



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

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### 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.

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### 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

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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

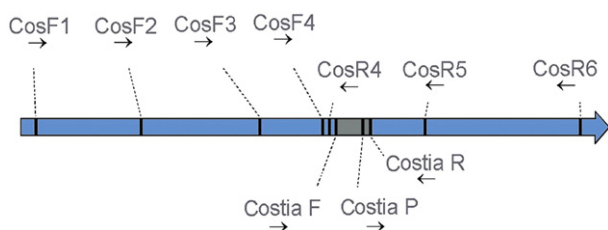
Partial SSU rDNA (18S rRNA gene) sequences of *Ichthyobodo* spp. available in GenBank (Table 1) were aligned using AlignX (Vector

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 primer 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>I. necator</i> s.s.	I	<i>Salmo salar</i>	FW	Norway	[9]
AY224691	<i>I. necator</i> s.s.	I	<i>Gasterosteus aculeatus</i>	FW	Norway	[9]
DQ414519	<i>I. necator</i> s.s.	I	<i>Oncorhynchus mykiss</i>	FW	Norway	[8]
GQ184295	<i>I. necator</i> s.s.	I	<i>Salmo salar</i>	FW	Norway	[11]
GQ184296	<i>I. necator</i> s.s.	I	<i>Salmo salar</i>	FW	Norway	[11]
GQ184297	<i>I. necator</i> s.s.	I	<i>Gasterosteus aculeatus</i>	FW	Norway	[11]
GQ184298	<i>I. necator</i> s.s.	I	<i>Salmo trutta</i>	FW	Norway	[11]
AY224685	<i>I. salmonis</i>	II	<i>Salmo salar</i>	SW	Norway	[9]
AY224686	<i>I. salmonis</i>	II	<i>Salmo salar</i>	FW	Norway	[9]
AY229972	<i>I. salmonis</i>	II	<i>Salmo salar</i>	FW	Norway	[9]
AY229973	<i>I. salmonis</i>	II	<i>Salmo salar</i>	SW	Norway	[9]
JF290203	<i>I. salmonis</i>	II	<i>Salmo salar</i>	BW	Norway	[10]
JF290204	<i>I. salmonis</i>	II	<i>Salmo salar</i>	FW	Norway	[10]
JF290205	<i>I. salmonis</i>	II	<i>Salmo salar</i>	SW	Norway	[10]
AY224689	<i>Ichthyobodo</i> sp.	III	<i>Oncorhynchus masou</i>	FW	Japan	[9]
AY224690	<i>Ichthyobodo</i> sp.	IV	<i>Gadus morhua</i>	SW	Norway	[9]
AY255800	<i>Ichthyobodo</i> sp.	IV	<i>Gadus morhua</i>	SW	Norway	[9]
AY028448	<i>I. necator</i> s.l.	V	<i>Morone</i> hybrid	FW	USA	[26]
AY297476	<i>Ichthyobodo</i> sp.	V	<i>Xiphophorus helleri</i>	FW	USA	[7]
AY224692	<i>Ichthyobodo</i> sp.	VI	<i>Apistogramma</i> sp.	FW	Brazil	[9]
AY224688	<i>Ichthyobodo</i> sp.	VII	<i>Cyprinus carpio</i>	FW	South Africa	[9]
AY297478	<i>Ichthyobodo</i> sp.	VII	<i>Carassius auratus</i>	FW	USA	[7]
AY297479	<i>Ichthyobodo</i> sp.	VII	<i>Cyprinus carpio</i>	FW	USA	[7]
AY297480	<i>Ichthyobodo</i> sp.	VII	<i>Cyprinus carpio</i>	FW	USA	[7]
AY297481	<i>Ichthyobodo</i> sp.	VII	<i>Ictalurus punctatus</i>	FW	USA	[7]
AY297482	<i>Ichthyobodo</i> sp.	VII	<i>Paralichthys olivaceus</i>	SW	Japan	[7]
AY224687	<i>Ichthyobodo</i> sp.	VIII	<i>Carassius auratus</i>	FW	Singapore	[9]
AY297477	<i>Ichthyobodo</i> sp.	VIII	<i>Cyprinus carpio</i>	FW	Greece	[7]
AY297483	<i>Ichthyobodo</i> sp.	IX	<i>Oncorhynchus mykiss</i>	FW	USA	[7]
DQ414520	<i>I. hippoglossi</i>	X	<i>Hippoglossus hippoglossus</i>	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  $\mu$ L 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 1): Isolation of total RNA from animal tissue. All RNA samples were eluted by adding 70 (2  $\times$  35)  $\mu$ L DEPC-treated water to the spin column.

Addition of nuclease (DNase, RNase) is optional according to the protocols (E.Z.N.A. and QIAamp) 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  $\mu$ L<sup>-1</sup>) and purity ( $A_{260}/A_{280}$  ratio) were measured with use of NanoDrop® ND-1000 spectrophotometer. Accuracy for the spectrophotometer is  $\pm 2$  ng  $\mu$ L<sup>-1</sup>.

## 2.3. PCR and sequencing

PCR reaction mixtures (total 25  $\mu$ L per reaction) contained 2.5  $\mu$ L (final concentration 1  $\times$ ) 10 $\times$  Thermopool Reaction buffer (New England

BioLabs®), 0.2  $\mu$ L (1 U) Taq polymerase (New England BioLabs®), 1.0  $\mu$ L forward primer (final concentration 0.4  $\mu$ M), 1.0  $\mu$ L reverse primer (final concentration 0.4  $\mu$ M), 2.5  $\mu$ L dNTP (final concentration 100  $\mu$ M each), 15.75  $\mu$ L nuclease free water and 2.0  $\mu$ L 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  $\mu$ L as standard sample volume of nucleic acid in a final volume of 12.5  $\mu$ L 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  $\mu$ L total volume reactions: 6.25  $\mu$ L 2 $\times$  RT-PCR Buffer, 1.0  $\mu$ L forward primer, 1.0  $\mu$ L reverse primer, 0.22  $\mu$ L hydrolysis probe (TaqMan), 0.25  $\mu$ L 25 $\times$  RT-PCR Enzyme Mix, 1.78  $\mu$ L nuclease-free water, 2.0  $\mu$ L 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'-AATAGGAGGTCTGCGAACC	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'-TTCCTGTACTGTGAAGTTCC	21	1353–1333	59	58
CosR6	Reverse	5'-GTTGACCTGTATCCAACCTGG	20	1872–1853	59	58
Costia F	Forward	5'-ACGAACCTTATCGGAAGGCA	19	1036–1054	64	60
Costia R	Reverse	5'-TGAGTATTCACCTYCCGATCCAT	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 log<sub>10</sub> 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<sup>-1</sup>) were measured by NanoDrop® using AE buffer as a “blank”

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

Equation	Formula	Description
Eq. (1)	$E = [(e^{10/(-\text{slope})} - 1) * 100\%$	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 $x = \log_{10}(x)$ , in a 10-fold serial dilution of templates in the real-time PCR amplifications.
Eq. (3)	$N = (C * V / Mw) * N_A$	Numbers of target molecules in a sample ( $N$ ) calculated given the Avogadro constant ( $N_A$ ), concentration ( $C$ ; gL <sup>-1</sup> ), volume ( $V$ ; L), mass (g) and mol. weight ( $Mw$ ; gmol <sup>-1</sup> )

**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<sup>-1</sup>) were calculated using Avogadro constant ( $N_A$ :  $6.022 \times 10^{23}$  mol<sup>-1</sup>), mol. weight ( $MW$ , g mol<sup>-1</sup>) and nucleic acid concentrations ( $C$ , ng µL<sup>-1</sup>). 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

in the calibration. A standard sample volume of 2 µL per reaction was used in the real-time PCR analysis. Mol. weight (g mol<sup>-1</sup>) was calculated using the oligo calculation tool from Genscript [27]. Avogadro 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.

## 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)) × =  $\log_{10}(3) = 0.477$ ).

### 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 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	<i>Gadus morhua</i> <sup>a</sup>	Adult	06.06.2007	O	EtOH	T	69°39'N, 18°57'E
F2	<i>Gadus morhua</i> <sup>a</sup>	Juvenile	09.03.2006	O	EtOH	H	60°38'N, 04°48'E
F3	<i>Gadus morhua</i> <sup>a</sup>	Adult	16.03.2010	O	EtOH	H	60°38'N, 04°48'E
F4	<i>Gadus morhua</i> <sup>a</sup>	Juvenile	26.10.2005	G,M	EtOH	N	66°01'N, 12°13'E
F5	<i>Gadus morhua</i> <sup>a</sup>	Juvenile	03.10.2007	I,M,O I,M,O	EtOH	T	69°39'N, 18°57'E
F5	<i>Gadus morhua</i> <sup>a</sup>	Juvenile	03.10.2007		EtOH	T	69°39'N, 18°57'E
F6	<i>Salmo salar</i> <sup>a</sup>	Adult	18.03.2011	O	EtOH	H	60°15'N, 05°34'E
F7	<i>Salmo salar</i> <sup>b</sup>	Parr	12.06.2009	I,M,O	smear	SF	60°53'N, 07°15'E
F8	<i>Salmo salar</i> <sup>a</sup>	Adult	17.07.2010	R	EtOH	H	60°15'N, 05°34'E
F9	<i>Salmo salar</i> <sup>b</sup>	Parr	13.07.2009	I,M,O	EtOH	MR	62°48'N, 08°43'E
F10	<i>Salmo salar</i> <sup>b</sup>	Parr	01.09.2010	I,M,O	EtOH	H	60°06'N, 05°14'E
F11	<i>Salmo trutta</i> <sup>b</sup>	Parr	12.06.2009	I,M,O	smear	SF	60°53'N, 07°15'E
F12	<i>Oncorhynchus mykiss</i> <sup>b</sup>	Parr	25.04.2003	I,O	EtOH	H	70°31'N, 05°30'E
W1	<i>Gasterosteus aculeatus</i> <sup>c</sup>	Adult	26.07.2010	R	EtOH	A	59°28'N, 10°37'E
W2	<i>Gadus morhua</i> <sup>a</sup>	Adult	08.10.2008	O	smear	H	60°16'N, 05°13'E
W3	<i>Hippoglossus hippoglossus</i> <sup>a</sup>	Adult	10.10.2010	R	EtOH	F	70°34'N, 25°26'E
W4	<i>Hippoglossus hippoglossus</i> <sup>a</sup>	Adult	10.10.2010	R	EtOH	F	71°10'N, 25°53'E
W5	<i>Phoxinus phoxinus</i> <sup>b</sup>	Juvenile	25.07.2010	R	EtOH	Op	60°52'N, 09°42'E
W6	<i>Pollachius pollachius</i> <sup>a</sup>	Juvenile	06.10.2010	R	EtOH	H	60°16'N, 05°13'E
W7	<i>Salmo salar</i> <sup>b</sup>	Adult	08.12.2009	R	frozen	H	60°38'N, 06°09'E
W8	<i>Salmo salar</i> <sup>b</sup>	Adult	09.12.2009	R	frozen	MR	62°38'N, 08°07'E
W9	<i>Salmo salar</i> <sup>b</sup>	Adult	08.07.2011	R	EtOH	R	59°09'N, 06°10'E
W10	<i>Salmo trutta</i> <sup>b</sup>	Adult	03.07.2008	R	EtOH	He	61°51'N, 10°34'E
W11	<i>Gobius niger</i> <sup>a</sup>	Adult	02.07.2011	R	EtOH	H	60°15'N, 05°19'E
W12	<i>Pomatoschistus microps</i> <sup>c</sup>	Adult	05.07.2011	R	EtOH	H	60°13'N, 05°23'E

<sup>a</sup> Seawater.

<sup>b</sup> Freshwater.

<sup>c</sup> Brackish water.

**2.9. Data analyses**

The standard curves of the 10-fold serial dilutions were made as scatter plots between C<sub>T</sub> 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<sup>2</sup>) was >0.99. Formulae used in reproducibility tests and for quantification are shown in Table 3.

**3. Result**

**3.1. Reproducibility**

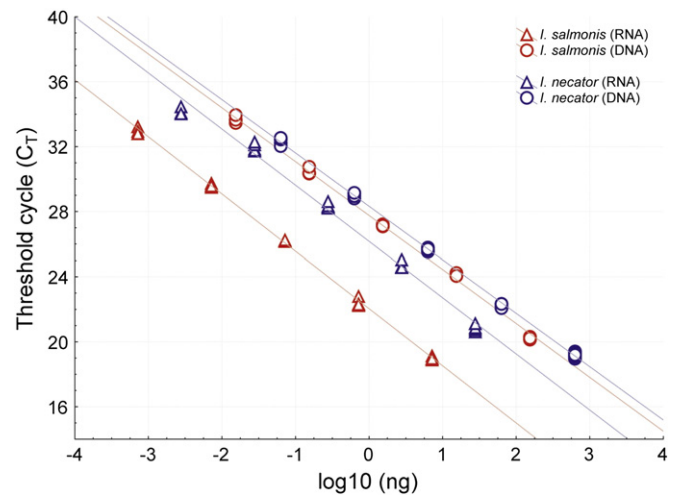
To determine the reproducibility of the Costia-assay, serial dilutions of nucleic acid extractions of salmonid gills containing different

**Table 6**

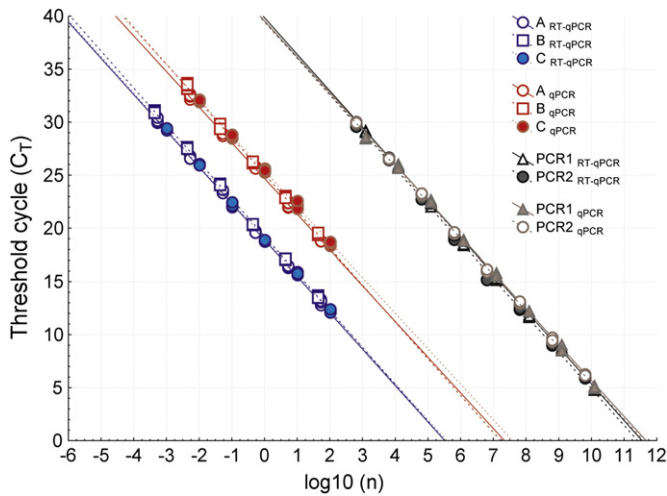
Efficiency test of *Ichthyobodo* specific real-time PCR assay. Linear regression analyses of standard curves from 10-fold serial dilutions of DNA and RNA extracted from *Ichthyobodo* infected gills from Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Concentration (C; ng μL<sup>-1</sup>) 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<sub>T</sub> values as a function of log<sub>10</sub> (ng) in the RT-qPCR amplification are used for calculation of efficiency (E%) and coefficient of determination (R<sup>2</sup>).

Host	Species	Template	C	Abs	Regression line	E%	R <sup>2</sup>
Salmon	<i>I. salmonis</i>	DNA	77	1.9	C <sub>T</sub> = 27.76 – 3.32x	100	0.999
Salmon	<i>I. necator</i>	DNA	316	1.9	C <sub>T</sub> = 28.33 – 3.29x	102	0.999
Salmon	<i>I. salmonis</i>	RNA	36	1.8	C <sub>T</sub> = 22.04 – 3.52x	92	0.998
Rainbow trout	<i>I. necator</i>	RNA	139	2.2	C <sub>T</sub> = 26.17 – 3.46x	95	0.991

*Ichthyobodo* species were used to generate standard curves analyzed by linear regression. The tests show acceptable values in coefficient of determination (R<sup>2</sup>) 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.



**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  $\mu$ L) 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  $1.25 \times 10^{10}$  and  $6.45 \times 10^9$  target copies per template volume (2  $\mu$ L) 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  $\leq 36$ . 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

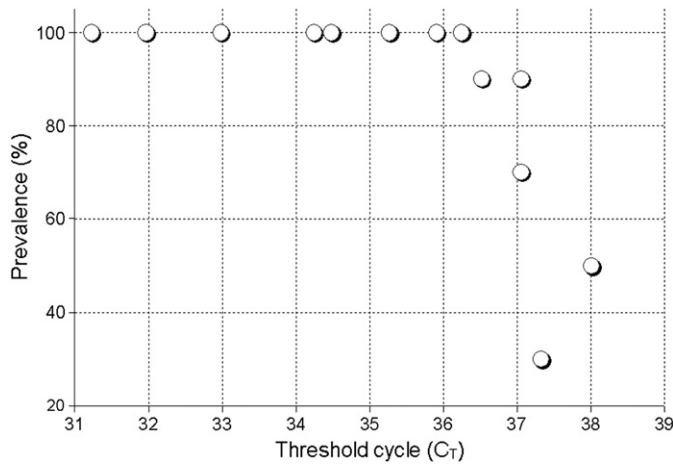
The Costia-assay was tested on samples from different hosts using RT-qPCR settings (Tables 5 and 8). Positive RT-qPCR analyses showed a range in  $C_T$  values from 9 to 28, from 9 different host species. Samples with  $C_T > 23$  required nested- or semi-nested PCR to obtain sequences for identification. 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  $\mu$ L.  $C_T$  values (mean;  $n$  replicates) for 2  $\mu$ L template in the amplification tests are given. Nucleic acid concentrations ( $C$ ;  $\text{ng } \mu\text{L}^{-1}$ ) 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/ $\mu$ L) 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  $\mu$ L 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	<i>G. morhua</i> <sup>a</sup>	Skin	225	11	443,423	<i>Ichthyobodo</i> sp. XI	JQ613335
F2	Atlantic cod	<i>G. morhua</i> <sup>a</sup>	Gill	386	17	4736	<i>Ichthyobodo</i> sp. IV	JQ613336
F3	Atlantic cod	<i>G. morhua</i> <sup>a</sup>	Gill	148	17	12,353	<i>Ichthyobodo</i> sp. IV	JQ613337
F4	Atlantic cod	<i>G. morhua</i> <sup>a</sup>	Gill	378	18	2483	<i>Ichthyobodo</i> sp. IV	JQ613338
F5	Atlantic cod	<i>G. morhua</i> <sup>a</sup>	Gill	85	13	309,448	<i>Ichthyobodo</i> sp. IV	JQ613339
F5	Atlantic cod	<i>G. morhua</i> <sup>a</sup>	Skin	261	17	7005	<i>Ichthyobodo</i> sp. IV	JQ613340
F6	Atlantic salmon	<i>S. salar</i> <sup>a</sup>	Gill	28	13	939,396	<i>I. salmonis</i>	JQ613341
F7	Atlantic salmon	<i>S. salar</i> <sup>b</sup>	Skin	298	17	6135	<i>I. salmonis</i>	JQ613342
							<i>I. necator</i> (co-infection)	JQ613343
F8	Atlantic salmon	<i>S. salar</i> <sup>a</sup>	Gill	176	18	5334	<i>I. salmonis</i>	JQ613344
F9	Atlantic salmon	<i>S. salar</i> <sup>b</sup>	Skin	100	19	4820	<i>I. salmonis</i>	JQ613345
F10	Atlantic salmon	<i>S. salar</i> <sup>b</sup>	Gill	923	16	3858	<i>I. necator</i>	JQ613346
F11	Brown trout	<i>S. trutta</i> <sup>b</sup>	Skin	148	9	2,557,026	<i>I. necator</i>	JQ613347
F12	Rainbow trout	<i>O. mykiss</i> <sup>b</sup>	Gill	176	11	566,876	<i>I. necator</i>	DQ414519
W1	Stickleback	<i>G. aculeatus</i> <sup>c</sup>	Gill	225	21	565	<i>Ichthyobodo</i> sp. XII	JQ613348
W2	Atlantic cod	<i>G. morhua</i> <sup>a</sup>	Gill	309	13	85,123	<i>Ichthyobodo</i> sp. IV	JQ613349
W3	Atlantic halibut	<i>H. hippoglossus</i> <sup>a</sup>	Gill	262	24	66	<i>I. hippoglossi</i>	JQ613350
W4	Atlantic halibut	<i>H. hippoglossus</i> <sup>a</sup>	Gill	367	25	24	<i>I. hippoglossi</i>	JQ613351
W5	Eurasian minnow	<i>P. phoxinus</i> <sup>b</sup>	Gill	320	22	204	<i>Ichthyobodo</i> sp. XIII	JQ613352
W6	Pollack	<i>P. pollachius</i> <sup>a</sup>	Gill	135	23	248	<i>Ichthyobodo</i> sp. IV	JQ613353
W7	Atlantic salmon	<i>S. salar</i> <sup>b</sup>	Gill	296	21	429	<i>I. salmonis</i>	JQ613354
							<i>I. necator</i> (co-infection) <sup>d</sup>	
W8	Atlantic salmon	<i>S. salar</i> <sup>b</sup>	Gill	141	20	1755	<i>I. salmonis</i>	JQ613355
							<i>I. necator</i> (co-infection) <sup>d</sup>	
W9	Atlantic salmon	<i>S. salar</i> <sup>b</sup>	Gill	309	26	15	<i>I. necator</i>	JQ613356
W10	Brown trout	<i>S. trutta</i> <sup>b</sup>	Skin	329	23	102	<i>I. necator</i>	JQ613357
W11	Black goby	<i>G. niger</i> <sup>a</sup>	Gill	212	26	21	<i>Ichthyobodo</i> sp. XIV	JQ613358
W12	Common goby	<i>P. microps</i> <sup>c</sup>	Gill	98	28	12	<i>Ichthyobodo</i> sp. XII	JQ613359

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



tissue from which nucleic acids were extracted (100  $\mu$ L). 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 (*Ichthyobodo* sp. XI). Other new sequences (species) found, *Ichthyobodo* sp. XII (sticklebacks and common goby, brackish water) and *Ichthyobodo* 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.

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