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Research Article

Development of sensitive and specific molecular tools for the efficient detection and discrimination of potentially invasive mussel species of the genus *Perna*

P. Joana Dias[1,2]*, Seema Fotedar[1], Jonathan P. A. Gardner[3] and Michael Snow[1]

1 *Western Australia Fisheries and Marine Research Laboratories, Department of Fisheries, PO Box 20, North Beach 6920 WA, Australia*
2 *School of Animal Biology, University of Western Australia, 35 Stirling Highway, Crawley 6009 WA, Australia*
3 *Centre for Marine Environmental and Economic Research, School of Biological Sciences, Victoria University of Wellington, PO Box 600, Wellington 6140, New Zealand*

E-mail: Joana.Dias@fish.wa.gov.au (PJD), Seema.Fotedar@fish.wa.gov.au (SF), Jonathan.Gardner@vuw.ac.nz (JPAG), Michael.Snow@fish.wa.gov.au (MS)

**Corresponding author*

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Abstract

Marine mussels of the genus *Perna* include three species: *P. canaliculus*, *P. viridis* and *P. perna.* While *P. canaliculus* appears to be greatly restricted to its endemic range of New Zealand, *P. perna* and *P.viridis* introductions have been recorded outside their native ranges in several regions of the globe. Such introductions have often resulted in significant negative ecological, economic and social impacts. *Perna perna* and *P.viridis* are exotic to Australia and are listed under the Australian Government National System for the Prevention and Management of Marine Pest Incursions as high priority species. Rapid detection of marine pests such as *Perna* species remains fundamental to their effective containment and control. The present study reports on the development and validation of both conventional and real-time PCR assays suited to the rapid identification and discrimination of juvenile and adult specimens of *P. viridis*, *P. canaliculus* and *P. perna*. The development of a sensitive high-throughput real-time PCR assay offers further potential for the efficient detection of the presence of single *Perna* specimens in mixed populations of native mussel species, and for early detection of larval stages in ballast water and plankton samples. This assay offers considerable advantages over traditional identification methods and represents an important step in developing capacity for efficient identification and management of *Perna* species incursions in Australian waters.

Key words: *Perna*; invasive; PCR; real-time PCR; Western Australia

Introduction

Marine mussels of the genus *Perna* are currently recognized as three species, *P. canaliculus* (Gmelin 1791), *P. viridis* (Linnaeus 1758) and *P. perna* (Linnaeus 1758) (Siddall 1980; Vakily 1989; Wood et al. 2007). The New Zealand green-lipped mussel *P. canaliculus*, an important aquaculture species, is endemic to New Zealand, extending from the far north of the North Island to as far as Stewart Island in the south (Jeffs et al. 1999; Powell 1979; Wood et al. 2007) (Figure 1). The Asian green mussel *P. viridis* is native to Asian waters, occurring extensively throughout the Indo-Pacific region from the Persian Gulf throughout India and South East Asia (Baker et al. 2007; Siddall 1980) (Figure 1). The brown mussel *P. perna* is native to most of the Atlantic coast of South America (Beauperthuy 1967; Siddall 1980), to the south coast of Portugal (Lorenço et al. 2012) through much of the African coastline (Nordsieck 1969; Siddall 1980) and to Sri Lanka (Sadacharan 1982; Wood et al. 2007) (Figure 1). In southwest India a fourth species *Perna indica* (Kuriakose 1980; Rao 1974), classified for many years as *P. perna* (Vakily 1989), was recently suggested to be a distinct species within the genus (Divya et al. 2009, 2010). The taxonomic status of this species is, however, recognised as requiring further clarification.

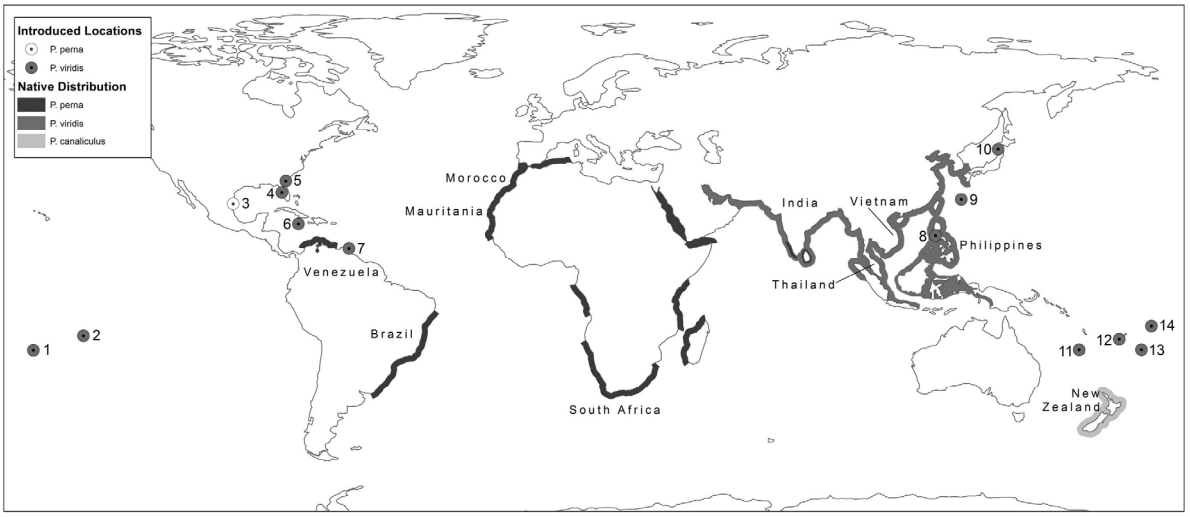

Figure 1. Geographic distribution of mussel species of the genus *Perna*. *Perna perna, Perna viridis* and *Perna canaliculus* native distributions are represented in solid lines and were adapted from similar figures from Siddall (1980), Vakily (1989) and Wood et al. (2007), with an added extension of *P. perna* distribution to the south coast of Portugal (Lourenço et al. 2012), the west coast of India (Vakily 1989), (Divya et al. 2010) and Sri Lanka (Sadacharan 1982). Locations where *P. perna* and *P. viridis* are known to have subsequently become established following introduction are marked with dots: 1. Cook Islands, 2. Tahiti (French Polynesia), 3. Mexico, 4. Florida (USA), 5. Georgia (USA), 6. Jamaica, 7. Trinidad, 8. Philippines, 9. Okinawa (Japan) 10. Japan, 11. New Caledonia, 12. Fiji, 13. Tonga and 14. Western Samoa. Countries from which samples were collected are named in the map. Details regarding collection points within these countries can be found in Table1.

While the only two *P. canaliculus* occurrences outside New Zealand, as far as the authors are aware, have been reported from South Australia (SA) and eradicated (in the 90's, Justin McDonald *personal communication*, and more recently, Wiltshire et al. 2010), *P. perna* and *P. viridis* introductions have been recorded outside their native ranges in several regions. *Perna perna* has been established in the western Gulf of Mexico since the 90's (Hicks and McMahon 2002; Hicks and Tunnel 1993, 1995) and was recently removed following its incursion in an oil rig in New Zealand waters (Hopkins et al. 2011a). A series of *P. viridis* introductions, resulting from shipping, fisheries and aquaculture activities, have been reported to occur in China and Japan since the 60's (Habe 1976; Hanyu and Sekiguchi 2000; Hanyu et al. 2001; Ye 1997; Yoshiyasu et al. 2004). *Perna viridis* introductions for aquaculture have also occurred in numerous Pacific islands (Bell et al. 1983; Coeroli et al. 1984; de Gaillande 1979; Eldredge 1994; Vereivalu 1990), the Caribbean (Agard et al. 1993; Hicks 2001) and Atlantic coasts of North and South America (Benson et al. 2001; Penchaszadeh and Velez 1996; Power et al. 2004; Rylander et al. 1996) (Figure 1). The successful establishment of *P. viridis* in these areas has had concerning economical and ecological impacts. The species' fast growth rate and lack of competitors has led to populations of *P. viridis* rapidly outcompeting native species at introduced locations (Hicks 2001; Hicks et al. 2001; Ingrao et al. 2001). Heavy fouling of man-made structures such as aquaculture cages, buoys, boats and water intake pipes of power stations has resulted in costly maintenance to shipping and other coastal industries (Rajagopal 1991; Rajagopal et al. 2006).

In Australia, *P. viridis* was introduced to Trinity Inlet, Cairns, Queensland in 2001 via ship hull biofouling. Successful eradication efforts were put in place following the detection of breeding individuals in this area in 2002 (Hayes et al. 2005). *Perna viridis* incursions (detection of individuals but not of established breeding populations) are frequent as this species is among the most commonly identified alien invasive species within the biofouling community of vessels entering Australian waters (McDonald 2012; Piola and McDonald 2012). *Perna perna* and *P. viridis* are listed under the Australian Government National System for the Prevention and Management of Marine Pest Incursions (DAFF 2010) as high priority species. The eradication of *P. viridis* from Australia (Hayes et

al. 2005) and of *P. perna* from New Zealand are among the few examples of successful eradication of marine pests worldwide. Such processes are extremely complex and expensive, as well as highly dependent on the early detection of the pest species in order to avoid irreversible establishment and dispersal (Elton 1958; Hayes et al. 2005; Hopkins et al. 2011a,b; Willan et al. 2000).

The early detection of marine pest species can only be accomplished through the establishment of baseline surveys (Campbell et al. 2007; Hewitt and Martin 1996, 2001), repeated monitoring of high risk areas (Hewitt et al. 2004), and screening and identification of a wide range of taxa using methods which are typically time-consuming and expensive (Bott et al. 2010; Hayes et al. 2005). As taxonomic expertise declines globally (Hopkins and Freckleton 2002; Kim and Byrne 2006), molecular techniques like Polymerase Chain Reaction (PCR) and real-time PCR are recognised as important complementary methods in species identification, as well as very attractive alternative tools in marine pest research and monitoring programs (Blanchet 2012; Bott and Giblot-Ducray 2011; Holland 2000; Mountfort and Hayden 2007; Wood et al. 2013). The use of DNA-based techniques has proven to be of particular relevance in the identification of marine invertebrate species, whose morphologic characters are frequently plastic, are influenced by environmental factors, may be lacking or not distinctive at early life stages and may often only be recognised by highly trained taxonomists (Deagle et al. 2003; Dias et al. 2009; McBeath et al. 2006; Siddall 1980; Willis et al. 2011).

PCR, although widely used and useful in species identification, is a qualitative technique that requires a certain initial amount of tissue sample for DNA extraction and the post-processing of samples for analysis. This has motivated some authors to apply hydrolysis probe based real-time PCR to the identification and quantification of marine invertebrate species whose initial life stages consist of planktonic larvae (Dias et al. 2009; McBeath et al. 2006; Pan et al. 2008; Vadopalas et al. 2006). This one-step, fully automated methodology allows detection to occur during the reaction, reducing the time, labour and costs arising from post processing of samples in agarose gels. By using highly specific fluorescent probes, the sensitivity and specificity is also greatly improved in comparison to conventional PCR, allowing for the detection from samples containing a significantly lower initial concentration of target DNA. Because the intensity of fluorescence during the exponential amplification phase, measured when it first rises above background level or Critical Threshold (Ct), is directly correlated with initial template quantity, real-time PCR has also been used quantitatively (for a review of this technology see Vasalek and Repa 2005).

The present work was initiated with the objective of developing a potentially high-throughput real-time PCR assay capable of rapidly detecting and discriminating the mussel species *P. perna*, *P. canaliculus* and *P. viridis*. The adoption of this assay by the Western Australian biosecurity program represents an important step towards developing capacity for the rapid and efficient identification and management of *Perna* species incursions in Australian waters.

Methodology

2.1. Sample origin and DNA extraction

In this study we used DNA extracts from 31 individuals of mussels *P. perna*, *P. viridis* and *P. canaliculus* sampled in the study of Wood et al. (2007) and four *P. perna* adductor muscle tissue reference samples obtained from the Marine Invasive Taxonomic Service (MITS) in New Zealand. DNA extracts from the study of Wood et al. (2007) were kept stored at -80ºC, transported to the WA Fisheries and Marine Research Laboratories in a cool package and stored at -20ºC until use. No field study was conducted and therefore no specific permits were required. Locations from where all 35 samples were obtained in previous study by Wood et al. (2007) and by MITS can be found in Table 1 and are indicated in Figure 1. From the four mussel adductor muscle samples, 5 mg of tissue was sub-sampled to an eppendorf tube, homogenized in 200 µl of lysis buffer (Fisher Biotec) using a micropestle and incubated overnight with 20 µl of proteinase K (Fisher Biotec) at 60ºC. DNA was extracted using a Fisher Biotec FavorPrep Tissue Genomic DNA Extraction Mini Kit, following the manufacturer's instructions. All DNA samples were stored at -20ºC until further use.

2.2. Primer design and PCR

P. perna, P. viridis and *P. canaliculus* mitochondrial DNA sequences available from the work of Blair et al. (2006) (nad4 gene spanning the IGS to the cox1 gene; GenBank DQ343568

to DQ343611), and *Perna* individuals sampled in the work of Wood et al. (2007) (cox1 region; DQ917582 to DQ917618) were aligned and screened for primer and probe candidate regions. Five sets of primer combinations were designed using the Primer Express version 3.0 software (Applied Biosystems). Generic primers, capable of amplifying targets from all members of the genus, were designed based on conserved regions of the sequences. These (conserved) primer regions flanked variable regions that were suited for the further design of species-specific probes. Primer specificity was tested against each *Perna* species through PCR amplification in 25 µl reaction mixtures containing 2 µl (10-20 ng) DNA, 1.25 mM of each dNTP, 62.5 mM $MgCl_2$, 2.5 µl of 10x reaction Buffer, 2.5 µM of each primer, one unit of *Taq* DNA polymerase (Fisher Biotec) and PCR-grade water (Fisher Biotec). PCR conditions consisted of an initial incubation at 94ºC for 5 min, followed by 30 cycles at 94ºC for 30 s, 56ºC for 30 s, 70ºC for 90 s, and a final extension step of 72ºC for 5 min in an Applied Biosystems (ABI) 2720 thermal cycler. A negative control, with no template DNA added, was included in all PCR assays. PCR products were separated by electrophoresis using 1.5% agarose (Fisher Biotec) gels stained with ethidium bromide (Fisher Biotec) alongside a 100 bp molecular weight marker (Axygen Biosciences) and visualised under UV light.

2.3. Sequencing

The most suitable set of primers across the three species (plus a set of flanking primers) were selected and used in the sequencing of the target region across all 35 *Perna* individuals. Sequencing of unpurified PCR products was performed using the service provided by the Australian Genome Research Facility (AGRF) in Perth. All samples were sequenced in both directions and consensus sequences generated using Sequencher 5.0 (Gene Codes Corporation). Sequences were aligned and analysed in the BioEdit 7.1.3.0 Sequence Alignment Editor (Hall 1999), using CLUSTAL W (Thompson et al. 1994) with default parameters.

2.4. Real-time PCR

Three specific hydrolysis TaqMan®-MGB (FAM, VIC and NED) probes (one for each *Perna* species) were designed using the Primer Express version 3.0 software (Applied Biosystems), based on the preliminary specificity trials described above and sequences obtained for all 35 individuals. To help guarantee the specificity of the method, all the designed primers and probe sequences were subjected to a BLAST search, to check the GenBank database for any similar sequences and potential cross-reactions. Probe specificity was further analysed across all 35 individuals of the three *Perna* species, using both single-probe and multiplex assays. Multiplex assays were performed on an ABI Step One Plus™ real-time PCR system using a cycling profile of 50ºC for 2 min (AmpErase® uracil N-glycosylase incubation), 95ºC for 20 s (DNA polymerase activation) followed by 45 cycles of 95ºC for 1 s (denaturation) and 60ºC for 20 s (annealing / extension). Reactions were conducted in a final volume of 20 µl containing 1 µl of DNA template, 1x TaqMan® Fast Advanced master mix (Applied Biosystems), 900 nM of each primer and 200 nM of each TaqMan® probe (Applied Biosystems). Efficiencies of primers and probes – Efficiency (%) = $[10^{(-1/\text{slope})}]-1 \times 100$ - were assessed using standard curves based on both triplicate single-probe and triplicate multiplex reactions of 10-fold dilutions of DNA samples of each *Perna* species.

Results

3.1. PCR

From the five sets of generic primers designed to target regions conserved across the three *Perna* species, the set that gave the best results was the one flanking the variable IGS region. The forward primer (Fw A) was designed at the 3' end of the nad4 gene and the reverse primer (Rev A) at the beginning of the cox1 gene (Table 2). This set not only allowed for the amplification of all three species with a single pair of primers, but also generated a species-specific size product for each species - *P. perna* 281 bp, *P. canaliculus* 249 bp and *P. viridis* 201 bp - due to the high variability of the IGS region (Figure 2). This assay thus has the potential to be used as a stand-alone assay to differentiate species of the *Perna* genus in the absence of access to real-time PCR systems.

3.2. Sequencing of the marker region

Sequencing of the region spanned by the Fw A/Rev A set of primers (primer region inclusive) was possible for 27 of the 35 individuals using either a single set of flanking primers (nad4 fw2

Table 1. Details of mussel samples used in this study: sample collection locations, names, voucher references and GenBank accession numbers.

Species	Location	Name	Voucher Reference	GenBank Accession Number
Perna canaliculus	Houhora, New Zealand	Hou13	JPA Gardner-001-VUWNZ	KF242420
	Castlepoint, New Zealand	Cap1	JPA Gardner-002-VUWNZ	KF242421
	Gore Bay, New Zealand	Gob1	JPA Gardner-003-VUWNZ	KF242422
	Fiordland, New Zealand	Fio18	JPA Gardner-004-VUWNZ	KF242423
Perna perna	Eastern South Africa	Af1	JPA Gardner-005-VUWNZ	KF242424
	Eastern South Africa	Af2	JPA Gardner-006-VUWNZ	KF242425
	Eastern South Africa	Af3	JPA Gardner-007-VUWNZ	KF242426
	Port Elizabeth, South Africa	SA1	MITS 35586-1	KF242427
	Port Elizabeth, South Africa	SA2	MITS 35586-2	KF242428
	Port Elizabeth, South Africa	SA3	MITS 35586-3	KF242429
	Port Elizabeth, South Africa	SA4	MITS 35586-4	KF242430
	Cumana, Venezuela	V1	JPA Gardner-008-VUWNZ	KF242431
	Cumana, Venezuela	V2	JPA Gardner-009-VUWNZ	KF242432
	Santa Catarina, Brasil	ScF1	JPA Gardner-010-VUWNZ	KF242433
	Santa Catarina, Brasil	ScF2	JPA Gardner-011-VUWNZ	KF242434
	Sao Paulo, Brasil	SPF1	JPA Gardner-012-VUWNZ	KF242435
	Sao Paulo, Brasil	SPF2	JPA Gardner-013-VUWNZ	KF242436
	Temara, Morocco	Mor1	JPA Gardner-014-VUWNZ	KF242437
	Temara, Morocco	Mor2	JPA Gardner-015-VUWNZ	KF242438
	Temara, Morocco	Mor3	JPA Gardner-016-VUWNZ	KF242439
	Temara, Morocco	Mor4	JPA Gardner-017-VUWNZ	KF242440
	Cansado, Mauritania	Pi1	JPA Gardner-018-VUWNZ	KF242441
	Cansado, Mauritania	Pi2	JPA Gardner-019-VUWNZ	KF242442
	Cansado, Mauritania	Pi3	JPA Gardner-020-VUWNZ	KF242443
Perna viridis	Chennai, India	Chen1	JPA Gardner-021-VUWNZ	KF242444
	Chennai, India	Chen2	JPA Gardner-022-VUWNZ	KF242445
	Chennai, India	Chen3	JPA Gardner-023-VUWNZ	KF242446
	Southern India	Vi1	JPA Gardner-024-VUWNZ	KF242447
	Southern India	Vi2	JPA Gardner-025-VUWNZ	KF242448
	Philippines	Phil1	JPA Gardner-026-VUWNZ	KF242449
	Philippines	Phil2	JPA Gardner-027-VUWNZ	KF242450
	Thailand	Thai1	JPA Gardner-028-VUWNZ	KF242451
	Thailand	Thai2	JPA Gardner-029-VUWNZ	KF242452
	Nha Trang, Vietnam	Viet1	JPA Gardner-030-VUWNZ	KF242453
	Nha Trang, Vietnam	Viet2	JPA Gardner-031-VUWNZ	KF242454

Table 2. List of primers and TaqMan®-MGB probes. Primer and probe name, sequence, melting temperature (Tm), GC content (%), length (bp) and attributed dye (probes).

Oligos	Name	Sequence 5'-3'	Tm C	% GC	Length (bp)	Dye
Flanking primers for sequencing	nad4 fw2	CATGGKYTRTGYTCYTCTGGRA	60.7	50	22	-
	COI rev5	TAATYAAAATATCAACWGCMGGYCCAGTA	61.9	34	29	-
PCR and real-time PCR	Fw A	CTTAGTGGCATTAATTCGDAATCC	59.2	39	24	-
	Rev A	CAAAGTACCAATATCTTTATGATTRGTWGA	57.5	28	30	-
TaqMan®-MGB probes	*P. canaliculus*	AGCATTTAATAGAGTAGAGCTA	68	32	22	FAM
	P. viridis	ACTCAAACAACAAAGTAAAC*	69	30	20	VIC
	P. perna	AACCATCGACTCAATTAA*	71	33	18	NED

Note: oligos marked with an asterix (*) were designed in the lagging DNA strand.

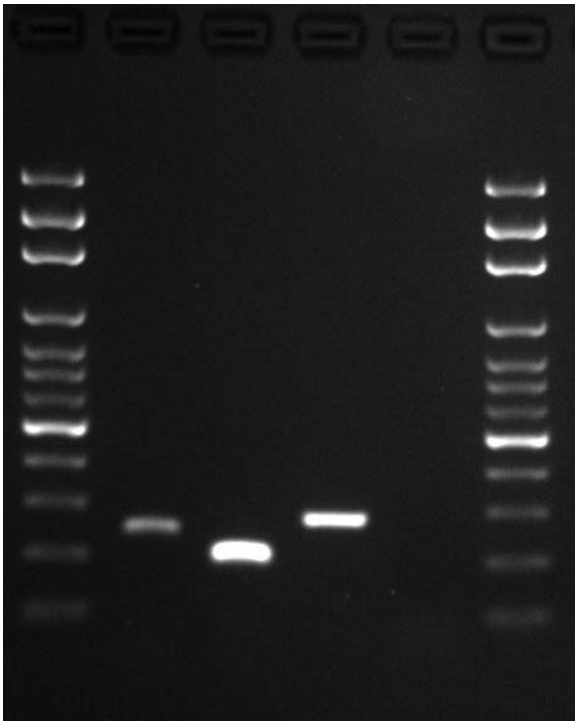

Figure 2. Agarose gel showing *Perna* species-specific band length. *P. canaliculus* 249 bp (Pc), *P. viridis* 201 bp (Pv) and *P. perna* 281 bp (Pp) species-specific PCR products are shown along a negative control and flanked by a 100 bp molecular weight marker (Axygen Biosciences).

and COI rev5, Table 2), or a combination of four primers (Fw A and COI rev5, nad4 fw2 and Rev A) with subsequent alignment of the two fragments for the generation of a final consensus sequence. For the remaining 8 individuals for which complete sequence was not obtained (Mor2, Mor3, Mor4, Chen2, Phil1, Phil2, Thai1 and Viet1) only the sequences between the target primers (Fw A and Rev A) were included in the general alignment. No effort was made to extend these sequences due to constraints in the amount of each DNA extract available. The sequences obtained were used for the basis of primer optimization and species-specific probe development around the variable IGS region. All sequences were deposited in GenBank (see Table 1 for accession numbers).

3.3. Real-time PCR method specificity and efficiency

Results from the GenBank BLAST searches of all the designed primers and probe sequences indicated these to be specific to the targeted *Perna* species. One should note that the target marker region had not been previously sequenced for *P. viridis* in the work of Blair et al. (2006) and was not available in the GenBank database for this or any closely related species.

Primers and probes were designed to be able to detect and differentiate between *P. perna*, *P. canaliculus* and *P. viridis* and were specific for these species. Detections from both single-probe and multiplex assays across all 35 individuals used in this study (Cts 18-34) were obtained for the species-specific DNA in the reactions. No detections were obtained from negative controls. In order to obtain accurate, reproducible and comparable results, real-time PCR reaction efficiency should be as close to 100% (slope of -3.33) as possible (Pfaffl 2004; Valasek and Repa 2005). Standard curves based on either single or multiplex probe reactions of *P. canaliculus*, *P. viridis* and *P. perna* DNA extracts revealed slope values between –3.8 (Efficiency= 83%) and -3.6 (Efficiency= 90%) (Figures 3 and 4). There was high correlation between cycle number and dilution factor, R^2 = 0.996 - 0.999 for all dilution series (Figures 3 and 4). Triplicate single-probe reactions yielded similar Ct values, with SD± 0.0 to 0.7 across all dilution series (standard deviations of triplicate Ct values were not included on Figures 3 and 4 as they are too small to be visualised).

Discussion

In this study, we have developed both a conventional PCR and real-time PCR assay capable of rapidly detecting and discriminating the mussel species *P. perna*, *P. canaliculus* and *P. viridis*. The simultaneous identification of all three species based on a single marker can save time and costs, offering considerable advantages over previous PCR identification approaches. The specificity and efficiency of the real-time PCR method indicates its potential for detection of *Perna* species in pooled tissue samples, and at larval and juvenile stages. This method could therefore save further time and costs of processing samples individually and prove of most value in the identification of *Perna* species at early life stages, when morphological characters can be particularly difficult to distinguish between species.

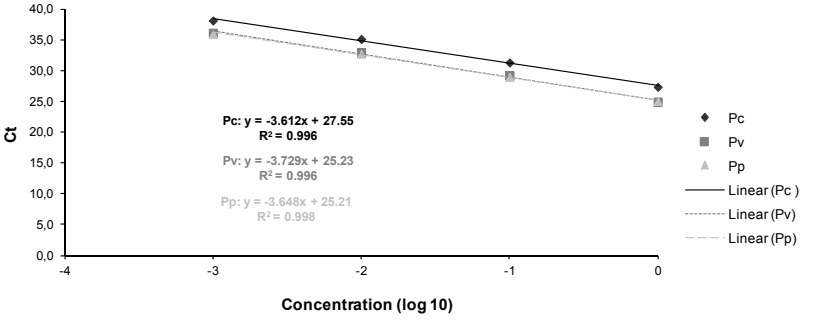

Figure 3. Efficiency of real-time PCR single-probe reactions. Average cycle threshold values obtained through single-probe real-time PCR reactions of 10 fold dilutions of total DNA extracted from adult *P. perna* (Pp), *P. canaliculus* (Pc) and *P. viridis* (Pv). Slope values giving reaction efficiency of Taqman®-MGB probes for each species are shown on the graphic. Standard deviations (SD) are ±0.2, ±0.7 and ±0.3 for *P. perna* (Pp), *P. canaliculus* (Pc) and *P. viridis* (Pv) respectively and are too low to be visualised in the figure.

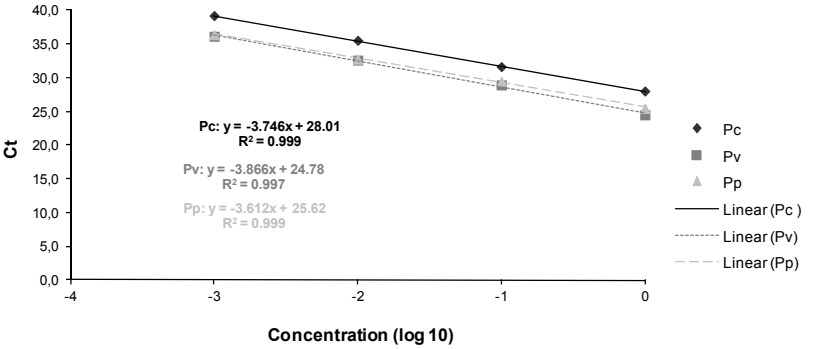

Figure 4. Efficiency of real-time PCR multiplex reactions. Average cycle threshold values obtained through multiplex real-time PCR reactions of 10 fold dilutions of total DNA extracted from adult *P. perna* (Pp), *P. canaliculus* (Pc) and *P. viridis* (Pv). Slope values giving reaction efficiency of Taqman®-MGB probes for each species are shown on the graphic. Standard deviations (SD) are ±0.6, ±0.5 and ±0.3 for *P. perna* (Pp), *P. canaliculus* (Pc) and *P. viridis* (Pv) respectively and are too low to be visualised in the figure.

4.1. PCR

Previously available assays for the molecular identification of *Perna* species include that used by Holland et al. (1999), which relied on chromosome morphology to confirm the identification of *P. perna* introduction in the Gulf of Mexico. Santaclara et al. (2006) also described a method in which *Perna* species can be identified through a combined approach of either of two PCR methods (Kenchington et al 1995; Perez et al. 2004) combined with Restriction Fragment Length Polymorphism (RFLP). Blair et al. (2006) developed a PCR method consisting of a combination of a general forward and three distinct reverse primers (designed in the mtDNA nad4 and IGS regions), each giving a size-specific band for each species. Although more straight-forward than the previously described techniques, this method requires conducting three separate reactions to identify each *Perna* individual. These authors attempted to design a reverse primer common to all three species but were unable to sequence the IGS region of *P. viridis*, and suggested that this could be due to a different mitochondrial gene order in this species. In our work, we were able to generate sequence across the IGS region for all species, which was confirmed to be of different size for each species and considerably smaller in *P. viridis*. The use of a single set of primers capable of amplifying species-specific fragments in a single reaction (Figure 2) greatly simplifies the identification process and costs associated with it. However, when using the PCR primers developed in the present work, one should note that the species-specific fragments are less than 50 bp different in size from each other (*P. canaliculus* 249 bp, *P. viridis* 201 bp and

P. perna 281 bp) and therefore the use of longer electrophoresis time and positive controls for the three species is strongly recommended.

4.2. Real-time PCR

The real-time PCR developed in the present study is able to detect and discriminate among all three *Perna* species. The real-time PCR efficiency obtained across a wide series of 10-fold dilutions of DNA of each *Perna* species was high (> 80%). Although our results indicate a loss of 4% efficiency when changing from a single-probe to a multiplex reaction, this seems to be an acceptable compromise considering the time and cost advantages of the multiplex assay. Considerable effort was made to obtain *Perna* species samples from as many populations as possible across the globe. Such samples allowed us to perform a comprehensive analysis of the IGS region intra and inter-species variability and better design species-specific probes. Based on such knowledge, and when applied to pest species detection from ships (fouling, ballast water) entering the waters of any nation, the real-time PCR method developed in this work is expected to be able to detect incursions of non-native *Perna* species from nearly every region of the globe. Nevertheless, one should note that the specificity of the method has been checked against sequences of organisms deposited in GenBank. Cross-reaction with species not previously sequenced remains possible and therefore electrophoresis (to check for right product size) and sequencing of *Perna* positives is strongly recommended, especially in a biosecurity context.

4.3. Future applications to biosecurity surveillance

Future applications of this method include the detection of *Perna* species from plankton and bulk tissue samples. The method specificity and efficiency outlined in this study represents a first positive step towards further potential applications such as the detection and relative quantification of larval stages in water samples. The development of such an application would allow important detection and/or monitoring to take place from ballast water of vessels and of plankton from, for example, high risk areas (ports, marinas) or following incursion/eradication events of *Perna* species. Pooling small tissue samples from up to 30 individual mussels for a single DNA extraction and real-time PCR reaction detection has been performed confidently for the efficient detection of closely related mussel species (*Mytilus* spp.) in previous studies (Dias et al. 2008). Such an approach enables the analysis of 30 samples in a single real-time PCR reaction. Although sample preparation is inevitably longer, each assay can include up to 94 reactions (plus two controls) in a 96-well plate, meaning that the pooling of 30 samples per reaction would allow for the analysis of 2820 mussels in a 33 min run, allowing detection results to be generated in the same day. The development of such rapid and specific methods could prove most valuable in assisting with the visual identification of pest species (especially at early stages) during biofouling inspections. Verifying the presence/ absence of suspected pests in a short time frame (same day) could at times avoid the unnecessary cleaning of vessels, considerably reducing the time and costs associated with these inspections.

Taqman, single-probe, real-time PCR assays for the detection of some of the bivalve species of biosecurity concern to Australia, namely *P. canaliculus*, *Corbula gibba* and *Musculista senhousia* have been recently developed and/or optimized by Bott and Giblot-Ducray (2011) at the South Australian Research and Development Institute (SARDI). These assays have proved efficient, time and cost effective in the screening of these pest species from both tissue and plankton samples. In order to establish the molecular capacity for pest detection, the costs of acquiring the necessary equipment and the relatively lengthy process of training staff, developing, testing and validating assays is inevitable, representing the major drawbacks of such applications. However, we believe such drawbacks can be overcome by the benefits of having these tools in place, not only for the regular monitoring of pest species, but also in the face of an incursion event.

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