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Presence of selected pathogens on the gills of five wrasse species in western Norway

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ABSTRACT: The objective of this study was to identify gill pathogens in Labridae (wrasse) species used as cleaner fish to control salmon louse in western Norwegian aquaculture. Wrasse are often moved over long distances, raising issues of fish health, welfare and pathogen transmission. Histological examination and real-time RT-PCR analysis of the gills from *Centrolabrus exoletus*, *Ctenolabrus rupestris*, *Labrus bergylta*, *L. mixtus* and *Symphodus melops* revealed several pathogens: a new species of *Ichthyobodo*, *Paramoeba perurans*, microsporidia, trichodinids, *Hatschekia* spp., *Candidatus* Similichlamydia labri and 2 putative new species of *Chlamydiae*. *Cand.* S. labri or closely related bacteria were present on most wrasse specimens. Epitheliocysts on the gills of *L. mixtus* contained large inclusions (120 µm) with actiniae radiating from the inclusion membrane. A possible member of the *Candidatus* family Parilichlamydiaceae was present at a high prevalence on the gills of *L. mixtus, L. bergylta* and *C. rupestris*. Sequencing the 16S rRNA gene showed 93.9% similarity to *Cand.* S. labri and 96.8% similarity to *Cand.* Parilichlamydia carangidicola from the gills of *Seriola lalandi*. This bacterium probably represents a new species within the order *Chlamydiales,* family *Cand.* Parilichlamydiaceae. The other *Chlamydiae* detected on gills of *S. melops* could represent a new species in *Cand.* genus Syngnamydia. *Ichthyobodo* sp. and *Paranucleospora theridion* were detected on the gills of nearly all individuals, while *Paramoeba* spp. were detected on the gills of *L. bergylta* and *L. mixtus*. Trichodinids, microsporidia and parasitic copepods had low prevalence. Viral haemorrhagic septicaemia virus was not detected.

KEY WORDS: Labridae · Cleaner fish · Biocontrol · Salmon louse · *Ichthyobodo* · *Paramoeba* · *Hatschekia* spp. · *Candidatus* Similichlamydia labri · Microsporidia · Trichodinids · Fish welfare

INTRODUCTION

The salmon louse *Lepeophtheirus salmonis*, which feeds on mucus, skin and blood of its host, is a serious problem to farming of Atlantic salmon *Salmo salar* in Norway (Heuch et al. 2005). The reproduction of *L. salmonis* in salmon farms and the spread of larvae into the surrounding sea are also recognized as a threat to wild salmonids, namely *S. salar* and *Salmo trutta*, along the Norwegian coast (Krkošek et al. 2013). Several strategies, including the use of cleaner fish, are being employed to reduce the consequences of *L. salmonis* (Torrissen et al. 2013). Salmon farmers in western Norway are mainly using 4 wrasse species (Labridae): rock cook *Centrolabrus exoletus*, goldsinny *Ctenolabrus rupestris*, ballan wrasse *Labrus bergylta* and corkwing wrasse *Symphodus melops*. Collection of wild wrasse species and culturing of *L. bergylta* for their use as cleaner fish, and movement of these species along the coast of Norway, raises questions about the health of wrasses in the wild and the possible transmission of pathogens between local wrasse populations. Cultured wrasse species may carry pathogens such as *Paramoeba perurans* and viral haemorrhagic septicaemia virus (VHSV); hence, movement of these wrasse species may represent a risk of introducing these pathogens into salmon cages (Karlsbakk et al. 2013, Munro et al. 2015,

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Wallace et al. 2015). Other gill pathogens that may occur on the gills of wrasse species include parasitic copepods, monogeneans, trichodinids, flagellates, microsporidians and bacteria (Karlsbakk et al. 1996, Askeland 2002, Treasurer 2012).

Recently, 2 new *Chlamydiae* species, *Candidatus* Similichlamydia labri (family *Cand*. Actinochlamydiaceae) and *Cand*. Syngnamydia salmonis (family *Simkaniaceae*), associated with epitheliocystis, were described from the gills of *L. bergylta* and *S. salar*, respectively, expanding the number of species and geographical distribution of bacteria within the *Chlamydiales* (Nylund et al. 2015, Steigen et al. 2015). Epitheliocystis has been seen in a high number of fish species; in most cases, these show a chlamydia-like intracellular development and rRNA gene sequences, suggesting members of the order *Chlamydiales* (Paperna & Sabnai 1980, Bradley et al. 1988, Crespo et al. 1990, Lewis et al. 1992, Nylund et al. 1998, Draghi et al. 2004, Nowak & LaPatra 2006, Horn 2008, Karlsen et al. 2008, Polkinghorne et al. 2010, Corsaro & Work 2012, Schmidt-Posthaus et al. 2012, Camus et al. 2013, Fehr et al. 2013, Steigen et al. 2013, 2015, Stride et al. 2013a,b,c, Nylund et al. 2015). *Beta*- and *Gammaproteobacteria* have also been connected to epitheliocystis in farmed fish (Toenshoff et al. 2012, Mendoza et al. 2013, Katharios et al. 2015, Contador et al. 2016, Seth-Smith et al. 2016), expanding the diversity of epitheliocystis agents.

To gain more knowledge on naturally occurring gill pathogens in different wrasse species, a study of selected pathogens was started in 2011. This study shows that *Chlamydiae* (several genetically distinct strains) and a putative new species of *Ichthyobodo* are relatively common on the gills of most wrasse species in western Norway. The morphology of epitheliocysts, the inclusions and the bacteria from *L. mixtus* are described and compared to *Candidatus* S. labri from *L. bergylta* (Steigen et al. 2015). The prevalence and occurrence of other pathogens such as *Paramoeba* spp., trichodinids, microsporidians and VHSV is also presented, and their potential importance for wild wrasse and farmed Atlantic salmon is discussed.

MATERIALS AND METHODS

Fish

Five different species of wrasse (*Centrolabrus exoletus*, *Ctenolabrus rupestris*, *Labrus bergylta*, *L. mixtus* and *Symphodus melops*) were collected during May and June 2012 in Raunefjorden close to Bergen, western Norway (Table 1). The fish were mature or had just spawned. A second sampling period was in October 2012 and a third was in May 2013. A small sample of *L. mixtus* was also collected in September 2014. The sampling was done using fyke nets, and live individuals were brought to the laboratory at the University of Bergen. All fish were treated according to the Norwegian Animal Welfare Act (01.01.2010), and all samples were taken from newly killed fish (Table 1). Tissues were fixed and preserved in Karnovsky fixative at −4°C, or stored for a very short period at −20°C before extraction of RNA or DNA which was stored at −80°C. Samples for real-time reverse transcription (RT)-PCR and sequencing were taken from the first gill arch, while the second and third arches were used for histology.

The wrasse species were identified based on morphology, and the identity of a selection of these was confirmed by sequencing of the cytochrome oxidase subunit I gene (*cox1*, 448 nt) using the primers (bar coding) Cox-F1 (TAA AGA YAT TGG CAC CCT YWA TC) and Cox-R1 (GGA GGT TTC ATG TTA RYR ATG G). The primers were designed to match conserved regions using an alignment of all available Cox I sequences from wrasse.

Inspection of gills for macroscopic and microscopic parasites

The left side of the fish and the gill arches on the left side of all specimens were checked for presence of parasitic copepods (*Caligus centrodonti, C. elongatus* and *Hatschekia* sp.) and gross pathology. This was performed as a visual recording of the presence of these parasites on the skin and gills *in situ*. The parasites were identified using Kabata (1992). The numbers of these macroparasites on each individual and the prevalence among the fish sampled were not recorded. Microparasites were detected by real-time RT-PCR and histological examinations.

Histology

Gill tissues were fixed in Karnovsky fixative. The gill tissues from 15 specimens (3 ind. species^{-1}) were processed as described by Steigen et al. (2013) and were used for histological studies and transmission electron microscopy.

Table 1. Overview of material collected in 2012–2014: wrasse species, collection month, number (N), weight, length and the number of fish positive for the following: 2 different species of *Chlamydiae* (*Candidatus* Similichlamydia labri and Clade A), *Ichthyobodo* sp., and *Paranucleospora theridion*. All fish were also tested for viral haemorrhagic septicaemia virus, but none returned positive results

Species	Month	N	Weight (g)	Length (cm)	*Cand.* S. labri	Clade A	*Ich.* sp.	*P. theridion*
Centrolabrus exoletus	May 2012	8	21.4	12.1	8	0	7	8
	Jun 2012	13	24.9	11.8	13	0	13	12
	Jun 2013	4	22.5	11.8	4	0	4	4
	Total	25			25	0	23	24
Ctenolabrus rupestris	May 2012	4	19.5	11.0	4	4	4	4
	Jun 2012	12	15.4	11.0	12	12	12	9
	May 2013	25	13.9	10.5	25	25	23	23
	Total	41			41	41	39	33
Labrus bergylta	Jun 2012	44	349.1	27.7	44	39	39	39
	Oct 2012	4	33.7	31.0	4	4	4	4
	May 2013	15	205.0	23.9	15	15	15	2
	Total	63			63	58	58	45
L. mixtus	May 2012	6	69.3	17.8	6	6	6	6
	Jun 2012	6	82.3	17.8	6	6	6	6
	Oct 2012	7	136.7	22.4	7	7	7	7
	May 2013	3	56.0	17.7	3	3	3	2
	Sep 2014	7	156.7	21.6	7	7	6	7
	Total	29			29	29	28	28
Symphodus melops	May 2012	39	41.3	13.3	37	7	34	32
	Jun 2012	4	38.5	13,9	4	0	4	4
	Oct 2012	16	52.8	15.1	16	0	14	15
	May 2013	11	49.1	15.0	11	2	11	7
	Total	70			68	9	63	58

PCR/real-time PCR

RNA and DNA were extracted from individual gill samples (first gill arch) of all specimens from each species sampled (see Steigen et al. 2013), and RNA and DNA were stored at −80°C. The RNA used for real-time RT-PCR was quantified by Nanodrop, and the amounts used were always as recommended (around 600 ng) by the producer of the AgPath-ID™ One-Step kit (ThermoFisher Scientific). Real-time RT-PCR was used for the detection of epitheliocystis agents belonging to the *Cand.* family Actinochlamydiaceae (Steigen et al. 2013), *Ichthyobodo* spp. (Isaksen et al. 2012), *Paramoeba* spp. (Table 2), VHSV (Duesund et al. 2010) and *Paranucleospora theridion* (syn.: *Desmozoon lepeophtheirii*) (Nylund et al. 2011). The assay for VHSV detects North Atlantic genotypes I, II and III.

The real-time assays published for the first time in this study were optimized as

Table 2. Taqman real-time RT-PCR assays used during the current study: Pspp (target: small subunit, SSU) for detection of *Paramoeba* spp; ChV (target: SSU) for detection of *Candidatus* Similichlamydia labri; ChW (target: SSU) for detection of a putative new species of *Chlamydiae* (family *Candidatus* Parilichlamydiaceae) present on the gills of wrasse. Elongation factor of the wrasse species (ElaUni; target: elongation factor) acts as an internal control for the quality of RNA extracted. The primers ElaUni-F and ElaUni-R can be used for all wrasse species except *Symphodus melops* (primers: ElaUni-Fsm and ElaUni-Rsm)

Primer/ probe	Sequence (5′–3′)	Assay efficiency
Pspp-F	TTG TCA GAG GTG AAA TTC TTG GAT T	
Pspp-probe	ATG AAA GAC GAA CTT CTG	1.97
Pspp-R	TGA AAA CAT CTT TGG CAA ATG C	
ChV-F	GGG ACY CCG AGA GGR ACC TT	
ChV-probe	TRY GAG CGG CCT GTG	1.96
ChV-R	ARG CCA TTA CCY TAC CAA CAA GCT	
ChW-F	GGT GCT AAT ACC GAA TGT TGT TGA	
ChW-probe	ATT TTC GAT GCC GAA	1.87
ChW-R	GGA GTC CCC CGC TTT CAT	
ElaUni-F	CCC CTC CAG GAT GTC TAC AAA	
ElaUni-Fsm	CCC CTG CAG GAY GTC TAC AAA	
ElaUni-probe	ATY GGY GGT ATT GGA AC	1.87
ElaUni-Rsm	MAC ACG ACC GAC GGG TAC W	
ElaUni-R	AAC ACG RCC SAC GGG KAC W	

recommend for Taqman assays using different concentrations of primers and probes. Amplification efficiency ($E = [10^{1/(-slope)}] - 1$) was calculated for all new assays using a dilution series of RNA obtained from positive homogenate (Müller et al. 2002). Each dilution was tested in triplicate, and the average cycle threshold (Ct) values were used to generate the standard curves. During real-time RT-PCR screening, the wrasse host house-keeping gene (elongation factor [ELF] assays; Table 2) was used as an internal control for the quality of the RNA extraction. New extractions of RNA from back-up tissues, followed by new real-time RT-PCR screening were carried out if initial results gave a Ct value >20 for ELF. It was not possible to use normalized expression to compare pathogen loads between the different wrasse species due to differences in the expression of ELF in gills of the different host species. Hence, we used Ct values (target: small subunit, SSU) as a relative measure of the amount of the wrasse *Chlamydiae* species. Non-template real-time PCR controls and non-template RNA-extraction controls for all assays were included in each real-time run.

DNA was used for sequencing of signature sequences from *Ichthyobodo* sp., *Paranucleospora theridion* and *Paramoeba perurans*. Sequencing of the partial SSU RNA gene of *Ichthyobodo* spp., *Paramoeba* spp. and *P. theridion* present on the gills of the wrasse species was performed from a selection of samples positive by real-time PCR, using primers published by Isaksen et al. (2012) and Nylund et al. (2008, 2010). The partial rRNA genes and the internal transcribed spacer (ITS) region of possible new *Chlamydiae* species were amplified by PCR and sequenced using degenerate primers targeting these regions in members of *Chlamydiales* and *Cand.* families Actinochlamydiaceae and Parilichlamydiaceae (Table 3). Amplified PCR products were sequenced from a selection of wrasse species, positive by RT-PCR. Two putative new species of *Chlamydiae* were detected, and the sequences were deposited in GenBank. A real-time RT PCR assay (ChW) (Table 2) was developed based on the 16S rRNA gene sequence of 1 of the putative new species. This assay did not amplify *Cand.* S. labri and gave a positive result only when the putative new *Chlamydiae* species was present (Table 1).

Table 3. Primers used for PCR and sequencing of the 16S, ITS and 23S rDNA (partial) from *Chlamydiae* obtained from the gills of 5 different wrasse species. Primers 16sigF and 806R are general *Chlamydiae* primers; ChPL-F1, ChPL-R2 and ChPL-R3 target *Cand.* Similichlamydia labri; ChSL-F1, ChSL-R2 and ChSL-R3 target Clade A *Chlamydiae*; and Ch-ITS-F1 targets *Cand.* Actinochlamydia

Primer	Sequence (5′–′3)	Target	Publication
16sigF	CGG CGT GGA TGA GGC AT	16S	Draghi et al. (2004)
806R	GGA CTA CCA GGG TAT CTA AT	16S	Draghi et al. (2004)
ChPL-F1	GGT GCG TAG GCG GCT GAG	16S	This study
ChSL-F1	GGT GCG TAG GCG GCC ATA C	16S	This study
Ch-ITS-F1	GGA ATT GCT AGT AAT GGC G	16S/ITS	Steigen et al. (2015)
ChPL-R2	TGA TCT TAT TTT ACG GAT CAG	ITS	This study
ChSL-R2	TCT TTT CAT CTT TAA AAG AGT C	ITS	This study
ChPL-R3	AGG ATA CCA ACT TGT GCT TTC	23S	This study
ChSL-R3	GAT ACC AAC TAA GAA AAC CC	23S	This study

Phylogeny of *Chlamydiae* species identified from wrasse

Preliminary species identification using sequence data from *Chlamydiae*-positive samples was performed by searching the public GenBank database for homologous sequences using BLAST (2.0). The Vector NTI Suite software package was used to generate multiple alignments with related sequences. Selected sequences from all existing families of the order *Chlamydiales*, available on the EMBL nucleotide database, were also included in pairwise sequence comparisons (from *Oreochromis niloticus, Serranus scriba* and *Cyclopterus lumpus*). The multiple sequence alignment editor GeneDoc (available at http://genedoc.software.informer.com) was used for manual adjustment of the sequence alignments (gaps in the alignments were removed). The best-fit nucleotide substitution model for the dataset was GTR+I+G identified by Modeltest 3.6 (Posada & Crandall 1998), and this was implemented in the analysis. The maximum likelihood tree was generated and bootstrapped (50 000 puzzling steps) in TREE_PUZZLE 5.2 (current version, available at www.tree-puzzle.de). Phylogenetic trees were drawn using TreeView (Page 1996).

RESULTS

All wrasse specimens collected were taken alive to the laboratory. The fish looked healthy except for slight scratches on fins and skin related to netting. The identification of the wrasse species was based on morphology and was confirmed by se-

quencing of the mitochondrial *cox1* gene (448 nt from 46 individual sequences). We did not find any within-species variation in *cox1* gene sequences used for the bar coding, confirming our morphological determination of the species. A phylogenetic tree showing relationships between the wrasse species is shown in Fig. S1 of the Supplement at www.int-res.com/articles/suppl/d128p021_supp.pdf.

Real-time PCR assays

Ct values obtained for the ELF were relatively stable within the gills of the individual wrasse species, but were different between species. The mean Ct values obtained from the gills of *Labrus mixtus, L. bergylta, Ctenolabrus rupestris, Centrolabrus exoletus* and *Symphodus melops* were 14.0, 15.3, 19.0, 19.1 and 17.0, respectively.

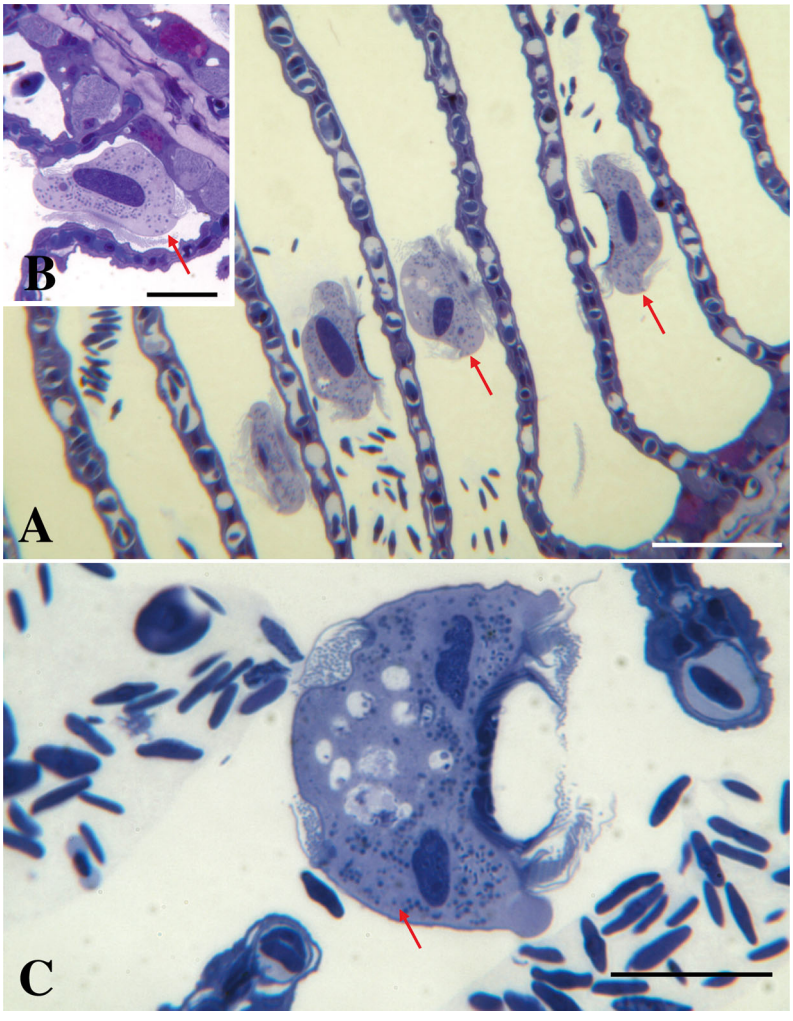

Fig. 1. (A) Presence of trichodinids (arrows) on the gills of *Ctenolabrus rupestris*. Scale bar = 100 µm. (B) Magnification of the trichodinid (arrow). Scale bar = 20 µm. (C) *Trichodina* sp. (arrow) on the gills of *Symphodus melops*. Scale bar = 20 µm

Parasites and VHSV

Crustacean parasites were observed both on the skin (*Caligus centrodonti*) and gills (*Hatschekia* sp.) of *L. bergylta* only. Gill pathology (hyperplasia and necrosis) was observed at the attachment sites for the latter.

Trichodinids were relatively abundant on histological sections of gills from *Ctenolabrus rupestris, L. mixtus* and *S. melops,* but no pathology was associated with their presence (Fig. 1).

Ichthyobodo spp. showed close to 100% prevalence (real-time RT-PCR screening) on all 5 wrasse species (Table 1). Partial 18S rDNA sequences of *Ichthyobodo* sp. (accession nos. KF179521–KF179526), obtained from the gills of *L. mixtus* (n = 2), *L. bergylta* (n = 3) and *S. melops* (n = 1), could represent a putative new species within this genus. The partial 18S sequences (1745 nt) of the *Ichthyobodo* sp. from these wrasse showed 96.6, 97.0 and 95.0% identity to *I. salmonis* (Norway), *Ichthyobodo* sp. from *Oncorhynchus masou* (Japan) and *Ichthyobodo* sp. from *Gadus morhua* (Norway), respectively. The SSU gene from *Ichthyobodo* spp. from the gills of *C. exoletus* and *C. rupestris* was not sequenced. The density of these *Ichthyobodo* spp., based on real-time RT-PCR data, was low, and no gill pathology connected to this parasite was observed.

Light microscopy detected the presence of *Paramoeba* spp. on the gills of some specimens (Fig. 2A,B). This was confirmed by real-time RT-PCR on the gills of 17 individuals of 47 tested *L. bergylta* and on 5 of 22 tested *L. mixtus*. The other 3 wrasse species (*C. rupestris* [n = 25], *C. exoletus* [n = 16] and *S. melops* [n = 31]) were all negative for the presence of *Paramoeba* spp.; *P. perurans* was identified by sequencing (accession no. KF179520) on the gill of *L. bergylta*

only. No gill pathology was observed in the tissue sections from the gills of *L. bergylta* or *L. mixtus* despite the presence of *Paramoeba* spp.

The microsporidian *Paranucleospora theridion* (syn.: *Desmozoon lepeophtheirii*) was detected in the gills of nearly all host individuals, with the lowest prevalence (71.4%) on the gills of *L. bergylta.* The SSU (rRNA) of *P. theridion* was sequenced from parasites in the gills of *C. exoletus* (accession no. KR187183) and *L. mixtus* (KR187184, KR187185; Table 1). The densities of this parasite were low on the gills of all wrasse species, based on the obtained real-time RT-PCR values (Ct > 21) (Nylund et al. 2011). Spores of the parasites could not be observed in the tissue sections of the gills (n = 3 fish species^{-1}).

Histological examination of gills from a selection of the collected wrasse species (n = 3 fish species^{-1}) showed that a single individual of *L. bergylta* was positive for the presence of a xenoma-forming microsporidian (Fig. 2C,D). Hyperplasia was seen in areas with xenoma.

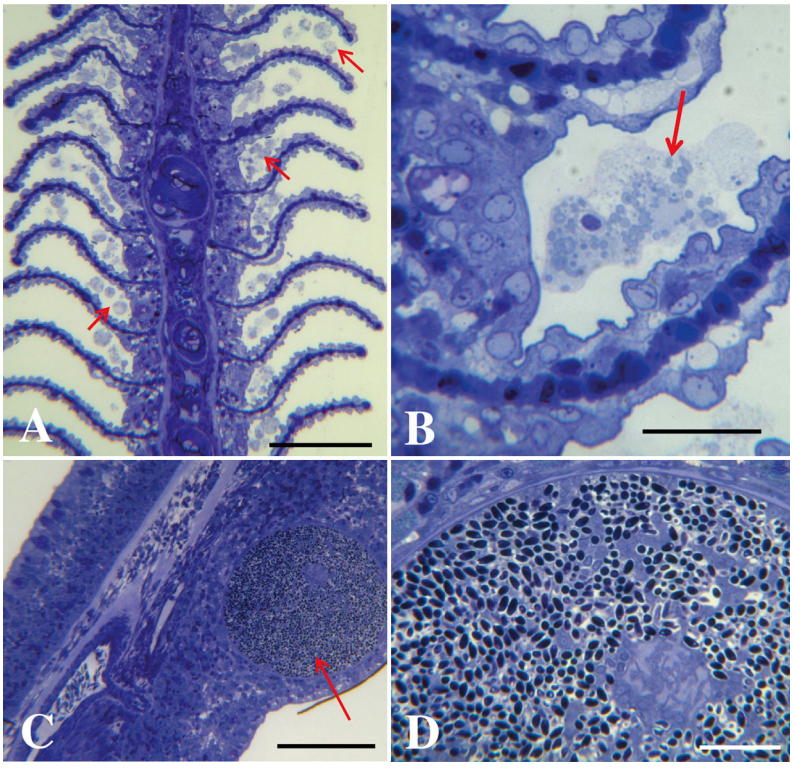

Fig. 2. (A) *Paramoeba perurans* (arrows) on the gills of *Labrus bergylta*. Scale bar = 100 µm. (B) Magnification of *P. perurans* (arrow). Scale bar = 20 µm. (C) Xenoma-forming microsporidian (arrow) on the gills of *L. bergylta*. Scale bar = 100 µm. (D) Magnification of the xenoma showing presence of microspores. Scale bar = 20 µm

All wrasse species were tested individually (real-time RT-PCR) for presence of VHSV and were found to be negative (Table 1).

Gill *Chlamydiae*

Screening of gill tissues (real-time RT-PCR) from all 5 wrasse species with respect to presence of members of the *Cand.* family Actinochlamydiaceae showed close to 100% prevalence in all species (Table 1). Only 2 specimens of *S. melops*, collected on 23 May 2012, were negative for this group of bacteria (i.e. prevalence = 97.1%). The 2 *Labrus* species carried the highest amount of *Actinochlamydiaceae* based on comparative real-time RT-PCR results (and standardised amount of RNA analysed per sample; Fig. 3). One group of *Chlamydiae* 16S rRNA gene sequences (1464 nt) obtained from all 5 wrasse species showed >98.9% identity to *Cand.* Similichlamydia labri, 97.0% identity to *Cand.* S. latridicola, 97.5% identity to *Cand.* S. laticola and 95.0% identity to *Cand.* Actinochlamydia clariae (Table 4). The second group of wrasse *Chlamydiae* (referred to as Clade A), showed a high prevalence (92.1–100.0%) and load (Ct values < 20) on the gills of *L. bergylta, L. mixtus* and *C. rupestris*, a low prevalence on *S. melops* (12.9%) and were absent from the gill of *C. exoletus* (Table 1, Fig. 3). Members of Clade A showed 96.6 to 96.8% identity to *Cand.* Parilichlamydia carangidicola based on 1103 nt from the 16S rRNA gene. The 16S sequence identity of members of Clade A to the *Cand.* species A. clariae, S. labri and Piscichlamydia salmonis were 93.0, 93.9 and 86.7%, respectively (Table 4). One specimen of *S. melops* was also positive for a possible new member of the *Chlamydiales* family *Simkaniaceae* (accession no. KC608868; Fig. 4).

The partial 16S gene from members of *Cand.* S. labri or closely related to this species was sequenced from 26 of the wrasse specimens with 3 or more sequence isolates from each wrasse species, while 11 sequences of the 16S gene were obtained from wrasse carrying members of Clade A (see Figs. 4 & 5). These sequences were used in a phylogenetic analysis, which included members from each of the families in the order *Chlamydiales* (Fig. 4). The phy-

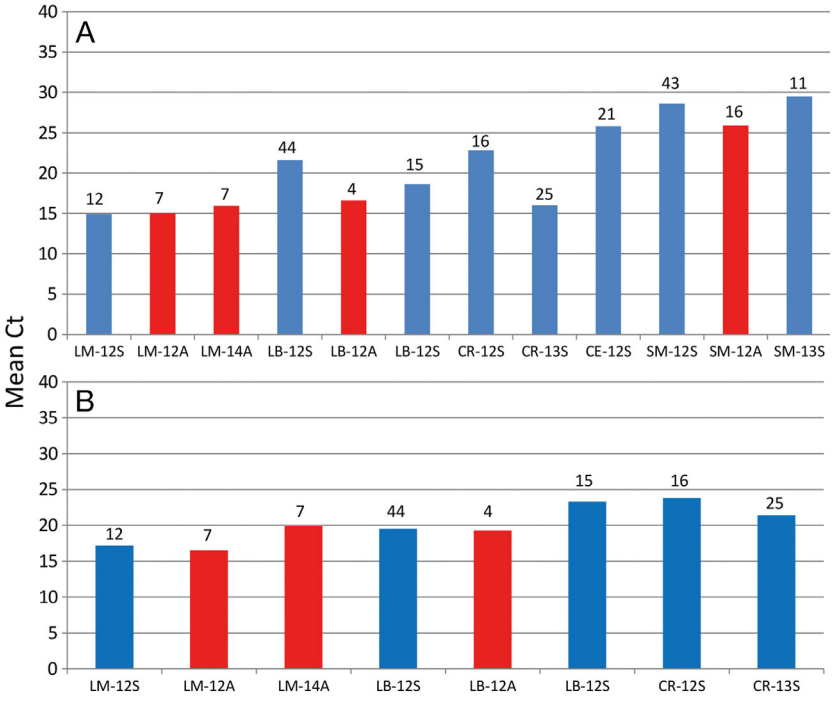

Fig. 3. Mean Ct values resulting from real-time RT-PCR analysis of gills from the wrasse species with respect to presence of *Chlamydiae* showing 16S rDNA sequence similarity to (A) *Candidatus* Similichlamydia labri and (B) Clade A *Chlamydiae*. LM: *Labrus mixtus*; LB: *L. bergylta*; CR: *Ctenolabrus rupestris*; CE: *Centrolabrus exoletus*; SM: *Symphodus melops*. Collection times were 12S: spring/summer 2012; 13S: spring/summer 2013; 12A: autumn 2012; 13A: autumn 2013; 14A: autumn 2014. The number at the top of each column represents number of fish. Blue columns: spring/summer catch; red columns: autumn catch

logeny showed that the majority of *Chlamydiae* 16S sequences isolated from the different wrasse species grouped into a distinct clade together with *Cand.* S. labri, while the other 16S rRNA gene sequences (n = 11) constituted a distinct clade with the closest affinity to *Cand.* P. carangidicola.

Six additional sequences obtained from the gills of *Oreochromis niloticus* (accession nos. JQ480302, JQ480303, KT158464), *Serranus scriba* (KF805948) and *Cyclopterus lumpus* (KF805949, KF805950) also belonged to this major branch in the phylogeny, which consisted of 2 *Cand.* families, Actinochlamydiaceae and Parilichlamydiaceae (Fig. 4). The *Cand.* S. labri-like sequences and the Clade A sequences grouped within the former and latter families, respectively. This branch in the order *Chlamydiales* contained 16S rRNA sequences from the gills of both fresh- and seawater fishes.

The ITS region (177 nucleotides) and the first 400 nt of the 23S rRNA gene were sequenced from the *Chlamydiae* present on the gills from all 5 wrasse species, from *C. lumpus, S. scriba, O. niloticus* and from *Cand.* A. clariae from African catfish. The partial 16S rRNA gene (1502 nt), the ITS (177 nt) and the partial 23S rRNA gene (396 nt) were used in a second phylogenetic analyses of the *Chlamydiae* from the wrasse species (Fig. 5). Using all of the above gene regions (2102 nt) did not change the relationship between the wrasse *Chlamydiae*, but gave a slightly better separation of the wrasse *Chlamydiae* and the *Chlamydiae* from other fish species (cf. Figs. 4 & 5).

Clade A was distinct for all sequences. To clarify this, alignments of *Cand.* S. labri and Clade A from the wrasse species are shown in Fig. S2 of the Supplement.

Inclusion morphology in *L. mixtus*

Epitheliocysts from *L. mixtus* were mostly located basally between the secondary gill lamellae. The largest size of the cysts measured was about 120 µm, and the shape of the cysts varied from round/oval to irregular (Fig. 6). A high number of actinae (more than 200 were counted on 1 section through an inclusion) radiated from the inclusion membrane. On semi sections, the inclusion filled nearly the whole host cell in large cysts, while the smaller inclusions were surrounded by a lighter stained area.

Studies of the ultrastructure of the larger epitheliocysts showed that the inclusion membrane was associated with a thick layer (ca. 35–100 nm thick, thicker near the base of the actinae) of electron-dense material (probably proteins) that was also present in the actinae (Figs. 7 & 8). Actinae extending from the inclusion membrane were highly abundant, very irregular in transverse sections and seemed to be branching. Abundant mitochondria and amorphous material were located between the actinae. In the smaller cysts, the actinae seemed to end before they reached the host cell surface. The volume from the end of the actinae to the cell membrane in these host cells was packed with endoplasmic reticulum and mitochondria. The endoplasmic reticulum seemed to contain material with the same electron density as that found in the actinae. Bundles of filaments (prob-

Table 4. Sequence similarity (%) of the partial *Chlamydiae* 16S rRNA gene obtained from gills of different fish species: Clade A from wrasse in the current study (1465 nt, accession no. KC469554), *Cand.* Similichlamydia labri (1464 nt, KC469556), *Cand.* S. latridicola (1396 nt, JQ687061) from *Latris lineata, Cand.* S. laticola (1402 nt, KF219613) from *Lates calcarifer, Cand.* Actinochlamydia clariae (1464 nt, JQ480300) from *Clarias gariepinus*, *Cand.* P. carangidicola (1103 nt, JQ673516) from *Seriola lalandi* and *Cand.* P. salmonis from *Salmo salar* (1470 nt, AY462244). *Chlamydiae* sequences obtained from the gills of *Oreochromis niloticus, Cyclopterus lumpus* and *Serranus scriba* are also included

Host/seq. ID	Clade A	*Cand.* S. labri	*Cand.* S. latridicola	*Cand.* S. laticola	*Cand.* A. clariae	*Cand.* P. carangidicola	*Cand.* P. salmonis
Labrus bergylta							
KC469561	100.0	93.9	93.7	93.7	93.0	96.8	86.7
KC469554	100.0	93.9	93.7	93.7	93.0	96.8	86.7
KC469555	100.0	93.9	93.7	93.7	93.0	96.8	86.7
KC469563	100.0	93.9	93.7	93.7	93.0	96.8	86.7
KC469559	100.0	93.9	93.7	93.7	93.0	96.8	86.7
KC469560	100.0	93.9	93.7	93.7	93.0	96.8	86.7
KT030895	99.9	93.9	93.8	93.2	92.9	96.8	86.8
KT030892	100.0	93.9	93.7	93.7	93.0	96.8	86.7
KT030894	93.2	99.3	97.2	97.4	94.6	93.1	86.8
L. mixtus							
KT030898	99.9	93.8	93.6	93.7	92.9	96.6	86.8
KT030896	100.0	93.6	93.7	93.8	92.9	96.8	86.8
KC469545	93.7	99.7	97.2	97.6	94.9	93.5	86.7
KC469565	93.7	99.7	97.2	97.5	94.9	93.5	86.7
KC469566	93.7	99.7	97.2	97.6	94.9	93.5	86.7
Centrolabrus exoletus							
KC469549	93.9	99.0	97.1	97.6	95.5	92.9	86.6
Ctenolabrus rupestris							
KT158463	99.5	93.6	93.3	93.5	92.7	96.6	86.5
KC469551	93.7	99.0	97.0	97.6	94.5	93.2	86.4
KC469552	93.9	98.9	97.0	97.4	94.5	93.0	86.4
Symphodus melops							
KC469568	94.0	99.1	97.3	97.6	94.7	93.1	86.6
Cyclopterus lumpus							
KF805949	94.2	97.7	97.6	97.5	95.2	93.8	86.8
Serranus scriba							
KF805948	94.8	96.3	96.4	96.4	94.3	93.8	86.5
Oreochromis niloticus							
KT158464[a]	93.0	95.1	95.1	94.6	94.6	92.6	86.7
JQ480303	93.2	95.3	95.5	95.1	94.8	93.0	86.4

[a]KT158464, obtained from the gill of *O. niloticus* collected in Lake Victoria, is identical to JQ480302 obtained from the gills of *O. niloticus* collected at a farm site in Namiyenge (Uganda)

ably actin) were also present in the host cell cytoplasm (Fig. 8C). Chloride cells, in contact with infected host cells, seemed to lose their cell membrane in the contact area, which suggested that they are incorporated as a part of (merged with) the infected cell. The chloride cells on the gill of *L. mixtus* contained high amounts of mitochondria.

The gill inclusions in *L. mixtus* were filled with highly polymorphic bacteria that varied from coccoid shapes to larger irregularly shaped morphs. The bacteria were surrounded by electron-dense amorphous material. Despite the variation in the morphology of the bacteria inside the inclusions, their cytoplasm seemed to be very similar, consisting of amorphous material with a slightly higher electron density towards the surrounding bacterial cell membrane. When containing the central part of the nucleoid, the smaller coccoid morphs measured 0.5–0.7 µm in mean diameter. They were all smaller than what has been termed reticulate body-like morphs in the cysts found in *Clarias gariepinus* (Steigen et al. 2013).

Fig. 4. Phylogenetic tree showing the relationships between the wrasse *Chlamydiae* and other members of *Chlamydiales* based on 16S rDNA only. *Cand.* Piscichlamydia salmonis is used as the outgroup. The analysis is based on 1103 nt from the 16S rRNA gene excluding parts of the loops. The scale bar shows the number of nucleotide substitutions as a proportion of branch length

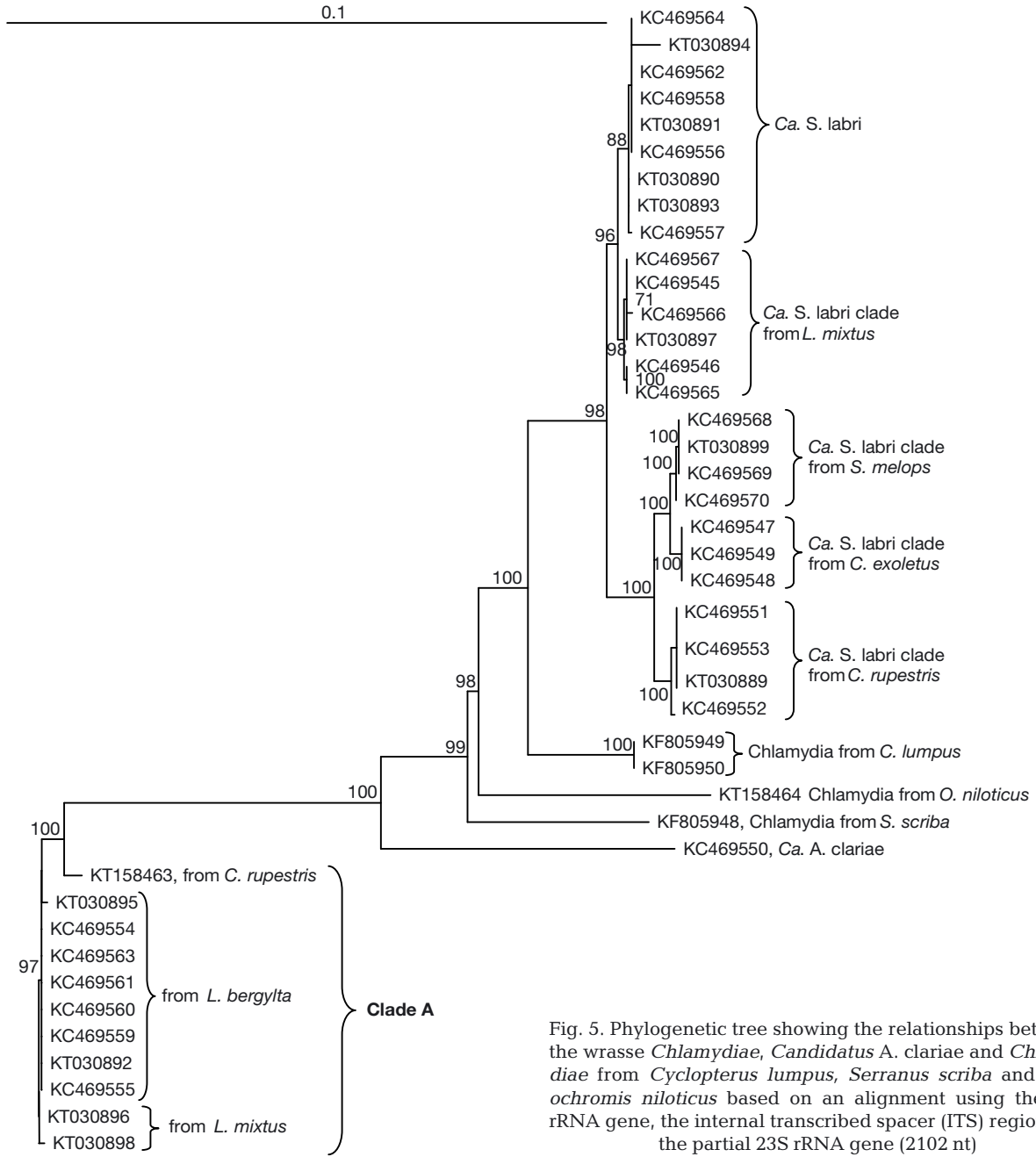

Fig. 5. Phylogenetic tree showing the relationships between the wrasse *Chlamydiae*, *Candidatus* A. clariae and *Chlamydiae* from *Cyclopterus lumpus, Serranus scriba* and *Oreochromis niloticus* based on an alignment using the 16S rRNA gene, the internal transcribed spacer (ITS) region and the partial 23S rRNA gene (2102 nt)

DISCUSSION

This study includes only a limited number of possible pathogens that could be present on the gills of wrasse in Norway. Trichodinids are not considered as parasites, but may constitute a problem in rearing tanks with high densities of wrasse (A. Nylund pers. obs.). Trichodinids occurred at high abundance on the gills of some individuals of the wild-caught wrasse used in this study, but no pathology was associated with the presence of these ciliates.

The prevalence of *Ichthyobodo* spp. was very high on the gills of all 5 wrasse species, but their importance as pathogens is not addressed in this study. *Ichthyobodo* spp. have been associated with serious diseases in other fish species (Isaksen 2013) and could be of importance in dense populations of wrasse in culture facilities and in salmon cages. However, in this study, no gill lesions were observed in connection with the presence of *Ichthyobodo* spp. on the wrasse gills. The *Ichthyobodo* sp. from 3 wrasse species, based on the rRNA gene, represents a puta-

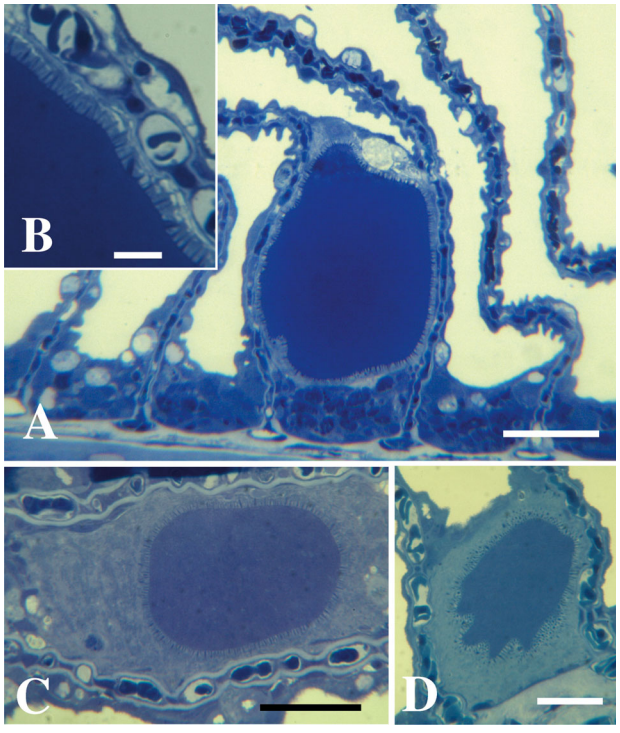

Fig. 6. Epitheliocystis on the gills of *Labrus mixtus*. (A) Large cyst located basal to the secondary lamellae. Actinia are radiating from the inclusion. No visible response can be seen in the surrounding tissue. Scale bar = 40.0 µm. (B) Magnification of the actinia radiating from the inclusion wall. Scale bar = 20 µm (C) Inclusion with a high number of radiating actiniae (about 200) surrounded by a large pale-staining area. Scale bar = 25.0 µm. (D) Asymmetric inclusion. Scale bar = 20 µm

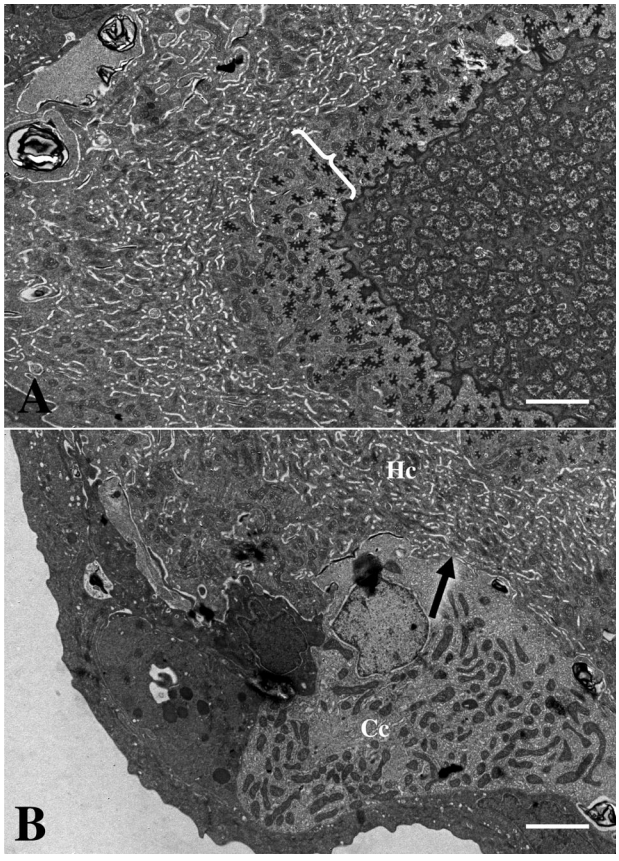

Fig. 7. Epitheliocysts in *Labrus mixtus*. (A) Inclusion containing pleomorphic bacteria. A high number of actinae extend from the inclusion membrane and into the host cell cytoplasm. The cytosol closest to the inclusion, between the actinae, contains mitochondria and amorphic material, while the cytosol peripheral to the inclusion contains high amounts of endoplasmic reticulum and mitochondria (bracket). Scale bar = 2.0 µm. (B) Chloride cell (Cc) containing a large amount of mitochondria, associated with a host cell (Hc) containing an inclusion. Part of the cell membrane is lost between the infected cell and the chloride cell (arrow). Scale bar = 2.0 µm

tive new and different species compared to the 2 species described from Atlantic salmon (Isaksen et al. 2010, 2011). Hence, use of wrasse as cleaner fish in salmon farms may not represent a risk of introduction and transmission of this parasite to the salmon population. Nothing is known about the prevalence of this putative new species of *Ichthyobodo* in other wild populations of wrasse along the Norwegian coast.

Paramoeba perurans has already been associated with mortalities in culture facilities of *Labrus bergylta* (Karlsbakk et al. 2013) and is also recognized as a serious pathogen in salmon farms worldwide (Young et al. 2008, Crosbie et al. 2012). However, we did not observe any pathology on the gills of the 2 *Labrus* spp. from which the SSU rDNA sequence of *P. perurans* was obtained. Experimental studies will have to be performed to determine if wrasse can be important for the introduction to and transmission of *P. perurans* in salmon farms. The geographical distribution of *P. perurans* in Norwegian wrasse populations is not known.

The xenoma-forming microsporidian is possibly a *Loma* species (cf. Askeland 2002), and other members of this genus are associated with disease and mortality (Kent et al. 1989, Brown et al. 2010); however, there are no reports of diseases in wrasse associated with this parasite in Norway.

The abundance of the copepod parasite *Hatschekia* sp. was relatively low on the examined individuals, and the only changes in the infected hosts were at the attachment sites of the parasite.

VHSV was not detected in any of the wrasse species even though this virus has been detected in other wild fish species along the Norwegian coast and in wrasse in salmon farms in Shetland, Scotland

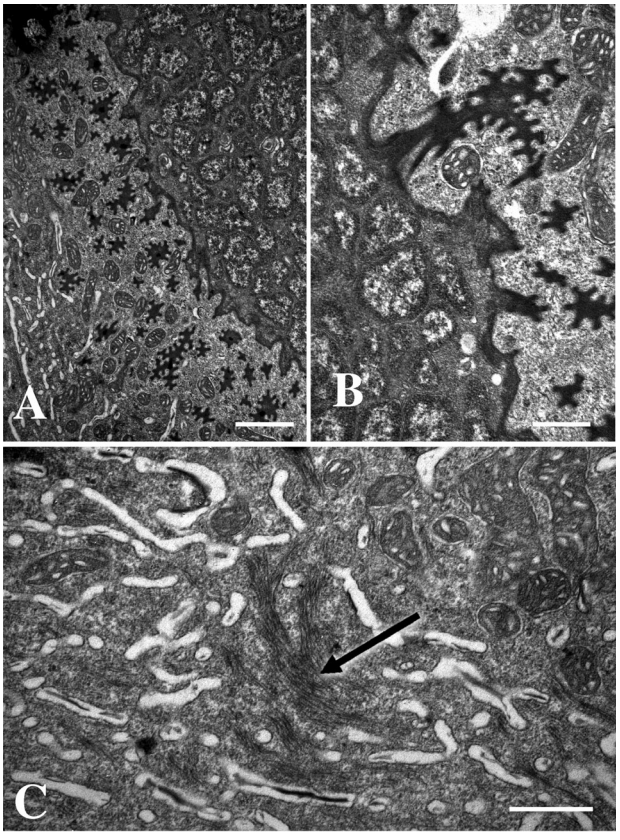

Fig. 8. Epitheliocysts in *Labrus mixtus*. (A) Transverse section of several irregularly shaped actinae radiating from the inclusion membrane. Scale bar = 1.0 µm. (B) The actinae seem to be branching in the host cell cytosol. Scale bar = 0.5 µm. (C) Large amounts of filaments (arrow) accumulating in some of the infected host cells in the area consisting of endoplasmic reticulum and mitochondria. Scale bar = 0.5 µm

(Duesund et al. 2010, Johansen et al. 2013, Sandlund et al. 2014, Munro et al. 2015, Wallace et al. 2015). The wrasse in Shetland were infected with VHSV genotype III, while only genotype I has been detected in wild fish along the Norwegian coast (Duesund et al. 2010, Johansen et al. 2013, Sandlund et al. 2014, Wallace et al. 2015). VHSV genotype III was detected in a few seawater rainbow trout farms in western Norway in the period 2007–2008, but it has not been possible to identify the source of the virus in these farms (Duesund et al. 2010). Genotype III seems to have a natural distribution in the Skagerrak, the North Sea and around the British Isles (Einer-Jensen et al. 2004, Snow et al. 2004, Wallace et al. 2015).

The number of available 16S rRNA sequences from bacteria has increased rapidly during the last decade. These signature sequences are always included in the description of new species of bacteria, i.e. every species description contains a phylogenetic analysis of the type strain based on 16S rRNA gene sequence even though high conservation of rRNA reduces its discrimination power and may not separate between closely related species. DNA–DNA hybridization is considered the 'gold standard' for species delimitation, but due to the workload involved it was suggested that such experiments need only be performed for isolates that share 16S rRNA gene sequence similarities above 97% (Stackebrandt & Goebel 1994). However, based on experience, this recommendation was changed in 2006. It was suggested that a 16S rRNA gene sequence similarity threshold range of 98.7–99.0% should be set as a level before DNA–DNA reassociation experiments are necessary (Stackebrandt & Ebers 2006). In a later study, based on the genomes of 6787 prokaryotes belonging to 22 phyla, it was shown that 98.65% 16S rRNA gene sequence similarity can be used as a threshold for separating species (Kim et al. 2014). This suggested change in threshold for species differentiation will have implications for description of bacteria that are not cultivable at present, and therefore mostly excludes DNA–DNA reassociation experiments and many phenotypic tests.

All members of *Chlamydiae* detected on the gills of different fish species, including those from the wrasse species, cannot be cultivated, which means that the species descriptions must be based on 16S rRNA gene sequences and core genes (such as housekeeping genes), inclusions, bacterial morphology and host species (Greub 2013). The characterized *Candidatus* species within *Chlamydiales* obtained from fish are *Cand*. Piscichlamydia salmonis, *Cand*. Clavichlamydia salmonicola, *Cand*. Actinochlamydia clariae, *Cand.* Parilichlamydia carangidicola, *Cand*. Renichlamydia lutjani, *Cand*. Syngnamydia venezia, *Cand*. Syngnamydia salmonis, *Cand*. Similichlamydia latridicola, *Cand*. Similichlamydia laticola and *Cand*. Similichlamydia labri (Draghi et al. 2004, Karlsen et al. 2008, Corsaro & Work 2012, Fehr et al. 2013, Steigen et al. 2013, 2015, Stride et al. 2013a,b,c, Nylund et al. 2015). In addition to these *Candidatus* species, there is a large number of *Chlamydiae*-related 16S rRNA gene sequences available that have been obtained from fish suffering from epitheliocystis. This suggests a high diversity and presence of a wide range of new species within *Chlamydiales* associated with fish (Meijer et al. 2006, Draghi et al. 2007, Polkinghorne et al. 2010, Corsaro & Work 2012, Camus et al. 2013, Lai et al. 2013, Nylund et al. 2015).

The 16S rRNA gene sequences of 1 group of gill *Chlamydiae* from *Ctenolabrus rupestris, Symphodus*

melops, Centrolabrus exoletus and *Labrus mixtus* show >98.9% similarity to *Cand*. S. labri described from *L. bergylta* in western Norway (Steigen et al. 2015). A strict interpretation of the criteria for separation of *Chlamydiae* species based on 16S rDNA suggests that these bacteria, from the different wrasse species, belong to the species *Cand.* S. labri described from *L. bergylta* (cf. Everett et al. 1999, Bush & Everett 2001, Kim et al. 2014, Steigen et al. 2015). However, when the phylogenetic analysis is expanded to include the ITS and partial 23S regions in addition to the 16S, they are separated into distinct and well supported clades reflecting the host species. If future research, including other *Chlamydiae* gene sequences, should show that *Chlamydiae* from the different wrasse species are distinct species then the use of 16S rRNA gene sequences for demarcation or separation of intracellular bacteria like *Chlamydiae* will have to be reconsidered (cf. Stackebrandt 2009).

The other group of *Chlamydiae* obtained from *L. bergylta* and *L. mixtus*, Clade A (Figs. 5 & 6) represent a putative new species in the *Cand.* genus Parilichlamydia. The closest relative to Clade A is *Cand*. P. carangidicola (accession no. JQ673516) from Australian waters. Other putative new species of *Chlamydiae*, based on 16S rRNA gene sequences only, are present on the gills of *Oreochromis niloticus,* collected in and around Lake Victoria, *Serranus scriba* from the Mediterranean Sea and *Cyclopterus lumpus* from western Norway. This shows the wide geographical distribution of these closely related *Chlamydiae*.

It is worth noting that the overall phylogeny of wrasse (Figs. S1 & S2) fits with the phylogeny of the putative *Chlamydiae* species and Clade A, indicating host-species specificity of the *Chlamydiae*.

Gill-associated *Chlamydiae* occurred at high prevalence in all 5 wrasse species from the west coast of Norway. However, this paper describes the morphology of cysts, inclusions and associated bacteria from the gills of *L. mixtus* only. We did not observe cysts on the gills of *C. rupestris, C. exoletus* and *S. melops*, which could be due to a relative low density of bacteria in these wrasse species (inferred from RT-PCR Ct values). The cysts and the inclusions on the gills of *L. mixtus* showed similarities to that of *Cand.* Similichlamydia labri from *L. bergylta* and *Cand*. A. clariae described from the gills of *Clarias gariepinus* in Uganda (Steigen et al. 2013, 2015). However, the inclusions and the epitheliocysts were larger in *L. mixtus*, reaching 120 µm in diameter, compared to that observed in *L. bergylta* and *C. gariepinus*. They all share actiniae radiating from the inclusion, but the number of actiniae was much higher in epitheliocysts from *L. mixtus*. Large cysts were also described on the gills of *Latris lineata* and *Lates calcarifer* infected with *Cand*. S. latridicola and *Cand*. S. laticola, respectively (cf. Stride et al. 2013b,c), but actiniae cannot be seen on any of the pictures included, and the ultrastructure/morphology of the bacteria inside the inclusions was not presented. A comparison of the bacteria within the cysts of *L. mixtus,* described in the current study, with these 2 *Candidatus* species can only be based on the partial 16S rDNA. This is of course assuming that the *Chlamydiae* sequences obtained here from infected *L. mixtus* represented those bacteria observed in cysts in this host, as an *in situ* hybridization assay was not used to confirm the link.

Steigen et al. (2013) discussed the possible function of the actiniae radiating from the inclusion membrane in *Actinochlamydiaceae*. It was suggested that they may have a function both in the uptake of nutrients for the growing bacteria inside the inclusion and for the expansion of the growth environment for these intracellular bacteria. The actiniae radiating from the inclusions in *C. gariepinus* are seen penetrating the cell membrane of neighbouring cells (Steigen et al. 2013), while this is less obvious in the *L. mixtus* inclusions presented here. However, the cell membranes of neighbouring chloride cells to the epitheliocysts in *L. mixtus* seemed to fuse with the cell membranes of the infected host cells. This resulted in large host cells containing abundant amounts of mitochondria and endoplasmic reticulum and may explain why the epitheliocysts were enlarged to 120 µm in diameter in this host species. Inclusion of neighbouring cells as part of the epitheliocysts, a sort of syncytium formation, has not been observed in other members of *Chlamydiae* outside the *Cand*. family Actinochlamydiaceae.

None of the wrasse *Chlamydiae* have been detected in farmed salmonids (results not shown) and as such they will probably not represent a problem for salmon culture. Movement of wrasse along the Norwegian coast, as cleaner fish in salmon farming, could transmit these bacteria to other wild populations of wrasse. Prevalence of these *Chlamydiae* in other wrasse populations are, however, unknown, which means that it is not possible to evaluate the consequences of such transmissions.

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