

Development of quantitative PCR assay for detection of the trematode parasite *Proctoeces maculatus* in the blue mussel *Mytilus edulis*

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ABSTRACT: The digenean trematode *Proctoeces maculatus* is an important parasite of the blue mussel *Mytilus edulis*. The parasite reduces mussel quality and yield, negatively impacting mussel aquaculture efforts. Typically, the trematode is detected by visual observation. To provide a better diagnostic tool able to detect this parasite at any life stage and at low intensities, we designed a species-specific molecular assay to detect *P. maculatus* in *M. edulis* tissue. Primers targeting the 18S nuclear ribosomal DNA (rDNA) from *P. maculatus* were used to develop an end-point polymerase chain reaction assay and a quantitative polymerase chain reaction (qPCR) assay. Analytical specificity of the assays was demonstrated using DNA from 4 other digenean trematodes. The qPCR assay was linear from 6.79×10^2 to 6.79×10^7 copies of the cloned target DNA and had a conservative detection limit of 68 copies. The qPCR assay detected single cercariae, and the number of isolated cercariae was linearly correlated with the threshold cycle (C_T). Diagnostic sensitivity of the PCR-based methods was 100%. The assays also detected the parasite in 6 additional samples from the 57 tested through microscopy. We used the assays to verify the presence of encapsulated sporocysts in the mantle and to document infected mussels from Dover, New Hampshire, extending the previously described northern range of the species. Thus, this work has important implications for detection of the parasite in aquaculture and in monitoring its potential spread with climate change.

KEY WORDS: Digenean · Aquaculture · Bivalve · Marine · Northwest Atlantic

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INTRODUCTION

The blue mussel *Mytilus edulis* Linnaeus, 1758 is an economically and ecologically important mollusc, as it is a dominant commercially harvested bivalve in temperate areas of both hemispheres (Seed & Suchanek 1992) and it supports a large aquaculture industry in Europe and North America. Trematodes, parasitic copepods, and shell-boring polychaetes are the primary parasites of blue mussels and have been studied extensively (e.g. Lauckner 1983, Bower 2010). One parasite species of increasing concern to mussel aquaculture is the digenetic trematode *Proctoeces maculatus* (Looss, 1901) that parasitizes *M. edulis*

and various other invertebrate and vertebrate host species. These trematodes are predominately found in the mantle tissue of bivalves (see Fig. 1), where they cause partial or complete castration of the host (Uzmann 1953, Stunkard & Uzmann 1959, Machkevsky 1985, Feng 1988, Teia dos Santos & Coimbra 1995, Kim et al. 2006, Antar & Gargouri 2015). High intensity infections can be seen macroscopically (Canzonier 1972) (see Fig. 1); such mussels were said to have 'orange sickness' (Cole 1935) due to the orange color often exhibited by the larval stage of the parasite.

Infection by *P. maculatus* decreases mussel quality and causes thinning of the mantle tissue (Cole 1935)

and visible abscesses (Sunila et al. 2004). The parasites deplete glycogen and triglyceride levels in the mantle and hepatopancreas of infected mussels (Dennis et al. 1974, Machkevsky & Shchepkina 1985) and cause major organ damage as they spread throughout the mussel (Machkevsky & Gaevskaja 2008). Intense infections cause mussels to gape and detach from the substrate (Machkevsky & Gaevskaja 2008). Thus, infected mussels may be unable to handle the stress of harvesting and processing involved in aquaculture (Cole 1935, Canzonier 1972). Infection slows mussel growth (Machkevsky 1988) and at the highest intensity levels, ultimately leads to mortality (Machkevsky 1982, Machkevsky & Gaevskaja 2008). *P. maculatus* was implicated in mass mortalities of *Mytilus galloprovincialis* Lamarck, 1819 in Laguna Vaneta, Italy (Munford et al. 1981), and in the Black Sea (Machkevsky & Parukhin 1981), where the disease is referred to as 'proctecosis' (Machkevsky & Gaevskaja 2008).

P. maculatus has been found along the Gulf Coast (Wardle 1980) and east coast of the USA as far north as Woods Hole, MA. After numerous attempts by previous researchers to find *P. maculatus* at sites north of Woods Hole were unsuccessful (Uzmann 1953, Pondick 1983), it was concluded that Woods Hole represented the northern geographic range limit of the parasite on the east coast of the USA. Pondick (1983) proposed that this was because the colder water temperatures north of Cape Cod act as a barrier for *P. maculatus*. Although *P. maculatus* has been found in many other localities worldwide (Bray 1983), considerable taxonomic confusion exists surrounding the species, and it likely comprises a species complex (Valdivia et al. 2010, Antar & Gargouri 2015).

P. maculatus does not display the typical trematode life cycle, which usually involves a gastropod intermediate host in which asexually reproducing larval forms (sporocysts) are found, a second intermediate host in which cercariae encyst as metacercariae (or encystment may occur on vegetation), and a definitive vertebrate host where sexual reproduction of adult trematodes occurs (e.g. Galaktionov & Dobrovolskij 2013, Antar & Gargouri 2015). Instead, *P. maculatus* can complete its entire life cycle within the mussel (Stunkard & Uzmann 1959, Lang & Dennis 1976), which likely becomes infected through accidental ingestion of trematode eggs (Lang & Dennis 1976). Miracidia hatch from the eggs and develop into mother sporocysts. Mother and daughter sporocysts are found primarily within the venous sinuses of the mantle, and large numbers also may be present

in the digestive organs (Stunkard & Uzmann 1959, Tripp & Turner 1978, Machkevsky & Gaevskaja 2008). Cercariae released from daughter sporocysts may infect new hosts through direct penetration of neighboring mussels (Lang & Dennis 1976). Thus, a single mussel can harbor any life stage(s) of the parasite. In tropical regions, adult *P. maculatus* reside in the hindguts of tropical and subtropical marine fish from the families Sparidae and Labridae (Bray 1983) after the fish consume infected mussels. However, in temperate waters fish hosts are less likely to be involved (Lang & Dennis 1976), and the trematode exhibits the abbreviated (progenetic) life cycle (Poulin & Cribb 2002).

Trematode infections are typically detected by visual observation of sporocyst, cercarial and/or adult stages through light microscopy. This presents a diagnostic challenge if only recently ingested eggs or miracidia are present. In addition, low intensity infections (1 to 100 sporocysts mussel⁻¹) of mussel tissue are practically indistinguishable from that of healthy individuals (Machkevsky & Gaevskaja 2008). Sporocysts in low intensity infections are difficult to see without experience studying *P. maculatus*, especially in the case of young sporocysts, which are smaller and lack the characteristic movement exhibited by mature sporocysts. In some instances, *P. maculatus* elicits a hemocytic response in the host mussel (Sunila et al. 2004) during which the parasites may be encapsulated and destroyed (Teia dos Santos & Coimbra 1995, Villalba et al. 1997). The parasites are nearly impossible to identify in the fresh tissue of these mussels, as the elongated sporocysts become round and opaque, bearing little resemblance to living sporocysts.

Molecular techniques offer greater sensitivity (Ndao 2009) and can detect the presence of trematodes at any life stage (Le et al. 2012, Liu 2012, Antar & Gargouri 2015). Both end-point polymerase chain reaction (PCR) and quantitative real-time PCR (qPCR) provide superior reproducibility and efficiency compared to visual detection (Weiss 1995, Ndao 2009) and have proven effective for parasite characterization and identification (Polley & Thompson 2009). Antar & Gargouri (2015) recently used 28S rDNA to confirm *P. maculatus* in *M. galloprovincialis* and fish hosts from Tunisia. Such molecular methods can also be used to detect DNA from dead pathogens as long as the target nucleotide sequence is intact (Josephson et al. 1993). This makes parasite detection possible even in hosts that have mounted a successful immune response, as shown by Phelan et al. (2016), who amplified DNA from encapsulated metacercar-

ial cysts (brown bodies) in polychaetes. This is important because the host response to *P. maculatus* is highly variable between mussels (Figueras et al. 1991), with some mussels able to destroy the parasite while others may have high intensity infections (Machkevsky & Gaevskaja 2008).

The purpose of this work was to develop a fast and inexpensive molecular diagnostic method for detection of *P. maculatus*. We sequenced the nuclear small subunit rDNA (18S rDNA) from *P. maculatus* in blue mussels from Long Island, NY, and used this to design primers specific to the 18S rDNA from *P. maculatus*. These primers were used to develop a qPCR assay for the detection of *P. maculatus* in *M. edulis* from the east coast of the USA. The diagnostic sensitivity of the assay was tested with mussels that had low intensities of *P. maculatus*, and analytical specificity was tested using DNA from mussels parasitized with the trematode *Himastha quissetensis* (Miller & Northup, 1926) and with genomic DNA from the digenean trematodes *H. quissetensis*, *Zoogonus lasius* (Leidy, 1891), *Gynaecotyla adunca* (Linton, 1905), and *Austroilharzia variglandis* (Miller & Northup, 1926).

MATERIALS AND METHODS

Collection and microscopy

Mytilus edulis specimens were sampled from Reynolds Channel, Point Lookout, NY (40° 35' 38" N, 73° 35' W), Jones Bay, Jones Beach, NY (40° 35' 24" N, 73° 33' 09" W), Shinnecock Bay, Hampton Bays, NY (40° 50' 29" N, 72° 29' 54" W), Cat Cove, Salem, MA (42° 31' 44" N, 70° 52' 17" W), Piscataqua River, Dover, NH (43° 07' 11" N, 70° 49' 38" W), Damariscotta River, Walpole, ME (43° 56' N, 69° 34' 52" W), and Frenchman's Bay, Lamoine, ME (44° 27' N, 68° 16' W). Parasite prevalence was determined by observation of the parasite in the mantle tissue under a dissecting

microscope for mussels from Jones Beach, Hampton Bays, Salem, and Lamoine. *Proctoeces maculatus* was identified based on morphological characteristics as described by Stunkard & Uzmann (1959). Prior to examination, a standardized sample (6 mm diameter, 56.5 mm²) was excised from the mantle near the gills and stored for molecular analyses at -20°C. In order to measure intensity, samples from Jones Beach were squashed between a clear slide and plastic coverslip with an adhesive 20 × 20 mm microscope slide-grid (Electron Microscopy Sciences). Intensity was quantified by counting the number of sporocysts viewed within the sample using a compound light microscope. The entire sample was then removed from the slide and stored for molecular analyses at -20°C. For mussels in which sporocysts were not counted, intensity was rated on a semi-quantitative scale. Mussels in which only a few sporocysts were found were considered to have a low infection level, mussels in which dispersed or patches of sporocysts were found were considered to have a medium infection, and mussels were considered to be heavily infected if sporocysts permeated the entire mantle. Cercariae were obtained from daughter sporocysts identified through light microscopy. A micro-scalpel was used to open the sporocysts and release the cercariae. Number of cercariae were counted and isolated by pipetting, and stored at -20°C.

DNA isolation and cloning

Sporocysts of *P. maculatus* were isolated from the mantle tissue of mussels collected from Point Lookout. DNA was isolated from the sporocysts using the DNeasy Blood and Tissue Kit (Qiagen), following the manufacturer's instructions. The 18S rDNA sequence was amplified through PCR using the WormA and WormB primers (Littlewood & Olson 2001) (Table 1). The 50 µl reaction contained Phusion HF reaction buffer (New England Biolabs), 1 unit of Phusion DNA

Table 1. Primers used for sequencing and detection of *Proctoeces maculatus*. Positions are relative to the 18S ribosomal DNA sequence for *P. maculatus* (GenBank accession no. KR052815)

Name	Sequence (5'-3')	Length (nt)	Position	Reference
Pmac18S-F	GTT GCT ACG ACT TGT ACT TG	20	595-614	Present study
Pmac18S-R	TGC AAA CGA GTC CTA ATG GA	20	717-697	
18S-WormA	GCG AAT GGC TCA TTA AAT CAG	21	1-21	Machida & Knowlton (2012)
18S-WormB	CTT GTT ACG ACT TTT ACT TCC	21	1863-1843	
18S1	CTG GTG CCA GCA GCC GCG GYA A	22	494-515	Littlewood & Olson (2001)
18S2_RC	TCC GTC AAT TYC TTT AAG TT	20	1172-1153	

Polymerase (New England Biolabs), 200 μM of deoxynucleoside triphosphates (dNTPs), 0.5 μM of forward and reverse primers, and 1 μl of genomic DNA. The thermocycling conditions were 98°C for 30 s; 35 cycles of 98°C for 15 s, 50°C for 20 s, and 72°C for 1 min; followed by 72°C for 10 min. The amplified DNA was inserted into a pCR-Blunt vector (Invitrogen) and transformed into GC10 Thunderbolt Electrocompetent cells (Sigma). Plasmids were isolated using the FastPlasmid Mini Kit (5 PRIME) and quantified using UV spectroscopy with a Synergy-HT microplate reader (BioTek). Copy numbers were calculated using the size of the plasmid and insert.

Chelex DNA isolation

For the purpose of developing a standardized molecular assay for *P. maculatus*, the standardized samples of mantle tissue were squashed and the number of sporocysts and adults per sample were counted as described above. DNA from these samples was isolated using Chelex[®]-100 resin (Walsh et al. 1991) following the protocol from Phelan et al. (2016), which was used to successfully extract DNA from encapsulated trematode cysts. Samples were removed from the slides and placed in 300 μl 10% Chelex. Samples were homogenized with 0.5 mm glass beads before adding 0.166 μg μl^{-1} Proteinase K. A 1 h incubation was performed at 60°C, followed by 10 min at 95°C. Samples were centrifuged at 18000 $\times g$ for 3 min. The supernatant containing the DNA was removed and the DNA was quantified using a Synergy-HT microplate reader (BioTek). For qPCR, cercariae isolated from daughter sporocysts were counted and DNA was isolated from pooled numbers ranging from 1 to 10 with Chelex as above.

Sequencing

Sanger sequencing of plasmid DNA from 5 clones was performed by Macrogen using the M13 universal primers and 18S1 and 18S2_RC (Machida & Knowlton 2012) universal primers for metazoan species (Table 1). Sequences were edited and assembled using BioEdit (Hall 1999). The final sequence was submitted to GenBank under the accession number KR052815. ClustalX executed in MEGA6 (Tamura et al. 2013) was used to align the sequence with published sequences of *P. maculatus* (GenBank accession no. AY222161) from Mississippi, USA, and *P. maculatus* (GenBank accession no. AJ224459) from

Queensland, Australia, as well as the congeners *Proctoeces* sp. (GenBank accession no. JQ782520) and *P. lintoni* (GenBank accession no. EU423077).

Primer design for end-point and qPCR

ClustalX executed in MEGA6 (Tamura et al. 2013) was used to align sequences of the genus *Proctoeces* with the closely related digenean trematodes *Felodistomum agnotum* Nicoll, 1909 (labeled as *Stringophorus agnotus* in GenBank; accession no. Z12599), *Olssonium turneri* Bray & Gibson, 1980 (GenBank accession no. AJ287548), *Stringophorus margolisi* Bray, 1995 (GenBank accession no. AJ287578), *Tergerstia laticollis* (Rudolphi, 1819) (GenBank accession no. AJ287580), *Felodistomidae* sp. (GenBank accession no. FJ595659), *Complexobursa* sp. (GenBank accession no. AJ224462), *Coomera brayi* Dove & Cribb, 1995 (GenBank accession no. AJ224469), *Stringophorus furciger* (Olsson, 1867) (GenBank accession no. Z25818), and *Felodistomum fellis* (Olsson, 1868) (GenBank accession no. Z12601). Primers with sequences specific to *P. maculatus* were designed from the most variable regions within this alignment (Table 1). Specificity of the primer pair was confirmed through the use of NCBI BLAST nucleotide sequence similarity search. Candidate primer sequences were screened using the IDT OligoAnalyzer 3.1 web application (Owczarzy et al. 2008).

End-point PCR

Four candidate primers were tested for efficacy and specificity through PCR using genomic DNA from *P. maculatus*, *M. edulis*, *H. quissetensis*, *Z. lasius*, *G. adunca*, and *A. variglandis*. PCR was performed in 25 μl reactions containing OneTaq Quick-Load Master Mix with Standard Buffer (New England Biolabs), 20 ng of Chelex-purified DNA, and 0.2 μM of forward and reverse primers. The 18S1 and 18S2 primers (Machida & Knowlton 2012) were used for positive controls and a no-template control was included for all reactions. The primer pair Pmac18S-F and Pmac18S-R (Table 1) had the least non-target amplification and was chosen for further analysis. Thermocycling conditions were optimized in order to maximize primer specificity. The optimized conditions were 94°C for 2 min; 35 amplification cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 30 s; followed by 68°C for 10 min. The limit of detection was assessed with DNA extracted from standardized

(56.5 mm²) samples of mantle tissue containing visually counted sporocysts (n ranging from 0 to 258). PCR products were amplified from 1, 1:10, 1:50, and 1:100 dilutions of sample DNA. End-point PCR reactions were performed in duplicate.

qPCR

qPCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosciences). All reactions were performed in triplicate using the Pmac18S-F and Pmac18S-R primers (Table 1). The 25 μ l reactions contained Power SYBR Green PCR Master Mix (Applied Biosciences), 1 μ l of a 1/10th dilution of Chelex-purified DNA (approximately 20 to 100 ng μ l⁻¹), and 0.2 μ M of forward and reverse primers. The thermocycling conditions were 95°C for 10 min and 45 amplification cycles of 95°C for 20 s, 60°C for 1 min, and 72°C for 30 s. A melt curve analysis was performed following PCR amplification.

A standard curve was generated using 10-fold serial dilutions of plasmid DNA containing the 18S rDNA target region. Dilutions ranged from 6.79×10^7 to 6.79 copies per 25 μ l reaction and were prepared using molecular-grade water. A new standard curve was used for each qPCR assay. qPCR was performed using DNA isolated from known quantities of cercariae and from DNA from mantle tissue containing either known or unknown numbers of sporocysts. Due to the high DNA concentration of DNA isolated from samples of mantle tissue (~200 to 1000 ng l⁻¹), 1:10 dilutions were used in the qPCR assay. The number of parasites per sample was adjusted for the dilution factor during analyses. All qPCR reactions were performed in triplicate.

RESULTS

Microscopy

Sporocysts of *Proctoeces maculatus* were visually identified in 30 out of 50 (60%) of the mussels from Jones Beach, NY, 4 out of 27 (14.8%) of the mussels from Hampton Bays, NY, 3 out of 41 (7.3%) of the mussels from Salem, MA, and 4 out of 66 (6%) of the

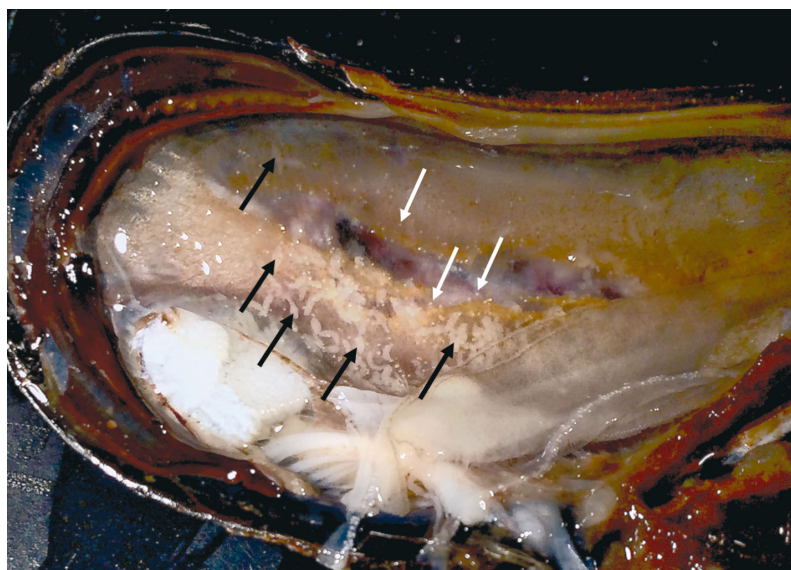


Fig. 1. Sporocysts of *Proctoeces maculatus* (black arrows) and metacercarial cysts of *Himasthla quissetensis* (white arrows) in the mantle of *Mytilus edulis*

mussels from Dover, NH. The parasite was not found in any of the mussels (n = 104) sampled from Maine. One mussel from Jones Beach seemed to contain a high intensity of encapsulated sporocysts which appeared as circular, opaque blisters in the mantle. The mantle of this mussel had the same thin and translucent appearance as mussels found with high intensity trematode infections. Metacercarial cysts of *Himasthla quissetensis* (identified by the presence of collar spines in the metacercariae; see Stunkard 1938, his Fig. 5) were found in mussels from Hampton Bays, Salem, Dover, Walpole, ME and Lamoine, ME (Fig. 1). The cysts were found primarily in the labial palps, with extensive infections extending into the mantle.

End-point PCR specificity and sensitivity

The primer pair Pmac18S-F and Pmac18S-R (Table 1) successfully amplified a 123 bp region of the 18S rDNA without non-target amplification. During early trials, the primers also resulted in amplification of *H. quissetensis* rDNA; thermocycling conditions were then optimized to increase specificity. Using the Pmac18S-F and Pmac18S-R primers with an annealing temperature of 60°C yielded amplification of *P. maculatus* samples without amplification of non-target species.

PCR was carried out using genomic DNA from *P. maculatus*, *Mytilus edulis*, *H. quissetensis*, *Z. lasius*,

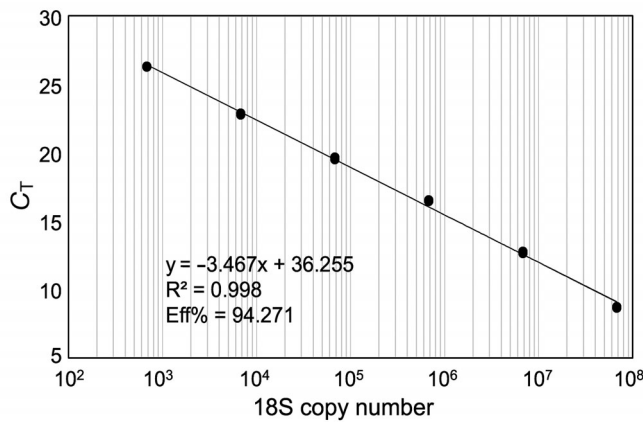


Fig. 2. Quantitative PCR standard curve generated using 10-fold serial dilutions of *Proctoeces maculatus* 18S rDNA in the pCR-Blunt vector. Dilutions ranged from 6.79 to 6.79 × 10⁷ copies reaction⁻¹. All reactions were performed in triplicate. The curve is linear from 6.79 × 10² to 6.79 × 10⁷ copies reaction⁻¹. The x-axis uses a log₁₀ scale; C_T: threshold cycle

G. adunca, and *A. variglandis* and the Pmac18S-F and Pmac18S-R primers (Table 1). Amplification only occurred in the sample containing DNA from *P. maculatus*, confirming diagnostic specificity. The diagnostic sensitivity of the end-point PCR assay was 0.8 sporocysts 56.5 mm⁻² using DNA extracted from standardized samples of mantle tissue containing a known quantity of sporocysts (a 1/10 dilution of sample that contained 8 sporocysts as confirmed by microscopy).

qPCR limit of detection

The limit of detection (analytical sensitivity) was assessed through a standard curve using triplicate reactions of 10-fold serial dilutions of plasmid DNA containing the 18S insert. Dilutions ranged from 6.79 to 6.79 × 10⁷ copies of the 18S rDNA gene. The assay was linear (R² = 0.998) from 6.79 × 10² to 6.79 × 10⁷ copies reaction⁻¹ (Fig. 2). The dilution containing a calculated 67.9 copies was also detected 100% of the time, with an average threshold cycle (C_T) value of 29.4, showing a conservative detection limit of 68 copies of the 18S rDNA.

Parasite quantification through qPCR

qPCR was performed on samples containing DNA from known quantities of cercariae and from samples of mantle tissue containing visually counted sporo-

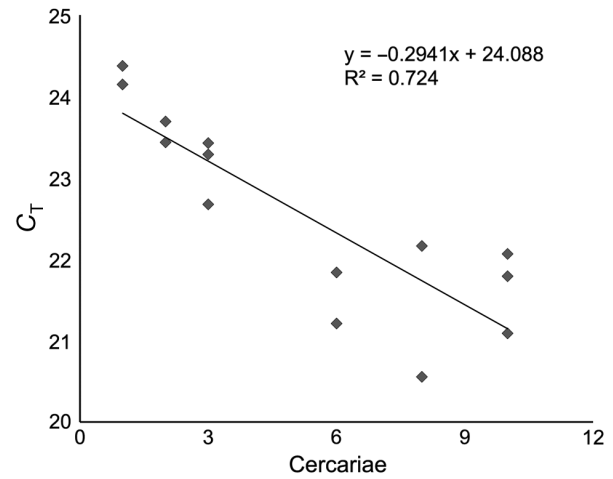


Fig. 3. Linear regression of isolated cercariae of *Proctoeces maculatus* with quantitative PCR threshold cycle (C_T). Regression analysis was linear from 1 to 10 cercariae (p < 0.001). Samples containing a single cercaria were detectable by qPCR. All reactions were performed in triplicate

cysts. All tested quantities of cercariae (1 to 10) were detected through qPCR. The number of cercariae was linearly correlated with the C_T (R² = 0.724, p < 0.001) (Fig. 3). The qPCR assay had an analytical sensitivity of 1 cercaria, which it detected 100% of the time. The average 18S rDNA copy number for 1 cercaria was 26873 (SD = 2010 for triplicate reactions of n = 2). There was no linear relationship between the number of sporocysts in a sample of mantle tissue and C_T value.

Assay application

Chelex-purified DNA from 57 mussels that had been visually assessed for the presence of *P. maculatus* was amplified through end-point PCR using the Pmac18S primers. The qPCR assay was performed on 28 of these same samples. *P. maculatus* was detected in 20 of 57 (35.08%) mussels through microscopy and 26 of 57 (45.6%) mussels using the molecular assays. The qPCR results confirmed the presence of *P. maculatus* in every mussel in which the parasite was identified via microscopy, and in 1 mussel in which *P. maculatus* was neither detected through end-point PCR nor microscopy. The end-point PCR method failed to detect *P. maculatus* in 2 samples in which the parasite had been identified via microscopy, but the parasite was detected in these samples through qPCR. One sample appeared to contain dead, encapsulated sporocysts, and presence of *P. maculatus* in this mussel was verified through end-point PCR.

Neither the PCR nor the qPCR assay showed evidence of detection for mussels that contained metacercarial cysts of *H. quissetensis* but had been determined negative for *P. maculatus* through microscopy. Late amplification ($C_T > 33$) occurred in some samples that contained only mussel DNA, as well as in no-template controls. Since the dilutions at the limit of detection (68 copies of the 18S) also had a C_T value below 30, only samples with C_T values below 33 were considered positive by qPCR. Melt curve analysis returned a single product peak with melting temperatures ranging from 81.39 to 81.82°C (Fig. 4), confirming diagnostic specificity. There were no discrepancies among triplicate qPCR reactions. All reactions considered positive were positive for all replicates.

DISCUSSION

We developed a sensitive and specific qPCR assay for the detection of *Proctoeces maculatus* in *Mytilus edulis* and compared it to both end-point PCR and visual methods of prevalence assessment. The qPCR assay was able to detect *P. maculatus* in tissue from one mussel in which the parasite was not detected by other methods. The limit of detection through qPCR was 68 copies of the 18S rDNA per reaction, or, more biologically relevant, a single cercaria. Both the limit of detection and the occurrence of amplification in no template controls were used to define the C_T cutoff for *P. maculatus* positive samples, which are recom-

mended methods for cutoff selection in diagnostic qPCR (Caraguel et al. 2011). Only samples with a C_T below 33 (using 45 cycles for qPCR) were considered positive for *P. maculatus* by qPCR. This C_T cutoff is conservative, yet low enough to protect against false positives due to contamination or amplification artefacts in this assay.

The PCR assay proved to be specific, as DNA from the trematode species *Himasthla quissetensis*, *Zoogonus lasius*, *Gynaecotyla adunca*, and *Austroilharzia variglandis* was not detected. Additionally, mussels containing only metacercarial cysts of *H. quissetensis* were not detected through qPCR. The melt curve analysis, used to assess qPCR assay specificity (Reischl 2006, Andree et al. 2011), returned a single peak, confirming the presence of only one amplification product. This is important since *H. quissetensis* can be common in mussel samples (Stunkard 1938).

The analytical sensitivity was determined by a standard curve using 10-fold serial dilutions containing 6.79 to 6.79×10^7 copies of the 18S target DNA. The assay was linear down to 6.79×10^2 copies. Dilutions containing 68 copies of the 18S DNA also amplified in 100% of runs, showing a limit of detection of 68 copies. The next dilution in the series (7 copies) was never detected but the true limit of detection likely lies somewhere between 7 and 68. While the assay was not linear down to 68 copies, using low-retention tubes may help to increase linearity. The use of low-retention plastics has been shown to

greatly improve quantification of target gene copy numbers below 100 (Ellison et al. 2006). These were not used in the reactions described in this paper, but the future use of low-retention tubes for plasmid and sample DNA storage and dilutions would theoretically increase the sensitivity and linearity of the assay. This may be useful in the detection of the parasite in a heavily diluted sample with very low intensity, but increased sensitivity is not typically necessary for the detection of a multicellular organism when using a multi-copy target gene such as 18S rRNA (Richard et al. 2008). In fact, the assay detected a single cercaria 100% of the time.

Both molecular methods had higher diagnostic sensitivity than microscopy. *P. maculatus* was detected in 26 of 57 (45.6%) mussels using the com-

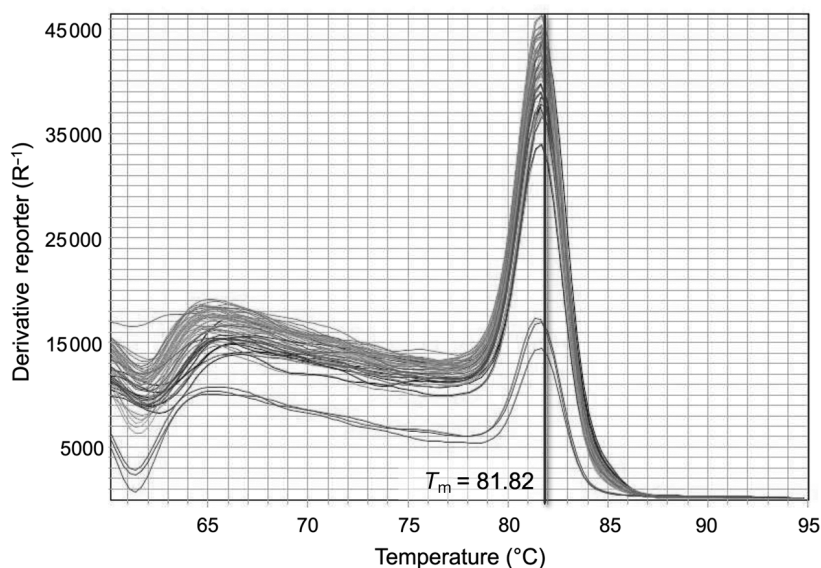


Fig. 4. Melt curve analysis for quantitative PCR (qPCR) assay using Pmac18S primers to amplify a region of the 18S rDNA from *Proctoeces maculatus*. T_m , melting temperature

Table 2. Comparison between microscopy, end-point PCR, and quantitative (qPCR) for detection of *Proctoeces maculatus* in *Mytilus edulis*. A total of 28 samples were tested using all 3 methods and an additional 29 were tested using only end-point PCR and microscopy. Mussels were scored as positive (+) or negative (–) for each method depending on whether the parasite was detected. A negative (–) qPCR result represents no amplification detected within 30 amplification cycles

Assay	Result combination							
	+	+	–	–	–	+	–	–
Microscopy	+	+	–	–	–	+	–	–
PCR	+	–	+	–	–	+	+	–
qPCR	+	+	+	+	–			
No. of mussels	13	2	1	1	11	5	4	20

bined PCR-based methods (Table 2), and in 20 of 57 (35.08 %) using microscopy. This may have been due to low intensity infections, or the presence of a life cycle stage that is difficult to detect visually, such as the miracidial stage. One of the samples appeared to contain encapsulated sporocysts, and the end-point PCR assay confirmed the presence of *P. maculatus* in this sample. PCR-based detection is an alternative to microscopy that can be used in place of, or in addition to, visual methods to verify findings.

We designed our end-point PCR method to be inexpensive, scalable, and relatively easy to perform for the purpose of making it available to researchers with basic molecular biological skills and resources, but it does have some limitations. DNA from 2 mussels in which the parasite was found via microscopy did not amplify through end-point PCR assays but did amplify through qPCR, which demonstrates that the end-point PCR assay has limited diagnostic sensitivity compared to the qPCR assay. Additionally, *P. maculatus* was detected through end-point PCR in a sample containing a DNA equivalent of 1 sporocyst, but went undetected in the 2 samples known to contain a larger number of sporocysts, suggesting that the efficacy of the end-point PCR assay was unrelated to the intensity of infection and could be related to the DNA extraction efficiency instead. The sensitivity of PCR-based detection methods is known to be influenced by the DNA extraction method (Bletz et al. 2015). We chose Chelex because of its cost and ease of use but acknowledge that other methods would likely yield higher quality DNA with less potential PCR inhibition. The Qiagen DNeasy Blood and Tissue Kit, for example, has been shown to be very efficient (Bletz et al. 2015) and could be used for future DNA extractions, but this would increase the cost and processing time of the assay considerably. Addi-

tionally, DNA extraction methods are unable to completely separate DNA from PCR inhibitors (Liu et al. 2009, Nagle et al. 2009). For this reason, dilutions were used in the PCR assays in order to decrease PCR inhibition. Although most samples amplified using both 1:10 dilutions and undiluted DNA, some samples only amplified using one or the other. For future use of the end-point qPCR assay it is recommended to use both undiluted DNA and 1:10 dilutions, or to use a secondary purification along with both dilutions as was done by Liu et al. (2009). In the qPCR assay all samples amplified from the 1:10 DNA dilutions, so the use of 1:10 dilutions is recommended. While there were no false negative results from the qPCR assays, estimation of the true diagnostic sensitivity of the assay will require testing of more samples. Additionally, false negatives are theoretically possible when sampling mussels with localized infections. Increasing the amount of host tissue used in an assay can help reduce the frequency of false negatives due to sampling error (Iwanowicz et al. 2015). As a preventative measure, the whole mantle could be used instead of, or in addition to, a sample of mantle tissue, as was done by Liu et al. (2009) with *Mercenaria mercenaria* (Linnaeus, 1758) infected with QPX. Alternatively, DNA from a cross-section that includes samples of each mussel tissue (as is prepared for histopathological examinations) could be used in molecular assays.

The qPCR assay was able to detect a single cercaria, which is perhaps more biologically relevant than the sensitivity as determined by dilution of the cloned 18S rDNA gene. There was a linear relationship between the number of cercariae and the C_T . Conversely, there was no correlation between C_T and the number of visually counted sporocysts within a sample of mantle tissue. This was not surprising due to the high variability in the number of daughter sporocysts or cercariae contained within mother or daughter sporocysts. However, the results were also consistent with the end-point PCR data, as some of the samples containing a large number of sporocysts only amplified using the undiluted DNA and not the 1:10 dilutions. This is likely related to the efficiency of DNA extraction, on which accurate quantification through qPCR is dependent (Bletz et al. 2015). This assay is meant for the detection of *P. maculatus* in low intensity infections or in infection verification when identification via microscopy is not possible, therefore quantification is not necessary. However, use of another DNA extraction method could improve analytical sensitivity and increase the accuracy of quantification.

The detection of *P. maculatus* in mussels from Dover, NH extends the geographical range of the parasite ~180 km farther north than the previously described northernmost limit of Woods Hole, MA (Uzmann 1953, Stunkard & Uzmann 1959, Pondick 1983). Presence of *P. maculatus* had previously been assessed at sites north of Woods Hole; Uzmann (1953) sampled over 2000 mussels sampled from Newburyport and Gloucester, MA, none of which were infected. In addition, Pondick (1983) examined populations of dog whelks *Nucella lapillus* (Linnaeus, 1758) from 8 sites along the coast of New England for *P. maculatus*. *P. maculatus* was found in *N. lapillus* from Rhode Island (Block Island and Point Judith, Narragansett) and Connecticut (Avery Point) at prevalences of up to 4.7%, but not in the locations in Massachusetts (Manomet, Cape Cod Bay) or Maine (Eastport, New Harbor, and Pemaquid Point). Pondick (1983) concluded that the colder water temperatures north of Cape Cod act as a barrier for *P. maculatus*. This could have been the case in 1983, but increasing water temperatures may have broken this barrier, allowing for the northward expansion of *P. maculatus*. A recent modeling study showed that sea surface temperatures (SSTs) in the Northeast Atlantic are increasing almost 3 times faster than the global average (Saba et al. 2016). Furthermore, SSTs in the Gulf of Maine are rising faster than 99% of the world's ocean waters (Pershing et al. 2015).

Due to the effects of climate change, northward range expansions have already occurred in other trematode species, and as global temperatures rise, even greater range expansions are expected (Mas-Coma et al. 2008, 2009, Huntley et al. 2014). For some parasites, climate change is also predicted to lead to greater disease impact due to an increase in local prevalence (Mas-Coma et al. 2008, Huntley et al. 2014, Williams & Boyko 2016). This is likely related to higher transmission rates caused by an increase in cercarial production response to warming water temperatures (Poulin 2006). The prevalence of *P. maculatus* in Salem, MA and Dover, NH is currently low, but prevalence may increase in the future.

While further studies are necessary in order to assess the current distribution of *P. maculatus*, the observed range extension may impact mussel aquaculture along the east coast of the United States. *P. maculatus* has had damaging effects on aquaculture of *Mytilus galloprovincialis* in the Black Sea, which led to efforts to improve methods for diagnosis and control (Machkevsky & Gaevskaja 2008). The present distribution of *P. maculatus* already overlaps areas of mussel aquaculture in Rhode Island and

Massachusetts. While we did not find *P. maculatus* in any of the mussels sampled from locations in Maine, parasites were found in mussels from the Piscataqua River in Dover, New Hampshire, which lies at the boundary between the 2 states. If the parasite moves northward with climate change (as found in other parasites; e.g. Harvell et al. 2002, Marcogliese 2008, Burge et al. 2014), it may begin to invade this important region for mussel production. One earlier study of mussel parasites found that offshore and off-bottom mussel culture reduced parasite load, presumably because intermediate hosts such as snails are restricted to nearshore benthic sites (Buck et al. 2005). Because *P. maculatus* can complete its entire life cycle within its bivalve host, both nearshore, benthic, suspended and offshore mussels may be vulnerable to infection.

The range expansion of *P. maculatus* and potential increase in local prevalence are likely to have detrimental effects on aquaculture of *M. edulis*. Blue mussel aquaculture has been steadily increasing over the last 3 decades, with global production reaching 184433 tons in 2014, translating to almost US \$ 444 million in revenue (FAO 2014). *P. maculatus* has been recently included in a list of economically important parasites of blue mussels (Shinn et al. 2015). Assuming fatality of mussels following infection by *P. maculatus*, Shinn et al. (2015) calculated that at an average prevalence of 0.68% would have caused damages totaling US \$ 1.07 million. Even if infections are not fatal, *P. maculatus* can severely reduce salability of mussels due to the apparent 'orange sickness' and can reduce profitability by negatively impacting reproduction. Machkevsky & Gaevskaja (2008) reported that the disease reduced productivity of mussel farms in the Black Sea due in part to the unmarketability of diseased mussels. Additionally, the reduced reproductive capabilities of cultured mussels infected with *P. maculatus* adversely affects the profitability of mussel farms due to a decrease in spat (Machkevsky & Gaevskaja 2008), as this causes production to decline. Sunila et al. (2004) reported that the high fall prevalence of *P. maculatus* (60%) off the Connecticut coast of Long Island Sound would limit potential for mussel culture to the seasons with low disease prevalence. The range expansion of *P. maculatus* may lead to limits on aquaculture farther north and the species should be monitored as proposed for other parasites and pathogens (Groner et al. 2016, Maynard et al. 2016).

Consistent mussel sampling is recommended to monitor the potential range expansion and to institute any management practices that might help pre-

vent the spread of *P. maculatus* (Machkevsky & Gaevskaja 2008). The PCR-based methods will be useful as alternative or additional method for parasite detection as they allow for higher sensitivity in testing. The assays also have potential use for testing water samples. This would likely be most effective during spawning when mussels release parasites along with gametes (Machkevsky 1985). Spawning and partial parasite expulsion can also be artificially induced by increasing water temperature (Machkevsky 1985). Additionally, Polley & Thompson (2009) proposed that molecular techniques should be used to determine new parasite distributions that result from climate change. The molecular assay we have developed provides a powerful tool in detecting the parasite, thus enabling new studies on the biology and impacts of this ecologically and commercially important species.

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