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Development of real-time PCR assays for discrimination and quantification of two *Perkinsus* spp. in the Manila clam *Ruditapes philippinarum*

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ABSTRACT: The Manila clam *Ruditapes philippinarum* is infected with 2 *Perkinsus* species, *Perkinsus olseni* and *P. honshuensis*, in Japan. The latter was described as a new species in Mie Prefecture, Japan, in 2006. Ray's Fluid Thioglycollate Medium (RFTM) assay has been most commonly used to quantify *Perkinsus* infection. However, this assay cannot discriminate between species that resemble one another morphologically. We developed real-time PCR assays for the specific quantification of *P. olseni* and *P. honshuensis*. DNA was extracted using Chelex resin. Cultured *P. olseni* and *P. honshuensis* cells were counted and spiked into uninfected clam gill tissue prior to DNA extraction to generate standard curves, which allowed quantification based on the PCR cycle threshold values. We compared the RFTM assay with both real-time PCR assays by quantifying *Perkinsus* spp. in gill tissue samples from the same individual clams obtained from various localities in Japan. Infection intensities estimated by both assays were significantly correlated ($r^2 = 0.70$). Our results suggest that the prevalence and infection intensity of *P. honshuensis* are much lower than for *P. olseni* in Manila clams.

KEY WORDS: *Perkinsus olseni* · *Perkinsus honshuensis* · qPCR · *Ruditapes philippinarum* · Manila clam · RFTM

INTRODUCTION

In Japan, the annual catch of the Manila clam *Ruditapes philippinarum* was between 100 000 and 160 000 metric tons from the 1960s to early 1980s, peaking at 160 000 tons in 1983. Since then, the annual catch has decreased dramatically and has remained below 40 000 tons in the last decade (Annual Statistics of Fishery and Fish Culture, Ministry of Agriculture, Forestry and Fisheries, Japan). Matsukawa et al. (2008) suggested that the decline was caused by overfishing, degeneration of sediments, mass depletion of larvae, and/or feeding damage by predatory animals. However, the exact cause(s) remain unclear. We hypothesized that infection with the protozoan parasites *Perkinsus olseni* and/or *P. honshuensis* has played an important role in the depletion of Manila clam populations.

The genus *Perkinsus* includes protozoan endoparasites that infect marine mollusks and are likely closely related to dinoflagellates (Saldarriaga et al. 2003). *Perkinisus marinus* was at least partly responsible for mass mortalities and stock depletion of the eastern oyster *Crassostrea virginica* on the east coast of the United States (Andrews & Hewatt 1957, Ewart & Ford 1993). A second species, *P. olseni*, was also implicated in the mass mortalities experienced by abalones *Haliotis* spp. in Australia and the gloved

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carpet shell clam *Ruditapes decussatus* in Spain and Portugal (Ruano & Cachola 1986, Azevedo 1989, Goggin & Lester 1995). In East Asia, several researchers have suggested that infection with *P. olseni* caused mass mortalities and stock depletion of Manila clams in some regions of Korea and Japan (Hamaguchi et al. 1998, Park & Choi 2001). Because of their potential risk to the health of aquatic mollusks, the 2 *Perkinsus* species are listed pathogens requiring notification to the World Organization for Animal Health (OIE) (World Organization for Animal Health 2010).

Until recently, it was thought that the Manila clam was infected only with *Perkinsus olseni* in Japan. However, Dungan & Reece (2006) described *P. honshuensis*, a new species, in Manila clams collected from Mie Prefecture, Japan. *P. olseni* is distributed throughout the tropical Pacific Ocean, Australia, the North Island of New Zealand, Vietnam, Korea, Japan, China, Portugal, Spain, France, Italy, and Uruguay. *P. olseni* also infects a wide range of hosts, including clams, oysters, pearl oysters, abalones, and possibly other bivalve and gastropod species (Azevedo 1989, Goggin & Lester 1995, Hamaguchi et al. 1998, Park & Choi 2001, Casas et al. 2002, Villalba et al. 2004, Cremonte et al. 2005). *Perkinsus* infections have been reported in Manila clams throughout Japan, except for regions of northern and eastern Hokkaido (Hamaguchi et al. 2002, Nishihara 2010). However, previous reports of *Perkinsus* infection in Manila clams from Japan have not distinguished between the 2 species. Thus, their relative distribution is largely unknown and the impact of each species on their host has not been sufficiently evaluated particularly in field surveys.

Perkinsus spp. are most commonly detected and quantified using Ray's Fluid Thioglycollate Medium (RFTM) assay (Ray 1952, 1966). However, this method cannot distinguish between *Perkinsus* species because of their morphological similarity. In addition, the RFTM assay requires a long culture period (>4 d). Recently, Takahashi et al. (2009) developed a PCR-RFLP method to distinguish *P. olseni* and *P. honshuensis*, demonstrating that both species are present in western Japan. Unfortunately, it is not possible to quantify the intensity of infection using PCR-RFLP.

We aimed to develop real-time PCR assays for the discrimination and quantification of *Perkinsus olseni* and *P. honshuensis* to investigate their distribution in wild Manila clams. We compared the results obtained using real-time PCR and RFTM in naturally infected clams.

MATERIALS AND METHODS

Clams

We purchased uninfected Manila clams from the Akkeshi fisheries cooperative association. The clams were collected from the tidal flat at Akkeshi, Hokkaido, Japan, an area that is free of *Perkinsus* spp. We purchased naturally infected Manila clams that were collected in Aichi Prefecture from a local retailer in Tokyo. In addition, we collected naturally infected Manila clams from 4 locations: Ariake Basin, Kumamoto Prefecture; Lake Hamana, Shizuoka Prefecture; an unnamed bay, Mie Prefecture, where *P. honshuensis* was first described; and the Marine Park in Kanagawa Prefecture.

Parasites

We purchased trophozoites of *Perkinsus olseni* (ATCC PRA-181) and *P. honshuensis* (ATCC PRA-177) from the American Type Culture Collection and cultured them in *Perkinsus* broth medium (PBM) (ATCC medium 1886) as follows. We added 1 ml of old culture to 10 ml fresh medium at 2 to 3 wk intervals, and incubated the medium at 25°C. Cultures of *P. olseni* and *P. honshuensis* were filtered through 10 µm nylon mesh to remove aggregated trophozoites. The filtrate was then centrifuged at 275 × *g* for 5 min and the pelleted cells were resuspended in 3 ml modified phosphate buffered saline (MPBS: 0.2 g KCl, 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 20.5 g NaCl, 1 l distilled water [DW], 720 mOsm), which was made isotonic with PBM by increasing NaCl in Dulbecco's phosphate buffered saline. Stock solutions of trophozoites of *Perkinsus* species were prepared as described above throughout this study. We quantified the cell density of the resuspended cells using a hemocytometer. All counts were conducted in triplicate and the mean value was used for analysis.

Ethanol-fixed trophozoites of a range of *Perkinsus* species (Table 1) were provided by Chris Dungan (Cooperative Oxford Laboratory, Maryland Department of Natural Resources) for use in the primer specificity test.

Real-time PCR

We developed 2 real-time quantitative PCR protocols for the discrimination and quantification of *Perkinsus olseni* and *P. honshuensis* genomic DNA

Table 1. Isolates of *Perkinsus* spp. used to test the specificity of the primer sets. ATCC: American Type Culture Collection; C: clonal strain; P: polyclonal strain

Isolate identity	Isolate code	ATCC no.	Clonality	Source host	Source state	Source country
Perkinsus olseni	PaG3F	PRA-31	P	*Ruditapes decussatus*	Galicia	Spain
	NZMSAs-5/C9	PRA-205	C	*Austrovenus stutchburyi*	North Island	New Zealand
	Mie-4g/F6	PRA-179	C	*Ruditapes philippinarum*	Mie	Japan
	Mie-13v/H8	PRA-183	C	*Ruditapes philippinarum*	Mie	Japan
	Mie-5mg	PRA-180	P	*Ruditapes philippinarum*	Mie	Japan
	Mie-5mg/F8	PRA-181	C	*Ruditapes philippinarum*	Mie	Japan
Perkinsus honshuensis	Mie-3g	PRA-176	P	*Ruditapes philippinarum*	Mie	Japan
	Mie-3g/H8	PRA-177	C	*Ruditapes philippinarum*	Mie	Japan
Perkinsus marinus	CRTW-3he	50439	P	*Crassostrea virginica*	Maryland	USA
Perkinsus chesapeaki	CHBRMa-14	PRA-65	P	*Mya arenaria*	Maryland	USA

targeting the internal transcribed spacer (ITS) region. The primer sets (Table 2) were designed using BioEdit (Ibis Biosciences) based on the known sequences of the genus *Perkinsus* registered in GenBank (www.ncbi.nlm.nih.gov/genbank/). Real-time PCR assays were performed in 8-strip tubes using a MiniOpticon real-time PCR detection system (Bio-Rad Laboratories Japan). Each tube (20 µl) contained 10 µl of SsoFast EvaGreen Supermix (Bio-Rad Laboratories Japan), 0.1 µl of each primer (100 µM), 1 µl of template DNA and 8.8 µl of ultrapure water. The thermal cycling conditions were: 1 cycle of polymerase activation at 98°C for 2 min; 40 cycles of amplification at 98°C for 2 s and 60°C for 5 s; 1 cycle of annealing at 60°C for 5 min; and melting curve analysis at 60 to 98°C in which the temperature was increased by 0.1°C every 5 s to determine the purity of PCR products. We included negative controls in all PCR runs (ultrapure water only and extract of uninfected clam only) to rule out DNA contamination.

Primer specificity

Genomic DNA was extracted from 10 isolates of *Perkinsus* spp. (Table 1) using a 10% (w/w) Chelex (Bio-Rad Laboratories Japan) resin solution (Barber & Erdmann 2000, De Faveri et al. 2009). Briefly, Chelex solution was prepared by suspending molecular biology grade Chelex 100 resin (200–400 dry mesh, 75–150 µm wet beads; Biorad-Laboratories Japan) in ultrapure water. A small volume (<20 µl) of ethanol-fixed parasite cell pellet was transferred into a 1.5 ml microtube. We then added 1 ml of 10% Chelex solution to each tube and incubated the mixture at 95°C in a dry bath for 45 min. The concentrations of all DNA extracts were determined using a spectrophotometer. To test the specificity of the primer sets designed for *P. olseni* and *P. honshuensis*, we performed real-time PCR on the extracted DNA. The amount of template DNA was between 100 and 200 ng per reaction.

Table 2. Primer sets used in this study for *Perkinsus* spp.

Target species	Primer name	Oligonucleotide sequence (5′–3′)
P. olseni	OF	Forward: CTT AAC GGG CCG TGT TA
	OR	Reverse: CAT AAC GAA CTA TCT CCG AAG
P. honshuensis	HF	Forward: CTG CCT GGC AGG TGA T
	HR	Reverse: CGA ATT GGC TCA ATA AAT TG

Effect of clam tissue on PCR amplification

To test the influence of clam tissue on PCR amplification, we compared PCR amplification between 2 groups of samples. We prepared a serial dilution series of *Perkinsus* trophozoite cultures (5.0×10^6 to 5.0 cells per ml). The first group consisted of DNA samples extracted from a 300 µl trophozoite suspension. The second group consisted of DNA samples extracted from uninfected clam gill tissues inoculated with the trophozoite suspension. The gills were excised from uninfected clams collected at Akkeshi and minced with clean scissors. Thirty milligrams (wet mass) of the minced gill tissue was transferred into a 1.5 ml microtube, to which 300 µl of the serially diluted trophozoite suspension was added. The mixture was then centrifuged at 800 × *g* for 5 min to obtain gill tissue spiked with trophozoites. The tissues were then extracted with Chelex resin as

described above. We carried out the real-time PCR runs using the DNA solutions as template. The experiment was repeated for both species.

Comparison of DNA extraction methods

We spiked uninfected clam gill tissues with serially diluted trophozoites of each *Perkinsus* species (4.0×10^7 to 4.0×10^2 cells g^{-1} wet tissue mass). DNA was then immediately extracted from the tissues using one of 3 methods: QIAamp DNA Mini Kit (Qiagen), QIAamp DNA Stool Mini Kit (Qiagen), or Chelex resin. We extracted DNA from 25 and 50 mg of the spiked gill tissues using the QIAamp DNA Mini Kit (following the protocol for tissue extraction) and the QIAamp DNA Stool Mini Kit (following the protocol for isolation of DNA from stools for pathogen detection), respectively. The final solution volume was 200 µl for both methods. We also extracted DNA from 30 mg of spiked gill tissue using a 10% Chelex resin solution as described above. We carried out the real-time PCR runs using the DNA solutions as a template.

Preparation of standard samples

We prepared standard samples for real-time PCR by spiking the gill tissue of uninfected clams with cultured trophozoites. Serial dilutions of both species were prepared over a range of 7 orders of magnitude (5.0×10^6 to 5.0 cells ml^{-1}). We then added 300 µl of each dilution to a 1.5 ml tube containing clam gill tissue (30 mg). The tubes were centrifuged at 800 × *g* for 5 min, after which the supernatant was removed. DNA was extracted from these samples with a 10% Chelex resin solution as described above. We prepared 3 serial dilutions from 3 concentrated trophozoite samples for each *Perkinsus* species and used real-time PCR to generate a standard curve. We performed linear regression analysis to determine the relationship between *Perkinsus* spp. cell density and threshold cycle (C_T) values generated by the real-time PCR assay.

We evaluated whether real-time PCR can be used to accurately quantify the number of *Perkinsus honshuensis* cells when the level of infection is significantly lower than that of *P. olseni*. We performed real-time PCR on samples that were prepared as follows: Four sets of 30 mg gill tissues (wet mass) were removed from uninfected Manila clams, minced with sterile scissors, and transferred to a 1.5 ml microtube. We then added 300 µl of serially diluted *P. honshuensis* trophozoites (4.0×10^6 to 4.0 cells per ml) to each tissue sample. In addition, we added 300 µl of *P. olseni* trophozoite suspensions (1.0×10^7 cells per ml) to 2 of the 4 tissue samples. The mixture was briefly vortexed and centrifuged at 800 × *g* for 5 min. The supernatant was discarded and DNA was immediately extracted with Chelex resin as described above.

Comparison of tissue preservation method

To evaluate the effect of the method of tissue preservation prior to DNA extraction, we prepared standard curves using spiked gill tissue samples that were processed using one of 3 methods. Spiked standard samples prepared in the same way as described above were subjected to DNA extraction immediately after preparation, frozen at −20°C overnight, or fixed in 500 µl of 95% ethanol at −20°C overnight before DNA extraction. The ethanol-fixed samples were centrifuged (800 × *g*, 5 min) to remove ethanol before DNA extraction. DNA extraction was performed with Chelex resin as described above. We prepared 2 serial dilutions generated from 2 different trophozoite stocks and subjected them to real-time PCR to obtain the standard curve.

Comparison of tissue types

We spiked minced gill tissue or minced whole soft body with 10-fold serially diluted trophozoites of the 2 *Perkinsus* species. The spiked samples were subjected to DNA extraction with Chelex resin followed by real-time PCR. We prepared 2 serial dilutions generated from 2 different trophozoite stocks. The serial dilutions were then subjected to real-time PCR to obtain standard curves.

Comparison of the real-time PCR and RFTM assays

We quantified the trophozoites of the 2 *Perkinsus* species in naturally infected Manila clams using both real-time PCR and the RFTM assay. Both sides of the gills were excised from Manila clams (n = 16 from Aichi Prefecture, n = 22 from the Ariake Basin, n = 18 from Lake Hamana, n = 16 from the location at which *P. honshuensis* were first described in Mie Prefecture, and n = 20 from the Marine Park). The whole gill leaves from one side of the body were weighed and processed immediately using RFTM following the method of Ray (1952, 1966), as modified by Choi

et al. (1989). In brief, gill tissue samples were placed in screw-capped test tubes containing 5 ml of RFTM (14.9 g fluid thioglycollate medium, 10 g NaCl, 500 ml DW). We added 500 units ml^{-1} penicillin G potassium and 500 µg ml^{-1} streptomycin sulfate to inhibit bacterial growth. The samples were then incubated at 25°C in the dark for 4 to 7 d; the length of the incubation period (within this range) had little effect in our preliminary study. Following incubation, the media was centrifuged at 275 × *g* for 5 min and the resulting pellets were lysed in 10 ml of 2 M NaOH at 60°C until the tissue was fully lysed. The lysates were washed twice in DW. After the final centrifugation step, the pellets were resuspended in 1 ml DW. The suspensions were 10-fold serially diluted in a 96-well microplate. Hypnospores in the well were stained with a drop of Lugol's iodine solution and counted under an inverted microscope.

A sample of the gill tissue from the opposing side was transferred into a 1.5 ml microtube, homogenized with a Nippi Bio-Masher II, and weighed (<40 mg). Total DNA was extracted from the homogenates with Chelex resin and subjected to both real-time PCR assays for quantification of the 2 *Perkinsus* species. A standard curve was generated using Manila clam gill tissues spiked with 10-fold serially diluted trophozoites of the 2 *Perkinsus* species. We compared the estimates of infection intensity from the RFTM assay and both real-time PCR assays using linear regression analysis and a Bland-Altman plot.

RESULTS

Primer specificity

Only the target species exhibited a positive reaction during real-time PCR, with the exception that the primer sets designed for *Perkinsus olseni* and *P. honshuensis* both yielded a positive reaction to DNA extracted from the *P. honshuensis* strain (PRA 176) (Table 3). PRA 176 is a polyclonal strain isolated from a clam that was collected from the site in Mie Prefecture, where both species are present (Dungan & Reece 2006). The C_T values for this strain were 15.7 and 26.7 for the primer sets designed for *P. honshuensis* and *P. olseni*, respectively.

Table 3. PCR amplification of genomic DNA from isolates of *Perkinsus* species with primer sets specific to *P. olseni* and *P. honshuensis*. ATCC: American Type Culture Collection; C: clonal strain; P: polyclonal strain; C_T: threshold cycle; NA: not amplified in 40 cycles

Species	ATCC no.	Clonal strain	C_T value for each primer set	
			P. olseni	*P. honshuensis*
P. olseni	PRA-31	P	14.7	NA
	PRA-205	C	16.9	NA
	PRA-179	C	12.2	NA
	PRA-183	C	17.5	NA
	PRA-180	P	13.6	NA
	PRA-181	C	13.5	NA
P. honshuensis	PRA-176	P	26.7	15.7
	PRA-177	C	NA	15.7
P. marinus	50439	P	NA	NA
P. chesapeaki	PRA-65	P	NA	NA

Effect of clam tissue on PCR amplification

DNA samples extracted from clam gill tissues spiked with trophozoites had higher C_T values by 3 to 5 units than those extracted from trophozoites alone (Fig. 1).

DNA extraction methods

We did not detect an amplification signal following real-time PCR on *Perkinsus honshuensis* DNA extracted with the QIAamp DNA Mini kit. DNA of the 2 *Perkinsus* species extracted with the QIAamp DNA stool kit yielded a little higher C_T values than for DNA extracted with Chelex resin (Fig. 2). The C_T values for DNA extracted with Chelex resin were the lowest among the 3 extraction methods for both *Perkinsus* species.

Preparation of standard samples

The C_T values obtained from the standard samples exhibited a clear linear correlation with trophozoite density in both species ($r^2 > 0.96$; Fig. 3). We occasionally observed non-specific amplification of DNA, even in the negative controls after 40 cycles. DNA amplification was detected within the range of 1.5×10^6 to $1.5 \times 10^{0.5}$ cells for both *Perkinsus olseni* and *P. honshuensis* per 30 mg of wet gill tissue. This equates to 5.0×10^7 to $5.0 \times 10^{1.5}$ trophozoites g^{-1} wet gill tissue. We observed very little variation in the ranges between PCR runs.

The C_T values for *Perkinsus honshuensis* DNA were very similar between corresponding samples despite the presence of *P. olseni* trophozoites (Fig. 4).

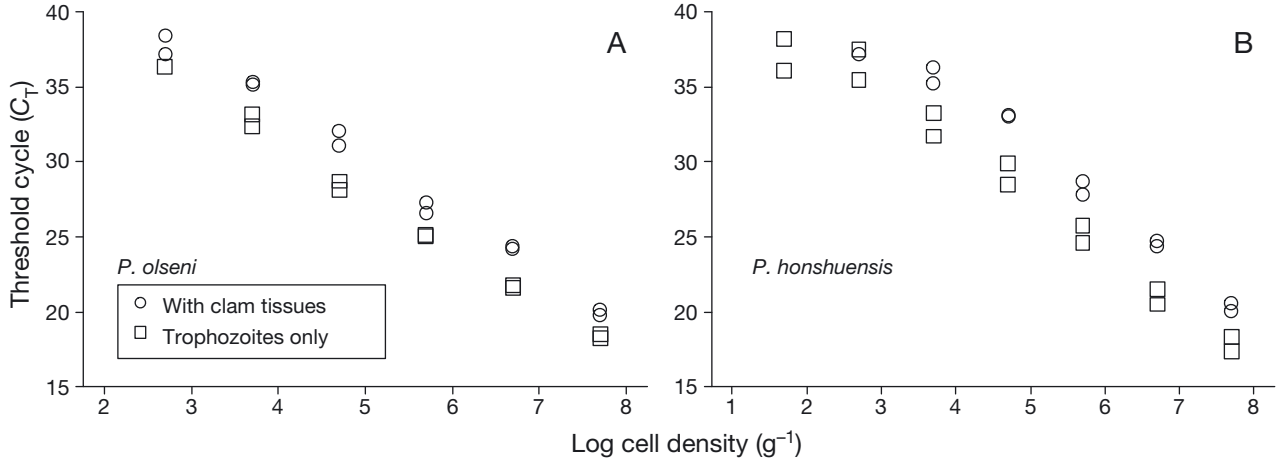

Fig. 1. Comparison of threshold cycle (C_T) values between DNA solutions extracted from trophozoites only and clam gill tissues spiked with trophozoites in (A) *Perkinsus olseni* and (B) *P. honshuensis*

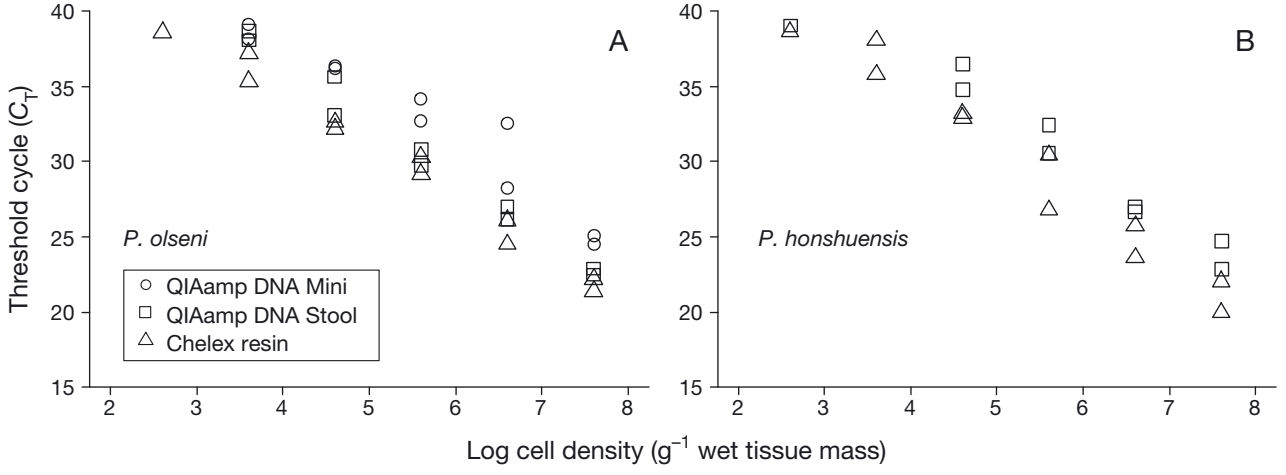

Fig. 2. Comparison of threshold cycle (C_T) values for DNA extracted using one of 3 methods from clam gill tissue spiked with trophozoites of (A) *Perkinsus olseni* and (B) *P. honshuensis*

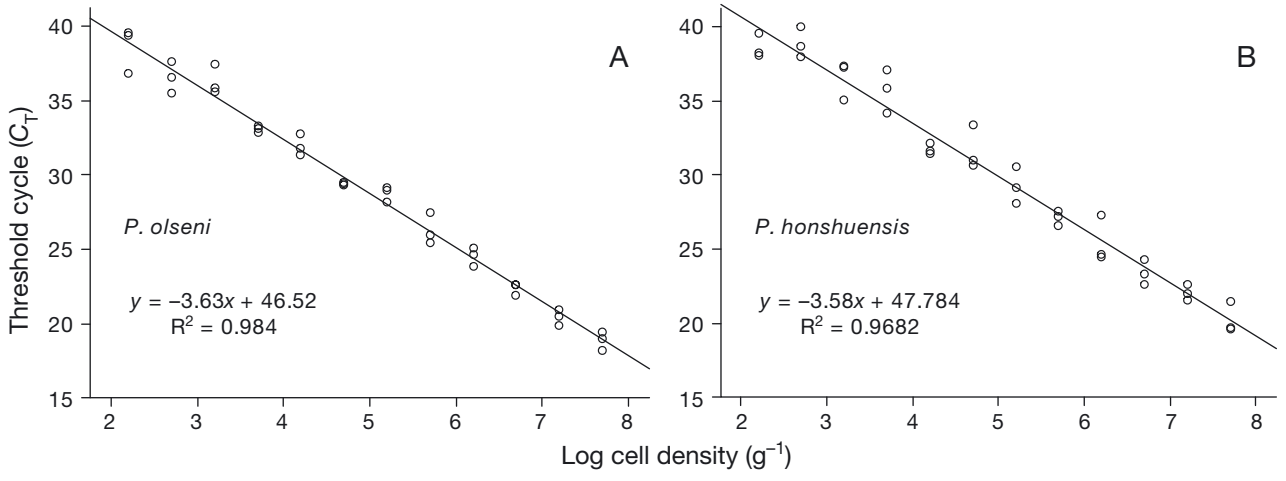

Fig. 3. Standard curve representing the relationship between cell density (cells g^{-1} wet gill) and threshold cycle (C_T) values of real-time PCR of (A) *Perkinsus olseni* and (B) *P. honshuensis*

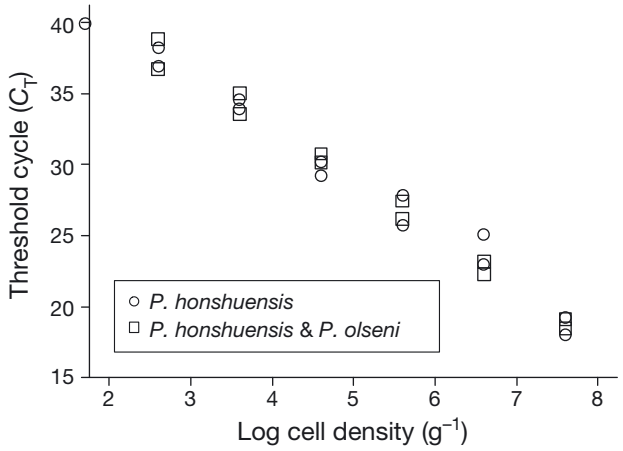

Fig. 4. Threshold cycle (C_T) values for DNA extracted from clam gill tissues spiked with *Perkinsus honshuensis* only and tissues spiked with both *P. honshuensis* and *P. olseni*

Comparison of preservation method and tissue type

The method of tissue preservation had little effect on the C_T value for both *Perkinsus* species (Fig. 5). Similarly, there was little difference between standard samples prepared with gill tissue or whole body tissue for both *Perkinsus* species (Fig. 6).

Comparison of real-time PCR and RFTM assays

All of the 92 Manila clams we examined were positive in the RFTM assay. Conversely, 64 clams were positive only for *Perkinsus olseni*, 2 were positive for only *P. honshuensis*, 13 were positive for both *P. olseni* and *P. honshuensis*, and 13 were negative for

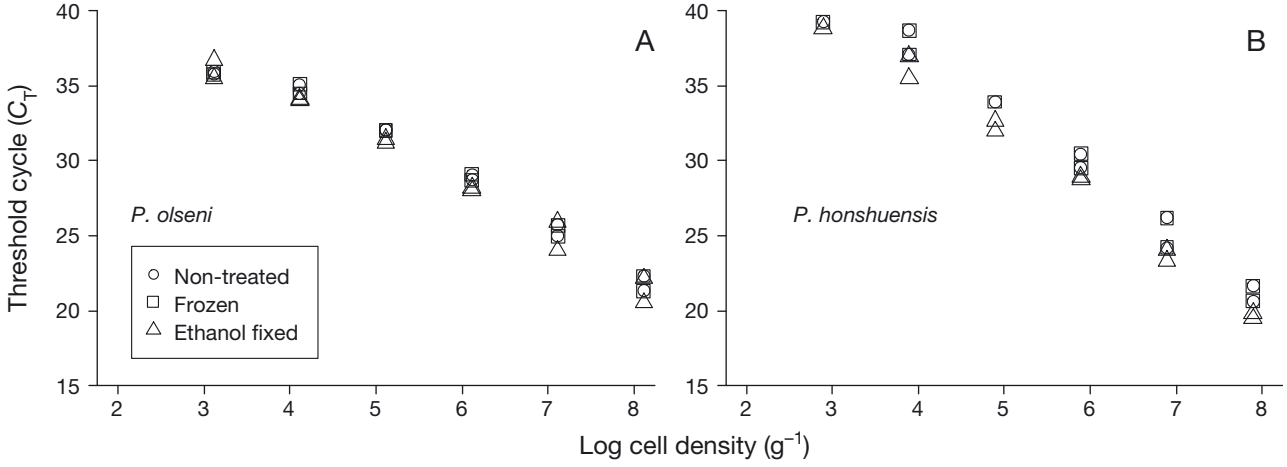

Fig. 5. Comparison of threshold cycle (C_T) values of cell density (g^{-1} wet gill) of (A) *Perkinsus olseni* and (B) *P. honshuensis* extracted from gills that were preserved using one of 3 methods: non-treated, frozen, and ethanol fixed

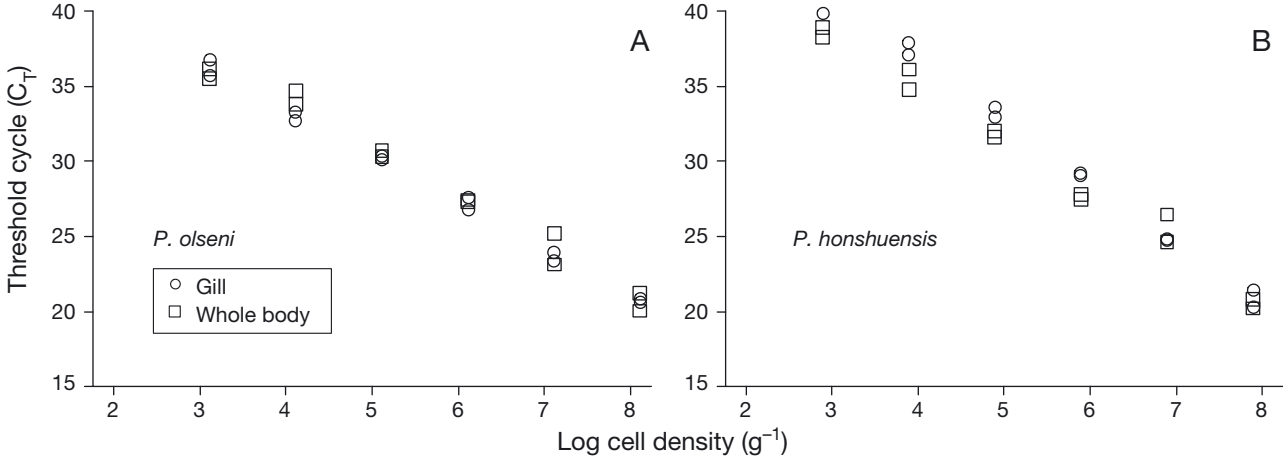

Fig. 6. Threshold cycle (C_T) values of cell density (g^{-1} wet gill) of (A) *Perkinsus olseni* and (B) *P. honshuensis* extracted from the gill or whole body

both *P. olseni* and *P. honshuensis* in the real-time PCR assays (Table 4). The prevalence of *P. olseni* was much higher than for *P. honshuensis* at all localities. Similarly, the infection intensity was between 16 and 40 000 times higher for *P. olseni* than for *P. honshuensis* in the clams that were positive for both species in real-time PCR (Fig. 7). The infection intensity of *P. honshuensis* (5.6×10^3 cells g^{-1}) tended to be higher in clams that were collected at the site where *P. honshuensis* was first documented. There was a significant linear correlation between the combined (both species) infection intensity determined by real-time PCR and RFTM ($r^2 = 0.70$, $p < 0.05$), after removal of PCR-negative clams from the analysis (Fig. 8A). In the Bland-Altman plot, the differences between the infection intensities determined by the 2 methods were independent of the infection intensity ($r^2 = 0.0136$, $p > 0.05$). Furthermore, the mean log intensity obtained using real-time PCR was significantly higher (0.15) than that obtained using RFTM (paired *t*-test, $p < 0.05$; Fig. 8B).

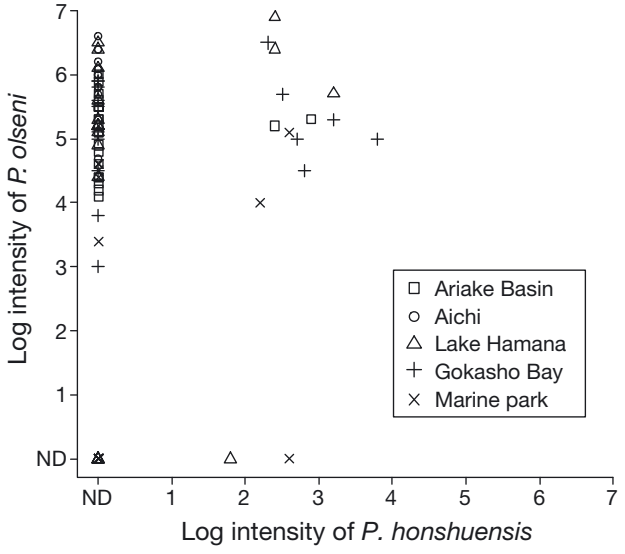

Fig. 7. Infection intensity of *Perkinsus olseni* and *P. honshuensis* in Manila clams from various locations measured by real-time PCR. Each symbol represents the value for an individual clam. ND: not detected

DISCUSSION

In general, studies of infectious diseases require methods for the identification and quantification of pathogens. Two species of the genus *Perkinsus* (*P. olseni* and *P. honshuensis*) have been found in Manila clams in Japan, often co-infecting single clams (Dungan & Reece 2006, Takahashi et al. 2009). These 2 species cannot be distinguished morphologically (Dungan & Reece 2006), thus necessitating the development of methods for their discrimination and quantification. Takahashi et al. (2009) developed a PCR-RFLP method that could be used to discriminate the 2 species and document their presence, but not for quantification. To our knowledge, the present study is the first report of real-time PCR methods for discriminatory quantification of *P. olseni* and *P. honshuensis*.

We distinguished the 2 species using primer sets that targeted sequences unique to *P. olseni* or *P. honshuensis* in the ITS regions either side of the 5.8S ribosomal DNA region. Interestingly, the primer set OF/OR, designed for *P. olseni*, also amplified *P. honshuensis* PRA-176. However, the C_T values differed considerably between the primer sets (15.7 and 26.7 for HF/HR and OF/OR, respectively). Assuming that the amplification efficiency for both primer sets was 2.0, we estimate that strain PRA-176 contains 2000-fold more *P. honshuensis* than *P. olseni*. *P. honshuensis* PRA-176 is a polyclonal strain from which the monoclonal strain *P. honshuensis* PRA-177 was derived. Given that *P. honshuensis* PRA-177 was not amplified using the OF/OR primer set, we conclude that *P. honshuensis* PRA-176 was contaminated with a small number of *P. olseni* cells.

Clam tissues contain compounds that often inhibit PCR reactions (Nakatsugawa 2007). Thus, it is important to optimize DNA extraction for PCR to reduce the inhibitory effect. We tested 3 extraction methods and found that 10% Chelex resin solution was the most effective at minimizing the inhibitory effect on real-time PCR. However, DNA samples extracted from clam gill tissues spiked with trophozoites

Table 4. Infection intensity (cells g^{-1} wet gill) of *Perkinsus olseni* and *P. honshuensis* at each site, measured by real-time PCR

Site	No. clams tested	*P. olseni*			*P. honshuensis*		
		No. infected clams	Infection intensity Min.	Max.	No. infected clams	Infection intensity Min.	Max.
Ariake Basin	22	22	1.3×10^4	9.5×10^5	2	2.8×10^2	7.8×10^2
Aichi Prefecture	16	16	5.4×10^4	3.6×10^6	0	0	
Lake Hamana	18	14	2.6×10^4	7.9×10^6	3	2.3×10^2	1.7×10^3
Mie Prefecture	16	16	9.5×10^2	2.9×10^6	6	1.9×10^2	5.6×10^3
Marine Park	20	9	2.4×10^3	1.6×10^5	3	1.2×10^2	4.3×10^2

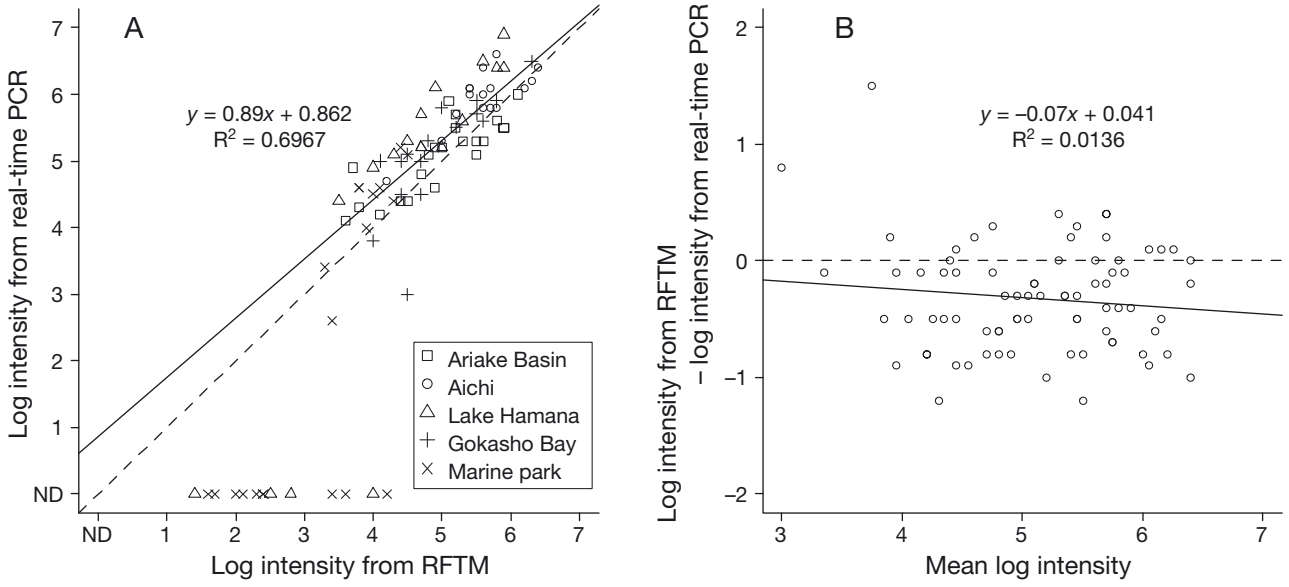

Fig. 8. (A) Relationship between the combined infection intensity (cells g^{-1} wet gill) of *Perkinsus olseni* and *P. honshuensis* determined by Ray's Fluid Thioglycollate Medium (RFTM) assay and by real-time PCR. The solid and dashed lines represent the correlation between the 2 intensities and the line $x = y$, respectively. ND: not detected. (B) Bland-Altman plot of the intensities determined by the 2 methods. The difference in log intensity obtained by the 2 methods was plotted against the mean log intensity obtained by the 2 methods. The solid and dashed lines represent the correlation between the difference and mean intensity and the line $y = 0$, respectively

yielded higher C_T values than those extracted from trophozoites only. Therefore, we used DNA extracted from gill tissue spiked with a known amount of *Perkinsus* trophozoites as standards for the real-time PCR assays.

Although spin-column methods have been used frequently to extract DNA from bivalves, we were unable to amplify DNA from samples extracted using the QIAamp DNA Mini kit, which uses spin-column methodology. The OIE manual (World Organization for Animal Health 2010) recommends the use of spin-column methodology for genus-specific PCR detection of *Perkinsus* species using the genus-specific primer set (PerkITS-85/PerkITS-750). Given this, we performed real-time PCR using the genus-specific primer sets on samples extracted with the QIAamp DNA Mini kit and Chelex resin. Samples extracted with the QIAamp DNA Mini kit were also successfully amplified, though the C_T values were higher by 2.1 to 4.3 units than those for DNA extracted with Chelex resin (data not shown). The PCR primers and conditions, which differ from those for genus-specific conventional PCR, and the presence of inhibitory chemicals that were not removed by the QIAamp DNA Mini kit may explain the failure to amplify DNA that was extracted using this method. The QIAamp DNA Stool kit also uses an absorption resin, InhibitEx tablet, to remove PCR inhibitors. Given that Chelex resin and the QIAamp DNA Stool kit showed higher efficiency of DNA amplification than the QIAamp DNA Mini kit, the PCR inhibitor in clam tissue may be substance(s) absorbed onto resin, e.g. some kind of metallic ion. In this study, we adopted Chelex resin as a standard method because of the efficiency of PCR amplification and the simplicity of the protocol.

The PCR reaction yielded a linear standard curve using DNA extracted from clam gill tissues spiked with known numbers of trophozoites, suggesting that the assay may be used for the quantification of both *Perkinsus olseni* and *P. honshuensis*. Furthermore, the assay was able to successfully quantify the infection intensity of *P. honshuensis* in the presence of *P. olseni* trophozoites.

DNA amplification was detected within the range of 5.0×10^7 to $5.0 \times 10^{1.5}$ trophozoites g^{-1} wet gill tissue for both *Perkinsus olseni* and *P. honshuensis*. In wild Manila clams, the highest infection intensity of *Perkinsus* species is generally approximately 10^7 cells g^{-1} wet gill tissue (Choi et al. 2002, Park et al. 2008, Yoshinaga et al. 2010), which is within the range that can be quantified using our method. Takahashi et al. (2009) showed that the lower limit of detection by PCR-RFLP was 100 cells per 10 mg tissues (10 000 cells g^{-1} tissue) for *P. olseni* and one cell (100 cells g^{-1} tissue) for *P. honshuensis*. Therefore, both assays are comparable in sensitivity.

There was little difference in the quantification of infection intensity by real-time PCR between the

frozen, ethanol-fixed, and fresh standard samples spiked with *Perkinsus* trophozoites. Moreover, we found no difference in the sensitivity of the assays between samples of gill tissue and whole body. Thus, our results suggest that both frozen and ethanol-fixed samples of the gill or whole body can be used to quantify infection intensity using these real-time PCR assays.

There was a linear correlation of the infection intensity of wild Manila clam samples between the real-time PCR and RFTM assays. However, the values obtained by real-time PCR were higher than those obtained by RFTM. This difference may be explained in 2 ways: (1) some existing *Perkinsus* trophozoites did not mature into hypnospores in the RFTM assay or (2) the DNA of dead cells is amplified by PCR.

Although the detection efficiency was high for the real-time PCR assays, some samples that tested positive in the RFTM assay were negative in the real-time PCR assays, particularly for clams with low infection intensities. Assuming all the trophozoites are enlarged in RFTM, the assay is able to detect the presence of even a single cell. Conversely, DNA is only extracted from a fragment of the tissue (<40 mg) prior to real-time PCR. Because *Perkinsus* spp. cells tend to be unevenly distributed in Manila clam tissues, the lack of amplification may be attributable to the low volume of tissue used for DNA extraction. Thus, an increase in the lower threshold of detection by real-time PCR likely requires an increase in sample volume. These real-time PCR assays are well suited for field research but less suited for diagnostic purposes. The level of infection with *P. honsehuensis* was much lower than that of *P. olseni* at all the locations examined in this study, consistent with the observations of Takahashi et al. (2009). *P. olseni* is likely predominant in the Manila clam populations examined in this study. Interestingly though, the infection intensity of *P. honshuensis* tended to be higher in clams that were collected from the site at which this species was first described, perhaps explaining why Dungan & Reece (2006) were able to isolate *P. honshuensis* in their earlier study.

The PCR primer set for *Perkinsus honshuensis* was designed based on the DNA sequence of a clonal isolate from only one locality. In addition, we have not checked the specificities of the primers we developed in this study for *P. olseni* and *P. honshuensis* against *P. beihaiensis*, as a cultured strain of *P. beihaiensis* is currently available. The specificities and efficacies of the primers may need to be tested when additional cultures of *P. honshuensis* and *P. beihaiensis* become available.

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