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Identification and quantification of two species of oyster larvae using real-time PCR*

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Abstract – A real-time polymerase chain reaction (PCR) assay was developed for the identification and quantification of two oyster species: *Ostrea edulis* and *Crassostrea gigas*. Two sets of primers and TaqMan-MGB probes were designed, based on partial sequences of the 16S rRNA gene. An amplification positive control system was also located in the 18S rRNA gene sequences. Closely related species of oysters and other bivalves, known to co-occur with the target species in European waters, were used to test the assay for cross-reactivity. The assay designed was specific for the target species and no signal or no significant signal was detected for all non-target species tested. The high sensitivity of this method was demonstrated since it is possible to detect just one larva (150–200 μm size) of each species even when it is present with others. Furthermore, this assay provided an acceptable quantification of the number of spiked larvae (1, 10 and 100 larvae) in plankton samples employing a standard curve for larvae.

Keywords: Real-time PCR / Oyster larvae identification / Species identification / 16S rRNA

1 Introduction

The northwest of Spain (Galicia) is one of the regions in the world where the cultivation of bivalves has gained significant importance, due to its suitable environmental and geographical conditions which provide high yields in this area (Figueiras et al. 2002). The flat oyster, *Ostrea edulis* Linnaeus 1758, is an autochthonous species in this area. Nowadays, natural production of this species is almost symbolic due to its high susceptibility to *Bonamia* parasitosis (Iglesias et al. 2005). The high mortality rates of *O. edulis* led to the consequent introduction of the Pacific oyster, *Crassostrea gigas* (Thunberg 1793), mainly from France (Iglesias et al. 2005; Mirella da Silva et al. 2005), which is now the main oyster cultivated in Galicia. This signifies that in the same area it is possible that both species co-occur.

Identification of plankton larvae, particularly bivalves species, is a difficult task, mainly due to their small size (<500 μm) and the great morphological similarity among the different species in the early stages of the biological cycle (Garland and Zimmer 2002). The ability to differentiate between bivalve species in their early larval phases allows for a more comprehensive knowledge of larval dispersal pathways, population connectivity and gene flow. Such knowledge would provide the information needed for proper fisheries and management of wild and cultured marine resources in regions like Galicia, or countries where production of bivalve molluscs is an important economic resource.

Classical methodology for identifying bivalve larvae relies on observation of morphological characters by optical microscopy. Such methodology is typically time consuming and often requires taxonomic expertise as larval phases of bivalves often do not show clear morphological differences. Therefore, due to time constraints, the number of samples which can be analyzed by this procedure are generally low and this hampers monitoring works or studies which require high sampling.

Immunological techniques currently offer a method for identifying plankton larvae. Some authors have successfully applied polyclonal antibodies for larval identification in plankton samples (Paugam et al. 2000; Paugam et al. 2003) but cross-reactions with non-target species. The use of monoclonal antibodies is an alternative method which has been successfully applied in the identification of mussel species (Lorenzo-Abalde et al. 2005; Pérez et al. 2009).

There are other recent methods of species identification based on fluorescent in situ hybridization with DNA probes, FISH-CS (Le Goff-Vitry et al. 2007; Henzler et al. 2010) and

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image analysis (Thompson et al. 2012; Goodwin et al. 2014). In situ hybridization has been effective in zooplankton larvae identification but some aspects, such as autofluorescence, need improvement since they could compromise the effectiveness of labeling techniques by yielding false positives. Although image analysis methods like ShellBi attain high accuracy in the identification of larvae reared in the hatchery, the effect of different growth conditions (temperature and salinity) on shell formation between larvae reared in the hatchery and in the field cause a significant decrease in accuracy (30% if larvae are not grown in similar conditions to those used in the larvae classification). Therefore, improvements in image analysis are needed for application to field samples.

Real-time PCR technique (RT-PCR) has emerged as a powerful and rapid tool for species identification. A number of other nucleic acid amplification methods have also been developed, including: species-specific oligonucleotide probes for shellfish larvae identification (Bell and Grassle 1998); RFLP analysis (Toro 1998; Hosoi et al. 2004; Wang et al. 2006); RAPDs (André et al. 1999); multiplexed species-specific PCR (Hare et al. 2000; Bendezu et al. 2005) and nested PCR (Patil et al. 2005). However, none of these has so far been implemented to the same extent as RT-PCR due to its highly sensitive and rapid quantitative detection ability.

In real time PCR technique fluorescence dyes or probes are introduced into the reaction allowing the PCR product formed during the amplification process to be visualized by monitoring the fluorescence signal emitted. There are two types of RT-PCR analysis depending on the fluorescence source. The simplest type involves the use of intercalating dyes such as SYBR Green. These molecules bind to double-strand DNA, producing an increase in fluorescence which correlates with the amount of dsDNA present. The major drawback is that any double stranded product, including unspecific products or primer-dimers, will be detected and false positives can thus occur. The second type is a more specific method for detecting the accumulation of an amplicon because this involves the use of fluorescent probes that are designed to be complementary to a target sequence within the amplicon. There are several types of probes, such as molecular beacons, scorpions and TaqMan probes (hydrolysis probes). The most commonly used, TaqMan probes, are labelled with reporter and quencher fluorophores in the 5′ and 3′ ends, respectively. Reporter fluorescence is reduced by the quencher as long as the probe is intact, regardless of whether it is attached to its target. When Taq polymerase with a 5′ nuclease activity begins to add nucleotides and hydrolyzes the probe attached to the template DNA, the quencher is separated from the reporter, thereby enabling the emission of fluorescence which is then registered and analyzed by the real time PCR software. The most common method used to analyse the experimental data is the Threshold Cycle Method whereby a fluorescence threshold value, within the exponential phase of the amplification curve, is selected. The PCR cycle at which the sample curve exceeds this fluorescence threshold is the Ct value. This data is used to compare all samples.

Real-time PCR has been successfully used for identifying and quantifying phytoplankton species (Hosoi-Tanabe and Sako 2005), shellfish (Dias et al. 2008) and other marine invertebrate larvae (Vadopalas et al. 2006; Pan et al. 2008; Wight et al. 2009; Smith et al. 2012). Furthermore, in a previous study (Quinteiro et al. 2011), a real-time PCR assay was used for the identification and quantification of Manila clam larvae (*Ruditapes philippinarum*) and successful results were obtained in terms of specificity and sensitivity. Taking into account this background work, the development of a reliable real-time PCR assay for efficient and specific identification and quantification of *O. edulis* and *C. gigas* larvae in plankton samples is proposed.

2 Materials and methods

2.1 Samples collection

Adult samples of *C. gigas*, *C. angulata* and *O. edulis* were collected from several locations as show in Table 1. Further specimens belonging to other adult bivalve species, 99 specimens from 41 species, were obtained from local markets, collected from several locations on the Galicia coast and donated by other research institutions and universities (Table 1).

Plankton samples were collected by CETMAR (Technological Centre of the Sea, Vigo, Spain) from different locations along the northwest coast of Spain in 2009 and 2010. Sampling was done using double oblique tows equipped with a 40-μm mesh at a depth of 10 m. The samples (400–500 L) were filtered again through 40-μm mesh upon return to the laboratory and any retained material was suspended in 20 ml of sterile seawater. Bivalve larvae were isolated using sugar gradient centrifugation (Pérez et al. 2009), suspended in seawater and stored at −20 °C.

C. gigas and *O. edulis* larvae (150–200 μm size) were obtained from single-species experimental cultures at the CIMA-Corón (Center for Marine Research). These larvae were counted under binocular lenses (NIKON SMZ-2T) and transferred by pipette to 1.5-ml microfuge tubes with 20 μl of ethanol (33%). Standard samples containing larvae from 1 to 128 (1, 2, 4, 8, 16, 32, 64 and 128) of each species were used to generate standard quantification curves. Three samples consisting of 1, 10 and 100 cultured larvae of *C. gigas* and *O. edulis* were further isolated and used to spike the CETMAR plankton samples that contained around 250 larvae of other bivalve species, mostly *Mytilus galloprovincialis.*

2.2 DNA extraction

DNA from most adult bivalve samples (with the exception of *C. gigas*) was extracted from 0.2 g of adductor muscle, mantle or foot tissues after overnight digestion in a thermo shaker at 56 °C with 860 μl of lysis buffer (1% SDS, 150 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl pH 8), 100 μl of 5 M guanidium thiocyanate and 40 μl of proteinase K (≥20 Unit mg^{-1}). After 3 h of digestion, extra proteinase K (40 μl) was added to the solution and it was left overnight. DNA was then isolated using the Wizard DNA Clean-Up System kit (Promega) following manufacturer's instructions. *Crassostrea gigas* adult samples were processed with the EZNA Mollusk kit (OMEGA bio-tek). A piece of 0.2 g of adductor muscle was digested

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Table 1. Tissue samples from bivalve species used for the study. IFREMER, France. XG: Consellería do Mar, Xunta de Galicia (Spain).

Species	Key	*N*	Source
Order Mytiloida			
Mytilus edulis	MEDU	2	
Mytilus galloprovincialis	MGAL	1	This work
Xenostrobus securis	XSEC	1	
Order Ostreoida			
Crassostrea angulata	CANG	10	This work
Crassostrea gasar	CGAS	1	IFREMER
Crassostrea gigas	CGIG	19	This work
Crassostrea hongkongensis	CHON	1	Applied Marine Biology Lab, South China Sea Institute of Oceanology (China)
Crassostrea sikamea	CSIK	1	Tohoku National Fisheries Research Institute (Japan)
Crassostrea virginica	CVIR	1	University of Georgia (USA)
Ostrea angasi	OANG	1	IFREMER
Ostrea chilensis	OCHI	1	Southern University of Chile
Ostrea conchaphila	OCON	1	IFREMER
Ostrea edulis	OEDU	31	This work
Ostrea lurida	OLUR	1	Oregon State University (USA)
Ostrea stentina	OSTE	1	IFREMER
Order Pectinoieda			
Aequipecten opercularis	AOPE	1	This work
Chlamys varia	CVAR	1	XG
Limaria hians	LHIA	1	This work
Pecten maximus	PMAX	1	XG
Order Veneroida			
Acanthocardia echinata	AECH	1	This work
Callista chione	CCHI	1	This work
Cerastoderma edule	CEDU	1	XG
Clausinela fasciata	CFAS	1	This work
Dreissena polymorpha	DPOL	1	
Donax trunculus	DTRU	1	
Ensis ensis	EENS	1	
Ensis silicua	ESIL	1	
Gari depressa	GDEP	1	XG
Glycimeris pilosa	GPIL	1	
Laevicardium crassum	LCRA	1	
Ruditapes decussatus	RDEC	1	
Ruditapes philippinarum	RPHI	1	
Scrobicularia plana	SPLA	1	
Solen marginatus	SMAR	1	This work
Spisula solida	SSOL	1	
Venerupis pullastra	VPUL	1	XG
Venerupis rhomboideus	VRHO	1	XG
Venerupis aurea	VAUR	1	
Venus casina	VCAS	1	This work
Venus gallina	VGAL	1	
Venus verrucosa	VVER	1	

in 350 μl of ML1 buffer supplied with the Kit and 40 μl of proteinase K ($\geq$20 Unit mg^{-1}). After 3 h of digestion, another 40 μl of proteinase K was added and it was left overnight. DNA was then isolated following manufacturer's instructions.

DNA extracts from adult samples were quantified by UV-spectrometry at 260 nm and by Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) for dsDNA quantification with a VersaFluor Fluorometer (Bio-Rad). DNA concentration was adjusted to 25 ng μl^{-1} for use in subsequent real-time PCR reactions.

Samples of cultured *C. gigas* and *O. edulis* larvae were washed in sterile Milli-Q water for 20 min at room temperature to eliminate the ethanol, then the larvae were isolated by removing the supernatant liquid after centrifugation at 10 000 *g*, 1 min. DNA extraction from larvae was carried out using EZNA Mollusc kit (OMEGA bio-tek) following manufacturer's instructions. Finally, DNA was eluted in 50 μl of elution buffer.

The quality of DNA extracts, from adult and cultured samples, was evaluated by the UV ratio 260 nm/280 nm, obtaining

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values between 1.8 and 2 for adult samples and between 1.4 and 1.9 for cultivated larvae.

2.3 DNA amplification and sequencing

The universal primers 16Sa: 5′CGCCTGTTTAACAAAAACAT3′ and 16Sb: 5′ACGTGATCTGAGTTCAGACCGG3′ (Palumbi et al. 1991) were used to amplify a fragment of approximately 490 bp of the mitochondrial 16S rRNA gene for the *C. gigas*, *C. angulata* and *O. edulis* species and another 18 bivalve species (Table 2). PCR was performed on a final volume of 25 μl using PuReTaq^{TM} Ready-To-Go^{TM} PCR beads (GE Healthcare), 2.4 μM of each primer, water and DNA. Amplification conditions consisted of an incubation step of 94 °C for 3 min, followed by 35 cycles of 94 °C for 40 s, 50 °C for 40 s, 72 °C for 40 s and a final extension step of 72 °C for 7 min.

PCR products were treated with 3 μl ExoSAP-IT (Ammersham Biosciences) for deactivating dNTPs and hydrolysing single strand DNA in a two step incubation, first at 37 °C for 30 min and then at 80 °C for another 15 min. Sequencing reactions were prepared with the ExoSAP-IT treated PCR products and Big Dye (Applied Biosystems) following manufacturer's instructions. Sequencing reactions consisted of an incubation step of 3 min at 94 °C, followed by 25 cycles of 10 s at 96 °C, 5 s at 50 °C and 4 min at 72 °C. Products were purified using a standard ethanol precipitation, and the pellet obtained stored at −20 °C. Sequencing was carried out in an ABI PRISM 310 DNA Sequencer (Applied Biosystems). The resulting sequences were analysed using BIOEDIT (Hall 1999) software and then aligned with other sequences available from Genbank (Table 2) using CLUSTAL (Thompson et al. 1997) software.

2.4 Probe and primers design

Two sets of primers and TaqMan-MGB probes were designed for *C. gigas* and *O. edulis* based on the alignment of 16S rRNA gene sequences from 33 bivalve species (Table 2) using Primer Express (version 2.0) software (Applied Biosystems) and following the standard parameters. The specificity of primers and probes were evaluated via BLAST (Johnson et al. 2008). A positive control system for bivalve taxa was also designed in the 18S rRNA gene sequences from the Genbank (Table 2) to discard possible false negatives for non-target species with the oyster specific systems. The 5′ end of the CGIG/ANG16S_P, OEDU16S_P and BIV18S_P probes were labelled with the fluorescent reporter dyes VIC, FAM and NED respectively.

2.5 Real-time PCR conditions

All real-time PCR reactions were performed in a total volume of 20 μl consisting of 10 μl of TaqMan Fast Universal PCR Master Mix no UNG Amperase (2X), 1 μl of each primer and probe with a final concentration as described in Table 3, water and 2 μl of DNA (25 ng μl^{-1}) for inclusivity (target species) and exclusivity (non-target species) assays; for larvae quantification assays 2 μl of DNA from each extract sample were added. Reactions were conducted in triplicate on an ABI 7500 Fast (Applied Biosystems) real-time PCR machine at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. In all real-time PCR assays three non target control (NTC) wells were included to discard false positives due to contamination. The average Ct value, calculated for each target species, *C. gigas*, *C. angulata* and *O. edulis* (inclusivity assay) was compared with that of all non-target species (exclusivity assay), using a normal distribution *t*-test with different variances (Yuan et al. 2006). The confidence interval (α = 0.05) was calculated for the average Ct value of target and non target species.

3 Results

3.1 DNA sequencing and Real time PCR system design

The mitochondrial 16S rRNA region, flanked by the 16Sa and 16Sb primers, was successfully amplified and sequenced for several bivalve species (Table 2). All data obtained in the present work, together with sequences available on GenBank, were used for the design of a specific real time PCR system for *C. gigas* and *O. edulis* species.

The alignment of 16S rRNA sequences of the bivalve species listed in Table 2 point to the existence of many inter-specific nucleotide differences between the group composed of the *O. edulis, C. gigas and C. angulata* sequences and the rest of bivalves sequences used in the alignment (Table 4 and Table S1, online-only material). No inter-specific nucleotide differences were found between the *C. gigas* and *C. angulata* sequences in this region. The absence of inter-specific variability in these two latter species confirms observations from previous studies about the close phylogenetic relationship that exists between these two taxa (Boudry et al. 2003; Reece et al. 2008). Consequently, one system was designed for *Ostrea edulis* and another for the closely related *C. gigas* and *C. angulata*. The high inter-specific variability between *O. edulis* and *C. gigas/C. angulata* sequences with all other bivalve sequences permits the location of potential target sites for designing a real-time PCR system for both groups. Only a few of such potential sites were selected, specifically those that permitted location of specific real-time PCR systems with an adequate score for design parameters (primers and probes melting temperature, GC content, amplicon length, nucleotide composition and secondary structure) included in the Primer Express software. The alignment of the 18S rRNA sequences are used for the design of the positive control system for bivalve taxa (Table S2). No intra-specific variability affecting specificity was detected in any of the systems; this allows identifications without the incidence of false negative results. The sequences of the primers and probes of each of the systems developed are shown (Table 3).

3.2 Real-time PCR setup

The optimal concentration of primers and probes was determined experimentally for each system by taking into account combinations of primers and probe that produced the

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Table 2. Sequences used to design the BIV_18S, CGIG/ANG_16S and OEDU_16S systems. GenBank accession numbers in bold letters correspond to sequences obtained during this work. V: Veneroida, P: Pectinoida, O: Ostreoida, M: Mytiloida.

Species	Order	Key	Accession number 18S rRNA sequences	Accession number 16S rRNA sequences
Acanthocardia echinata	Veneroida	AECH		**JF808174**
Acanthocardia tuberculata	V	ATUB	AM774522	
Aequipecten opercularis	Pectinoida	AOPE		**JF808175**
Anomia ephippium	P	AEPH	AF120535	
Callista chione	V	CCHI	AJ007613	AJ548772
Cardites antiquata	V	CAUT	AF120550	
Cerastoderma edule	V	CEDU	AM774520	**JF808177**
Chamelea striatula	V	CSTR	DQ279943	
Chlamys hastata	P	CHAS	L49049	
Chlamys islandica	P	CISL	L11232	
Chlamys varia	P	CVAR		AJ586481
Clausinela fasciata	V	CFAS		**JF808178**
Crassostrea angulata	Ostreoida	CANG		**JF808176**
Crassostrea gasar	O	CGAS		**JF808179**
Crassostrea gigas	O	CGIG	AB064942	**JF808180**
Crassostrea hongkongensis	O	CHON		**JF808181**
Crassostrea sikamea	O	CSIK		**JF808182**
Crassostrea virginica	O	CVIR	X60315	**JF808183**
Donax trunculus	V	DTRU		EF417553
Dosinia corrugata	V	DCOR	EF426290	
Dosinia exoleta	V	DEXO		**JF808184**
Ensis ensis	V	EENS	AF120555	AJ548775
Ensis siliqua	V	ESIL		AJ586470
Eucrassatella cumingii	V	ECUM	AM774479	
Gari elongata	V	GELO	AM774532	
Glycymeris insubrica	Arcoida	GINS	AF207647	
Limaria hians	Limoida	LHIA		**JF808185**
Lutraria lutraria	V	LLUT	AM774553	
Mytilus edulis	Mytiloida	MEDU	AY527062	NC_006161
Mytilus galloprovincialis	M	MGAL	L33451	NC_006886
Nucula sulcata	Nuculoida	NSUL	DQ279937	
Ostrea chilensis	O	OCHI		**JF808186**
Ostrea edulis	O	OEDU	L49052	**JF808187**
Ostrea lurida	O	OLUR		**JF808188**
Ostrea stentina	O	OSTE		**JF808179**
Panopea abrupta	Myoida	PABR	AM774514	
Pecten maximus	P	PMAX	L49053	EU379454
Pharus legumen	V	PLEG	AM774510	
Pholas dactylus	Myoida	PDAC	AY070122	
Ruditapes decussatus	V	RDEC		AJ294949/**JF808190**
Ruditapes philippinarum	V	RPHI	AM774568	
Solen marginatus	V	SMAR		AJ586473
Solen vaginoides	V	SVAG	AM774507	
Spisula solida	V	SSOL	L11266	**JF808191**
Venerupis aurea	V	VAUR		AJ294950
Venerupis pullastra	V	VPUL		AJ417845
Venerupis rhomboideus	V	VRHO		AJ417848
Venerupis saxatilis	V	VSAX	AM774571	
Venus casina	V	VCAS		**JF808192**
Venus gallina	V	VGAL		**JF808193**
Venus verrucosa	V	VVER	AJ007614	AJ294947

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Table 3. Primers and TaqMan-MGB probe sequences.

TaqMan System	Primer/Probe	Primer Sequence 5′ → 3′	Amplicon length (bp)	Primer and Probe concentration (nM)
CGIG/ANG 16S	CGIG/ANG16S_F	GGGCGCCTAGAAAGCAAGT	62	300
	CGIG/ANG16S_R	ATCGGGTCAAATCCGGAAAG		300
	CGIG/ANG16S_P	VIC-AACCTTTCTGAATAACTAAC-MGB		200
OEDU 16S	OEDU16S_F	GGCGCCCCACCTAAAAAT	62	900
	OEDU16S_R	AGACCCCGTGCAACTTTTAAAG		900
	OEDU16S_P	FAM- TGAAACTCCTAAACAAGTTG-MGB		225
Positive control	BIV_18S_F	AGCCACACGAGATTGAGCAAT	57	300
	BIV_18S_R	GCGGCCCCGAACATCTA		900
	BIV_18S_P	NED-ACAGGTCTGTGATGCC-MGB		200

lowest Ct value and the highest final fluorescence value. Such concentrations were used to carry out all assays (Table 3).

Real-time PCR efficiency was assessed through seven (CGIG/ANG_16S and BIV_18S) and six (OEDU_16S) 10-fold DNA dilutions starting at 50 ng. Efficiency curves showed a slope of −3.34, −3.41 and −3.35 for CGIG/ANG_16S, OEDU_16S and BIV_18S, respectively. Efficiency of the systems, calculated as $E = [10^{(-1/\text{slope})} - 1] \times 100$, was found to be 99%, 96%, 99% for CGIG/ANG_16S, OEDU_16S and BIV_18S, respectively. Values very close to 100% efficiency for which the DNA amount in each PCR cycle (n) is twice the amount of the previous cycle ($n - 1$), supporting the correct guidelines for real time PCR assays (Chemistry Guide, Applied Biosystems 2005).

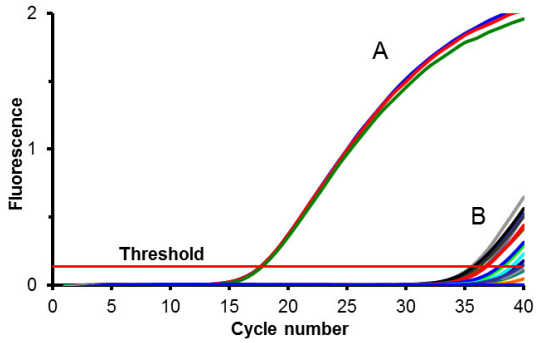


Fig. 1. Graphic representation of the cross-reaction assay to *Crassostrea gigas*/*C. angulata*, CGIG/ANG_16S system. A: Amplification pattern for target species. B: Amplification pattern for non-target species.

3.3 Specificity

The CGIG/ANG_16S and OEDU_16S systems were tested for specificity and cross-reactivity with the bivalve species listed in Table 5. The CGIG/ANG_16S system, which amplifies a 62 bp fragment of the mitochondrial 16S rRNA gene, presents an average Ct value of 17.37 ± 0.27 for all *C. angulata* and *C. gigas* specimens used in the study, while no amplification or no significant signal was obtained from other bivalve species in the cross-reactivity analysis, including *O. edulis*, as can be seen in Table 5 and Figure 1 (Ct average of 39.49 ± 0.36 from 42 non-*C. gigas*/*C. angulata* specimens).

Similar results were obtained for the OEDU_16S system, for which the *O. edulis* samples gave an average Ct value of 17.79 ± 0.21 with no amplification or no significant signal obtained for most bivalve species in the cross-reactivity analysis, with the exception of *O. angasi* which presents a Ct value similar to that of *O. edulis*. Some research articles, including Jozefowicz and Ó Foighil (1998), Kenchington et al. (2002) and Hurwood et al. (2005) consider these two taxa as the same species. Following the suggestions in these papers, and based on the identical sequence shown by the two species in the 16S rRNA alignment (Table S1), Ct of *O. angasi* will be not included in the calculation of non target species Ct average (37.90 ± 2.59 for 43 non *O. edulis*).

The species used in the cross-reactivity test for the oyster specific systems were also tested with the bivalve, BIV_18S positive control system. They showed a positive amplification in all cases (Table 5) with a Ct average of 16.16 ± 0.97.

There is a statistically significant difference between the Ct value obtained for target species and that for the rest of the analyzed non target species ($p < 0.001$) for both systems.

3.4 Identification and quantification of oyster larvae

Standard quantification curves were established using DNA isolated from cultured larvae in order to investigate the potential of the CGIG/ANG_16S and OEDU_16S systems for quantification. The standard quantification curves were obtained for samples of 1 to 128 *C. gigas* and *O. edulis* larvae (Fig. 2). Detection as low as one single larva was possible with both systems. The potential for *C. gigas* and *O. edulis* larvae quantification from plankton samples was also tested. In order to do this, plankton samples were spiked with 1, 10 and 100 larvae of *C. gigas* and *O. edulis*. These were then homogenized and DNA extracted as described in the Materials and Methods section. The number of spiked target larvae was determined by extrapolation from each standard curve. Real and estimated numbers of larvae are highly correlated (Fig. 3).

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Table 4. Alignment of 16S rRNA partial sequences, showing the position of the *Crassostrea gigas/C. angulata*, CGIG/ANG_16S system.

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                          10         20         30         40         50         60         70         80         90
CGIG_JF808180     -TTTAGGTGG GGCGCCTAGA AAGCAAGTC- TAACCTTTC- ------TGAA T--AACTAAC TCTTTCCGGA TTTGACCCGA TTATATTCGA
CANG_JF808176     -......... .......... .........- .........- ------.... .--.......Y .......... .......... ..........
AECH_JF808174     -...T.T... ...AA.A.CG G.-G..--T- A.C.TCCGT- -------T.. .TA...G..G ATCCATAT-- -A.T.TGAT. A---------
AOPE_JF808175     --..G.C... ...AG.A.A. G......ATC AG..TC..TC TTT-TC.TTG .TTG.TAG-- -ACGA..CAC ..ACTT-TAT ...GTG--AT
CCHI_AJ548772     -...T.T... ..TAA.A.TG G.CT..AAA- G...G.C..- -------.TT. .TAT.TA..A ATCC.AT--- -....TAGA. A---------
CEDU_JF808177     -....AT... ...AAT.GCG G.-A...AT- A.C.TCCG.- -------TT- .TATGTG.GG ATCCATTA-- -C.T.TGA-. .---------
CFAS_JF808178     -...T.T... ...AATA-T. ..C...AAT- A.CGG...A- ------ATTG .TA..TG..G ATCC.T---- -....AAGA. A---------
CGAS_JF808179     -......... ......A.A. G..G..ACTA .......C.GC -------..T- .TA.G....T ....A.T... G......T.. C.TA.G....
CHON_JF808181     -......... ........?. ...T.....- .........- -------... .--..T..GT .......... ..C....... ..........
CSIK_JF808182     -......... .......... ...T...C.- .........- -------...G .--....... .......... .......... ..........
CVAR_AJ586481     T...G.C... ...AG.A.AG .G....AA.T AGC...C.TT ATT-CGAA.. ACGG.TGCGT .ACGA..CAC AGGA.TGTAT G.TGTG--AC
CVIR_JF808183     -...G..... ........A? G..T..AATT .......C.GT -------.... .TAC.GA..T ...AA.TA.. .......T.. C.TC.G....
DEXO_JF808184     -C..TAT... ...AATA-T. ..T...AAT- A.CGG...AT ------AA.. AAT......G ATCC.TT--- ..A..AAGA. A---------
DTRU_EF417553     -...T.TC.. ...AGA.G.G ...AG..AT- A.TGTC...- ------.T.C .AT..TAC.G ATCCAG.A-- -.G.CTGAA. A---------
EENS_J548775      -....AC... ...AGT.GAG ..C...TAA- AT.GT.C.TT ------GTT- -ATT-TA..G ATCCGGT--- ...AC.GAAC A---------
ESIL_AJ586470     -....AC... ...AGT.GAG ..C...AAA- AT.GT.C.TT ------GTT- -ATT-TG..G ATCCGGT--- ...AC.GATC A---------
LHIA_JF808185     -..AG.C... ...AG.C... TG.G..TGAA A.TA.CA.AC GAGAGACTT. AAA.G.GGG. GAGC.TTTA. CGATC.A.T. GGTC.G.GA.
MEDU_NC006161     -...T.A... .AGAT.AGT. G.AAT.AGT- CTT.TAC.AT ------CAT. .TA.T..T.. .AG.ATTTCC .AACTTTAT. .---------
MGAY_NC006886     -...T.A... .AGAT.AGT. G.AAG.AGT- CTT.TAC.AT ------CAT. .TA.T..T.. .AG.ATTTCC .AACTTTAT. .---------
OCHI_JF808186     -......... ........CT G..T..TAT- C.....C.G- -------..T. C--GGTA..- A.....AA.T A.A....... CA..G.-...
OEDU_JF808187     -......... ........TT ...T..TAT- .......AG- -------..T. C--G.TA..- A.....AA.T A.A....... CA..G.-...
OLUR_JF808188     -......... ........CT G..T..TA.- ......C.G- -------..T. .--..AA..- A.....A..T G.A....... CAT.G.-...
OSTE_JF808189     -......... ........CT G..T..TAT- ......C.G- -------..T. .--..TA..T A.....AA.T ..A....... CAT.G.-...
PMAX_EU379454     T...G.C... ...AG.A.AG G.....AG.T AG..TCC.TT AGGGTG.A.. ACAG.TGTGT .ACGA..CAC AAAC.T-TA. GGTGTG--AT
RDEC_JF808190     -.G.G.T... ..AAAGCTAG .TTT...AA- A.CAAG.CT- -------AT. ATAGG.G..G ATCC...T-- -CG.GAGAT. A---------
RDEC_AJ294949     -.G.G.T... ..AAAGCTAG .TTT...AA- A.CAAG.CT- -------AT. ATAGG.G..G ATCC...T-- -CG.GAGAT. G---------
SMAR_AJ586473     -.....C... ...AGT.CCG ..CA..TAA- A..GT...GT ------GTGT AATTGTG..G ATCCAATA.. .A..TTGAAT A---------
SSOL_JF808191     -...T.T... ...AA.AG.. ..AG..AAA- G.TTT....- ------.T.T A-T..TGG.G ATC...T--- -....GTGA. .---------
VAUR_AJ294950     -GG...T... ..A.AGCTA. .CTTT..AG- A.TAAG..T- --------TG GGAG..AG.G ATCC..TC-- -GGAGAGAA. ----------
VCAS_JF808192     -...T.T... ...AA.A-TT GGTT..AAT- A.CGAC..A- ------ATTT .TA...AT.G ATCC.TTT-- G?...AAGA. A---------
VGAL_JF808193     -...TAT... ...AATA-T. ..T...AAT- A.CGG...A- ------AT.. .TAG.TA..G ATCC.T---- G.C..AAGA. A---------
VPUL_AJ417845     -GG...T... ..A.AGCT.. GCTTT..AG- A.TAGG...- --------T. AAAG..A.GG ATCC..TTT- -AGAGAGAA. A---------
VRHO_AJ417848     -.G...T... ..AAAG.TAG GCTTG..AT- A.TAAGC.T- --------TT .AAGGTG..G ATCC.TTT-- -.GAGAGAA. ----------
VVER_AJ294947     -...T.T... ...AATA-T. G.C...AAT- A.CGG...A- ------ATT. .TT..TA..G ATCC.TT--- G....AAGA. A---------
```

Table 5. Results of cross-reaction assays for CGIG/ANG_16S, OEDU_16S and BIV_18S systems. Average Ct value ± SD for three replicates. Average Ct value ± confidence interval ($\alpha = 0.05$) for oyster specific systems in bold letters. Ct value of 40 means no amplification.

Species	Sample	CGIG/ANG_16S	OEDU_16S	BIV_18S
Crassostrea angulata	CANG 1		33.45 ± 0.24	14.85 ± 0.11
Crassostrea angulata	CANG 2		31.40 ± 0.38	
Crassostrea angulata	CANG 3		29.23 ± 0.01	
Crassostrea gigas	CGIG 1	**17.37 ± 0.27**	39.77 ± 0.40	14.34 ± 0.03
Crassostrea gigas	CGIG 2		40	
Crassostrea gigas	CGIG 3		36.63 ± 0.82	
Crassostrea hongkongensis	CHON	40	40	16.05 ± 0.13
Crassostrea sikamea	CSIK	39.31 ± 0.43	40	14.50 ± 0.12
Crassostrea virginica	CVIR	40	40	14.81 ± 0.03
Ostrea edulis	OEDU 1	40		13.22 ± 0.03
Ostrea edulis	OEDU 2	36.45 ± 1.37	**17.79 ± 0.21**	
Ostrea edulis	OEDU 3	40		
Ostrea chilensis	OCHI	40	40	13.99 ± 0.09
Ostrea stentina	OSTE	40	40	15.05 ± 0.08
Ostrea angasi	OANG	40	18.86 ± 0.18	15.70 ± 0.03
Crassostrea gasar	CGAS	40	37.26 ± 0.66	14.12 ± 0.04
Ostrea conchaphila	OCON	40	40	14.57 ± 0.04
Mytilus edulis	MEDU	36.47 ± 1.03	35.27 ± 1.08	13.66 ± 0.08
Mytilus galloprovincialis	MGAL	36.45 ± 0.86	35.64 ± 0.62	13.83 ± 0.04
Xenostrobus securis	XSEC	40	37.23 ± 0.63	14.73 ± 0.13
Acanthocardia echinata	AECH	40	39.59 ± 0.71	24.52 ± 0.14
Aequipecten opercularis	AOPE	38.62 ± 0.73	38.16 ± 1.92	14.53 ± 0.06
Chlamys varia	CVAR	40	36.94 ± 0.76	13.71 ± 0.09
Limaria hians	LHIA	40	40	16.61 ± 0.03
Pecten maximus	PMAX	40	37.87 ± 1.18	14.06 ± 0.09
Callista chione	CCHI	40	39.86 ± 0.24	16.55 ± 0.13
Cerastoderma edule	CEDU	39.69 ± 0.54	37.20 ± 1.21	25.79 ± 0.13
Clausinela fasciata	CFAS	40	40	14.09 ± 0.03
Dreissena polymorpha	DPOL	40	38.91 ± 1.38	15.03 ± 0.13
Dosinia exoleta	DEXO	40	40	16.84 ± 0.24
Donas trunculus	DTRU	39.88 ± 0.20	36.12 ± 0.83	18.52 ± 0.14
Ensis ensis	EENS	40	40	15.59 ± 0.14
Ensis siliqua	ESIL	40	33.63 ± 0.43	15.74 ± 0.11
Gari depressa	GDEP	40	38.65 ± 1.58	18.92 ± 0.03
Glycymeris pilosa	GPIL	39.76 ± 0.41	36.01 ± 1.62	
Laevicardium crassum	LCRA	39.51 ± 0.85	38.22 0.44	26.14 ± 0.10
Ruditapes decussatus	RDEC	39.68 ± 0.56	40	15.21 ± 0.04
Ruditapes philippinarum	RPHI	40	38.71 ± 2.23	16.02 ± 0.04
Scrobicularia plana	SPLA	40	39.54 ± 0.79	17.47 ± 0.10
Solen marginatus	SMAR	40	37.45 ± 0.43	15.41 ± 0.08
Spisula solida	SSOL	40	40	18.58 ± 0.05
Venerupis pullastra	VPUL	40	38.14 ± 1.62	14.78 ± 0.03
Venerupis rhomboideus	VRHO	40	39.99 ± 0.02	16.33 ± 0.04
Venerupis aurea	VAUR	40	40	17.79 ± 0.32
Venus casina	VCAS	36.43 ± 0.29	33.87 ± 0.18	14.43 ± 0.03
Venus gallina	VGAL	40	40	15.81 ± 0.04
Venus verrucosa	VVER	39.80 ± 0.34	37.71 ± 0.16	14.51 ± 0.13

4 Discussion

As mentioned above, mollusc larvae are difficult to identify due to their small size and morphological similarity. Therefore, alternative techniques for their identification through morphological characteristics are required. To this end, in recent years alternative methods based on immunological and DNA techniques, FISH-SC, and image analysis have been applied. Although all of them, real time PCR included, have greatly improved larvae identification and quantification, none has been found to be entirely suitable.

Immunological techniques have been used to identify plankton larvae (Perez et al. 2009). There are, however, a number of disadvantages associated with such techniques, including the need to produce monoclonal antibodies, maintain hybridomas and the need for incubation steps, all of which results in longer analysis times. Furthermore, to obtain quality stained larvae, these have to be preserved in more restrictive

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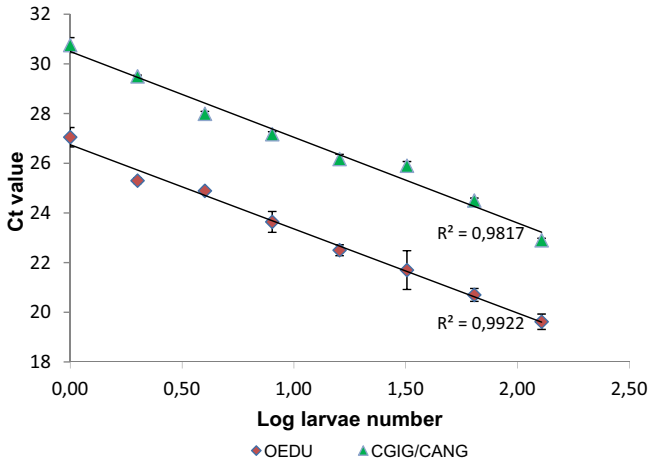


Fig. 2. Standard curve of *Crassostrea gigas/C. angulata,* CGIG/ANG_16S (triangle) and *Ostrea edulis*, OEDU_16S (diamond) systems showing Ct (threshold cycle) values plotted against logarithm of larvae number (1 to 128) analyzed in triplicate.

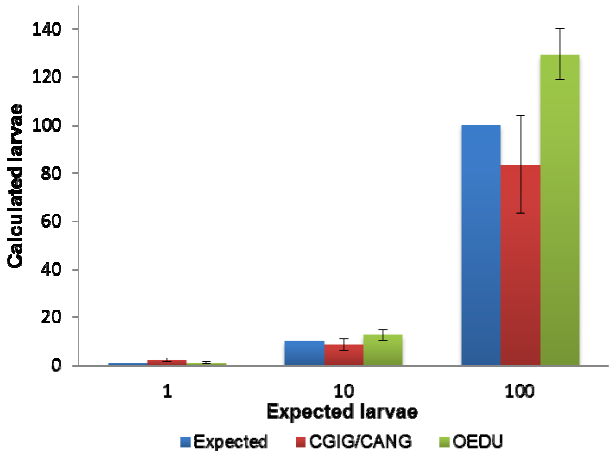


Fig. 3. Correlation between the expected and quantified skyped larvae in plankton samples.

conditions than those needed for DNA analysis. For example, ethanol cannot be used in immuno-detection as it affects the antigen and consequently the staining of the larvae (Perez et al. 2009).

Fluorescent in situ hybridization with DNA probes and image analysis (Le Goff-Vitry et al. 2007; Goodwin et al. 2014) have the drawbacks of autofluorescence and the effect of different growth conditions (temperature and salinity) on shell formation, respectively.

In this study, we have proposed a specific TaqMan real time system for identifying and quantifying oyster larvae from *C. gigas* and *O. edulis* species. The systems were designed for the 16S rDNA region where intra-specific variability was either absent or very low. The developed probe and primers have been shown to be highly specific and able to differentiate the species of interest from a large number of other bivalve species. Moreover, both systems showed high sensitive: the detection of only one larva in a plankton sample was possible.

The quantification method shown here is based on the use of standards and same sized spiked larvae. One may hypothesize that larval size may affect the number of mtDNA molecules per larva, and therefore different larval sizes may produce different responses to real time PCR. However, Vadopalas et al. (2006) have shown that pinto abalone larval quantification was not affected by differences in larval size. Further studies must nevertheless be conducted in order to verify whether there is any effect on quantification results across the entire range of oyster larval sizes. If so, a new protocol that separates larvae by size should be employed to circumvent this problem.

In this work, we have demonstrated that real-time PCR facilitates identification and quantification of two of the most important commercial oyster species. This is the first time that this technique has been used for this purpose. The high correlation observed between larvae number calculated by real-time PCR assay and the real larvae number in spiked plankton samples is truly outstanding. Although real-time PCR has been shown to be a valid technique for the aims proposed in this work, the size range of the quantified larvae cannot be provided. This disadvantage could be overcome by combining the real-time PCR technique with other techniques such as image analysis.

5 Conclusion

Molecular technologies like real-time PCR not only facilitate species identification but also reduce bivalve larvae analysis time. The present study proposes a DNA method which is rapid, one-step, time-saving and simpler than any other DNA technique previously described, for identifying and quantifying *Crassostrea gigas* and *Ostrea edulis* larvae. This method can be a useful tool for monitoring spawning in certain areas, even in areas where *C. gigas* and *O. edulis* are mixed with closely related species, because of the high specificity and very low cross-reaction of the proven TaqMan systems. In addition, the tool could be used in ecological studies such as those concerning the influence of different parameters on larvae population dynamics.

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Online-only materials

Table S1. Alignment of 16S rRNA partial sequences, showing the position of the *Ostrea edulis*, OEDU_16S system.
Table S2. Alignment of 18S rRNA partial sequences from GenBank, showing the position of bivalve, BIV_18S system.

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Table S1. Alignment of 16S rRNA partial sequences, showing the position of the *Ostrea edulis*, OEDU_16S system.

```
                          10        20        30        40        50        60        70        80        90       100       120       130       140       150       160
OEDU_JF808187   TA----GGCG CCCCACCTAA AAA------- ---------- ---------- -TCTGAAACT CC-------- ---------- ------TAAA CAA------- --GTTGA--C TTT------- ----------  AAAAGTTGCA CGGGG-TCTT
AECH_JF808174   .T----.TT. .....A.A.. .GT------- ---------- ---------- -...TTCG-- AA-------- ---------- -----T..C. AC.------- --A..TGAAA .AA------- --------AT  -C...C.CG. .....-....
AOPE_JF808175   .T----.CT. .....G.C.. C.-------- ---------- ---------- -----.C.AA AG-------- ---------- -----TGGT. AG.ATATTTC T-----CCA. ..-------- ------AGTT  ..C.AC.T.. .....-....
CANG_JF808176   ..----.... .......... ...------- ---------- ---------- -...TTT... ..-------- ---------- -----TG..T A..------- --..CA.--T ...------- ----------  C......... .....-....
CCHI_AJ548772   .T----.TTA .....A.A.. ..T------- ---------- ---------- -..AA..G-- AA-------- ---------- -----TA.T. ACTGACTTTC AAT.ATTAA. ...C------ -------TAA  TTC....CG. TA...-....
CEDU_JF808177   C.----ATT. .....AT... ..T------- ---------- ---------- -.TC------ GG-------- ---------- -----T..CC AT.------- --..G..AG. ..C------- --------AT  TC...C.CG. .....-....
CFAS_JF808178   .-----ATT. .....A.A.. .TT------- ---------- ---------- -...AA.TT-- A.-------- ---------- -----TA..C AC-------- -AACAATAAT ...------- --------AA  TT...C.CG. .A...-....
CGAS_JF808179   .T----.... .......... ...------- ---------- ---------- -...TTTG.C AT-------- ---------- -----T...T .T------- --.C.T.A-T ...------- ----------  C......... .....-....
CGIG_JF808180   ..----.... .......... ...------- ---------- ---------- -...TTT... ..-------- ---------- -----TG..T A..------- --...A.--T ...------- ----------  C......... .....-....
CHON_JF808181   ..----.... .......... ...------- ---------- ---------- -...TTT... T.-------- ---------- -----TG..T A..------- --...A.--T ...------- ----------  C......... .....-....
CSIK_JF808182   ..----.... .......... ...------- ---------- ---------- -...TTT... ..-------- ---------- -----TG..T A..------- --..CA.--T ...------- ----------  C......... .....-....
CVAR_AJ586481   .T----.CT. .....G.C.. ..CGCACTGA CCATTCTTCC CAACGACAAT TCTAC.C.AG AACCCCAAGC CTTGCATATT TATATTGCT. AT.ACATCTG TTA.A.GGAT ..CACGTTAT GGCAGGCATT  CCT.AC.T.. .....-....
CVIR_JF808183   ..----.... .......C.. ...------- ---------- ---------- -...TTT... TA-------- ---------- -----T...T TC.------- --.C.T.GCT ...------- ----------  C......... .....-....
DEXO_JF808184   .-----ATT. .....ATA.. G.T------- ---------- ---------- -.AAA..T-- TA-------- ---------- -----TA.CT TT-------- --A.AA.AAT ..C------- --------AA  TT..AC.CG. .A...-....
DTRU_EF417553   C.----TCT. ....GA.A.. .C.------- ---------- ---------- -.A.ACCG-- A.-------- ---------- -----T..G. A--------- -ATAGCCAAT AA.------- ---------A  CC...C.CA. .A...-....
EENS_J548775    C.----ACT. .....GT... .TT------- ---------- ---------- -.A.T.T.-- TA-------- ---------- -----AA..C TCT------- --T.AC.-AT ...------- ---------A  TT...C.CG. .A...-....
ESIL_AJ586470   C.----ACT. .....GT... .TT------- ---------- ---------- -.A.T.T.-- TG-------- ---------- -----AA.TC TTT------- --ACAT.-AT ...------- ---------A  TT...C.CG. .A...-....
LHIA_JF808185   C.TCTG.CT. .....G.CT. .GT------- ---------- ---------- -.TA..CTAA A.-------- ---------- -----TATTT TGT------- --C.A.CCAT .GC------- ----------  T....C.C.. .....-....
MEDU_NC_006161  CT----.ATC T....T.A.. .TC------- ---------- ---------- -GTAT..GAG ..-------- ---------- -----TG.G. G--------- ---C.CCAAT .AA------- --------GA  T....C.T.. TA...-....
MGAY_NC_006886  CT----.ATC T....T.A.. .TC------- ---------- ---------- -GTAT..GAG ..-------- ---------- -----TG.G. G--------- ---C.CCAAT .AA------- --------GA  T....C.T.. TA...-....
OANG_AF052063   ..----.... .......... ...------- ---------- ---------- -...TTT... ..-------- ---------- -----TG..T A..------- --...A.--T ...------- ----------  C......... .....-....
OCHI_AJ808186   ..----.... .......... ...------- ---------- ---------- -...T..G.. T.-------- ---------- ------...G T..------- --.C..-.-- ...------- ----------  .......... .....-....
OLUR_JF808188   ..----.... .......... ...------- ---------- ---------- -...TGGGT. T.-------- ---------- -----C.C.. T..------- --ACC..-.- ...------- ----------  .......... .....-....
OSTE_JF808189   ..----.... .......... ...------- ---------- ---------- -...T...T. T.-------- ---------- ------.T.. T..------- --ACC..-.- ...------- ----------  .......... .....-....
PMAX_EU379454   .T----.CT. .....G.C.. ..C-TGCT-A TTATTCACCC CT-------T C.GAA.C... AAGTTCTAGT GCTCCTCAAC GATTCGGCC. ...ATAGCCT T-----.GAA. ..CCCA--AT AACTTAAATT  TCT.AC.T.. .....-....
RDEC_JF808190   AG----CTTT .....A.C.C .CT------- ---------- ---------- -.TA.TTTTA AA-------- ---------- -----TA.T. ATCACACATA AAA..T.AAA CC.------- --------GA  TT...C.CA. TA...-....
SMAR_AJ586473   G.----ACT. .....G.... .CT------- ---------- ---------- -.A.TT.C-- A.-------- ---------- -----AA.CC ATT------- --C.GTGCA. ...------- ---------A  TC...C.CG. .A...-....
SSOL_JF808191   CT----.TT. .....A.A.. .CT------- ---------- ---------- -...ATTTT-A A.-------- ---------- -----TACCC A--------- --AC.A.AA. --.------- --------AA  TC...C.CAC .....-....
VAUR_AJ294950   AG----CT.T .....A...C CCT------- ---------- ---------- -...A.TTT-- AG-------- ---------- -----C---- ----CTATTT TCT.CTTAAA CC.------- --------AA  TT...C.CGG .A...-....
VCAS_JF808192   .-----.TT. .....A.A.. ..T------- ---------- ---------- -...A.TT-- AT-------- ---------- -----TAT.. AT.AA----- -ACAAACAAT C..------- --------AA  TT...C.CG. .A...-....
VGAL_JF808193   .-----ATT. .....ATA.. ..T------- ---------- ---------- -...A.TT-- GA-------- ---------- -----TAT.. A--------- --CAAACAAT ...------- --------AA  TT...C.CG. .A...-....
VPUL_AJ417845   AG----CT.T .....A...C C.T------- ---------- ---------- -...AACCT-- AA-------- ---------- -----TA.C. ACT-CTCCAA TCC.ACCAAA CC.------- --------AA  TT...C.CGG .A...-....
VRHO_AJ417848   A.----CTTT .....A...C .TT------- ---------- ---------- -.TGACT.AA AA-------- ---------- -----T---- ------TCTA TCA.CCTTAG ...------- --------AA  TT...C.CG. .A...-....
VVER_AJ294947   .-----ATT. .....A.A.. ..T------- ---------- ---------- -...A.TT-- AT-------- ---------- -----CA... A--------- --CAAATAAT ...------- --------AA  TT...C.CG. .A...-....
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Table S2. Alignment of 18S rRNA partial sequences from GenBank, showing the position of bivalve, BIV_18S system.

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                10        20        30        40        50        60        70        80
AEPH_AF120535   ACAAGTGGCG TTTAGCCACA CGAGATTGAG CAATAACAGG TCTGTGATGC CCTTAGATGT TCGGGGCCGC ACGCGCGCTA
ATUB_AM774522   .......... .......... ...A..G... .......... .......... .......... C......... ..AT.T....
CAUT_AF120550   .........T .......... .....C.... .......... .......... .......... .......... ..........
CCHI_AJ007613   .......... C......... .......... .......... .......... .......... .......... ..AT......
CEDU_AM774520   .......... .......... ...A.CG... .......... .......... .......... C......... ..AT.T....
CGIG_AB064942   .......... .......... .......... .......... .......... .......... .......... ..........
CHAS_L49049     .......... .......... .......... .......... .......... .......... .......... ..........
CISL_L11232     .......... .......... .......... .......... .......... .......... .......... ..........
CSTR_DQ279943   .......... C......... .......... .......... .......... .......... .......... ..AT......
CVIR_X60315     .......... .......... .......... .......... .......... .......... .......... ..........
DCOR_EF426290   .......... C......... .......... .......... .......... .......... .......... ..AT......
ECUM_AM774479   .........T .......... .....C.... .......... .......... .......... .......... ..........
EENS_AF120555   .......... .......... .......... .......... .......... .......... .......... ..A.......
GELO_AM774532   ...G...... .......... .......... .......... .......... .......... C......... ..AT......
GINS_AF207647   .........T .......... .......... .......... .......... .......... .......T.. ..AT......
LLUT_AM774553   ...G...... C......... .......... .......... .......... .......... .......... ..AT......
MEDU_AY527062   .......... .......... .......... .......... .......... .......... .......... ..........
MGAL_L33451     .......... .......... .......... .......... .......... .......... .......... ..........
NSUL_DQ279937   .......... .......... .......... .......... .......... .......... .......... ..........
OEDU_L49052     .......... .......... .......... .......... .......... .......... .......... ..........
PABR_AM774514   .......... .......... .......... .......... .......... .......... .......... ..A.......
PDAC_AY070122   ...G...... C......... .......... .......... .......... .......... .......... ..AT......
PLEG_AM774510   .......... .......... .......... .......... .......... .......... .......... ..A.......
PMAX_L49053     .......... .......... .......... .......... .......... .......... .......... ..........
RPHI_AM774568   .......... C......... .......... .......... .......... .......... .......... ..AT......
SSOL_L11266     ...G...... C......... .......... .......... .......... .......... .......... ..AT......
SVAG_AM774507   .......... .......... .......... .......... .......... .......... .......... ..A.......
VSAX_AM774571   .......... C......... .......... .......... .......... .......... .......... ..AT......
VVER_AJ007614   .......... C......... .......... .......... .......... .......... .......... ..AT......
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