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Development and validation of a quantitative PCR assay for *Ichthyophonus* spp.

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ABSTRACT: Members of the genus *Ichthyophonus* are trophically transmitted, cosmopolitan parasites that affect numerous fish species worldwide. A quantitative PCR (qPCR) assay specific for genus *Ichthyophonus* 18S ribosomal DNA was developed for parasite detection and surveillance. The new assay was tested for precision, repeatability, reproducibility, and both analytical sensitivity and specificity. Diagnostic sensitivity and specificity were estimated using tissue samples from a wild population of walleye pollock *Theragra chalcogramma*. *Ichthyophonus* sp. presence in tissue samples was determined by qPCR, conventional PCR (cPCR), and histology. Parasite prevalence estimates varied depending upon the detection method employed and tissue type tested. qPCR identified the greatest number of *Ichthyophonus* sp.-positive cases when applied to walleye pollock skeletal muscle. The qPCR assay proved sensitive and specific for *Ichthyophonus* spp. DNA, but like cPCR, is only a proxy for infection. When compared to cPCR, qPCR possesses added benefits of parasite DNA quantification and a 100-fold increase in analytical sensitivity. Because this novel assay is specific for known members of the genus, it is likely appropriate for detecting *Ichthyophonus* spp. DNA in various hosts from multiple regions. However, species-level identification and isotype variability would require DNA sequencing. In addition to distribution and prevalence applications, this assay could be modified and adapted for use with zooplankton or environmental samples. Such applications could aid in investigating alternate routes of transmission and life history strategies typical to members of the genus *Ichthyophonus*.

KEY WORDS: *Ichthyophonus* sp. · qPCR · Validation · Detection methods · *Theragra chalcogramma*

INTRODUCTION

Parasites and diseases can have considerable ecological and economic impacts on commercially important fish stocks, including poor product recovery and quality of end products (Kurochkin 1985). Ichthyophonosis, a disease of economic significance in both wild and cultured fisheries (McVicar 1999), is believed to cause mortalities in numerous fish species including yellowtail flounder *Limanda ferruginea*, rainbow trout *Oncorhynchus mykiss*, and Atlantic herring *Clupea harengus* (Sindermann 1958, Powles et al. 1968, McVicar 1982, Patterson 1996, Rahimian & Thulin 1996, Mellergaard & Spanggaard 1997). The effects of ichthyophonosis can vary among hosts, but include reduced swimming stamina, a reduction in mean age, and reduced seafood product quality (McVicar 1999, Hershberger et al. 2002, Kocan et al. 2004). Surveys of Atlantic herring (Sindermann & Chenoweth 1993) and yellowtail flounder (Powles et al. 1968, Ruggieri et al. 1971) have documented epizootics in the North Atlantic. However, in regions of the North Pacific, ichthyophonosis is considered to be an emerging disease

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affecting Chinook salmon *O. tshawytscha* (Kocan et al. 2004), Pacific herring *C. pallasii* (Marty et al. 1998), and walleye pollock *Theragra chalcogramma* (Eaton et al. 1991).

Ichthyophonosis is caused by protists of the genus *Ichthyophonus* in the class Mesomycetozoea, a group located at the divergence between animals and fungi (Mendoza et al. 2002). Two species exist within this genus, viz. the type species *I. hoferi*, described over a century ago (Plehn & Mulsow 1911), and *I. irregularis*, characterized more recently (Rand et al. 2000). Although the majority of worldwide reports from this genus have been identified as *I. hoferi*, there are indications that this species may represent a species assemblage of closely related organisms (Spanggaard et al. 1996, Rasmussen et al. 2010). Phylogenetic studies aim to further define this species complex, which may provide insight into phenotypic differences among isolates and challenge the notion of broad global distribution of this single species (Criscione et al. 2002, Rasmussen et al. 2010).

Ichthyophonus spp. infections can be detected by a number of diagnostic methods including, but not limited to, *in vitro* culture, histology, and conventional PCR (cPCR) (McVicar 1982, Whipps et al. 2006). *In vitro* explant culture (McVicar 1982, Kocan et al. 2004) is likely the most accurate detection technique for determining infection prevalence (Kocan et al. 2011) but is neither quantitative nor suitable for use at remote collection sites that fail to satisfy the requirement of a temperature-controlled environment. For *Ichthyophonus* spp., *in vitro* culture alone is not appropriate for detection in environmental samples, as morphologically similar species could be present, yielding false positive results.

Histology, like *in vitro* culture, provides visual confirmation of the organism but also provides information on host response to the parasite and allows for long-term storage (Rahimian 1998). Spherical bodies associated with infection, often called schizonts (previously 'resting spores'), are the most commonly observed stage in infected tissue (McVicar 1999, Kocan et al. 2010). Spherical schizonts vary in diameter from 10 to 250 µm and are multinucleated (McVicar 1999). Histology provides visual identification via microscopy; however, it is less sensitive than *in vitro* culture (Whipps et al. 2006). Therefore, detection of *Ichthyophonus* spp. by histology alone may yield false negative results, resulting in significant underestimates of parasite prevalence, especially in early infections and related to the patchy nature of schizont distribution within the host tissues (Kocan et al. 2011).

Whipps et al. (2006) developed and evaluated a cPCR assay for *Ichthyophonus hoferi* DNA as a tool to detect the presence of parasite DNA from preserved host tissues while also allowing for long-term sample storage. These authors demonstrated that the diagnostic specificity of the test was consistently greater than 94% regardless of infection intensity, while the diagnostic sensitivity of the test was much higher in fish with heavy infections than those with light infections (Whipps et al. 2006). Although Whipps et al. (2006) demonstrated that cPCR is a specific (94.0–100%) and moderately sensitive (47.8–100%) tool for detecting *I. hoferi* DNA, *in vitro* culture is still considered the most accurate technique for determining the best estimate of parasite prevalence (Kocan et al. 2011).

With advances in molecular biology, quantitative PCR (qPCR) has become a routine technique for pathogen detection and surveillance (Guimaraes et al. 2011). Because qPCR can quantify parasite DNA, such assays have the potential for enabling fine-scale pathogen monitoring within host species as well as in the environment (Guy et al. 2003, Guimaraes et al. 2011) while increasing through-put by obviating gel visualization needed for conventional molecular methods. Moreover, qPCR has greater analytical sensitivity than cPCR (Guimaraes et al. 2011) and could potentially detect more light infections, resulting in better estimates of prevalence. However, because both qPCR and cPCR target parasite DNA and do not visualize the parasite, these assays are a proxy indicator of parasite presence and require extensive developmental testing to ensure assay validity (Burreson 2008, Bustin et al. 2009). A qPCR assay may prove useful for *Ichthyophonus* spp. detection in novel hosts, putative transport hosts, and the environment, thus elucidating transmission, life history, and disease dynamics of this parasite, and assisting fisheries management. Therefore, the objectives of this study were to develop, optimize, and validate a qPCR assay for quantification of *Ichthyophonus* spp. DNA and contrast molecular and histological methods of parasite detection using an infected wild fish population, Alaskan walleye pollock.

MATERIALS AND METHODS

Host samples

In 2006 and 2007 between the months of June and July, walleye pollock were captured using bottom trawl gear during National Marine Fisheries Service annual stock assessment surveys in Alaska (USA) wa-

ters. Individuals collected in 2006 (N = 83) had a mean fork length of 55.2 cm (range 17–79 cm); in 2007 (N = 221) mean fork length was 48.3 cm (range 9–84 cm).

Two to 6 individuals were collected daily, and the weight, length, and sex of each were recorded. Fish remained on the boat deck at ambient temperature for approximately 1 h prior to sample collection (monthly average air temperature range was 5 to 9°C for collection months). Two tissues were excised from each fish: a portion of the ventricle of the heart and skeletal muscle near the dorsal fin (mainly white muscle within Sector 2 defined by Kocan et al. 2011). Each tissue type was removed with a separate set of clean tools, split into 2 samples, and preserved separately in 10% sodium acetate-buffered formaldehyde for histological processing and 100% ethanol for qPCR development and molecular analysis. Tool sets used on the boat were scrubbed with warm soapy water, soaked in 10% bleach, rinsed with fresh water, and allowed to air-dry.

Ichthyophonus spp.-specific qPCR development and validation

DNA extraction

Ethanol-preserved tissue samples were cut into quarters with a sterile scalpel; one-quarter of the tissue was minced and ca. 20 mg were used per extraction. Residual ethanol was allowed to evaporate on the bench (ca. 10 min). Between samples, tools were wiped with a Kimwipe, dipped in 10% bleach, and rinsed in tap water twice, and then the tools were rinsed in deionized water and dipped in alcohol and flamed twice. DNA was extracted from each minced sample using a DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's protocol, except that DNA was eluted in 100 µl.

Primer and probe design and optimization

ClustalX 2.0 (Larkin et al. 2007) was used to align published 18S ribosomal DNA (rDNA) sequences of members of the genus *Ichthyophonus* from multiple hosts from various regions and other related species (Table 1). Prospective primers and hydrolysis probes were designed using Primer3 0.4.0 (Rozen & Skaletsky 2000) based on all *Ichthyophonus* spp. 18S rDNA sequences publically available at the time of development (Table 1) and the consensus sequence of 10 clones of *Ichthyophonus* sp. 18S rDNA extracted from one walleye pollock host (GenBank accession no. JX509908–JX509918). Primer–probe sets were evaluated during assay development for specificity, dynamic range, efficiency, and correlation coefficient (Stratagene 2004). The primer–probe set vc7F–ICH27–vc5R (Table 2), yielding a 75 bp qPCR amplicon, was selected for further assay development. The hydrolysis probe was labeled with 6-FAM on the 5′ end and the quencher Iowa Black FQ on the 3′ end (Integrated DNA Technologies).

Annealing temperature was optimized using a gradient thermal cycler from 54 to 66°C (Eppendorf Mastercycler). Primer concentrations were optimized by testing 5 concentrations (50, 100, 300, 400, and

Table 1. Species and accession numbers used in sequence alignment of members of the genus *Ichthyophonus* and other related species; geographical region of host species included for *Ichthyophonus* spp.

Species	Host	GenBank accession no.
Pacific Ocean North America		
Ichthyophonus sp.	*Theragra chalcogramma*	JX509908–JX509918
Ichthyophonus hoferi	*Clupea pallasii*	AF467793–AF467798
Ichthyophonus hoferi	*Oncorhynchus tshawytscha*	AF467799–AF467802
Ichthyophonus hoferi	*Sebastes flavidus*	AF467785–AF467786
Ichthyophonus hoferi	*Sebastes alutus*	AF467787–AF467792
Atlantic Ocean North America		
Ichthyophonus hoferi	*Limanda ferruginea*	U25637
Ichthyophonus irregularis	*Limanda ferruginea*	AF232303
Atlantic Ocean Northern Europe		
Ichthyophonus hoferi	*Clupea harengus*	U43712
Related species		
Uncultured ichthyosporean		HQ219472
Amoebidium parasiticum		AF274051
Enterobryus sp. CMJ-2003		AY336701
Paramoebidium sp. CMJ-2003		AY336708
Sphaeroforma arctica		Y16260
Creolimax fragrantissima		EU124916
Pseudoperkinsus tapetis		AF192386
Dermocystidium sp. CM-2002		AF533950
Anurofeca richardsi		AF070445
Rhinosporidium seeberi		AF118851
Amphibiocystidium viridescens		EF493029
Sphaerothecum destruens		AY267344
Psorospermium haeckeli		PHU33180
Capsaspora owczarzaki		AY363957

Table 2. Primers and probes used in development and validation of the *Ichthyophonus* spp. quantitative PCR (qPCR) assay

Purpose	Type	Name	Sequence
qPCR assay	Probe	ICH27	5′-/6-FAM/ TAA GAG CAC CCA CTG CCT TCG AGA AGA /IABLFQ/-3′
qPCR assay	Forward primer	vc7F	5′-GTC TGT ACT GGT ACG GCA GTT TC-3′
qPCR assay	Reverse primer	vc5R	5′-TCC CGA ACT CAG TAG ACA CTC AA-3′
Plasmid template	Forward primer	PIchF1	5′-ACC CGA CTT CTG GAA GGG TTG-3′
Plasmid template	Reverse primer	PIchR3	5′-AGT ATG TGT TGC CAC GCG CT-3′

600 nM) in a 5 × 5 full factorial matrix format. Hydrolysis probe concentration was optimized using 100, 200, and 300 nM per reaction. Optimal concentrations were defined as those yielding the lowest quantification cycle (Cq) (Stratagene 2004).

qPCR conditions

qPCR reactions were prepared in 25 µl volumes consisting of 2 µl of extracted DNA template, *Ichthyophonus* spp.-specific primers and probe (400 nM vc7F and vc5R primers, 300 nM 6-FAM-labeled probe ICH27), 15 µg bovine serum albumin, 12.5 µl 2× SensiMix II Probe (Bioline), and 6.25 µl sterile deionized H_2O; during development, 2 µl deionized H_2O were replaced with exogenous internal amplification control (IAC) primers, HEX-labeled probe, and template (Nolan et al. 2006) to test for inhibition. Each 96-well reaction plate was comprised of serially diluted standards in triplicate, unknown samples in duplicate, and 6 no-template controls (NTC). The qPCR assay was conducted on an Mx3000P™ Real-Time PCR System (Agilent Technologies) and a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories) using the manufacturers' softwares. On both instruments, *Taq* was activated for 10 min at 95°C, followed by 40 amplification cycles at 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min.

Plasmid standard curve

During assay development, PCR primers PIchF1 and PIchR3 (Table 2) were used to amplify a 1530 bp fragment of *Ichthyophonus* sp. (from walleye pollock) 18S rDNA for insertion into a plasmid. 'Fragment B' (generated by primers ICH1F and ICH4R; Criscione et al. 2002) is nested within the 1530 bp fragment and is also suitable for use as a plasmid insert for the qPCR assay. Each fragment was purified (QIAquick PCR Purification Kit; Qiagen), inserted into a pCR®2.1-TOPO® plasmid vector, and cloned in TOP10 *Escherichia coli* (TOPO-TA Cloning® Kit; Invitrogen) according to the manufacturers' protocols. Recombinant plasmid DNA was purified (QIAprep Spin Miniprep Kit; Qiagen) and digested with the restriction enzyme *Not*1 to linearize the plasmid. The plasmid was quantified relative to standards (dsDNA Quantitation Kit; Invitrogen) using a microplate reader (Tecan); a stock solution of 1.5×10^7 plasmid copies μl^{-1} was prepared following the methods of Applied Biosystems (2003). The final plasmid standard curve consisted of seven 10-fold dilutions in Tris EDTA buffer (TE).

qPCR efficiency and linear dynamic range

Two separate plasmids were assembled (insert generated with primers PIchF1/PIchR3), and a standard curve was constructed from each of the 2 plasmids as described above. Dilution series from each plasmid containing 3 to 3×10^6 copies $reaction^{-1}$ were assayed in triplicate on 2 different plates with extra replicates for low copy number standards (20 replicates of 3 copies and 10 replicates of 30 copies). Efficiency (E) was calculated from the slope of the standard curve using the following formula $E = [10^{(-1/slope)}] - 1$; efficiencies between 90 and 110% were considered acceptable (Stratagene 2004). Standard curve precision was evaluated via coefficients of determination (R^2); an R^2 value greater than 0.985 was considered acceptable (Stratagene 2004). The linear dynamic range was determined by plotting Cq versus the dilution factor of the standard curve in a base10 semi-logarithmic graph, fitting the data to a straight line. The linear range of the plot is the dynamic range of the qPCR assay. Regression coefficients were compared following a statistical test outlined by Burns et al. (2005) to calculate the probability that differences between 2 coefficients are due to chance alone. Briefly, an F-variance ratio test was used; the null hypothesis assumes no significant

difference between 2 regression coefficients and p-values < 0.05 reject the null hypothesis (Burns et al. 2005).

Reproducibility and repeatability

Eight *Ichthyophonus* sp. positive walleye pollock samples were selected (4 heart muscle, 4 skeletal muscle), with *Ichthyophonus* sp. DNA concentrations spanning the operating range of the assay as described by OIE (2009). These 8 walleye pollock were identified as *Ichthyophonus* sp.-positive by both histology and qPCR. Data were collected for each sample, and an average percent coefficient of variation (CV) was calculated per tissue type.

To test reproducibility, samples and the plasmid standard curve were assayed on a plate in triplicate once per day for 5 d and were performed by 3 different operators. Reproducibility, or inter-assay variation, was determined for each sample by calculating CV of copy number sample means between runs (Bustin et al. 2009). CV was determined using the following calculation where 'plate mean' is the mean of triplicate reactions for each sample per plate, '5 d mean' is the mean of the plate means over 5 d, and SD is the standard deviation of the 5 d mean:

$$\frac{\text{SD}}{\text{5 d mean}} \times 100 = \%\text{CV}$$

In general, a mean inter-assay variability of 15 to 30% is realistic over a wide dynamic range (Pfaffl 2004).

To test repeatability, 8 replicates of each sample were assayed on 1 plate with the plasmid standard curve in triplicate. Repeatability, or intra-assay variation, was determined by calculating the percent CV for each sample using the following formula where 'plate mean' is the mean copy number of the 8 replicates, and SD is the standard deviation of the plate mean (Bustin et al. 2009):

$$\frac{\text{SD}}{\text{Plate mean}} \times 100 = \%\text{CV}$$

Generally, a CV of 10 to 20% is realistic for mean intra-assay variability (Pfaffl 2004).

Analytical sensitivity and specificity

The limit of detection, or analytical sensitivity (ASe), is the point where the assay measurement can no longer be considered valid due to loss of signal. ASe is defined as the lowest number of plasmid copies giving 50% positive results (OIE 2009). Replicates (N = 60–80) of low copy plasmid standards (1, 3, and 10 copies) from several plasmid standard curves were assayed on multiple plates over multiple days to determine the assay's ASe.

Analytical specificity (ASp) was evaluated at the genus level by measuring 3 component factors, i.e. selectivity, exclusivity, and inclusivity (OIE 2010), to determine the assay's ability to distinguish between *Ichthyophonus* spp. DNA and other assay components. Selectivity refers to the assay's ability to accurately quantify the DNA target in the presence of sample matrix interferents (OIE 2010). To confirm assay selectivity, one plasmid was used to construct multiple standard curves in 3 diluents, i.e. TE, DNA extracted from walleye pollock heart tissue, and DNA extracted from walleye pollock skeletal muscle. Each standard curve was assayed in triplicate to determine whether qPCR efficiency and precision were within acceptable ranges, 90 to 110% and >0.985, respectively (Stratagene 2004). Each standard curve in DNA diluents was assayed on the same plate paired with a standard curve in TE to compare regression coefficients following Burns et al. (2005). Additionally, an IAC (Nolan et al. 2006) was multiplexed in a subset of reactions to determine whether PCR amplification was inhibited (increased or absent Cq). Exclusivity at the genus level refers to the ability of the assay to detect genomic sequence unique to known members of the genus *Ichthyophonus* while excluding other potentially cross-reactive organisms (OIE 2010). Exclusivity was assessed by performing the qPCR assay with *Ichthyophonus* sp. DNA from walleye pollock and DNA from other non-*Ichthyophonus* spp. members of the class Ichthyosporea including *Creolimax fragantissima*, *Dermocystidium salmonis*, *Pirum gemmata*, *Sphaerothecum destruens*, and *Sphaeroforma nootkatensis* acquired from W. Marshall (University of British Columbia, Canada). To confirm the presence of Ichthyosporea material, each donated sample was assayed by cPCR with both ascomycete universal primers (SL1/cITS5; Kohlmeyer et al. 2005) and 18S rDNA universal primers (CS1/CAS2; Le Roux et al. 1999), as neither universal primer set was appropriate for all species tested. Inclusivity at the genus level, or the ability of this assay to detect several strains and species within the genus *Ichthyophonus*, was tested *in silico* using Amplify 3.1 (http://engels.genetics.wisc.edu/amplify/) on DNA sequences of all published isotypes of *Ichthyophonus* spp.

Diagnostic sensitivity and specificity

Preliminary estimates of diagnostic sensitivity (DSe) and specificity (DSp) were calculated following the work of Whipps et al. (2006). Heart and skeletal muscle tissues were tested for *Ichthyophonus* sp. presence using 2 existing methods: histology and cPCR. The 'true' infection status of a fish was determined by using a combination of these test results. A fish was categorized as 'infected' with *Ichthyophonus* sp. if either of the tissues tested positive with either test; a fish was categorized as 'uninfected' if all test results were negative. *Ichthyophonus* sp. infections were also categorized as light or heavy. Light infections were characterized as having only 1 tissue type positive and ≤1 schizont mm^{-2} of tissue by histology. Heavy infections where characterized as having >1 tissue type infected and >1 schizont mm^{-2} tissue identified by histology (Whipps et al. 2006). To calculate DSe and DSp, 'true' infection status and qPCR results (skeletal muscle, heart, and tissues combined) were organized in cross-classification table format.

cPCR–18S rDNA universal and *Ichthyophonus hoferi*-specific

To confirm the presence of amplifiable DNA extracted from each fish sample, a cPCR reaction was performed with 18S rDNA eukaryotic universal primers (CS1/CAS2; Le Roux et al. 1999). Reactions were prepared in 20 µl volumes and consisted of 1× GoTaq Flexi PCR buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM dNTP, 25 pmol of each primer, 0.025 U $µl^{-1}$ *Taq* DNA polymerase, and 0.8 µl of template DNA. Reactions were carried out using an MJ Research DNA Engine PTC-200 thermal cycler, with initial denaturation for 5 min at 94°C, followed by 30 amplification cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by final adenylation at 72°C for 10 min. All DNA samples and the plasmid standard curve were tested with an *I. hoferi*-specific cPCR assay using primers Ich7f and Ich6r, following published methods (Whipps et al. 2006). PCR products were separated by electrophoresis on 1 to 2% agarose gels, stained with ethidium bromide, and visualized using UV illumination.

Histology

Formalin-fixed tissues of heart and skeletal muscle were processed through paraffin blocks following standard procedures (Sheehan & Hrapchak 1980). Deparaffinized tissue sections were stained with hematoxylin and eosin Y (Luna 1968). The entire section of each tissue type was examined by light microscopy, and image analysis was performed using Nikon-Elements BR 3.0 software. Tissue area ($µm^2$) was first estimated using the calibrated auto-detect function. The same function was used to enumerate and estimate the area of *Ichthyophonus* sp. and associated granulomas within the tissue. These data were used to calculate the number and size distribution of schizonts per tissue area and compare histological results to qPCR copy number. *Ichthyophonus* sp. schizont area ($µm^2$) was normalized by natural log transformation, and estimates of diameter were calculated based on schizonts being circular, although it is recognized that most observed individuals were not perfect circles due to histological processing.

RESULTS

Ichthyophonus spp.-specific qPCR development and validation

qPCR efficiency and linear dynamic range

Regression coefficients of 2 plasmid standard curves assayed on 2 separate plates were not significantly different (Table 3). Both mean (99.3%) and individual reaction efficiencies of plasmid standard curves used for determining dynamic range were within the acceptable range of 90 to 110%; both mean (0.993) and individual assay precision based on coefficient of determination were greater than the acceptable limit of 0.985 (Table 3). Based on the reaction efficiency of multiple standard curves, the linear dynamic range of the assay was 3 to 3×10^6 copies.

Reproducibility and repeatability

The assay was reproducible. Mean inter-assay variability of copy number (CV) was 9.4% for skeletal muscle samples and 11.2% for heart muscle samples; for both sample types, variability was less than the goal of 15 to 30%. The mean intra-assay variability or repeatability of the qPCR assay was 8.2% for skeletal muscle samples and 11.2% for heart muscle samples, also within or below the target range for intra-assay variability of 10 to 20% (Table 4).

Table 3. *Ichthyophonus* spp. Quantitative PCR (qPCR) assay efficiency and precision of plasmid standard curves prepared in different diluents for (a) preliminary standard curve development, where 20 replicates were performed for 3-copy standards, 10 replicates for 30-copy standards, and 3 replicates for all other standards; (b) selectivity component of analytical specificity, where 3 replicates were performed for all standards. The linear range for all plasmid standard curves is 3 to 3×10^6 copies

Standard curve comparison	Diluent (ng DNA reaction^{-1})	Efficiency (%)	Precision (R^2)	p
(a) Multiple plasmid preparations in standard diluent	Tris EDTA buffer–plasmid 1	100.5	0.993	0.651
	Tris EDTA buffer–plasmid 2	98.1	0.993	
(b) Single plasmid preparation in different diluents to test effect of matrix interferents	Tris EDTA buffer	94.8	0.997	0.938
	Extracted skeletal muscle DNA (88)	95.4	0.995	
	Tris EDTA buffer	94.8	0.997	0.094
	Extracted heart DNA (172)	100.0	0.996	
	Tris EDTA buffer	99.1	0.994	0.815
	Extracted heart DNA (48)	100.4	0.995	

Table 4. *Theragra chalcogramma*. Reproducibility (inter-assay variation) and repeatability (intra-assay variation) of the *Ichthyophonus* spp.-specific quantitative PCR (qPCR) assay for a range of walleye pollock skeletal and heart muscle tissue

Sample ID no.	Reproducibility			Repeatability		
	Mean copies	SD	CV (%)	Mean copies	SD	CV (%)
Muscle						
2007-4133c	2.51×10^1	2.95×10^0	11.7	2.57×10^1	4.16×10^0	16.2
2007-4202c	3.67×10^2	1.80×10^1	4.9	3.63×10^2	2.35×10^1	6.5
2006-1024c	8.54×10^3	1.32×10^3	15.4	8.65×10^3	4.11×10^2	4.8
2006-1028c	3.69×10^4	2.08×10^3	5.6	3.84×10^4	2.06×10^3	5.4
		Mean	9.4		Mean	8.2
Heart						
2006-1066d	1.19×10^1	1.50×10^0	12.5	1.43×10^1	2.57×10^0	18.0
2007-4103d	2.30×10^2	2.21×10^1	9.6	2.36×10^2	3.81×10^1	16.2
2006-1034d	3.20×10^3	5.34×10^2	16.7	3.23×10^3	1.98×10^2	6.1
2007-4145d	3.73×10^4	2.29×10^3	6.1	3.45×10^4	1.54×10^3	4.5
		Mean	11.2		Mean	11.2

Analytical sensitivity and specificity

By OIE definition (OIE 2009), the ASe was 1 copy per reaction. The ability to detect low copy numbers ranged from 50% (40/80) for 1 copy, 80.8% (59/73) for 3 copies, and 100% (60/60) for 10 copies. However, the lowest limit of detection theoretically possible has been defined as 3 copies (Bustin et al. 2009). Consequently, we set the ASe of this assay to 3 copies, and unknowns were considered positive only if the average quantity of replicates was ≥3 copies.

ASp was defined by the selectivity, exclusivity, and inclusivity of the assay. Selectivity: For a linear range of 3 to 3×10^6 copies, efficiency and precision of plasmid standard curves prepared in different diluents were similar to standard curves prepared in TE (absence of matrix interferents) and within the acceptable ranges of 90 to 110% and >0.985, respectively (Table 3). The presence of walleye pollock heart and skeletal muscle extracted DNA with concentrations up to 172 ng per reaction did not measurably inhibit the assay (Table 3). The IAC (Nolan et al. 2006) Cq range was consistently less than 1.5 Cq and did not increase in the presence of sample matrix relative to no template controls. Exclusivity: *Ichthyophonus* sp. from walleye pollock was tested *in vitro*, while all other isotypes and species within the genus *Ichthyophonus* were tested *in silico*. Only DNA from the genus *Ichthyophonus* was amplified with the qPCR assay, illustrating its genus-level specificity. The presence of amplifiable DNA was confirmed in samples of the other tested members of the class Ichthyosporea using universal primers. Although *Amoebidium parasiticum* (GenBank accession no. AF274051) and an uncultured ichthyosporean clone (GenBank accession no. HQ219472.1) have the highest sequence similarity with *Ichthyophonus* spp. in the regions selected for the qPCR primers, neither was available to test for cross-reactivity *in vitro*, but no PCR amplicon was predicted based on *in silico*

PCR. Inclusivity: Based on tests performed *in silico* on published sequences of *Ichthyophonus* spp., we expect the qPCR assay to detect DNA from all known isotypes of members of the genus *Ichthyophonus.* With the exception of the isotype associated with the North Pacific rockfishes *Sebastes alutus* and *S. flavidus*, all sequences of published isotypes (GenBank search date 3 January 2013) are identical in the qPCR amplicon region. Therefore, this qPCR assay is likely appropriate for *Ichthyophonus* spp. DNA detection in various hosts worldwide, including mugilids from the Mediterranean, *Clupea harengus* from the European Atlantic, *Alosa sapidissima* and *Limanda ferruginea* from the North American Atlantic, freshwater rainbow trout from Idaho, and *C. pallasii*, *Oncorhynchus tshawytscha*, *A. sapidissima*, *Theragra chalcogramma*, and copper rockfish from the North American Pacific. The isotype associated with the North Pacific rockfishes *S. alutus* and *S. flavidus* varies by a single nucleotide in the forward primer sequence, vc7F; these isotypes would require testing *in vitro* to ensure inclusivity and await further examination. Because this assay is specific for members of the genus *Ichthyophonus*, specific molecular identification of isotypes and species would require sequencing.

Diagnostic sensitivity and specificity

Preliminary estimates of DSe and DSp following the methods of Whipps et al. (2006) are listed in Table 5. DSp was high (≥93.8%) regardless of tissue type, year, or infection level. DSe was ≥63.8% regardless of tissue type and year but varied greatly depending on infection level, with values ranging from 36 to 100% (Table 5). DSe was highest for any infection level when the qPCR assay was performed as a 2-tissue test (Table 5). For all detection methods, tests performed on skeletal muscle yielded more positive results than heart tissue (Table 6). Prevalence of *Ichthyophonus* sp. in walleye pollock in 2006 and 2007 was 27.7 and 23.5%, respectively, when all test results were combined, but prevalence estimations varied depending on detection method and tissue tested (Table 6).

cPCR–18S rDNA universal and *Ichthyophonus hoferi*-specific

All extracted fish samples produced a PCR product when assayed with 18S rDNA universal primers, indicating the presence of amplifiable DNA. *Ichthyophonus* sp. DNA was detected in walleye pollock by cPCR (Whipps et al. 2006). Concordance between the molecular assays was observed, as all fish samples that were scored negative by the new qPCR assay were also scored negative for parasite DNA by the cPCR assay of Whipps et al. (2006) from the same extraction. Of the 112 fish samples (skeletal muscle or heart) that were identified as positive by the qPCR assay, 66 (59%) were positive by cPCR. Samples with low copy number (<300 copies) were less likely to be

Table 5. *Theragra chalcogramma*. Diagnostic sensitivity (DSe) and specificity (DSp) of the *Ichthyophonus* spp. quantitative PCR (qPCR) test on skeletal muscle, heart tissue, and the 2 tissues combined for identification of parasite DNA in walleye pollock. DSe was calculated for light and heavy infections separately following Whipps et al. (2006). Values are percentages (95% binomial proportion CI)

Tissue / Year	Sensitivity: Combined results	Sensitivity: Light	Sensitivity: Heavy	Specificity
Skeletal muscle				
2006	73.7 (54–93)	54.5 (25–84)	100 (75–100)	93.8 (88–100)
2007	91.5 (84–99)	88.0 (75–100)	95.5 (87–100)	98.3 (96–100)
Heart				
2006	73.7 (54–93)	54.5 (25–84)	100 (75–100)	98.4 (95–100)
2007	63.8 (50–78)	36.0 (17–55)	95.5 (87–100)	98.2 (96–100)
Combined (2-tissue test)				
2006	94.7 (85–100)	90.9 (74–100)	100 (75–100)	93.8 (88–100)
2007	95.7 (90–100)	92.0 (81–100)	100 (89–100)	97.1 (95–100)

Table 6. *Ichthyophonus* sp. infecting *Theragra chalcogramma*. Prevalence in walleye pollock tissues as determined by 3 detection methods. Values are no. fish positive/no. fish examined (% positive; 95% binomial proportion CI). cPCR: conventional PCR; qPCR: quantitative PCR

Detection method / Tissue	2006	2007
Histology		
Skeletal muscle	14/83 (16.9; 8.8–24.9)	36/221 (16.3; 11.4–21.2)
Heart	4/83 (4.8; 0.2–9.4)	22/219 (10.0; 6.1–14.0)
cPCR		
Skeletal muscle	12/83 (14.5; 6.9–22.0)	29/221 (13.1; 8.7–17.6)
Heart	9/83 (10.8; 4.2–17.5)	16/218 (7.3; 3.9–10.8)
qPCR		
Skeletal muscle	18/83 (21.7; 12.8–30.6)	46/221 (20.8; 15.5–26.2)
Heart	15/83 (18.1; 9.8–26.4)	33/218 (15.1; 10.4–19.9)
All tests combined[a]	23/83 (27.7; 18.1–37.3)	51/217 (23.5; 17.9–29.1)

[a]Only included fish when results were available for all tests

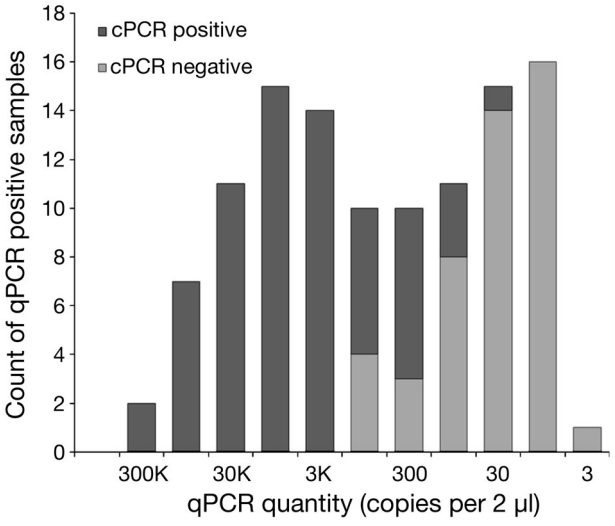

Fig. 1. *Theragra chalcogramma.* Proportion of quantitative PCR (qPCR) positive tissue samples from walleye pollock found positive with conventional PCR (cPCR). Groups arranged by decreasing *Ichthyophonus* sp. DNA starting quantity, estimated by qPCR. K: ×1000

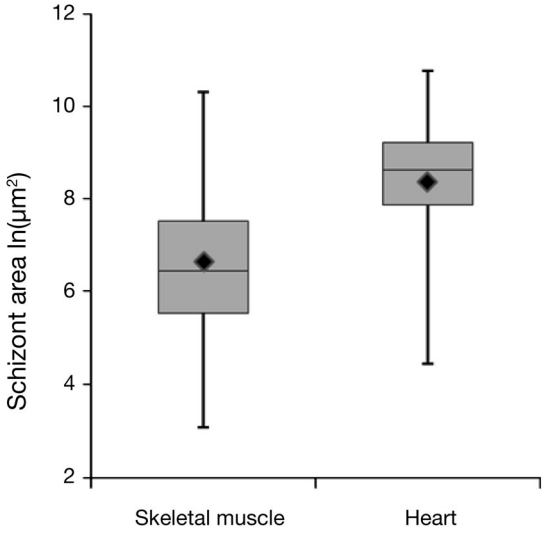

Fig. 2. *Ichthyophonus* sp. infecting *Theragra chalcogramma.* Degree of dispersion in size of all *Ichthyophonus* sp. schizonts measured from skeletal muscle (N = 1187) and heart tissue (N = 114) from walleye pollock histology samples (size represented as the natural log of schizont area in µm^2). Boxes represent quartiles; whisker ends represent the minimum and maximum area of all data; diamonds represent the mean area of all schizonts for each tissue type

detected by the cPCR method (Fig. 1), illustrating the improved sensitivity of the qPCR assay. Similarly, cPCR results from the plasmid standard curve produced a faint band for the 300-copy standard, but 30-copy and 3-copy standards did not produce a visible band.

Histology

Ichthyophonus sp. schizonts were visually identified in walleye pollock by histology; an individual's infection status varied depending on the tissue tested by histology (Table 6). When *Ichthyophonus* sp. was detected in a fish by both histology and qPCR, there was poor correlation between visually determined parasite density and estimated 18S rDNA copy number, with the exception of a few high-intensity infections in skeletal muscle (data not shown). *Ichthyophonus* sp. schizonts identified by histology in walleye pollock had similar size ranges in both skeletal muscle and heart tissues (Fig. 2). However, when comparing the median and average schizont size from all samples combined, schizonts from skeletal muscle were generally smaller than those examined in heart tissue (Fig. 2). An estimate of diameter from area returned similar results, where schizonts in skeletal muscle ranged from approximately 5 to 195 µm with a mean of 41.7 µm (SD = 35.8; N = 1187), while from all heart tissue examined, schizonts ranged in size from 10 to 246 µm with a mean of 88.5 µm (SD = 46.5; N = 114). Likewise, when a comparison of schizont size and abundance between tissue types from an individual fish was possible, schizonts of smaller size were generally more frequent in skeletal muscle as compared to a smaller quantity of larger-sized schizonts in heart tissue (Fig. 3).

DISCUSSION

The qPCR assay developed in this study is a new tool for future research on *Ichthyophonus* spp.-related issues, from prevalence estimations to transmission strategies. Through comprehensive validation of the assay, we found that the qPCR test was consistently specific for *Ichthyophonus* spp. DNA. DSp remained high either as a 2-tissue test (skeletal muscle and heart; Table 5) or a single tissue test (skeletal muscle or heart; Table 5), similar to the findings of Whipps et al. (2006) for the cPCR assay. Moreover, the evaluation of 3 components of ASp ensures confidence in positive results. This assay provides a more accurate estimate of prevalence than cPCR and histological methods for both tissue types tested (Table 6) while still allowing for chemical preservation of samples and long-term storage. Additionally,

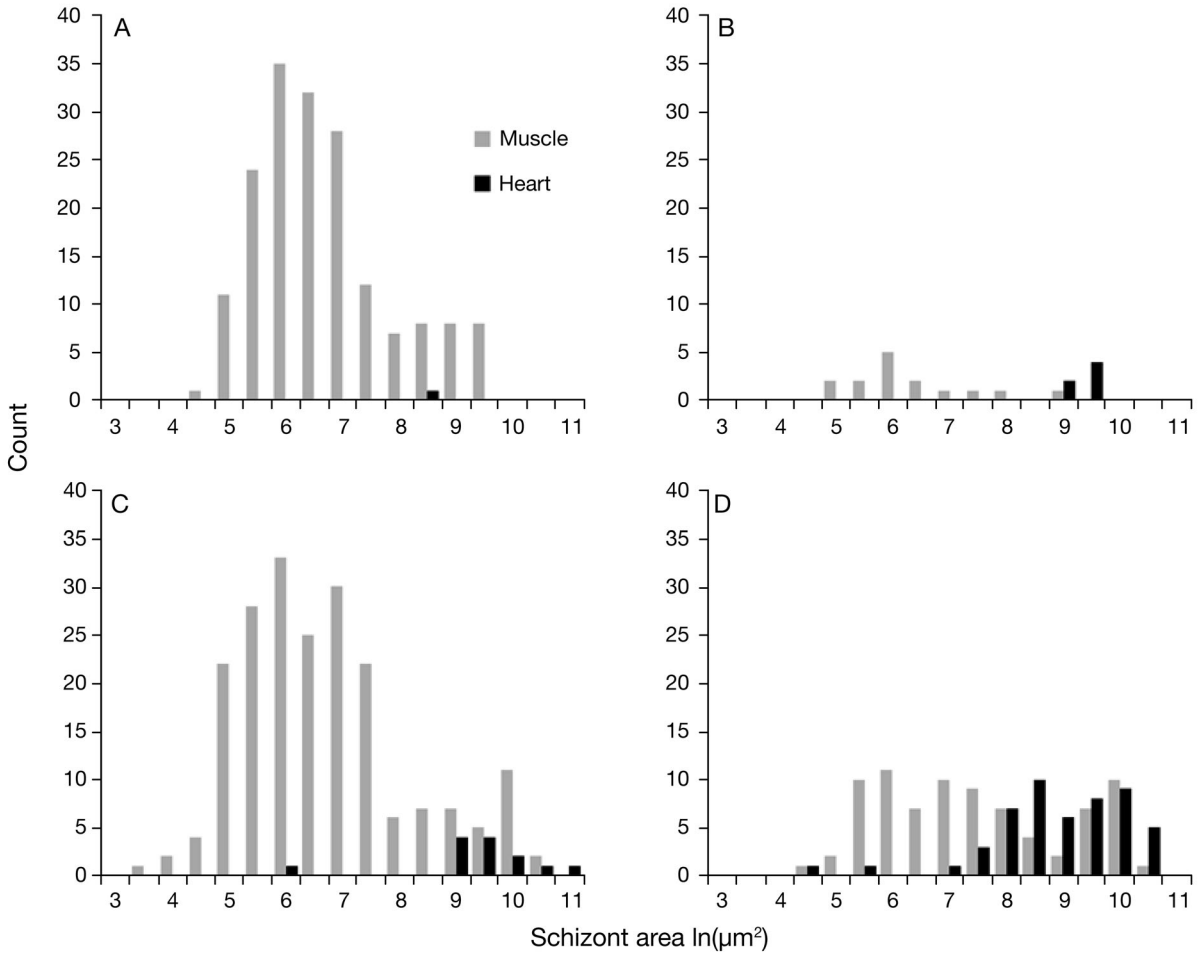

Fig. 3. *Ichthyophonus* sp. infecting *Theragra chalcogramma*. Count of *Ichthyophonus* sp. schizonts by size group ln(area in μm^2) in skeletal muscle and heart tissue from 4 walleye pollock: (A) fish fork length = 43 cm; (B–D) fish fork length = 50 cm

at an individual sample level, qPCR proved more effective than cPCR for identifying *Ichthyophonus* sp. DNA at low concentrations (Fig. 1).

The 100-fold increase in ASe of qPCR over cPCR (Fig. 1) likely aids in the detection of light infections. This notion was reflected in the increase in minimum DSe for light, heavy, and all infections with qPCR (Table 5) as compared to cPCR (Whipps et al. 2006). However, the DSe of the qPCR assay varied greatly depending upon whether the fish had light or heavy *Ichthyophonus* sp. infection status, consistent with the cPCR assay evaluation (Whipps et al. 2006). DSe was high for both tissue types in both years for heavy infections, but decreased dramatically in fish with light infections (Table 5). The notable discrepancy in DSe between tissue types of light infections in 2007 may be due to variation in the quantity, size, and distribution of schizonts in skeletal muscle as compared to heart tissue as seen by histology (Figs. 2 & 3). To avoid missing light infections with the qPCR assay and to obtain the best estimate of *Ichthyophonus* spp. prevalence, it is recommended that at least 2 tissue types are collected for analysis, if costs permit (Tables 5 & 6).

One benefit of PCR-based assays is the small amount of tissue necessary to test for parasite DNA presence in an individual. However, this benefit can become a limiting factor when the host individual is large and the parasite is unevenly distributed within the host or lacks specific tissue tropism. This problem regarding *Ichthyophonus* spp. detection has been well described by Kocan et al. (2011) in a 3-dimensional analysis of schizonts in Pacific herring skeletal muscle. *Ichthyophonus* spp. schizonts have variable distribution within host tissue as well as between individuals and host species, which likely contributes to differences in DSe among detection techniques (Kocan et al. 2011). To acquire the most accurate esti-

mate of prevalence, it is important to identify the best target tissue for different hosts. For example, in Chinook salmon, heart samples produced more positive results by all detection methods (Whipps et al. 2006), while in walleye pollock, skeletal muscle samples produced more positive results by all detection methods (Table 6).

The qPCR assay for *Ichthyophonus* spp. DNA detection has been validated as described previously, but questions still remain surrounding its comparability to non-molecular detection methods for this parasite group. For example, comparing parasite quantity estimations from qPCR and histology presents a challenge considering that *Ichthyophonus* spp. are multinucleated organisms of various sizes containing various nuclei. These issues of uneven parasite size and distribution were apparent in this study, as the count of *Ichthyophonus* sp. schizonts mm^{-2} did not correlate with qPCR copy numbers. Additionally, it is uncertain whether the qPCR assay is comparable to *in vitro* culture, currently the most accurate technique for estimating prevalence of this parasite in fish populations (Kocan et al. 2011). *In vitro* culture was not compared to qPCR in this study due to issues with sample transportation from remote locations, but it is likely that many of the potential sources for detection error discussed by Whipps et al. (2006) and Kocan et al. (2011) also apply to qPCR due to inherent issues with the process of DNA extraction of small samples. To more fully assess the DSe and DSp of this qPCR assay relative to the diagnostic standard, a lab-based comparison of *in vitro* culture and qPCR on captive Pacific herring is currently underway.

In vitro culture may be the most sensitive method of *Ichthyophonus* spp. detection because of enhancement of the live parasite during sample storage (Kocan et al. 2011) and also, in part, due to the ability to use a large piece of starting material without the need to subsample after field collection, a routine practice in PCR-based assays and histological sectioning. Techniques with potential for enhancing qPCR DSe include pooling samples from different organs, homogenization of larger samples, and processing multiple subsamples. Some of these options could reduce costs over processing heart and skeletal muscle separately as described in this study, but these practices should be approached with caution as assay sensitivity could be affected by either target DNA dilution or PCR inhibition. For example, a false negative result could occur if *Ichthyophonus* spp. target DNA from one organ is diluted beyond the assay's limit of detection with non-target DNA from pooling. PCR inhibition could also affect assay sensitivity if pooled organs possess inhibitory factors; therefore assay application to other organs or blood (as a non-lethal method) would require additional testing. Although qPCR is not likely a replacement for *in vitro* culture estimation of *Ichthyophonus* spp. prevalence in fish populations, this assay does provide a more accurate estimate of prevalence than cPCR and histological methods when culture is not feasible (Table 6). The sample collection method and chemical preservation used in the qPCR assay also provides some flexibility in remote locations when long-term, temperature-controlled storage and shipment are not reliable or available, considering that live cultures of *Ichthyophonus* spp. must be kept cool.

Beyond basic *Ichthyophonus* spp. prevalence estimation, the qPCR assay could be used for a number of different types of applications in both wild host populations and captive laboratory experiments. For example, transmission of *Ichthyophonus* spp. to planktivorous hosts has not been defined, and researchers have suggested that the parasite could be acquired directly from the water or via a yet-to-be indentified invertebrate intermediate host (Hershberger et al. 2002). *Ichthyophonus* spp. cell forms within intermediate hosts or the environment could be small or appear morphologically different from the large multinucleate schizonts frequently observed in fish tissue. Therefore, *Ichthyophonus* spp. from these sample types could be misidentified or disregarded without DNA detection used in combination with other tests (i.e. *in vitro* culture followed by cPCR). This new qPCR tool could be optimized to screen for DNA from members of the genus *Ichthyophonus* in zooplankton, water, sediment, and other samples in which axenic collection is not possible. A modified version of the qPCR protocol is currently being tested for use in mixed zooplankton samples, although as mentioned previously, the presence of inhibitors and modifications to DNA extraction methods could alter assay sensitivity. Additionally, qPCR may be useful in laboratory experiments where fish are infected by interperitoneal injection under controlled conditions. Inoculation doses can be challenging to estimate considering the highly variable size of *Ichthyophonus* spp. schizonts. By using qPCR to quantify gene copy number, the assay could prove useful in estimating and standardizing inoculation dose of *Ichthyophonus* spp., as well as aiding in the determination of infective units (P. Hershberger pers. comm.).

Development and validation of this novel qPCR assay provides researchers a new tool for examining *Ichthyophonus* spp. and could be used for a variety of applications. Parasite DNA detection with qPCR may identify novel hosts and population trends, leading to a better understanding of *Ichthyophonus* spp. dynamics and range. While results from environmental samples could further our understanding of transmission and life history of this organism. Considering *Ichthyophonus* spp. are parasites of many commercially important fish species, use of this new assay could supply the industry with information to better manage product recovery, fish waste, and resources in the North Pacific as well as worldwide.

PCR-based assays are increasingly being applied as complements or alternatives to conventional pathogen detection techniques such as histology and *in vitro* culture (Yang & Rothman 2004). Likewise, qPCR assays are rapidly replacing cPCR assays with advantages including rapid detection, quantification of gene copies, and the potential for increased sensitivity and specificity (Guimaraes et al. 2011). Although qPCR is widely used in pathogen detection (Klein 2002), it is essential that development of new assays include proper validation to ensure that the test meets or exceeds the sensitivity and specificity of existing detection methods, while being repeatable and appropriate for the application (OIE 2009).

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