a barcoding assay using novel Pectinidae family-specific primers, targeting a partial fragment of the highly variable 5' end of the 16S rRNA gene. Fresh, as well as processed, samples from 14 scallop species were analyzed, including six expensive scallops traded globally, such as *Mizuhopecten yessoensis*, *Placopecten magellanicus*, *Pecten maximus*, *Argopecten purpuratus*, *A. irradians*, and *Chlamys farreri*.

Materials and methods

Scallop sampling and DNA isolation

Fourteen scallop species representing ten genera were collected in Peru, Canada, the Philippines, China, Japan and England from wild locations and local markets (fresh and processed products; Table 1). When possible, more than one location per species was sampled. Adductor muscles were removed and preserved immediately in 95% ethanol. Except for *Amusium japonicum* and *Chlamys hastata* (one individual each), from two to eight individuals were analyzed for each species. DNA isolation from fresh tissue was performed according to standard phenol-chloroform protocol. As for the processed scallop samples, we followed the protocol described in Sokolov [27] with minor modifications by Marín et al. [4].

Complete mitochondrial 16S rRNA gene determination

The complete mitochondrial 16S rRNA gene (hereafter 16S gene) was determined in seven scallop species (*Amusium japonicum*, *Annachlamys macassarensis*, *Bractechlamys vexillum*, *Decatopecten radula*, *Chlamys hastata*, *Mimachlamys sanguinea*, and *Pecten maximus*) following the same strategy as Marín et al. [26] with a combination of degenerated and species-specific primers (complete primers listed in Table S1, Supporting information). PCR conditions were optimized according to the primer's annealing temperatures for each species (conditions are available upon request). When unexpected PCR products were visualized, the specific band was excised and purified using a QIAquick Gel Exraction Kit (Qiagen, Hilden, Germany). PCR amplicons were sequenced using an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, Hitachi, Japan). The start and end of the 16S genes were determined by multi-aligning complete bivalve mitogenome sequences retrieved from GenBank.

Pectinidae 16S gene 5' end primers design

To design robust primers that enable amplification of a partial fragment at the 5' end of the mitochondrial 16S gene in all species analyzed, the complete 16S genes developed in this study, plus six other complete scallop 16S genes available from mitogenomes deposited in GenBank database, were multi-aligned using MEGA 5.2.2 software [28]. The main criteria for primer design were a minimum length of 19 nt and no priming site polymorphism within the 8 nt from the 3' end of the primer. Accordingly, one forward Pect16BCF 5'-CGTACCTTTTGCATCATGG-3' and one reverse Pect16BCR 5'-GCACGATTTACCGCGTCCGTTTA-3' primers were developed based on highly conserved regions. Since a reliable DNA barcoding marker is supposed to be able to authenticate

processed food samples that have been through extreme conditions (e.g., high temperature and pressure) that can degrade the DNA, we designed the primers to amplify fragments no longer than 700 bp. The Sequence Manipulation Suite [29] was used to evaluate the quality and characteristics of the primers.

PCR, sequencing and primers performance

The optimal annealing temperatures for the novel 5' end 16S gene primers were determined using a gradient ASTEC G02 GeneAtlas thermal cycler. PCR conditions were as follows: 50 ng of template DNA; 40 µM dNTPs; 1X Ex *Taq* buffer (TaKaRa Bio, Shiga, Japan); 0.5 µM each primer; and 0.025 U Ex *Taq* polymerase (TaKaRa) in a total volume of 20 µl. PCR thermocycling conditions were: initial denaturation for 5 min at 94 °C; followed by 30 cycles of denaturation for 15 s at 94 °C; annealing for 30 s at 60 °C; and extension for 30 s at 72 °C; followed by a final extension for 7 min at 72 °C. PCR products were visualized in a 1.5% agarose gel and sequenced using an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems). In order to identify the possibility of pseudogene (i.e. numts) amplifications, all PCR products were carefully examined for the presence of unspecific or multiple bands, double or mixed peaks in electropherograms and unexpected phylogenetic placements. Additionally, to evaluate the family specificity of our primers, we tested them in one species of Ostreoidea and two species of Mytiloidea (two individuals each species), using additional reactions with universal primers as positive controls.

157 Interspecific variation at both extremes of the 16S gene

158 To compare the interspecific genetic variation between the 5' and 3' extremes of the 16S
159 gene, genetic distance values were calculated in partial sequences of 15 scallop species
160 using the Kimura-2 model (K2P) [30]. One individual sequence was used per species.
161 Partial sequences belonging to the 3' end were retrieved from the GenBank database.
162 Sequences representing the 5' end were obtained by amplifying our family-specific
163 primers in 14 scallop species, along with an additional scallop sequence from the
164 GenBank database, which was extracted from its complete mitogenome annotation
165 (Table 2). Mean variation, variable and conserved sites, and parsimony-informative sites
166 were estimated using MEGA software.

167

168 DNA barcoding analyses

169 A total of 69 scallop sequences were multi-aligned using ClustalW as implemented in
170 MEGA. The same software was used to construct a neighbor-joining tree with 1,000
171 bootstrap replicates for node support. We used the tree-based neighbor-joining
172 approach because is the most used in DNA barcoding references. Besides, the aim of
173 DNA barcoding is a fast and accurate species identification rather than phylogenetic
174 inferences [15]. The pearl oysters *Pinctada margaritifera* (NC_021638) and *P. maxima*
175 (NC_018752) were used as outgroups.

176

177 **Results**

Based on structural classification, the analyzed Pectinidae samples covered 9 genera, including *Amusium*, *Annachlamys*, *Argopecten*, *Bractechlamys*, *Decatopecten*, *Chlamys*, *Mimachlamys*, *Mizuhopecten*, *Pecten*, with 13 species. Additionally, five samples labelled as East Coast scallop were bought in a Canadian local market (fresh muscle) and later identified genetically as *Placopecten magellanicus*. The complete 16S gene sequences from seven scallop species determined in this study have been submitted to the GenBank/DDBJ/EMBL DNA databases with accession numbers from KF982785 to KF982791. Comparisons among the complete 16S gene sequences developed herein and those of eight different scallop species retrieved from GenBank (Table 2) showed that *A. irradians* and *C. farreri* have the smallest and largest 16S gene length (1,292 and 1,479), respectively. The overall A+T nucleotide composition content was A=27.27 and T=31.42, with relatively slight variation among species.

Primer performance

No secondary structures (i.e., hairpins) were found after primer quality analysis. Except for *A. purpuratus* and *A. irradians* (both species contain two nucleotide variations at their respective reverse priming sites), all the other species showed perfect matches or single nucleotide variation (Fig. 1). Our novel family-specific primers showed an amplification success rate of 100% in all 14 scallop species. PCR product sizes ranged from 610 bp in *Argopecten purpuratus* to 682 bp in *Decatopecten radulla*. All PCR reactions, including fresh and processed scallop products, were performed under highly stringent conditions resulting in one single specific PCR product in 14 scallop species (Fig. 2). Neither ambiguous nor double peaks results were detected after sequence

analysis. All sequences were submitted to GenBank/DDBJ/EMBL DNA databases under accession numbers KJ000134-KJ000202. Cross-amplification tests showed weak unspecific multiple products at 50 °C annealing and complete failure at 55 and 60 °C in *Crassostrea gigas*, *Mytilus trossolus* and *M. galloprovincialis* (data not shown).

Interspecific variability comparison between the 5' and 3' ends of the 16S gene

The matrix sizes for partial 5' and 3' end regions were 575 and 602 nt, respectively. In spite of the short difference in length size between both matrices (only 27 nt), the interspecific variation from both extremes of the 16S gene showed significant differences. Overall mean variation at the 5' end (0.4130) was twice as high as that of the 3' end (0.2272), which is reflected in the lower number of conserved sites at the 5' end (nearly half of the conserved sites found at the 3' end, Table 3). Within the 5' end region, 387 were variable sites and 318 were parsimony-informative. As for the 3' end, 225 parsimony-informative sites were found out of 280 variable sites. It is clear that the 5' end is the more divergent region among Pectinidae species.

Neighbor-joining and DNA barcoding analysis

The DNA barcoding analysis based on the 5' end of the 16S gene correctly identified 100% of the scallop specimens. The neighbor-joining tree clustered all conspecific species in the same monophyletic group with high bootstrap values (Fig. 3). The 14 species-specific clades spanned four subfamilies. Within the subfamily Chlamydinae, five species were recovered: *Mizuhopecten yessoensis*; *Chlamys hastata*; *Chlamys farreri*;

223 *Mimachlamys sanguinea*; *Mimachlamys nobilis*. The monospecific genus *Placopecten* was
224 included in the subfamily Palliolinae clade. Within the Pectininae subfamily, six species
225 were recovered: *Pecten maximus*; *Pecten albicans*; *Amusium japonicum*; *Annachlamys*
226 *macassarensis*; *Decatopecten radula*; *Bractechlamys vexillum*. Finally, from the basal
227 clade belonging to the Aequipectini group, two species were recovered: *A. irradians* and
228 *A. purpuratus*. The intraspecific 16S gene nucleotide variation ranged from 0 to 1%
229 (mean 0.19%) and the interspecific variation ranged from from 2.16 to 56.42% (mean
230 35.4%). Mean K2P interspecific genetic distance was 186-fold higher than the mean
231 intraspecific variation. The clear separation between mean intraspecific and
232 interspecific divergences, and between the maximum intraspecific and minimum
233 interspecific genetic divergence, indicates the presence of the so-called "barcoding gap".
234 In total, 181 conserved sites (31.7%) and 390 variable sites (68.3%) with 378
235 parsimony-informative sites (66.2%) were found in a sequence matrix of 571 nt.

236

237 **Discussion**

238 A Pectinidae DNA barcoding issue was addressed and analyzed in this study. A previous
239 report [13] tested the COI and 16S genes for barcoding suitability in Pectinidae. As a
240 result, both genes showed barcoding gaps, as well as advantages and disadvantages as
241 barcoding markers. The main pitfall of Folmer primers within Pectinidae was a total lack
242 of amplification. To overcome the limitations of Folmer primers, different studies have
243 reported new universal COI primers or improved versions of Folmer primers; e.g.,
244 marine invertebrates [31], marine metazoans [10], gastropods [32] and scallops [33].
245 However, most of these new universal primers are degenerated, which rises the chances

of unspecific annealing resulting in decreased yield of specific products [34]. Feng et al. [13] concluded that the 3' end of the 16S gene surpasses COI gene in terms of the universality of 16S gene priming sites. That conclusion is accurate, considering that many papers have reported a reduced performance of Folmer primers in different taxa [7-12]. However, it is well known that universal eukaryotic primers not only amplify target species but also their parasite DNA [35, 36], as well as pseudogenes [8]. In spite of this, in many genetic studies, researchers tend to re-use primers from other authors. Consequently, such primers become widely used even though they are not the most suitable markers for certain purposes [37]. A possible solution to avoid the co-amplification of non-targeted DNA is to use more specific primers [38]. PCR amplification using the family-specific primers developed in this study resulted in unambiguous PCR products. After DNA sequencing analysis, all sequences belonged to their target species. Thus, the high specificity of the novel primers reported herein will avoid any potential risk of false positives by cross-amplification.

Based on the 5' end of the 16S gene, a neighbor-joining analysis resolved the Aequipectini group as the most basal group, followed by the Pectininae, Palliolinae, and Chlamydinae as the most recently diverged subfamilies within Pectinidae. Our results are in agreement with a previous Pectinidae phylogenetic classification [21]. Since our primers successfully amplified DNA from members of the four Pectinidae subfamilies, clustered from the base to the top of the phylogenetic tree, we strongly believe that their amplification success in all Pectinidae species is assured. Remarkably, our primers were able to amplify good quality amplicons in all processed scallop products, including boiled-frozen, canned and simmered presentations. A PCR test for the family specificity of our primers resulted in no specific amplification at 50 °C and no amplification at all at

higher annealing temperatures in oysters and mussels. However we cannot rule out the possibility of amplification in closer sister taxa (i.e., Spondylidae, Propeamussidae and Limidae).

The higher interspecific variation found at the 5' end of the 16S gene in Pectinidae can be explained by the fact that different regions of the 16S gene evolve at different evolutionary rates [39]. Similarly, a recent study on higher taxa [40] reported a higher interspecific conservation rate at the 3' end of the 16S gene among 11 species from three different vertebrate groups. Besides, in the same study, a total of six regions within the complete 16S gene were found to be identical among the 11 species, four out of the six interspecific conserved regions were located at the 3' end.

As mentioned in the introduction, the 3' end of the 16S gene has been widely applied for phylogenetic and population genetic studies. However, in spite of the considerably good resolution for phylogenetic insights, the 3' end fragment includes relatively highly conserved regions, which may bias its performance as a barcoding marker, especially when analyzing species that are recently diverged. That can be the case for the congeneric species *M. nobilis* and *M. sanguinea*. Our interspecific comparison analysis of the 16S gene showed that partial sequences from the 3' end region of both *Mimachlamys* species are highly conserved, differing only in a single base (genetic distance 0.0018). On the contrary, partial sequences at the 5' end contained 10 nucleotide mutations, resulting in a genetic distance of 0.0216. The *Mimachlamys* congeneric genetic divergence found at the 5' end was 12-fold higher than that of the 3' end, enabling our barcoding assay to clearly differentiate both species. A neighbor-joining tree placed both *Mimachlamys* species in two discrete subclades within a monophyletic clade supported by a 100% bootstrap value. Thus, our comparative

results, with experimental evidence, have proven the limited potential of the 3' end for delimitation of closely related species.

The K2P congeneric distances between the two *Argopecten* (0.1932 to 0.2066) and two *Chlamys* species (0.1244 to 0.1272) based on the 5' end of the 16S gene are similar to the divergence found in other congeneric bivalve groups (e.g. *Brachidontes spp*., 0.118 to 0.151) [41]. On the other hand, congeneric distances between both *Pecten* species analyzed herein ranged from 0.0411 to 0.0513. *Pecten maximus* and *P. albicans* populations are distributed along the northeast Atlantic and southern Japanese seacoast, respectively. Similar low congeneric genetic distance estimates between allopatric *P.* maximus and *P. novaezelandiae* populations have been reported (0.0123 to 0.0207) based on the 3' end of the 16S gene [42]. Generally, the 16S gene has been found to evolve rapidly in marine bivalves. However, slow evolutionary rates of the mitochondrial 16S gene has been found in flat oysters [42, 43]. A possible relation between flat oyster incubatory habits and low fecundity with the reduced mitochondrial mutation rates has been proposed [43]. Since scallops are known as highly fecund organisms, whether the low mitochondrial divergence commonly found among *Pecten* species is a result of a recent lineage separation or because of an evolutionary slowdown mechanism has yet to be determined.

The only temporary shortcoming for using the 5' end may be that, due to the generalized use of universal primers, researchers rely upon database information based on the 3' end of the 16S gene, which has the widest taxonomic representation in the nucleotide database. However, we should emphasize that the increasing annotations of complete Pectinidae mitogenomes will eventually allow researchers to perceive the 5' end of the 16S gene as a highly useful marker. Furthermore, due to the high interspecific

variation at the end 5' of the 16S gene, our novel family-specific primers can be used in combination with species-specific primers for rapid Pectinidae species identification by single-step multiplex PCR.

This study has proved for the first time that the 5' end of the 16S rRNA gene of the family Pectinidae is so far the most appropriate marker for barcoding analysis. The novel DNA barcoding assay reported herein provides a reliable scallop species identification tool. Due to the robustness of our novel primers and the demonstrated high interspecific variation at the 5' end of the 16S rRNA gene, we propose this barcoding assay to be performed in further Pectinidae studies and encourage supplemental analysis involving more scallop species.

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Figure legends

Fig. 1 Priming site variability of Pectinidae for the primers Pect16BCF and Pect16BCR in 14 scallop species: *Amusium japonicum*; *Annachlamys macassarensis*; *Argopecten irradians*; *A. purpuratus*; *Bractechlamys vexillum*; *Decatopecten radula*; *Chlamys farreri*; *C. hastata*; *Mimachlamys nobilis*; *M. sanguinea*; *Mizuhopecten yessoensis*; *Pecten albicans*; *P. maximus* and *Placopecten magellanicus*

Fig. 2 Agarose electrophoresis of partial 16S rRNA gene PCR products in 14 scallop species: 1. *Argopecten purpuratus*, 2. *A. irradians*, 3. *Placopecten magellanicus*, 4. *Mimachlamys sanguinea*, 5. *M. nobilis*, 6. *Bractechlamys vexillum*, 7. *Mizuhopecten. yessoensis*, 8. *Chlamys hastata*, 9. *Pecten maximus*, 10. *P. albicans*, 11. *C. farreri*, 12. *Amusium japonicum*, 13. *Annachlamys macassarensis*, and 14. *Decatopecten radula*. M: 100 bp ladder, C-: negative control

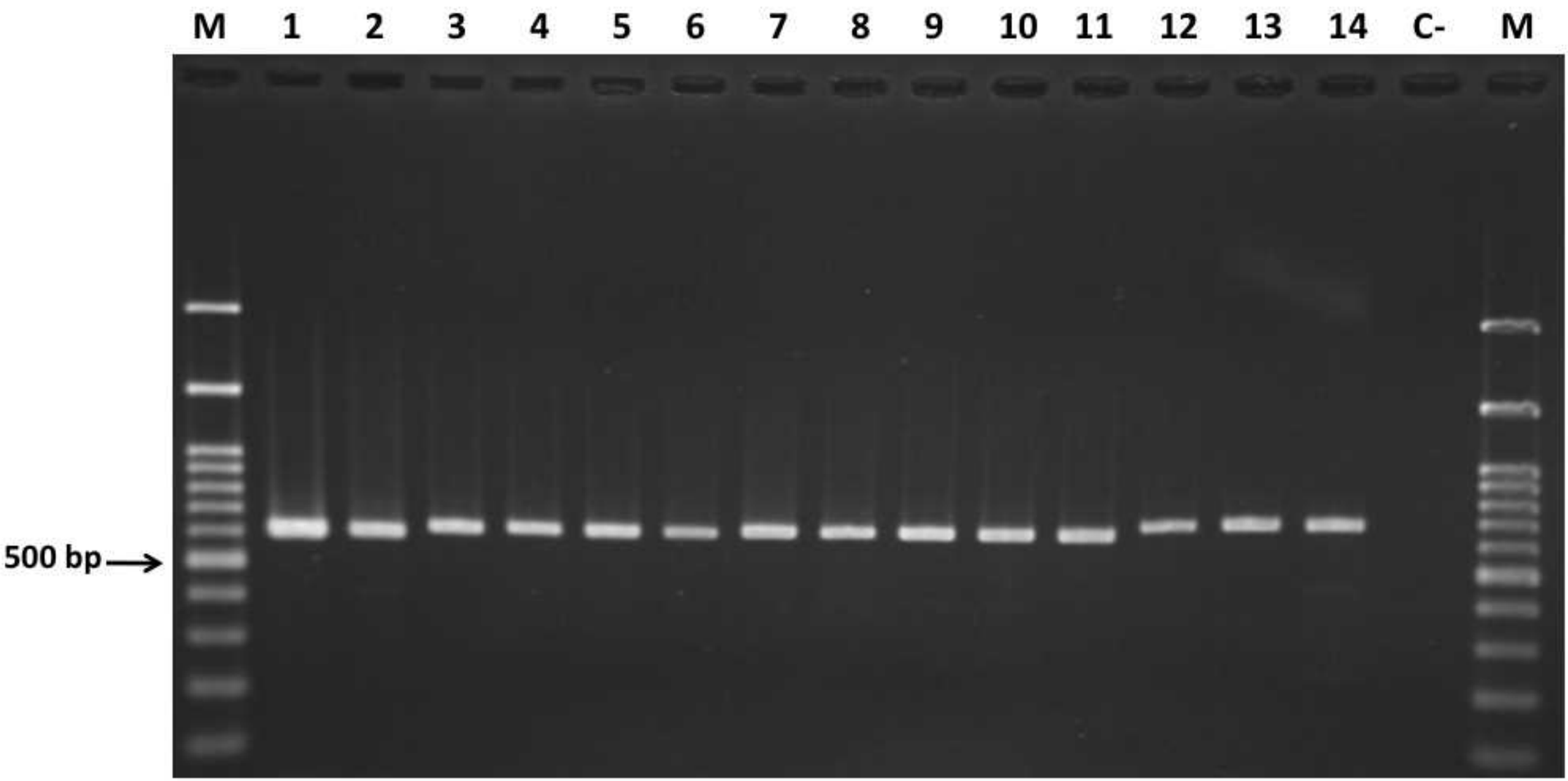
Fig. 3 Neighbor-joining phylogenetic tree based on partial sequences at the 5' end of the mitochondrial 16S rRNA gene in 14 scallop species. Bootstrap values at corresponding nodes are based on 1,000 replicates. Outgroup GenBank accession numbers NC_021638: *Pinctada margaritifera*, and NC_018752: *P. maxima*

Supporting information

Table S1 List of primers used in the amplification of the complete 16S rRNA mitochondrial gene in 7 scallop species

	Forward primer sequence																									
Pect16BCF				5'	**C**	**G**	**T**	**A**	**C**	**C**	**T**	**T**	**T**	**T**	**G**	**C**	**A**	**T**	**C**	**A**	**T**	**G**	**G**	3'			
A. japonicum	T	C	A	-	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	C	-
A. macassarensis	G	T	T	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	A	-
A. irradians	T	C	T	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	T	-
A. purpuratus	T	C	T	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	T	-
B. vexillum	T	C	T	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	-
D. radula	G	C	T	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	T	-
C. farreri	T	C	G	-	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	C	-
C. hastata	G	C	G	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	C	-
M. nobilis	C	T	T	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T	T	-
M. sanguinea	C	C	T	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T	T	-
M. yessoensis	C	G	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	C	-
P. albicans	A	C	T	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	T	A
P. maximus	A	C	T	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	T	A
P. magellanicus	G	T	T	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	T	-

	Reverse primer sequence (reverse complemented)																													
Pect16BCR				3'	**T**	**A**	**A**	**A**	**C**	**G**	**G**	**A**	**C**	**G**	**C**	**G**	**G**	**T**	**A**	**A**	**A**	**T**	**C**	**G**	**T**	**G**	**C**	5'			
A. japonicum	G	G	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A	G
A. macassarensis	A	G	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A	G
A. irradians	A	A	C	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	A	A	G
A. purpuratus	A	A	C	T	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	A	A	G
B. vexillum	A	G	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	A	G
D. radula	A	G	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	A	G
C. farreri	A	G	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A	G
C. hastata	A	G	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A	G
M. nobilis	A	A	C	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A	G
M. sanguinea	A	A	C	C	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A	G
M. yessoensis	A	G	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A	G
P. albicans	G	G	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	A	A	G
P. maximus	G	G	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	A	A	G
P. magellanicus	A	G	C	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A	G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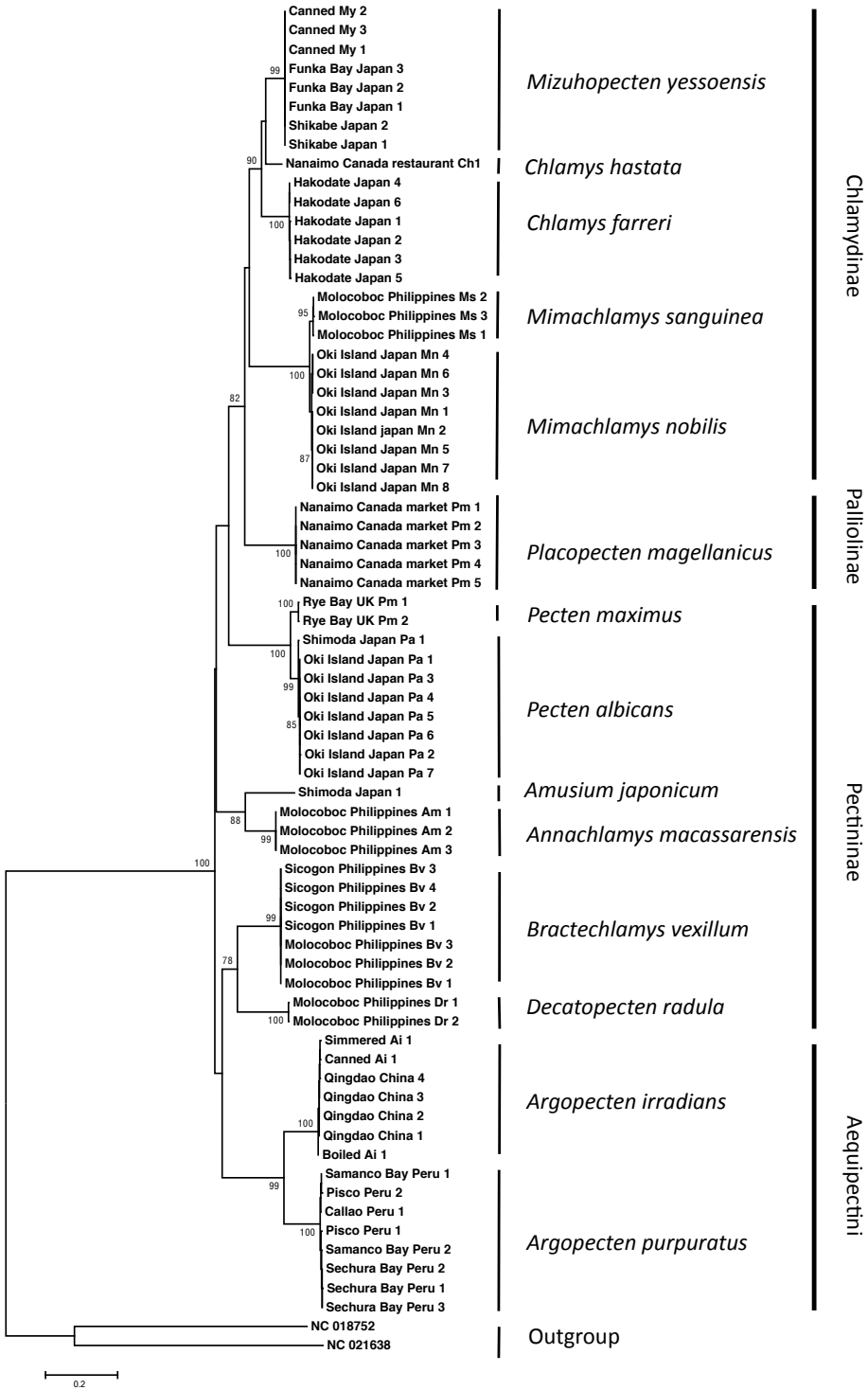
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Funka Bay Japan 1
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Hakodate Japan 4
Hakodate Japan 6
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Hakodate Japan 2
Hakodate Japan 3
Hakodate Japan 5
Molocoboc Philippines Ms 2
Molocoboc Philippines Ms 3
Molocoboc Philippines Ms 1
Oki Island Japan Mn 4
Oki Island Japan Mn 6
Oki Island Japan Mn 3
Oki Island Japan Mn 1
Oki Island japan Mn 2
Oki Island Japan Mn 5
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Nanaimo Canada market Pm 1
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Rye Bay UK Pm 2
Shimoda Japan Pa 1
Oki Island Japan Pa 1
Oki Island Japan Pa 3
Oki Island Japan Pa 4
Oki Island Japan Pa 5
Oki Island Japan Pa 6
Oki Island Japan Pa 2
Oki Island Japan Pa 7
Shimoda Japan 1
Molocoboc Philippines Am 1
Molocoboc Philippines Am 2
Molocoboc Philippines Am 3
Sicogon Philippines Bv 3
Sicogon Philippines Bv 4
Sicogon Philippines Bv 2
Sicogon Philippines Bv 1
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Molocoboc Philippines Bv 2
Molocoboc Philippines Bv 1
Molocoboc Philippines Dr 1
Molocoboc Philippines Dr 2
Simmered Ai 1
Canned Ai 1
Qingdao China 4
Qingdao China 3
Qingdao China 2
Qingdao China 1
Boiled Ai 1
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Pisco Peru 2
Callao Peru 1
Pisco Peru 1
Samanco Bay Peru 2
Sechura Bay Peru 2
Sechura Bay Peru 1
Sechura Bay Peru 3
NC 018752
NC 021638
0.2
Mizuhopecten yessoensis
Chlamys hastata
Chlamys farreri
Mimachlamys sanguinea
Mimachlamys nobilis
Placopecten magellanicus
Pecten maximus
Pecten albicans
Amusium japonicum
Annachlamys macassarensis
Bractechlamys vexillum
Decatopecten radula
Argopecten irradians
Argopecten purpuratus
Outgroup
Chlamydinae
Palliolinae
Pectininae
Aequipectini

Table 1 Pectinidae species (fresh and processed) analyzed in this study

Species	Sub family	Origin	Size bp	Sample size	Haplotypes	Genbank
Mizuhopecten yessoensis	Chlamydinae	Funka Bay, Japan (fresh)	652	3	2	KJ000180-KJ000187
		Shikabe, Japan (fresh)		2		
		Japanese local market (canned)		3		
Chlamys hastata	Chlamydinae	Seafood restaurant, Nanaimo, Canada (fresh)	653	1	1	KJ000166
Chlamys farreri	Chlamydinae	Hakodate, Japan (fresh)	655	6	4	KJ000160-KJ000165
Mimachlamys sanguinea	Chlamydinae	Molocoboc Island, Philippines (fresh)	644	3	3	KJ000177-KJ000179
Mimachlamys nobilis	Chlamydinae	Oki Islands, Japan (fresh)	645	8	2	KJ000169-KJ000176
Placopecten magellanicus	Palliolinae	Local market, Nanaimo, Canada (fresh)	637	5	2	KJ000196-KJ000200
Pecten maximus	Pectininae	Rye Bay, England (fresh)	654	2	2	KJ000201-KJ000202
Pecten albicans	Pectininae	Shimoda, Japan (fresh)	655	1	4	KJ000188-KJ000195
		Oki Islands, Japan (fresh)		7		
Amusium japonicum	Pectininae	Shimoda, Japan (fresh)	679	1	1	KJ000149
Annachlamys macassarensis	Pectininae	Molocoboc Island, Philippines (fresh)	682	3	2	KJ000150-KJ000152
Decatopecten radula	Pectininae	Molocoboc Island, Philippines (fresh)	682	2	2	KJ000167-KJ000168
Bractechlamys vexillum	Pectininae	Sicogon Island, Philippines (fresh)	648	4	1	KJ000153-KJ000159
		Molocoboc Island, Philippines (fresh)		3		
Argopecten irradians	Aequipectini	Qingdao, China (fresh)	611	4	5	KJ000142-KJ000148
		Japanese local market (boiled-frozen, canned, simmered)		3		
Argopecten purpuratus	Aequipectini	Sechura Bay, Peru (fresh)	610	3	7	KJ000134-KJ000141
		Samanco Bay, Peru (fresh)		2		
		Callao, Peru (fresh)		1		
		Pisco, Peru (fresh)		2		

Table 2 Complete 16S rRNA gene comparison in 15 scallop species

Species	**Location**	**Size (bp)**	**A+T content**	**Reference**	**GenBank accession**
Amusium japonicum	Japan	1,412	27.6+30.7	This study	KF982785
Annachlamys macassarensis	Philippines	1,424	26.1+32.0	This study	KF982786
Bractechlamys vexillum	Philippines	1,362	26.9+30.3	This study	KF982787
Decatopecten radula	Philippines	1,403	29.1+29.5	This study	KF982788
Chlamys hastata	Canada	1,415	27.1+30.6	This study	KF982789
Mimachlamys sanguinea	Philippines	1,363	27.0+33.4	This study	KF982790
Pecten maximus	England	1,383	27.5+31.3	This study	KF982791
Argopecten purpuratus	Peru	1,317	27.9+30.5	[26]	HQ677600
Pecten albicans	Japan	1,423	28.0+31.4	[4]	JN896624
Mimachlamys senatoria	China	1,379	24.8+34.2	[44]	NC_022416
Mizuhopecten yessoensis	China	1,424	27.6+30.2	[45]	FJ595959
Argopecten irradians	China	1,292	29.0+30.5	[46]	NC_012977
Chlamys farreri	China	1,479	27.0+31.2	[46]	EF473269
Mimachlamys nobilis	China	1,375	27.3+33.6	[45]	NC_011608
Placopecten magellanicus	Canada	1,387	26.2+31.9	[47]	NC_007234

Table 3 Interspecific variation comparison between the extremes 5' and 3' of the 16S gene in 15 scallop species. Genetic distances were calculated using the Kimura-2 model (Kimura, 1980 [30])

16S gene region	5' end	3' end
Primers 5'-3'	Pect16BCF CGTACCTTTTGCATCATGG Pect16BCR GCACGATTTACCGCGTCCGTTTA [This study]	16Sar CGCCTGTTTATCAAAAACAT 16Sbr CCGGTCTGAACTCAGATCACGT [16]
Matrix size bp	575	602
Mean variation	0.4130	0.2272
Variable sites	387	280
Conserved sites	184	315
Parsimony informative sites	318	225

The variable 5’ end of the 16S rRNA gene as novel barcoding tool for scallops (Bivalvia, Pectinidae)

Fisheries Science

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Table S1 List of primers used in the amplification of the complete 16S rRNA mitochondrial gene in 7 scallop species. "PCR" indicates primers that were used for PCR reactions and "SEQ" the ones used for sequencing

Primer	5'-3'	Reference	*B. vexillum*	*A. macassarensis*	*A. japonicum*	*C. hastata*	*M. sanguinea*	*D. radula*	*P. maximus*
ND1E	CGGCTTCGCCATGATCttyatygcnga	Marín et al. (2013a)	PCR	PCR	-	PCR	PCR	-	-
ND1F	TGTGCCGGAGCAGCttyccncgnta	Marín et al. (2013b)	-	-	PCR	-	-	PCR	PCR
CO1AB	GGTGCTGGGCAGCcayatnccngg	Marín et al. (2013a)	-	-	PCR	PCR	-	-	PCR
CO1BC	CCCGGGGGAAGCTCatrtcratnsc	This study	PCR	PCR	-	-	PCR	PCR	-
16Sar	CGCCTGTTTAACAAAAACAT	Palumbi et al. (1991)	PCR-SEQ	PCR-SEQ	PCR-SEQ	PCR-SEQ	PCR-SEQ	PCR-SEQ	PCR-SEQ
16SR	CCGRTYTGAACTCAGCTCACG	Puslednik et al. (2008)	PCR-SEQ	PCR-SEQ	PCR-SEQ	PCR-SEQ	PCR-SEQ	PCR-SEQ	PCR-SEQ
16Sarl2U	AAGTTACTCCGGGGATAACAGCGTT	This study	SEQ	SEQ	SEQ	SEQ	SEQ	-	-
16SRUniv	CTTCACGGGGTCTTCTCGTCTTTCT	This study	SEQ	SEQ	SEQ	SEQ	SEQ	SEQ	-
BvexiSRU	CTGTAAAAGATGACTCC	This study	SEQ	-	-	-	-	-	-
JapMaSRU	TAACCCACTCCAAAAAG	This study	-	SEQ	SEQ	-	-	-	-
CradUSRU	GATAGTTCCTTAAAACCC	This study	-	-	-	-	-	SEQ	-
HassaSRU	TGGTGTAACCCACTCC	This study	-	-	-	SEQ	SEQ	-	-
16SCC	GCGTAATCCGTCTTGACAGT	Marín et al. (2013a)	-	-	-	-	-	SEQ	SEQ
Palb16S	CTAAGTATAGCTCTTCGGTTGATG	Marín et al. (2013b)	-	-	-	-	-	-	SEQ