Parasitology International 61 (2012) 675–683

Contents lists available at SciVerse ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Molecular tools for the detection and identification of *Ichthyobodo* spp. (Kinetoplastida), important fish parasites

Trond E. Isaksen [a,*], Egil Karlsbakk [b], Oddvar Repstad [c], Are Nylund [c]

[a] Uni Research, Uni Environment, P.O. Box 7810, 5020 Bergen, Norway
[b] Institute of Marine Research, P.O. Box 1870 Nordnes, 5817 Bergen, Norway
[c] Department of Biology, University of Bergen, P.O. Box 7803, 5020 Bergen, Norway

ARTICLE INFO

Article history:
Received 24 May 2012
Received in revised form 1 July 2012
Accepted 9 July 2012
Available online 21 July 2012

Keywords:
Kinetoplastida
Ichthyobodo necator
Ichthyobodo salmonis
18S rRNA gene
Protozoa
Real time PCR

ABSTRACT

Ichthyobodo spp. are ectoparasitic flagellates of fish that may cause disease (ichthyobodosis), a common problem affecting the aquaculture industry worldwide. Ichthyobodosis in farmed fish is often associated with a range of other infectious agents and diagnosis in for example gill disease may be difficult. Sensitive and effective methods for detection and identification of *Ichthyobodo* spp. are needed to aid diagnosis of ichthyobodosis and epizootiological studies on *Ichthyobodo* spp. We have designed a specific quantitative real-time PCR assay targeting SSU rDNA for the detection of *Ichthyobodo* spp. infections. Also, several novel primer sets are presented for use in identification of *Ichthyobodo* spp. through PCR and sequencing. These PCR methods have been optimized and tested on samples from wild caught and farmed fish from different geographical areas in Norway. The real-time PCR assay has been tested for sensitivity and efficiency, and we present data demonstrating its use for absolute quantification of *Ichthyobodo salmonis* in tissue samples through RT-qPCR and qPCR. We demonstrate the use of the described set of molecular tools for the detection and sequencing of *Ichthyobodo* spp. from farmed and wild fish, and also show that they may aid the discovery of new *Ichthyobodo* species. The detection of light *Ichthyobodo* spp. infections through microscopy is time consuming and less sensitive compared to PCR methods. Initial real-time PCR testing and subsequent sequencing of positive samples is a powerful method that will increase diagnostic precision, aid carrier detection and promote species discoveries in the Ichthyobodonidae. Our preliminary observations indicate a high *Ichthyobodo* spp. diversity.

1. Introduction

In recent years, gill diseases have been reported as an increasing problem in Norwegian salmon production. Such diseases are commonly termed as proliferative gill disease (PGD), or when an inflammatory response is present as proliferative gill inflammation (PGI). PGI or PGD are unspecific diagnoses associated with a range of pathogens that can now be detected and identified with use of PCR methods [1,2]. Real-time PCR is a fast and highly sensitive method and several assays have been developed for the screening of potential pathogens in fish farms. Such studies have demonstrated that different species of bacteria causing epitheliocystis and microsporidian infections are particularly common in Atlantic salmon suffering from PGD or PGI in seawater. Infections by *Ichthyobodo* spp. are also detected in association with gill disease, but detection relies on light microscopy [1,3,4].

Until recently, the genus *Ichthyobodo* contained a single variable species, *Ichthyobodo necator*, identified from fish worldwide [5,6]. However, small subunit ribosomal RNA gene (SSU rDNA) sequences of flagellates from different hosts and environments have shown that *I. necator* actually represents several different species [7–9]. Among these, four have been identified from farmed salmonid and marine fish in Norway. *Ichthyobodo necator* was redescribed by Isaksen et al. [8] and the morphological conception of that species narrowed to a species well characterized by its SSU rDNA sequence. Also two additional species were described, the marine *Ichthyobodo hippoglossi* from farmed halibut (*Hippoglossus hippoglossus*) and the euryhaline *Ichthyobodo salmonis* from farmed Atlantic salmon (*Salmo salar*) [8,10]. Both *I. necator* and *I. salmonis* infect Atlantic salmon, and both species have been associated with ichthyobodosis and mortalities [10,11]. Clinical signs of ichthyobodosis are often easily recognized among tank-reared fish, including grayish coating on the dorsal surface of the fish and 'flashing' behavior [12]. The parasites are readily detected by light microscopy of fresh smears of skin or gills of heavily infected fish, but slight *Ichthyobodo* spp. infections may be very difficult to ascertain with

* Corresponding author at: Uni Research, Uni Environment, P. box 7810, 5020 Bergen, Norway. Tel.: +47 55 58 42 48.
E-mail addresses: trond.isaksen@uni.no (T.E. Isaksen), egil.karlsbakk@imr.no (E. Karlsbakk), oddvar.repstad@ewos.com (O. Repstad), are.nylund@bio.uib.no (A. Nylund).

1383-5769 © 2012 Elsevier Ireland Ltd. Open access under CC BY-NC-ND license.
doi:10.1016/j.parint.2012.07.006

this method [11]. Microscopy requires experience in recognizing both free and attached forms of these flagellates, and is highly time consuming when prevalence is low and infections slight.

In salmonid hatcheries the infections may be severe and cause an increase in mortality among fry when not treated [13,14]. In general, juvenile fish are more prone to develop ichthyobodosis than larger fish [11,15], but gill disease and mortality due to *Ichthyobodo* infections have also been observed among seawater reared post-smolt salmonids in both the North Atlantic and North-Pacific [3,16–18]. Despite frequent records of *Ichthyobodo* infections and disease among farmed fish, detection of *Ichthyobodo* spp. still relies on light microscopic methods including histology. According to the Norwegian Veterinary Institute (NVI), *Ichthyobodo* infections among farmed fish in Norway are most likely under-diagnosed due to the ineffective methods for detection [19]. A sensitive method for *Ichthyobodo* spp. detection is much desirable as an aid in both epizootiological studies, research on disease etiology and diagnostic work on diseases associated with numerous pathogens such as PGD.

The main aim of the present study was to develop effective and sensitive PCR methods for detection and subsequent identification of *Ichthyobodo* spp. A real-time PCR assay was designed based on SSU rDNA sequences of the different molecularly recognized *Ichthyobodo* species. The assay was tested for efficiency and the ability to quantify the parasites in samples. PCR primers are presented that can be used to amplify *Ichthyobodo* spp. SSU rDNA and thereby facilitate a molecular identification of *Ichthyobodo* species from infections through sequencing.

2. Materials and methods

2.1. Primer and probe design

Partial SSU rDNA (18S rRNA gene) sequences of *Ichthyobodo* spp. available in GenBank (Table 1) were aligned using AlignX (Vector NTI Suite 9.0.0) to reveal conserved areas suitable as target regions for specific primers and probes. Partial SSU rDNA sequences from several fish species were included to allow selection of primers specific for the collection of *Ichthyobodo* spp. Different primer sets were designed to amplify overlapping fragments of the SSU rRNA gene. A quantitative real-time PCR assay ("Costia assay") with hydrolysis probe was designed with the use of online real-time tool software "GenScript Real-time PCR (TaqMan) Primer Design" [20]. Settings for the PCR primers and the Costia-assay primers and probe were in accordance with the guidelines as described by Apte and Daniel [21] and as recommended by Invitrogen™ and Applied Biosystems™ [22–24]. Melting temperatures (T_m), secondary structures and the possibility for primer-dimers were tested with use of a software DNA calculator [25]. The specificity for all primer and probe sequences were also checked for sequence similarity with other sequences by use of NCBI BLAST® nucleotide sequence similarity search. Primers were manufactured by Sigma-Aldrich and the hydrolysis probe by Applied Biosystems™.

Based on the criteria for primer and probe design, several primers sets for PCR and sequencing and the Costia-assay targeting SSU rRNA genes of *Ichthyobodo* spp. were made (Fig. 1, Table 2). All Cos-primers show 100% identity with *Ichthyobodo* spp. sequences in BLAST search, but the primers CosF1, CosF2, CosF3 and CosR4 sequences also show 100% identity and similar *E*-value with a sequence of *Perkinsela*-like organism (GenBank ID: JN202437). Costia R, reverse primer designed for the qPCR assay had to be degenerated due to a mismatch between *I. necator* and *Ichthyobodo* spp. in a single position (position 1159, GenBank ID: AY224691); Y representing both C and T. BLAST results for Costia R (Y = C) show 100% identity with *I. necator* ($E = 0.006$), Costia R (Y = T) show 100% identity with the other *Ichthyobodo* sequences ($E = 0.006$). Costia R (Y = C or T) revealed no significant match for other sequences available in NCBI GenBank®. Costia F and Costia Probe sequences show 100% identity with *Ichthyobodo* spp.,

Table 1
Ichthyobodo spp. from different fish hosts. Partial sequence data (>1500 bp) of the 18S rRNA gene (SSU rDNA) of *Ichthyobodo* spp. stored in NCBI GenBank®. Sequences of 10 species are available, designated by roman numerals I–X (Des.), of which 3 species have been described and named; *Ichthyobodo necator* sensu stricto (s.s.), *I. salmonis* and *I. hippoglossi*. Sensu lato (s.l.), i.e. identified as *Ichthyobodo necator* in wide-sense prior to the splitting of that species based on SSU rDNA sequences. Host habitat (water); FW: freshwater, BW: brackish water, SW: seawater.

No.	Species	Des.	Host	Water	Country	Ref.
AY224691	*I. necator* s.s.	I	*Salmo salar*	FW	Norway	[9]
AY224691	*I. necator* s.s.	I	*Gasterosteus aculeatus*	FW	Norway	[9]
DQ414519	*I. necator* s.s.	I	*Oncorhynchus mykiss*	FW	Norway	[8]
GQ184295	*I. necator* s.s.	I	*Salmo salar*	FW	Norway	[11]
GQ184296	*I. necator* s.s.	I	*Salmo salar*	FW	Norway	[11]
GQ184297	*I. necator* s.s.	I	*Gasterosteus aculeatus*	FW	Norway	[11]
GQ184298	*I. necator* s.s.	I	*Salmo trutta*	FW	Norway	[11]
AY224685	*I. salmonis*	II	*Salmo salar*	SW	Norway	[9]
AY224686	*I. salmonis*	II	*Salmo salar*	FW	Norway	[9]
AY229972	*I. salmonis*	II	*Salmo salar*	FW	Norway	[9]
AY229973	*I. salmonis*	II	*Salmo salar*	SW	Norway	[9]
JF290203	*I. salmonis*	II	*Salmo salar*	BW	Norway	[10]
JF290204	*I. salmonis*	II	*Salmo salar*	FW	Norway	[10]
JF290205	*I. salmonis*	II	*Salmo salar*	SW	Norway	[10]
AY224689	*Ichthyobodo* sp.	III	*Oncorhynchus masou*	FW	Japan	[9]
AY224690	*Ichthyobodo* sp.	IV	*Gadus morhua*	SW	Norway	[9]
AY255800	*Ichthyobodo* sp.	IV	*Gadus morhua*	SW	Norway	[9]
AY028448	*I. necator* s.l.	V	*Morone* hybrid	FW	USA	[26]
AY297476	*Ichthyobodo* sp.	V	*Xiphophorus helleri*	FW	USA	[7]
AY224692	*Ichthyobodo* sp.	VI	*Apistogramma* sp.	FW	Brazil	[9]
AY224688	*Ichthyobodo* sp.	VII	*Cyprinus carpio*	FW	South Africa	[9]
AY297478	*Ichthyobodo* sp.	VII	*Carassius auratus*	FW	USA	[7]
AY297479	*Ichthyobodo* sp.	VII	*Cyprinus carpio*	FW	USA	[7]
AY297480	*Ichthyobodo* sp.	VII	*Cyprinus carpio*	FW	USA	[7]
AY297481	*Ichthyobodo* sp.	VII	*Ictalurus punctatus*	FW	USA	[7]
AY297482	*Ichthyobodo* sp.	VII	*Paralichthys olivaceus*	SW	Japan	[7]
AY224687	*Ichthyobodo* sp.	VIII	*Carassius auratus*	FW	Singapore	[9]
AY297477	*Ichthyobodo* sp.	VIII	*Cyprinus carpio*	FW	Greece	[7]
AY297483	*Ichthyobodo* sp.	IX	*Oncorhynchus mykiss*	FW	USA	[7]
DQ414520	*I. hippoglossi*	X	*Hippoglossus hippoglossus*	SW	Norway	[8]

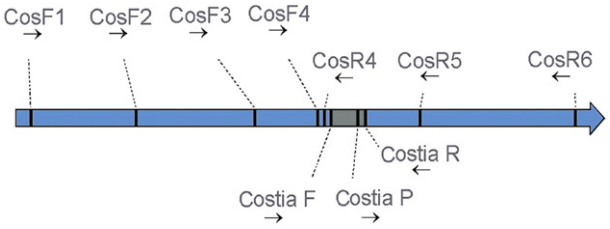

Fig. 1. Partial SSU rDNA sequence of *Ichthyobodo* spp. Primer sets for PCR and sequencing (Cos) and qPCR assay (Costia). Positions of *Ichthyobodo* spp. specific primers (forward, F; reverse, R) and probe (P) are marked. Amplification directions are marked with arrow. Software used: InforMax Vector NTI suite 9.0.0.

but also similar *E*-value and a 100% identity with a *Perkinsela*-like organism (GenBank ID: JN202437).

2.2. Nucleic acid extraction

RNA extractions from tissue samples are a necessity if other pathogens such as RNA viruses also are included in disease screening by real-time PCR. If DNA extraction kits are used, then the samples can be included in PCR and sequencing without a need for reverse transcription (cDNA synthesis). Therefore, both methods were chosen to examine the performance of the Costia-assay.

DNA and RNA were extracted from the samples with use of two available commercial spin-column based kits. For ethanol preserved samples, the ethanol was evaporated prior to the initial steps in the protocols of DNA or RNA extraction. Two commercial available nucleic acid extraction kits were tested. DNA was extracted with use of QIAamp® DNA Mini kit, using procedures as described in the spin column protocol "DNA Purification from tissues." All DNA samples were eluted by adding 100 μL Buffer AE to the spin column. This elution volume yields 92% according to the QIAamp DNA Mini and Blood Mini Handbook. RNA was extracted according to E.Z.N.A. protocol (E.Z.N.A. Total RNA Kit I): Isolation of total RNA from animal tissue. All RNA samples were eluted by adding 70 (2×35) μL DEPC-treated water to the spin column.

Addition of nuclease (DNase, RNase) is optional according to the protocols (E.Z.N.A. and QIAmp) and co-purification of DNA and RNA will not inhibit PCR. For simplicity, nuclease was not included in the protocols for nucleic acid extractions as described above; hence both DNA and RNA are present in the extracted samples. Nucleic acid concentrations (ng μL^{-1}) and purity (A_{260}/A_{280} ratio) were measured with use of NanoDrop® ND-1000 spectrophotometer. Accuracy for the spectrophotometer is ± 2 ng μL^{-1}.

2.3. PCR and sequencing

PCR reaction mixtures (total 25 μL per reaction) contained 2.5 μL (final concentration 1×) 10× Thermopol Reaction buffer (New England BioLabs®), 0.2 μL (1 U) Taq polymerase (New England BioLabs®), 1.0 μL forward primer (final concentration 0.4 μM), 1.0 μL reverse primer (final concentration 0.4 μM), 2.5 μL dNTP (final concentration 100 μM each), 15.75 μL nuclease free water and 2.0 μL DNA template. Standard PCR amplifications were performed on an Eppendorf Mastercycler® Gradient with thermal settings for 35 cycles after initialisation of 95 °C for 5 min: denaturation 95 °C for 45 seconds, annealing (T_a) 58 °C for 45 seconds, elongation 72 °C for 60–90 seconds. PCR reactions were completed with final elongation and hold of 72 °C for 10 min and 4 °C for a short time after last cycle.

Prior to sequencing, the PCR products were verified by gel analysis and purified with use of ExoSAP-IT®. The PCR templates were then sequenced using a BigDye Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems™). Sequencing was performed at University of Bergen (seqlab.uib.no). Sequences were assembled with ContigExpress (Vector NTI 9.0.0) and submitted to NCBI GenBank®.

2.4. Real-time PCR assays: Reverse transcription quantitative PCR (RT-qPCR) and qPCR

Master Mix (AgPath-ID™ One-Step RT-PCR kit) was prepared using 2 μL as standard sample volume of nucleic acid in a final volume of 12.5 μL master mix per reaction. Primer concentrations used in the master mix were optimized by testing concentrations varying from 50 to 900 nM and probe for concentrations between 25 and 225 nM. Primer and probe concentrations for optimal RT-qPCR analysis (amplification efficiency between 90 and 110% in preliminary tests) were set to 800 nM (for both forward and reverse) and 175 nM respectively. Components and amounts in the AgPath-ID™ Master Mix standardized for a 12.5 μL total volume reactions: 6.25 μL 2× RT-PCR Buffer, 1.0 μL forward primer, 1.0 μL reverse primer, 0.22 μL hydrolysis probe (TaqMan), 0.25 μL 25× RT-PCR Enzyme Mix, 1.78 μL nuclease-free water, 2.0 μL template (nucleic acid). Samples were distributed to a 96-well reaction plate (MicroAmp®). The real-time PCR reactions were performed and analyzed with Applied Biosystems™ 7500 Real-Time PCR System (standard) and software (SDS Software version 1.4). The thermal cycles were set according to the AgPath-ID™ protocol for 96-well system. Both DNA and RNA are present in the template (nuclease not added during extraction). Two different settings were performed to determine changes in sensitivity of the assay when cDNA was excluded from the template. For RT-qPCR assay: reverse transcription (RT) 45 °C for 10 min (cDNA synthesis, stage 1) and reverse transcriptase inactivation/initial denaturation 95 °C for 10 min (stage 2) following 40 cycles of amplification by 95 °C for 15 seconds and annealing (T_a) 60 °C for 45 seconds (stage 3). For the qPCR assay (only DNA), the RT-step (stage 1) was replaced with an initial 95 °C for 10 min (as an increased reverse transcriptase inactivating step). No-template-controls (NTC; nuclease free water) were included in all analyses.

Table 2
Primers and probe targeting 18S rRNA gene of *Ichthyobodo* spp. PCR primers ("Cos") are designed for sequencing and identification. The real-time PCR assay ("Costia") contains primer pair (F and R) and a hydrolysis probe giving an amplicon size of 136 bp. Melting temperatures (T_m) calculated with use of Sigma-Aldrich® "DNA calculator." Optimal annealing temperatures for combinations of Cos-primers in PCR reactions: $T_a = 58$ °C; for Costia primers and probe in real-time PCR runs: $T_a = 60$ °C. Positions according to GenBank ID: AY224691 (*I. necator*).

Name	Direction	Oligo sequence	Length	Position	T_m	T_a
CosF1	Forward	5′-AATAGGAGGTCTGCGAACG	19	26–44	62	58
CosF2	Forward	5′-CCTGAGAAACAGCTACCACT	20	377–396	59	58
CosF3	Forward	5′-CCGTCGTTATCACTGTGAAG	20	781–800	61	58
CosF4	Forward	5′-ACGTATCTGAGCGAGAGAGGT	21	993–1013	62	58
CosR4	Reverse	5′-CCGAGCGGTCTAAGAATTTC	20	1033–1014	63	58
CosR5	Reverse	5′-TTCCTGTACTGGTAAGGTTCC	21	1353–1333	59	58
CosR6	Reverse	5′-GTTGACCTGTATCCAACTGG	20	1872–1853	59	58
Costia F	Forward	5′-ACGAACTTATGCGAAGGCA	19	1036–1054	64	60
Costia R	Reverse	5′-TGAGTATTCACT**Y**CCGATCCAT	22	1171–1150	62/64	60
Costia Probe	Forward	5′ (FAM)-TCCACGACTGCAAACGATGACG-(TAMRA)	22	1125–1146	73	60

2.5. Reproducibility tests (efficiency)

Amplification efficiency (E) of the real-time PCR assay was tested on both *I. necator* and *I. salmonis*, since these represent distantly related species (see Todal et al. [9]). These different species are easy to identify and distinguish with use of the diagnostic primers, CoNec and CoEur, as described in Isaksen et al. [11]. The tests were performed on both DNA and RNA extractions using the RT-qPCR settings. Efficiency was determined with the use of the standard curves; 10-fold serial dilutions of nucleic acid samples were prepared, and a minimum of 3 replicates from 5 or more orders of magnitude of the templates were amplified. Threshold cycle values (C_T) from the replicates in the dilution series were plotted in order to generate a least squares standard curve. Slope- and R^2 (coefficient of determination) values were determined and the amplification efficiency (E%) calculated (Eq. (1), Table 3). Threshold and baseline values for analysis and efficiency testing on the ABI 7500 were set to 0.4 and "auto" respectively. This threshold line was within the exponential growth region of the amplification curve and appropriate for the widest range of C_T values obtained from analysis of the 10-fold serial dilutions.

2.6. Quantification

2.6.1. Counts of Ichthyobodo cells

Gills were collected from seawater reared salmon heavily infected with *I. salmonis*, stored in cold seawater and transported (c. 2 h) to the laboratory. Mucus with some tissue was scraped from a single primary filament onto a microscope slide. A droplet of water was added and a second, clean slide applied to the first slide. The two slides were then separated in producing two replicates *Ichthyobodo* smears. These were air-dried, and contained approximately the same amount of tissues. One smear was stained (Colorrapid-Set; Lucerna Chem AG, Switzerland) and all *I. salmonis* cells were counted with the use of a light microscope (1000× magnification). The smear from the duplicate slide was dissolved in ATL buffer (QIAamp kit) and transferred to a 2 mL microcentrifuge tube for DNA extraction. Three sets of duplicate smears were made (duplicate A, B and C). Ten-fold serial dilutions were made for each DNA extraction and standard curves with RT-qPCR and qPCR settings were made by plotting log10 of estimated numbers of *Ichthyobodo* cells against the corresponding C_T (Eq. (2), Table 3).

2.6.2. Copies of target sequences

PCR products (synthetic DNA), spanning the amplicon of the Costia-assay, were used to estimate the relationship between number of SSU rRNA gene copies and C_T values. Two sets of primers (Fig. 1, Table 4) were used to amplify different sequences within the SSU rDNA: CosF3/CosR6 (PCR1) and CosF4/CosR5 (PCR2). Hence one copy of the PCR1 or PCR2 DNA products contains one copy of the target SSU rDNA. The PCR products were purified using E.Z.N.A.® Cycle Pure Kit (Omega Bio-Tek) and eluted in 50 μL elution buffer. This elution yields 80–90% of bound DNA according to the manufacturer's handbook. Electrophoresis and gel analysis of the amplification products were performed to confirm correct sequence length. Concentrations of the PCR products (ng μL^{-1}) were measured by NanoDrop® using AE buffer as a "blank" in the calibration. A standard sample volume of 2 μL per reaction was used in the real-time PCR analysis. Mol. weight (g mol^{-1}) was calculated using the oligo calculation tool from Genscript [27]. Avogadros constant (N_A) was then used to calculate numbers of molecules (target copies) in the standard sample volume (Eq. (3), Table 3). Calculated amount of target sequences in PCR1 and PCR2 shown in Table 4.

Table 4
Synthetic DNA targeting known regions of the SSU rRNA gene of *Ichthyobodo* spp. Positions according to GenBank ID: AY224691 (*I. necator*). Amplicon sizes of the PCR products are given as base pairs (bp). Numbers of molecules (DNA oligos) per nanogram (N, copies ng^{-1}) were calculated using Avogadros constant (N_A: 6.022×10^{23} mol^{-1}), mol. weight (MW, g mol^{-1}) and nucleic acid concentrations (C, ng μL^{-1}). Purity (Abs; absorbance ratio of 260 nm and 280 nm) measured with use of spectrophotometer.

Template	Position	bp	MW	C	Abs	N
PCR1	781–1872	1092	3.39E+05	35	1.7	1.78E+09
PCR2	993–1353	361	1.12E+05	60	1.8	5.37E+09

2.7. Limit of detection (LOD)

2.7.1. Theoretical LOD

Ten-fold dilutions of the synthetic DNA (PCR 1 and PCR 2) were made for standard curve analysis as described above. Standard curves (Eq. (2), Table 3) based on log transformed numbers of molecules in the dilution series for PCR1 and PCR2 allow estimation of target copy numbers (Eq. (3), Table 3) corresponding to the measured C_T values. According to Bustin et al. [28], theoretical LOD (highest possible C_T value for a true positive sample) requires a minimum of 3 target copies in the template solution (theoretical LOD (Eq. (2))× = log10(3) = 0.477).

2.7.2. Experimentally determined LOD

Sensitivity for the Costia-assay is measured by the lowest amount of target copies that is detectable more than 90% of the time. The detection limit for the Costia-assay was determined experimentally based on analytical measurements. Positive *I. salmonis* DNA samples from the 10-fold dilution series used in the amplification efficiency test, showing low amounts of target copies ($C_T > 31$), were chosen as initial template and further diluted in 2-folds series. Ten replicates from each log were run with the given real-time PCR master mix and cycling conditions. Frequency of detection was plotted as a function of measured C_T values.

2.8. Validation of the PCR methods

A collection of skin and gill tissues from farmed and wild fish from Norway were studied, representing hosts from fresh-, brackish- and seawater. The material was collected from farmed fish where *Ichthyobodo* parasites had been observed by light microscopy or from fish where ichthyobodosis had been suspected due to clinical signs. The wild caught fish were randomly collected, no parasites or clinical signs of infections were observed. The tissue samples were verified by using the Costia-assay. Positive results were verified with use of the developed *Ichthyobodo* spp. specific primers for PCR and *Ichthyobodo* species were identified by sequencing. Hosts, sampling date and locations are listed in Table 5.

Table 3
Formulae used in real-time PCR analysis. Reproducibility tests and quantification determinations.

Equation	Formula	Description
Eq. (1)	$E = [(e^{\ln 10/-\text{slope}}) - 1]^* 100\%$	PCR amplification efficiency in percent (E), for "slope" = a in Eq. (2)
Eq. (2)	$C_T = b - a^* x$	Linear regression line. C_T values (threshold cycles) as a function of x, for $x = \log10(x)$, in a 10-fold serial dilution of templates in the real-time PCR amplifications.
Eq. (3)	$N = (C^* V/\text{Mw})^* N_A$	Numbers of target molecules in a sample (N) calculated given the Avogadros constant (N_A), concentration (C; gL^{-1}), volume (V; L), mass (g) and mol. weight (Mw; $gmol^{-1}$)

Table 5
Ichthyobodo spp. infections among farmed and wild fish. *Ichthyobodo* infected tissues sampled from different hosts in Norway. Farmed (F) and wild (W) caught fish. Counties: Finnmark (F), Troms (T), Nordland (N), Møre and Romsdal (MR), Sogn and Fjordane (SF), Hordaland (H), Rogaland (R), Oppland (O), Hedmark (He), and Austfold (A). Tissue samples stored in 70% ethanol (EtOH) as air-dried smears or frozen (−80 °C). Preliminary observations and remarks for the samples were noted as *Ichthyobodo* or *Ichthyobodo*-like parasites observed (O) in fresh smear, clinical signs of ichthyobodosis (I), increased mortality (M), gill disease suspected (G), random sampling (no preliminary observation, R).

No.	Host		Tissue	Sampling date	Notes	Storage	County	Map
F1	*Gadus morhua*[a]	Adult	Skin	06.06.2007	O	EtOH	T	69°39′N, 18°57′E
F2	*Gadus morhua*[a]	Juvenile	Gill	09.03.2006	O	EtOH	H	60°38′N, 04°48′E
F3	*Gadus morhua*[a]	Adult	Gill	16.03.2010	O	EtOH	H	60°38′N, 04°48′E
F4	*Gadus morhua*[a]	Juvenile	Gill	26.10.2005	G,M	EtOH	N	66°01′N, 12°13′E
F5	*Gadus morhua*[a]	Juvenile	Skin gill	03.10.2007	I,M,O I,M,O	EtOH	T	69°39′N, 18°57′E
F5	*Gadus morhua*[a]	Juvenile		03.10.2007		EtOH	T	69°39'N, 18°57′E
F6	*Salmo salar*[a]	Adult	Gill	18.03.2011	O	EtOH	H	60°15′N, 05°34′E
F7	*Salmo salar*[b]	Parr	Skin	12.06.2009	I,M,O	smear	SF	60°53′N, 07°15′E
F8	*Salmo salar*[a]	Adult	Gill	17.07.2010	R	EtOH	H	60°15′N, 05°34′E
F9	*Salmo salar*[b]	Parr	Skin	13.07.2009	I,M,O	EtOH	MR	62°48′N, 08°43′E
F10	*Salmo salar*[b]	Parr	Gill	01.09.2010	I,M,O	EtOH	H	60°06′N, 05°14′E
F11	*Salmo trutta*[b]	Parr	Skin	12.06.2009	I,M,O	smear	SF	60°53′N, 07°15′E
F12	*Oncorhynchus mykiss*[b]	Parr	Skin	25.04.2003	I,O	EtOH	H	60°31′N, 05°30′E
W1	*Gasterosteus aculeatus*[c]	Adult	Gill	26.07.2010	R	EtOH	A	59°28′N, 10°37′E
W2	*Gadus morhua*[a]	Adult	Gill	08.10.2008	O	smear	H	60°16′N, 05°13′E
W3	*Hippoglossus hippoglossus*[a]	Adult	Gill	10.10.2010	R	EtOH	F	70°34′N, 25°26′E
W4	*Hippoglossus hippoglossus*[a]	Adult	Gill	10.10.2010	R	EtOH	F	71°10′N, 25°53′E
W5	*Phoxinus phoxinus*[b]	Juvenile	Gill	25.07.2010	R	EtOH	Op	60°52′N, 09°42′E
W6	*Pollachius pollachius*[a]	Juvenile	Gill	06.10.2010	R	EtOH	H	60°16′N, 05°13′E
W7	*Salmo salar*[b]	Adult	Gill	08.12.2009	R	frozen	H	60°38′N, 06°09′E
W8	*Salmo salar*[b]	Adult	Gill	09.12.2009	R	frozen	MR	62°38′N, 08°07′E
W9	*Salmo salar*[b]	Adult	Gill	08.07.2011	R	EtOH	R	59°09′N, 06°10′E
W10	*Salmo trutta*[b]	Adult	Skin	03.07.2008	R	EtOH	He	61°51′N, 10°34′E
W11	*Gobius niger*[a]	Adult	Gill	02.07.2011	R	EtOH	H	60°15′N, 05°19′E
W12	*Pomatoschistus microps*[c]	Adult	Gill	05.07.2011	R	EtOH	H	60°13′N, 05°23′E

[a] Seawater.
[b] Freshwater.
[c] Brackish water.

2.9. Data analyses

The standard curves of the 10-fold serial dilutions were made as scatter plots between C_T values and replicates of the logarithmic amount of templates used in the RT-qPCR and qPCR amplifications. Outliers within the replicates resulting in standard deviation >0.25 were rejected. Linear regression lines for the standard curves were made and analyzed using Statistica® software. Accuracies for the *Ichthyobodo* assay were regarded acceptable for regression analyses when efficiency values were between 90 and 110% and the coefficient of determination (R^2) was >0.99. Formulae used in reproducibility tests and for quantification are shown in Table 3.

3. Result

3.1. Reproducibility

To determine the reproducibility of the Costia-assay, serial dilutions of nucleic acid extractions of salmonid gills containing different *Ichthyobodo* species were used to generate standard curves analyzed by linear regression. The tests show acceptable values in coefficient of determination (R^2) and amplification efficiencies (E) for both *I. salmonis* and *I. necator* with use of DNA and RNA extractions as templates. However, different methods for nucleic acid extractions (DNA- or RNA- extraction kit) apparently affect the amplification efficiency; the slope values being lower for DNA extracted samples with efficiency values equal or close to 100%. The amplification tests are summarized in Table 6 and standard curves shown in Fig. 2.

Table 6
Efficiency test of *Ichthyobodo* specific real-time PCR assay. Linear regression analyses of standard curves from 10-fold serial dilutions of DNA and RNA extracted from *Ichthyobodo* infected gills from Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Concentration (C; ng μL^{-1}) and purity (Abs; absorbance ratio between 260 nm and 280 nm) of DNA and RNA (nucleic acid) in the initial amount are given. Regression lines for C_T values as a function of log10 (ng) in the RT-qPCR amplification are used for calculation of efficiency (E%) and coefficient of determination (R^2).

Host	Species	Template	C	Abs	Regression line	E%	R^2
Salmon	*I. salmonis*	DNA	77	1.9	$C_T = 27.76 - 3.32x$	100	0.999
Salmon	*I. necator*	DNA	316	1.9	$C_T = 28.33 - 3.29x$	102	0.999
Salmon	*I. salmonis*	RNA	36	1.8	$C_T = 22.04 - 3.52x$	92	0.998
Rainbow trout	*I. necator*	RNA	139	2.2	$C_T = 26.17 - 3.46x$	95	0.991

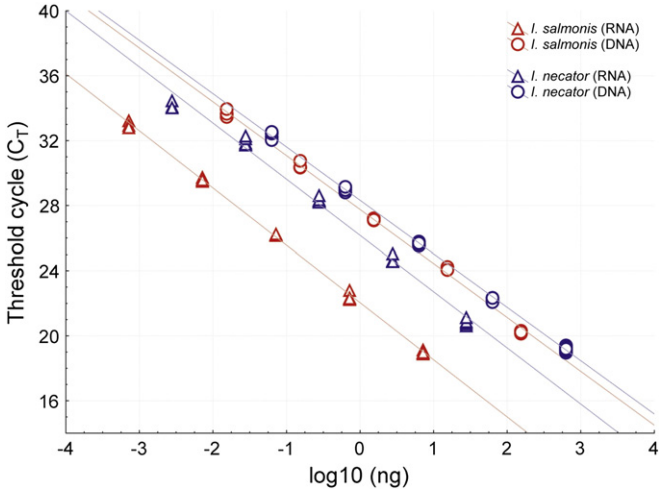

Fig. 2. Real-time PCR standard curves. Ten-fold dilution series with nucleic acid from *Ichthyobodo* spp. infected salmonids used as templates in the RT-qPCR runs. Starting amount of templates (ng) was 7.2 (*I. salmonis*, RNA), 154.0 (*I. salmonis*, DNA), 27.8 (*I. necator*, RNA) and 632 (*I. necator*, DNA). No amplification was detected for NTC (no template control) samples in these tests.

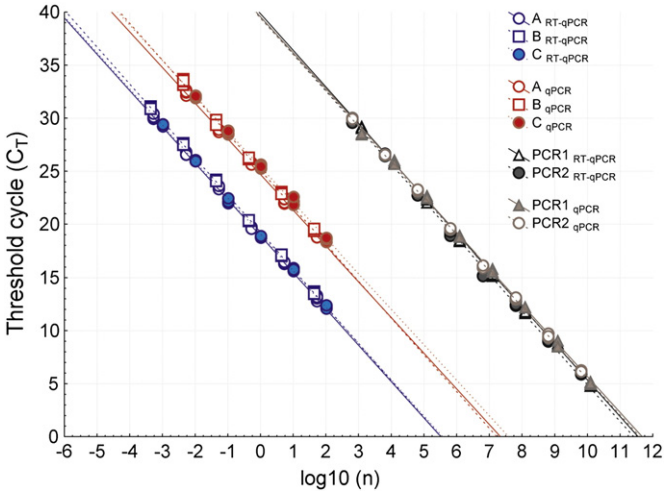

Fig. 3. Calibration curves for quantification of *Ichthyobodo* parasites by RT-qPCR and qPCR. Measured threshold cycles (C_T) as a function of known amounts of *I. salmonis* cells (A, B, C) and synthetic DNA (PCR1, PCR2). Ten-fold serial dilutions with a minimum of three replicates per log. Starting amount (n) for A, B and C was estimated to contain extracts from 54, 44 and 104 *Ichthyobodo* cells per template volume (2 μL) respectively. PCR1 and PCR2 are synthetic DNA oligos targeting 1092 bases (PCR1) and 361 bases (PCR2) of *Ichthyobodo* spp. 18S rDNA. Starting amount (n) for the PCR1 and PCR2 is 1.25×10^{10} and 6.45×10^{9} target copies per template volume (2 μL) respectively. Coefficient of determination; $R^2>0.99$ for all curves. No signals were detected in NTC samples (no template controls).

3.2. Quantification

Serial dilutions of DNA extractions (also including some RNA) containing estimated numbers of *I. salmonis* cells from smears (A, B and C) were analyzed with both RT-qPCR and qPCR settings for the Costia-assay. Linear regression analyses revealed acceptable amplification efficiencies for all runs, ranging from 94 to 96% (RT-qPCR) and 94 to 99% (qPCR). However, there is a shift in C_T values between the two different assays. By utilizing total nucleic acid (RNA and DNA) in RT-qPCR, detection of target in the real-time PCR reaction appear 1.7–1.8 log units earlier when compared to the qPCR tests that only amplify DNA from the nucleic acid extraction (Fig. 3). The relationships between C_T values and the estimated number of *Ichthyobodo* cells are shown in Table 7. Using Eq. (2) (Table 3), a single *I. salmonis* cell corresponds to C_T values ranging from 18.9 to 19.3 using RT-qPCR and from 24.7 to 25.4 with qPCR.

To estimate the numbers of SSU rRNA gene copies per *Ichthyobodo* cell, a calibration curve using synthetic DNA was generated. Two different synthetic DNAs (PCR1 and PCR2) were amplified as targets for the Costia-assay. Standard curves were generated with 10-fold dilution series and amplification efficiency tested with both RT-qPCR and qPCR settings. As expected there were no differences between RT-qPCR and qPCR runs since RNA was absent (Table 7; Fig. 3). Assuming that DNA from a single *I. salmonis* cell corresponds to C_T values within range 24.7–25.4 (see above), the predicted numbers of SSU rDNA copies per *I. salmonis* cell using the linear regressions for PCR1 and PCR2 (Table 7) was in the range 14930–24350 (PCR1) and 13200–21500 (PCR2).

3.3. Limit of detection (LOD)

Standard curves for the synthetic DNA with the Costia-assay were used to determine theoretical LOD. The linear regressions for PCR1 and PCR2 predict that 3 target copies correspond to $C_T=38$ (Table 3: Eq. (4), Table 7).

Experimental LOD was estimated using dilutions of nucleic acid extractions from salmon gills naturally infected with *I. salmonis* (i.e. a further dilution of *I. salmonis* DNA, Fig. 2). Reproducibility was tested on replicates ($n=10$) for each dilution using the Costia-assay with RT-qPCR settings. All replicates were positive at C_T values ≤ 36. The standard deviation exceeded 0.4 for C_T values above 34 and further dilution showed reduced reproducibility with no significant correlation between C_T values and concentrations of target (Fig. 4). The experimentally derived LOD ($C_T=36$) correspond to c. 12–18 target copies.

3.4. Validation of the PCR methods using natural infected tissue samples

The Costia-assay was tested on samples from different hosts using RT-qPCR settings (Tables 5 and 8). Positive RT-qPCR analyses showed a range in C_T values from 9 to 28, from 9 different host species. Samples with $C_T>23$ required nested- or semi-nested PCR to obtain sequences for identification. We were not able to obtain *Ichthyobodo* spp. sequences from samples with $C_T>28$, suggesting that the RT-qPCR assay (Costia-assay; designed for small amplicon size) was far more sensitive compared to the conventional PCR methods (Cos-primers; designed for larger amplicon sizes). The *Ichthyobodo* spp. sequences obtained in the present study represent 4 different sequences from farmed fish and 7 different sequences from wild caught fish. These include 4 novel sequences that may represent new species, designated species XI to XIV (Table 8). *Ichthyobodo* sp. XI was obtained from the skin of Atlantic cod, but diverged from other sequences from cod (*Ichthyobodo* sp. IV) by 4% (Tables 1 and 8). *Ichthyobodo* spp. XII and XIII originate from hosts caught in brackish- (common goby and stickleback) and freshwater (minnow) respectively. These showed less than 98% similarity with other *Ichthyobodo* sequences (spp. I–X). *Ichthyobodo* sp. XIV from the gills of the marine black goby diverged notably from other *Ichthyobodo* spp. with less than 93% similarity to the closest relatives.

Co-infections of *I. salmonis* and *I. necator* were detected in samples from salmon parr (F7, Table 8) and returning mature salmon (W7 and W8, Table 8) due to mixed signals in the sequencing chromatogram. The two species involved were subsequently identified from sequences obtained with species-specific primers for *I. salmonis* and *I. necator* (see Isaksen et al. [11]).

Table 7
Regression analyses. Standard curves from serial dilutions of different samples and real-time PCR assays (RT-qPCR and qPCR). Initial amount are given as numbers (N) of parasites (sample ABC) and 18S rDNA target copies (PCR1 and PCR2) per μL. C_T values (mean; n replicates) for 2 μL template in the amplification tests are given. Nucleic acid concentrations (C; ng μL^{-1}) and purity (Abs; absorbance ratio between 260 nm and 280 nm) measured by spectrophotometer. Linear regression (Linear) and amplification efficiency (E%) are given for the different samples and settings. Starting amount for PCR1 and PCR2 are 2 and 3 log dilutions of the original samples respectively; hence N and C for these are calculated values.

Sample	N	C	Abs	RT-qPCR (1)		qPCR (2)	
				C_T (1)	Linear (E%)	C_T (2)	Linear (E%)
A	26.73	41	2.0	13.1 (4)	$C_T=18.90-3.43x$ (96)	18.8 (2)	$C_T=24.69-3.36x$ (98)
B	21.97	72	2.1	13.6 (4)	$C_T=19.27-3.49x$ (94)	19.5 (3)	$C_T=25.08-3.47x$ (94)
C	52.10	94	2.1	12.2 (4)	$C_T=19.07-3.42x$ (96)	18.5 (3)	$C_T=25.41-3.35x$ (99)
PCR1	6.23E+09	3.5	–	5.0 (4)	$C_T=39.84-3.45x$ (95)	5.1 (3)	$C_T=39.56-3.39x$ (97)
PCR2	3.22E+09	0.6	–	6.0 (3)	$C_T=39.48-3.46x$ (95)	6.2 (3)	$C_T=39.42-3.40x$ (97)

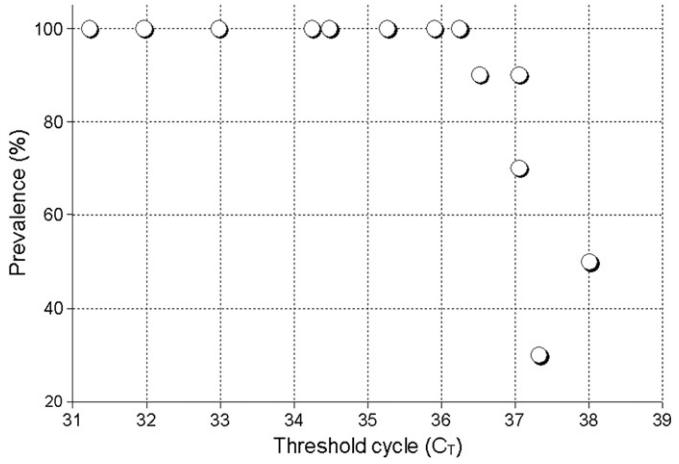

Fig. 4. Experimental limit of detection for the *Ichthyobodo* spp. RT-qPCR assay. Each point represents positive results from 10 replicates at different concentrations of nucleic acid extracted from *Ichthyobodo salmonis* infected salmon gills (*Salmo salar*). The relationship between prevalence of detection and C_T values are shown. Settings for C_T analysis: threshold 0.4 and auto baseline (ABI 7500). No amplification detected for the NTC (no template controls) samples.

4. Discussion

In the original work obtaining the first *Ichthyobodo* spp. sequences, the primers used were constructed based on alignments of SSU rDNA from a range of different kinetoplastids, and many of these primers are not specific for *Ichthyobodo* spp. [9,26]. Also, these primers show high variation in physical properties like melting temperature (T_m). At present there are 30 partial SSU rDNA sequences of *Ichthyobodo* spp. available in NCBI GenBank®, allowing us to design more *Ichthyobodo*-specific primers sets with similar T_m. The identification of conserved and variable regions in *Ichthyobodo* spp. SSU rDNA also allowed the development of a probe based real-time PCR assay ("Costia-assay") for the detection of all currently sequenced *Ichthyobodo* spp.

In BLAST searches, the Costia F primer matches 100% with some sequences of Perkinsela-like symbionts in amoebae and environmental (abyssal) kinetoplastids. Also the probe matches with some amoebal symbiont sequences. However the reverse primer (Costia R) only matches *Ichthyobodo* spp. sequences. Therefore, if such organisms should occur in the template used for real-time PCR analyses, some consumption of primer or probe could occur that might influence the efficiency of the assay. However, amplification should not occur.

The Costia-assay is shown to be reproducible and sensitive for different *Ichthyobodo* species, *I. necator* and *I. salmonis*. These two species are representatives from the two main clades revealed by phylogenetic analyses of *Ichthyobodo* spp., termed the A and B clades by Todal et al. [9]. Therefore, this assay will likely detect and amplify most *Ichthyobodo* spp. Determining the sensitivity of the Costia-assay, it was found that the theoretical limit of detection (LOD), 3 target copies of SSU rDNA, corresponds to a C_T value of 38. Hence C_T values above 38 may represent false positives and increase the possibility for Type I error (true negative considered as positive) if such test results are not rejected [29]. To minimize the chance of Type I error, a cut-off is recommended representing the C_T where samples are positive in more than 95% in a series of replicates [28]. We arrived at an experimental LOD at $C_T = 36$ representing 12 target copies of SSU rRNA for *I. salmonis* using RT-qPCR with the described baseline and threshold settings. However, based on DNA templates where the number of *I. salmonis* cells present were estimated, we found with qPCR that C_T values 30–31 represent a single *Ichthyobodo* cell present in the sampled

Table 8
Real-time PCR tests of *Ichthyobodo* infected fish from Norway. Farmed (F) and wild caught (W) fish. Host habitat (water); FW: freshwater, BW: brackish water, SW: seawater. Concentrations (C; ng/μL) of nucleic acid (DNA and RNA) extracted from host tissues were measured by NanoDrop® spectrophotometer. The purity (absorbance ratio: 260 nm/280 nm) between 1.9 and 2.1 for all samples. The RT-qPCR results are given as threshold cycle values (C_T) for target DNA (SSU rDNA) in 2 μL samples (ABI 7500 settings: threshold 0.4 and auto baseline). The C_T values only show tendencies; the values correlate negatively to the amount of target (nucleic acid from *Ichthyobodo* spp.). The *Ichthyobodo* species are identified by SSU rDNA sequences. Accession numbers (NCBI GenBank®) are given.

Code	Host		Tissue	C	C_T	N	Species	GenBank ID
F1	Atlantic cod	*G. morhua*[a]	Skin	225	11	443,423	*Ichthyobodo* sp. XI	JQ613335
F2	Atlantic cod	*G. morhua*[a]	Gill	386	17	4736	*Ichthyobodo* sp. IV	JQ613336
F3	Atlantic cod	*G. morhua*[a]	Gill	148	17	12,353	*Ichthyobodo* sp. IV	JQ613337
F4	Atlantic cod	*G. morhua*[a]	Gill	378	18	2483	*Ichthyobodo* sp. IV	JQ613338
F5	Atlantic cod	*G. morhua*[a]	Gill	85	13	309,448	*Ichthyobodo* sp. IV	JQ613339
F5	Atlantic cod	*G. morhua*[a]	Skin	261	17	7005	*Ichthyobodo* sp. IV	JQ613340
F6	Atlantic salmon	*S. salar*[a]	Gill	28	13	939,396	*I. salmonis*	JQ613341
F7	Atlantic salmon	*S. salar*[b]	Skin	298	17	6135	*I. salmonis*	JQ613342
							I. necator (co-infection)	JQ613343
F8	Atlantic salmon	*S. salar*[a]	Gill	176	18	5334	*I. salmonis*	JQ613344
F9	Atlantic salmon	*S. salar*[b]	Skin	100	19	4820	*I. salmonis*	JQ613345
F10	Atlantic salmon	*S. salar*[b]	Gill	923	16	3858	*I. necator*	JQ613346
F11	Brown trout	*S. trutta*[b]	Skin	148	9	2,557,026	*I. necator*	JQ613347
F12	Rainbow trout	*O. mykiss*[b]	Gill	176	11	566,876	*I. necator*	DQ414519
W1	Stickleback	*G. aculeatus*[c]	Gill	225	21	565	*Ichthyobodo* sp. XII	JQ613348
W2	Atlantic cod	*G. morhua*[a]	Gill	309	13	85,123	*Ichthyobodo* sp. IV	JQ613349
W3	Atlantic halibut	*H. hippoglossus*[a]	Gill	262	24	66	*I. hippoglossi*	JQ613350
W4	Atlantic halibut	*H. hippoglossus*[a]	Gill	367	25	24	*I. hippoglossi*	JQ613351
W5	Eurasian minnow	*P. phoxinus*[b]	Gill	320	22	204	*Ichthyobodo* sp. XIII	JQ613352
W6	Pollack	*P. pollachius*[a]	Gill	135	23	248	*Ichthyobodo* sp. IV	JQ613353
W7	Atlantic salmon	*S. salar*[b]	Gill	296	21	429	*I. salmonis*	JQ613354
							I. necator (co-infection)[d]	
W8	Atlantic salmon	*S. salar*[b]	Gill	141	20	1755	*I. salmonis*	JQ613355
							I. necator (co-infection)[d]	
W9	Atlantic salmon	*S. salar*[b]	Gill	309	26	15	*I. necator*	JQ613356
W10	Brown trout	*S. trutta*[b]	Skin	329	23	102	*I. necator*	JQ613357
W11	Black goby	*G. niger*[a]	Gill	212	26	21	*Ichthyobodo sp.* XIV	JQ613358
W12	Common goby	*P. microps*[c]	Gill	98	28	12	*Ichthyobodo* sp. XII	JQ613359

[a] Seawater.
[b] Freshwater.
[c] Brackish water.
[d] Amplified sequence <500 bp, not submitted to GenBank (NCBI).

tissue from which nucleic acids were extracted (100 μL). We have observed C_T values >31, which appear to represent a target DNA amount less than that in a single *Ichthyobodo* cell. Among possible explanations for this, the presence of nucleic acids from lysed *Ichthyobodo* cells in the samples is considered the most likely, but incomplete nucleic acid extractions may also produce such results.

Usually, there is far more rRNA than rRNA genes (rDNA) in a cell [30]; hence RT-qPCR is a more sensitive method than qPCR. In the present *I. salmonis* samples, RT-qPCR amplifying both RNA and DNA detected 60 times more template from nucleic acid extractions than qPCR. Calibration curves made for real-time PCR analyses shows that a single *I. salmonis* cell corresponds to $C_T = 19$ using RT-qPCR settings and $C_T = 25$ with use of qPCR. A quantification of *Ichthyobodo* cells with use of the Costia-assay is demonstrated for *I. salmonis*. Since this assay is designed to target all known *Ichthyobodo* species, it may be used to determine numbers of SSU rDNA copies in any sample of these. However the number of SSU rRNA genes may vary among species, so a quantification of SSU rDNA by qPCR cannot readily be converted to cell-numbers for other *Ichthyobodo* spp. Further insight into both intra- and interspecific variation in the number of genomic SSU rDNA copies in *Ichthyobodo* spp. is therefore needed (see Refs. [31–33]).

By comparing the linear relationship between C_T values (qPCR) and both numbers of *I. salmonis* cells and synthetic rDNA sequences, it was estimated that a single *I. salmonis* cell contains 13,000–25,000 copies SSU rDNA. A similar approach to determine numbers of rDNA copies in a cell have been used with the parasitic dinoflagellate *Hematodinium* sp. [34,35]. In *Hematodinium* sp. the number of SSU rDNA gene copies has been estimated to be within a range of 800–100,000 per cell [35,36]. There are no previous studies that have estimated the amount of rDNA and copies of rRNA in *Ichthyobodo* spp. cells, but the estimated number of rDNA copies per *I. salmonis* cell is high compared to other kinetoplastids [31].

Quantification by real-time PCR is a useful tool in estimating densities of these flagellates on hosts. An application of the Costia-assay may be the examination of microhabitat preferences for *Ichthyobodo* spp. on hosts, which in the past have been done with light microscopy [37]. Preferred sites vary with host species and also with life cycle stage within species. The qPCR may easily be applied also to water and sediment samples in epizootiological research. Standard veterinary screening for detection of *Ichthyobodo* spp. infections on fish farms also relies on light microscopy, either through examination of fresh smears prepared from external surfaces of individual fish or by histological study. Also, it may be difficult to detect the motionless trophozoites in fresh smears, and fixation and staining of smears are required in order to more precisely quantify the number of flagellates present using light microscopy (cf. Isaksen et al. [11]). These are time consuming and less sensitive methods for detection of pathogens compared to the use of real-time PCR assays, and there is a danger of confusing the potentially pathogenic *Ichthyobodo* spp. with for example the benign *Cryptobia* spp. Samples for molecular screening may be taken by non-specialist employees at farms and sent to laboratories for real-time PCR analyses, both for *Ichthyobodo* spp. and other pathogens.

Application of the Costia-assay has recently been tested and used in a multifactorial screening survey for factors that may influence the development of pancreas disease (PD) in farmed Atlantic salmon [38]. Tissue samples from seawater reared salmon ($N = 382$) were collected during a period from April to October in 2010 and screened for several pathogens; including Salmonid alphavirus (SAV) and *Ichthyobodo* spp. This study revealed that *I. salmonis* (identified by subsequent SSU rDNA sequencing) was common in gill samples and the most frequent pathogen among moribund fish. Intensities of *I. salmonis* infection as measured using relative quantification showed a significant positive correlation with SAV levels (causative agent for PD). Hence these flagellates may play a role in disease development and mortality in PD outbreaks. This application of the assay exemplifies an increasingly important usage of qPCR in fish disease studies; disentangling the role of different pathogens in complex multiagent infections associated with disease. Gill diseases (PGD, PGI) are often associated with a suite of different pathogens detected by qPCR, and the developed Costia-assay allows including also *Ichthyobodo* in such screening. Hence this tool should promote a better understanding regarding epizootiology and etiology of diseases in aquaculture.

We have also demonstrated that the Costia-assay may be used to detect *Ichthyobodo* spp. infections in different hosts. With subsequent PCR and sequencing the assay aids research into the poorly known diversity of *Ichthyobodo* spp. in for example marine fish. From naturally infected fish we identified two distinct *Ichthyobodo* species from skin or gills of Atlantic cod. One of these species have previously been identified from gill samples of juvenile cod in Western Norway (*Ichthyobodo* sp. IV Todal et al. [9]), while the second species detected in skin samples of large cod from Northern Norway is new (*Ichthybodo* sp. XI). Other new sequences (species) found, *Ichthyobodo* sp. XII (sticklebacks and common goby, brackish water) and *Ichthybodo* sp. XIII (minnow, freshwater) represent the first Eurasian *Ichthyobodo* spp. grouping in sub-lineage B2 in the phylogram shown by Todal et al. [9]. *Ichthyobodo* sp. XIV (black goby, seawater) appears to represent a new clade in the phylogeny of *Ichthyobodo*. Initial real-time PCR screening allows us to detect individual fish with *Ichthyobodo* infections, and pinpoint samples that may be sequenced. Hence in the search for new species, such molecular tools help direct attention to relevant fish hosts. These may consequently be targeted in field sampling securing material for the morphological characterization of new *Ichthyobodo* spp.

5. Conclusions

The Costia-assay, a real-time qPCR assay for the detection of *Ichthyobodo* spp., is presented and tested. The assay detects far less than a single cell-equivalent of rDNA. A novel primer suite is presented for PCR and sequencing and consequently identification of *Ichthyobodo* spp. infections detected with the Costia-assay. The Costia-assay may be applied together with other real-time PCR assays targeting different potential pathogens in complex infections. Also, this real-time PCR assay is useful in epizootiological studies and in basic research such as probing for new *Ichthyobodo* species.

Acknowledgments

The present study was funded by the Norwegian Research Council (Project no. 190448).

The authors wish to thank all that have contributed to the sampling necessary for our study, and in particular Erik Dahl-Paulsen, Eirik Hoel, Christian Koren, Linn-Maren Omdal, Kristin Ottesen, Øystein Staveland, Glenn A. Sundnes and the excellent anglers Geir Schriwer, Roald Qvale-Isaksen and Malene Qvale-Isaksen. Fish farms and fish health services that have contributed with fish samples to this study are AquaLab AS, E-CO Vannkraft (Aurland), EWOS, Fjord Forsøksstasjon Helgeland and Lerøy AS.

References

[1] Nylund S, Andersen L, Saevareid I, Plarre H, Watanabe K, Arnesen CE, et al. Diseases of farmed Atlantic salmon *Salmo salar* associated with infections by the microsporidian *Paranucleospora theridion*. Diseases of Aquatic Organisms 2011;94:41-57.
[2] Steinum T, Kvellestad A, Colguhoun DJ, Heum M, Mohammad S, Grontvedt RN, et al. Microbial and pathological findings in farmed Atlantic salmon *Salmo salar* with proliferative gill inflammation. Diseases of Aquatic Organisms 2010;91: 201-11.
[3] Poppe TT, Håstein T. Costiasis in Atlantic salmon (*Salmo salar* L.) in seawater. Norsk Veterinærtidsskrift, 94; 1982. p. 259-62. (in Norwegian).
[4] Rodger HD, Murphy K, Mitchell SO, Henry L. Gill disease in marine farmed Atlantic salmon at four farms in Ireland. Veterinary Record 2011.
[5] Lom J, Dyková I. Protozoan parasites of fishes. Amsterdam: Elsevier; 1992.

[6] Robertson DA. A review of *Ichthyobodo necator* (Henneguy, 1883) an important and damaging fish parasite. In: Muir JF, Roberts RJ, editors. Recent advances in aquaculture. London: Croom Helm; 1985. p. 1–30.
[7] Callahan HA, Litaker RW, Noga EJ. Genetic relationships among members of the *Ichthyobodo necator* complex: implications for the management of aquaculture stocks. Journal of Fish Diseases 2005;28:111-8.
[8] Isaksen TE, Karlsbakk E, Nylund A. *Ichthyobodo hippoglossi* n. sp. (Kinetoplastea: Prokinetoplastida: Ichthyobodonidae fam. nov.), an ectoparasitic flagellate infecting farmed Atlantic halibut *Hippoglossus hippoglossus*. Diseases of Aquatic Organisms 2007;73:207-17.
[9] Todal JA, Karlsbakk E, Isaksen TE, Plarre H, Urawa S, Mouton A, et al. *Ichthyobodo necator* (Kinetoplastida)—a complex of sibling species. Diseases of Aquatic Organisms 2004;58:9–16.
[10] Isaksen TE, Karlsbakk E, Watanabe K, Nylund A. *Ichthyobodo salmonis* sp. n. (Ichthyobodonidae, Kinetoplastida), an euryhaline ectoparasite infecting Atlantic salmon (*Salmo salar* L.). Parasitology 2011;138:1164-75.
[11] Isaksen TE, Karlsbakk E, Sundnes GA, Nylund A. Patterns of *Ichthyobodo necator* sensu stricto infections on hatchery reared salmon (*Salmo salar* L.) in Norway. Diseases of Aquatic Organisms 2010;88:207-14.
[12] Post G. Animal parasites of fishes. In: Post G, editor. Textbook of fish health. revised and expanded edition. Neptune City (USA): T.F.H. Publications, Inc.; 1987. p. 163-5.
[13] Henneguy LF. Sur un Infusoire flagelle, ectoparasite des Poissons. Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences 1883;96:658-60.
[14] Urawa S. Effects of rearing conditions on growth and mortality of juvenile chum salmon (*Oncorhynchus keta*) infected with *Ichthyobodo necator*. Canadian Journal of Fisheries and Aquatic Sciences 1995;52:18-23.
[15] Savage J. Notes on costiasis. Transactions of the American Fisheries Society 1935;65:332-3.
[16] Ellis AE, Wootten R. Costiasis of Atlantic salmon, *Salmo salar* L. smolts in seawater. Journal of Fish Diseases 1978;1:389-93.
[17] Urawa S, Kusakari M. The survivability of the ectoparasitic flagellate *Ichthyobodo necator* on Chum salmon fry (*Oncorhynchus keta*) in seawater and comparison to *Ichthyobodo* sp. on Japanese flounder (*Paralichthys olivaceus*). Journal of Parasitology 1990;76:33-40.
[18] Wood JW. Diseases of pacific salmon: their prevention and treatment. 3rd edition. Olympia, Washington: State of Washington. Department of Fisheries, Hatchery Division; 1979.
[19] Norwegian Veterinary Institute—Fish Health Report. Available at: http://www.vetinst.no/eng/Research/Publications/Fish-Health-Report (Accessed: 18 May 2012).
[20] GenScript—Real-time PCR (TaqMan) Primer Design. Available at: http://www.genscript.com/ssl-bin/app/primer (Accessed: 18 May 2012).
[21] Apte A, Daniel S. PCR primer design. In: Dieffenbach CW, Dveksler GS, editors. PCR primer: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2003. p. 61-74.
[22] Applied Biosystem™—Custom primer/probe design. Available at: http://www3.appliedbiosystems.com/WebTroubleshooting/PoorPCREfficiency/Custom_primer_probe_design.htm (Accessed: 18 May 2012).
[23] Invitrogen™—Top Ten Pitfalls in Quantitative Real-time PCR Primer Probe Design and Use. Available at: http://www.invitrogen.com/site/us/en/home/References/Ambion-Tech-Support/rtpcr-analysis/general-articles/top-ten-pitfalls-in-quantitative-real-time-pcr-primer.html.
[24] Invitrogen™—Real-time PCR: From theory to practice. Available at: http://tools.invitrogen.com/content.cfm?pageid=12257 (Accessed: 18 May 2012).
[25] Sigma-Aldrich®—DNA Calculator. Available at: http://www.sigma-genosys.com/calc/DNACalc.asp (Accessed: 18 May 2012).
[26] Callahan HA, Litaker RW, Noga EJ. Molecular taxonomy of the suborder Bodonina (order Kinetoplastida), including the important fish parasite, Ichthyobodo necator. Journal of Eukaryotic Microbiology 2002;49:119-28.
[27] GenScript—Oligo Calculation Tool. Available at: http://www.genscript.com/cgi-bin/tools/primer_calculation (Accessed: 18 May 2012).
[28] Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical Chemistry 2009;55:611-22.
[29] Burns M, Valdivia H. Modelling the limit of detection in real-time quantitative PCR. European Food Research and Technology 2008;226:1513-24.
[30] Prokopowich CD, Gregory TR, Crease TJ. The correlation between rDNA copy number and genome size in eukaryotes. Genome 2003;46:48-50.
[31] Torres-Machorro AL, Hernandez R, Cevallos AM, Lopez-Villasenor I. Ribosomal RNA genes in eukaryotic microorganisms: witnesses of phylogeny? FEMS Microbiology Reviews 2010;34:59-86.
[32] Greenwood SJ, Schnare MN, Cook JR, Gray MW. Analysis of intergenic spacer transcripts suggests 'read-around' transcription of the extrachromosomal circular rDNA in *Euglena gracilis*. Nucleic Acids Research 2001;29:2191-8.
[33] Perini F, Casabianca A, Battocchi C, Accoroni S, Totti C, Penna A. New approach using the real-time PCR method for estimation of the toxic marine dinoflagellate ostreopsis cf. ovata in marine environment. PLoS One 2011:1-6.
[34] Li CW, Shields JD, Miller TL, Small HJ, Pagenkopp KM, Reece KS. Detection and quantification of the free-living stage of the parasitic dinoflagellate *Hematodinium* sp in laboratory and environmental samples. Harmful Algae 2010;9:515-21.
[35] Nagle L, Place AR, Schott EJ, Jagus R, Pitula JS Messick G. Real-time PCR-based assay for quantitative detection of Hematodinium sp. in the blue crab *Callinectes sapidus*. Diseases of Aquatic Organisms 2009;84:79-87.
[36] Godhe A, Asplund ME, Harnstrom K, Saravanan V, Tyagi A, Karunasagar I. Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by real-time PCR. Applied and Environmental Microbiology 2008;74:7174-82.
[37] Urawa S. Epidermal responses of Chum Salmon (*Oncorhynchus keta*) fry to the ectoparasitic flagellate *Ichthyobodo necator*. Canadian Journal of Zoology 1992;70:1567-75.
[38] Repstad O. Kartlegging av patogendynamikken hos oppdrettslaks (*Salmo salar* L.) med diagnosen pankreassykdom (PD). University of Bergen, Department of biology; 2011 (in Norwegian).