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Exophiala angulospora causes systemic inflammation in Atlantic cod *Gadus morhua*

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ABSTRACT: Species of *Exophiala* are opportunistic fungal pathogens that may infect a broad range of warm- and cold-blooded animals, including salmonids and Atlantic cod. In the present study, we observed abnormal swimming behaviour and skin pigmentation and increased mortality in cod kept in an indoor tank. Necropsy revealed foci of different sizes with a greyish to brownish colour in internal organs of diseased fish. The foci consisted of ramifying darkly pigmented fungal hyphae surrounded by distinct layers of inflammatory cells, including macrophage-like cells. In the inner layer with many hyphae, the macrophage-like cells were dead. We observed no apparent restriction of fungal growth by the inflammatory response. A darkly pigmented fungus was repeatedly isolated in pure culture from foci of diseased fish and identified as *Exophiala angulospora* using morphological and molecular characters. This species has not been previously reported to cause disease in cod, but has been reported as an opportunistic pathogen of both marine and freshwater fish. Based on the morphology and sequence analysis presented here, we conclude that *E. angulospora* caused the observed chronic multifocal inflammation in internal organs of cod, leading to severe disease and mortality.

KEY WORDS: Macrophages · *Capronia coronata* · Herpotrichiellaceae · Black yeast

INTRODUCTION

Even though systemic mycoses are not common in aquaculture, a number of such diseases are described in fish (Roberts 2004). Members of the fungal group commonly referred to as 'black yeasts', more specifically the genus *Exophiala* (Herpotrichiellaceae, Chaetothyriales), cause both superficial and systemic, sometimes fatal, mycoses in a wide variety of warm- and cold-blooded animals (Langvad & Pedersen 1985, Otis et al. 1985, Orélis-Ribeiro et al. 2010, Li et al. 2011) including Atlantic cod (Blazer & Wolke 1979). In fact, *Exophiala* species are among the most commonly reported agents of systemic mycoses in fish, although the isolations of these species from non-fish substrates such as soil, sediments, nematode cysts, wood, plant material substrates, human hair and nails, and drinking water (Uijthof et al. 1997, Domsch et al. 2007, S. de Hoog pers. comm.) suggest they are opportunistic pathogens and not restricted to a pathogenic life-history strategy.

Among those *Exophiala* species reported to cause disease and mortality in fish are *E. pisciphila* (Fijan 1969, McGinnis & Ajello 1974). *E. psychrophila* (Pedersen & Langvad 1989), *E. salmonis* (Carmichael 1966), *E. xenobiotica* (Munchan et al. 2009), *E. angulospora* (Nyaoke et al. 2009), and a number of undescribed species (Alderman & Feist 1985, S. de Hoog pers. comm.). Rearing under artificial conditions appears to create a predisposition for infection by opportunistic fungal pathogens. For example, mycoses due to *E. angulospora* and other *Exophiala* spp. have been frequently reported in aquaria-maintained weedy sea dragons from a number of locations in the United States (Nyaoke et al. 2009). Pathological changes in *Exophiala*-infected fish have

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been reported to be dominated by necrosis (Nyaoke et al. 2009), mononuclear leukocytes (Munchan et al. 2009), and inflammation characterised as granulomatous in different fish species (Carmichael 1966, Richards et al. 1978, Langvad & Pedersen 1985, Kurata et al. 2008) including cod (Blazer & Wolke 1979). Fungal diseases usually do not cause high short-term losses, but may yield high accumulated losses over time.

In the present study, we isolated *Exophiala angulospora* from clinically diseased cod maintained in indoor tanks and showed that the fungus caused severe inflammation and increased mortality of infected animals.

MATERIALS AND METHODS

Fish samples. In November 2005, cod of about 150 to 250 g originating from a farm in the western part of Norway were transferred to an indoor tank for experimental purposes. The tank was supplied with continuously flowing sea water. After a few months, increasing numbers of fish showed clinical signs of disease. This included abnormal swimming behaviour and dark coloration of the skin of the caudal part, which most frequently appeared with a sharp demarcation to unaffected areas. During April–May 2006, moderate mortality was observed. At the time of sampling in June 2006, about 50% of the fish showed clinical signs of disease.

On 2 occasions, 4 fish with clinical signs of disease were killed by a blow to the head, brought on ice to the laboratory, and necropsied the next day (Samplings 1 and 2; Table 1). Tissue samples, as indicated in Table 1, were collected and microbiological smears were made. Based on the results from these cases, an additional 4 fish with clinical signs of disease, including one with rotating swimming behaviour and 2 apparently healthy fish, were killed with an anaesthetic overdose, necropsied on site, and material collected, as indicated in Table 1 (Sampling 3). Tissues were also frozen at –80°C for various analytical purposes. All fish samples used in the present study are listed in Table 1.

Histology. Tissue samples (Table 1), were fixed in neutral phosphate buffered 10% formalin, embedded in paraffin and 3 µm-thick sections were cut and stained with haematoxylin and eosin (H&E). Additional selected sections were stained with periodic acid-Schiff (PAS) for sugar residues present in cell walls and other fungal structures. Additional sections

Table 1. Cod material used in the present study along with obtained isolates of *Exophiala angulospora.* Cod individuals were either moribund (M) or apparently healthy (AH). Tissues investigated include brain (B), kidney (K), liver (L), spleen (S) and skin (Sk). VI numbers refer to the assigned strain number of the pure culture isolate of *E. angulospora* in the culture collection Mykoteket at the Norwegian Veterinary Institute. Missing isolates are either results of no growth (NG) or no isolation attempt (–). EMBL/GenBank accession numbers are given for ITS-sequences of the pure culture isolates

Sampling	Cod ID	Organs for histological examination	Gross anatomical distribution of mycotic lesions	*Exophiala angulospora* isolates	GenBank accession number
1	1.T1 (M)	K,L,S	K,S	VI 04031 (K) VI 04032 (L)	FR847107 FR847108
1	1.T2 (M)	–	K,S,L	–	–
1	1.T3 (M)	–	K,S	–	–
1	1.T4 (M)	–	K,S,L	–	–
2	2.T1 (M)	K,L,S	K,L	VI 03756 (K) VI 03757 (Sk) VI 03758 (K)	FR847101 FR847102 FR847103
2	2.T2 (M)	–	K	NG (K)	–
2	2.T3 (M)	K,L,S	K	VI 03759 (K)	FR847104
2	2.T4 (M)	K,L,S	K,L	VI 03760 (S) VI 03761 (K)	FR847105 FR847106
3	3.T1 (M)	K,L,S	K	NG[b]	–
3	3.T2 (M)	K,L,S	K,S,M	–	–
3	3.T3 (M[a])	K,L,S,B	K,S,L,B	NG[b]	–
3	3.T4 (M)	K,L,S	K,S	–	–
3	3.T5 (AH)	K,L,S	–	–	–
3	3.T6 (AH)	K,L,S	L	VI 05436[b] (L)	FR847109 FR847110[c] FR847111[c]

[a]Rotating swimming behaviour
[b]Isolation of fungi attempted from frozen material
[c]Sequence obtained directly from liver tissue (includes the DNA isolates 3.3.T6L1 and 3.3.T6L2)

of cod with inflammations due to infections with the bacteria *Listonella anguillarum* and atypical *Aeromonas salmonicida* were stained with PAS for the purpose of examining if inflammatory cells proved by non-fungal agents may contain positive material. Sections of the kidney of one fish were also stained with Martius scarlet blue (MSB) for detection of connective tissue fibers as described by Bancroft & Stevens (1996). Pigmentation of hyphae was studied in one unstained section.

Isolation of fungi. For the cod sampling 1.T1, microbiological smears were made with sterile inoculation loops from the kidney and liver onto Sabouraud's medium (SAB: 10 g l^{-1} neopeptone [Difco], 20 g l^{-1} glucose, 5 g l^{-1} bacto yeast extract [Difco], 20 g l^{-1} granulated agar [Difco], 1 ml l^{-1} chloramphenicol) adjusted to a pH of 6.0 to 6.5. Isolation plates were incubated in darkness at 25°C. This resulted in extensive growth of a darkly pigmented fungus. The internal organs from a second sampling of cod showing signs of disease (2.T1–T4) were therefore dissected aseptically, briefly surface sterilized in 70% ethanol for 30 s, rinsed in sterile Milli-Q water, cut into 2 mm pieces, and plated onto SAB medium. The isolation plates were incubated in darkness at 25°C. On-site isolation of fungi from the organs using microbiological smears of a third cod sampling (3.T1–T6) was attempted, but failed due to high levels of indoor fungal contaminants. Isolation of the fungus was therefore attempted from frozen organ material embedded in OCT-Tissue tek® medium (Chemi-teknik) according to the procedure described for 2.T1–T4. Additionally, 2 mm sub-samples of each organ were made for molecular analyses. From each isolation plate, one representative strain of the fungus that appeared pure culture was sub-cultured onto potato dextrose agar (PDA: 39 g l^{-1} potato dextrose agar [Merck]) for further morphological and molecular characterization. The isolates are maintained in the culture collection Mykoteket at the Norwegian Veterinary Institute.

To determine possible sources of infection, water samples were collected superficially and approximately at 1 m depth in the tank as well as from the inlet water and examined as described by Hageskal et al. (2007). Briefly, 100 ml from each water sample was filtered onto membrane filters that were transferred to dichloran 18% glycerol agar (DG18) plates (Hocking & Pitt 1980) and incubated at 20 ± 1°C for 2 wk. Colony-forming units (CFU) were enumerated weekly and expressed as CFU per 100 ml water sample. Dominant genera and species were determined based on morphology. Samples of fish feed stored both indoors and outdoors were also screened quantitatively and qualitatively for fungi according to the NMKL method 98 (Nordic Committee on Food Analysis 2005). Briefly, 40 g feed samples were added to 360 ml unbuffered peptone water and homogenized in stomacher bags for 2 min. A dilution series to 10^{-4} was prepared, and 0.1 ml of each dilution was plated on DG18 and incubated at 25 ± 1°C for 1 wk. Quantitative results were expressed as CFU g^{-1} feed sample, and dominant genera and species were determined based on morphology. Plates were examined for several months to detect both fast- and slow-growing fungi.

Morphological characterization. The macroscopic morphology of the fungus was examined in 15 to 30 d old colonies on PDA. Mounts of 7 to 10 d old slide-cultures grown on cereal agar (CA: 100 g l^{-1} Pablum, 20 g l^{-1} agar) were made in water, polyvinyl alcohol (PVA: 1.66 g polyvinyl alcohol in 10 ml water), or PVA with acid fuchsin (0.05 g acid fuchsin in 10 ml lactic acid and 1 ml glycerine mixed with 1.66 g polyvinyl alcohol dissolved in 10 ml water). Light micrographs (LM) were made using a Leica DC100 digital camera.

Molecular identification. A total of 9 fungal isolates (Table 1) representing one isolation plate each were identified based on sequences from the nuclear ribosomal internal transcribed spacer region (ITS) including ITS1, 5.8S, and ITS2. DNA was extracted from a 2 to 3 mm slice of fresh mycelium using the CTAB-protocol described by Gardes & Bruns (1993) with the modifications described in Vrålstad et al. (2009). The PCR reactions were run on a DNA Engine Tetrad® Peltier Thermal Cycler (PTC-225, MJ Research) using 1.7 µM of the primers ITS1 and ITS4 (White et al. 1990), 2 µl genomic DNA, PuReTaq Ready-To-Go™ PCR Beads (Amersham Biosciences), and Milli-Q water to a final reaction volume of 25 µl. The PCR-program included an initial denaturation (94°C for 4 min), 35 cycles of amplification (94°C for 30 s, 55°C for 30 s, 72°C for 1 min), and final elongation (72°C for 5 min). DNA was also extracted from frozen organ tissues with the same procedure, but PCR and sequencing were performed using the fungal specific primer ITS1F (Gardes & Bruns 1993) in combination with ITS4. The PCR products were visualized by gel electrophoresis on 1% agarose gels with ethidium bromide. PCR products were purified with ExoZap-IT (Amersham Biosciences) according to the manufacturer's protocol, and sequenced in both directions with their respective primers using the BigDye® Terminator version 3.1 Ready Reaction mix (Applied Biosystems) according to the manufacturer's instructions. The sequencing PCR-program consisted of initial denaturation (96°C for 1 min) and 40 cycles of 96°C for 10 s, 56°C for 5 s, and 60°C for 4 min. The products were purified with BigDye® XTerminator Purification Kit (Applied Biosystems, Life Technologies) according to the manufacturer's instructions, and analyzed on an ABI PRISM® 3100 Avant Genetic Analyzer (Applied Biosystems). Assembly and manual

editing of the sequence chromatograms were conducted in BioEdit version 7.0.1 (Hall 1999). Table 1 includes the EMBL/GenBank accession numbers of sequenced fungal isolates of this study.

Phylogenetic analysis. The ITS sequences of *Exophiala angulospora* isolates from the present study, and other members of the genus *Exophiala* and its associated teleomorph, *Capronia,* were assembled in a data matrix that was aligned using MAFFT version 6.717 (Katoh & Toh 2008). The subsequent alignment was manually verified, and the resulting matrices were subjected to maximum likelihood and Bayesian analyses. The Akaike information criterion in Modeltest (Posada & Crandall 1998) was used to determine the best-fit model of evolution for both maximum likelihood and Bayesian analyses. Maximum likelihood analyses used to determine the most likely tree and maximum likelihood bootstrap support for each dataset were conducted using GARLI version 1.0 (Zwickl 2006) with the selected models of evolution implemented. Bayesian analyses were conducted using MrBayes version 3.1 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003), with 2 independent runs of four Markov Chain Monte Carlo chains with 1.0×10^7 generations each, sampling trees every 1000th generation. A final standard deviation < 0.01 for the split frequency was taken as an indication that convergence had been achieved. The first 20% of sampled trees were discarded as burn-in and posterior probabilities for each node of the 50% majority rule consensus tree were recorded. All Modeltest, GARLI, and MrBayes analyses were performed at http://www.bioportal.uio.no.

RESULTS

Gross pathology

The clinically diseased fish had foci that ranged from a few mm to 2 cm and occurred in varying numbers in one or several organs such as the kidney (Fig. 1a), spleen, and liver (Fig. 1b), with the kidney being affected most frequently (Table 1). The foci were rounded with an indistinct border to adjacent tissue, and with a greyish to brownish colour on their external (Fig. 1b) and cut surfaces. In fish with dark coloration of the skin in the caudal part, pathological changes were detected in the caudal part of the kidney just cranial to the dark coloration. In one fish with dark coloration of the skin on one side of the head, an ipsilateral focus was found in the head kidney. The fish with rotating swimming behaviour had a small greyish area in the midbrain (Fig. 1c) and one apparently healthy fish had a focus in its liver.

Histopathology

The foci described above were dominated by light yellow to brown, septate, branched (Fig. 1d), and PAS-positive fungal hyphae surrounded by inflammatory cells that were organised in distinct layers. Altogether, this created an invasive appearance (Fig. 1e). In the innermost layer, denoted Layer 1, there were many ramifying PAS-positive hyphae, arranged in a star-shaped manner and surrounded by dead macrophage like (MLCs) (Fig. 2a,b). Layer 2 consisted of mostly MLCs with blastic nuclei, a few fungal hyphae (Fig. 2a), and a few flattened, concentrically arranged cells with condensed, elongated nuclei that are tentatively identified as fibrocyte-like cells (FLCs). A third peripheral layer included concentrically arranged FLCs (Fig. 2a) and the presence of collagen as indicated by MSB-staining. In some foci, we observed intra- and extracellular materials that stained homogenously for PAS (Fig. 2c). In the kidney of some fish, fungal hyphae were also detected in the excretory system and apparently invading the epithelium (Fig. 2d).

In the fish with rotating swimming behaviour, the small greyish area of the midbrain was located in the tectum opticum, and adjacent tissue contained hyphae and inflammatory cells. In the 2 apparently healthy fish, a few foci as described for the moribund fish were seen in the spleen and kidney or liver, respectively. Also, foci in control sections were layered in similar way, and PAS staining of material that was not consistent with fungus, was observed in Layer 2.

Microbial cultivation and morphological identification

Numerous uniform, black, slow-growing fungal colonies were obtained in pure culture from all but one of the isolation plates of foci from Samplings 1 and 2 (Table 1). Only indoor fungal contaminants were obtained from isolation plates that were inoculated on-site during Sample 3 (data not shown). However, fungal colonies that were morphologically identical to cultures from the other 2 samplings emerged in pure culture from the frozen liver tissue of one cod from that sampling (Table 1).

All isolates were consistent in morphology, producing floccose dark grey to olivaceous grey-brown colonies on PDA, attaining a diameter of 33 mm after 30 d (Fig. 3a) and becoming velvety with age. Conidiophores were darkly pigmented and branched or unbranched, bearing hyaline to pale olivaceous, flask-shaped to cylindric conidiogenous cells (Fig. 3b,c). Conidiation was annellidic, and also occurred occasionally at intercalary conidiogenus loci (Fig. 3d). Conidia were produced in

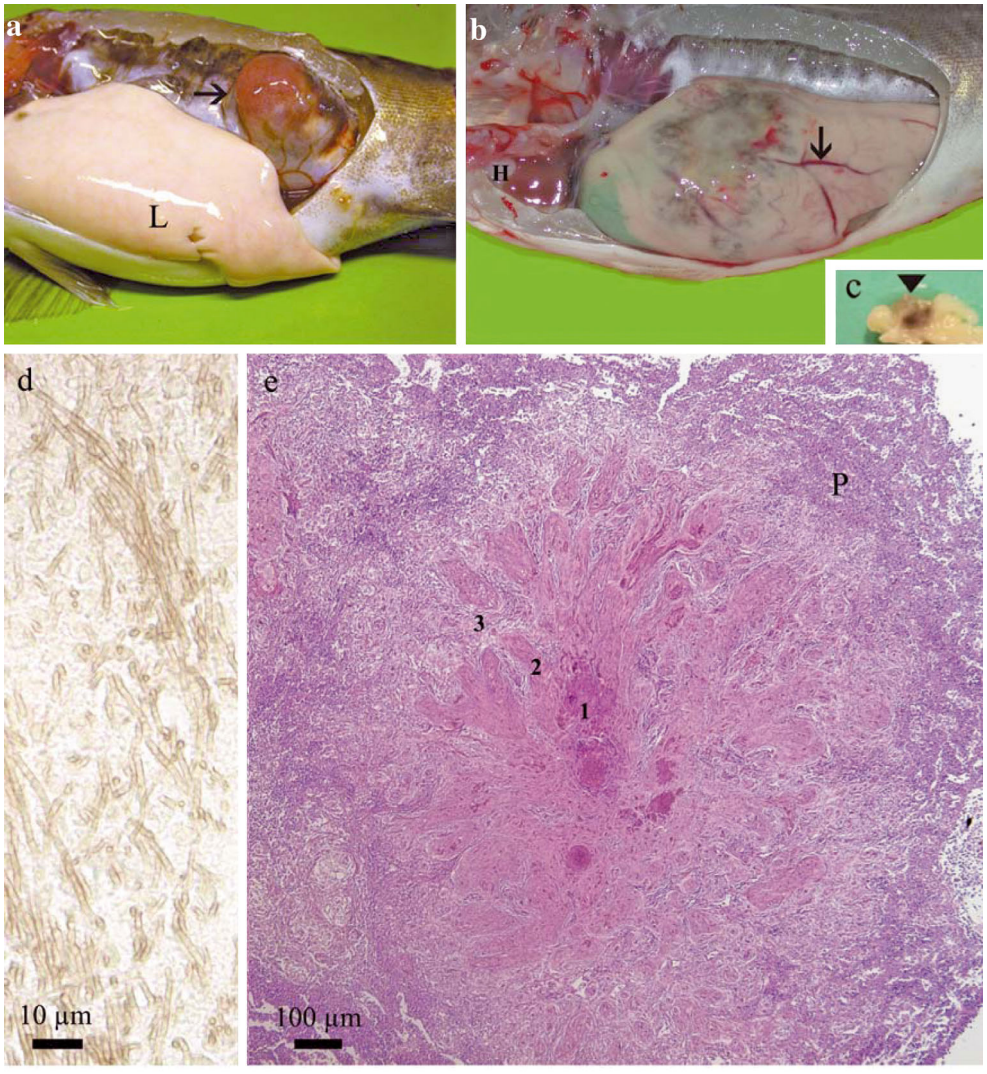

Fig. 1. *Gadus morhua*. Atlantic cod infected with *Exophiala angulospora*. (a–c) Gross pathology; (d,e) histological sections of kidney. (a) Large protruding focus in caudal part of kidney (arrow). L: liver. (b) Large greyish to brownish and partly greenish focus and blood-filled vessels in the liver (arrow). Note also the greenish color outside the focus in the most cranial part of the liver, as compared to normal colour in (a). H: heart. (c) Greyish to brownish focus affecting the whole tectum opticum of the midbrain (arrowhead). (d) Brownish hyphae in a focus. No stain. (e) Large, irregular and poorly-confined foci consisting of centrally-located hyphae and surrounded by inflammatory cells organized in distinct layers denoted layer 1, 2 and 3 as indicated. The invasiveness of hyphae in different directions has resulted in a ramified pattern of eosinophila due to dead cells around hyphae within the focus. Haematoxylin and eosin. P: parenchyma

masses, were single-celled, angular, or sometimes elliptic to subglobose, 2–4(–8) × 1.5–3 µm (Fig 3e). The diagnostic angular conidia allowed the morphological identification of the fungus as *Exophiala angulospora*.

Exophiala angulospora was not recovered from any of the water or feed samples. These were instead dominated by the genera *Penicillium* and *Cladosporium* in numbers that, at least in some samples, may indicate reduced hygienic quality of the water and feed (Table 2). The water and feed plates were also quickly overgrown, which in general hampers the detection of slower-growing fungal taxa.

Molecular identification and phylogeny

The sequenced isolates, one representative from each isolation plate, were in all cases identified as 99 to 100% similar across the ITS region to *Exophiala angulospora* and 99% similar to its sexual stage, *Capronia coronata*. The aligned matrix of ITS sequences included 495 characters for the 214 sequences representing 32 species of *Exophiala* and *Capronia*. The SYM+I+G model was selected by ModelTest as the best-fit model of evolution for the data and was implemented in both maximum likelihood and Bayesian inference analyses.

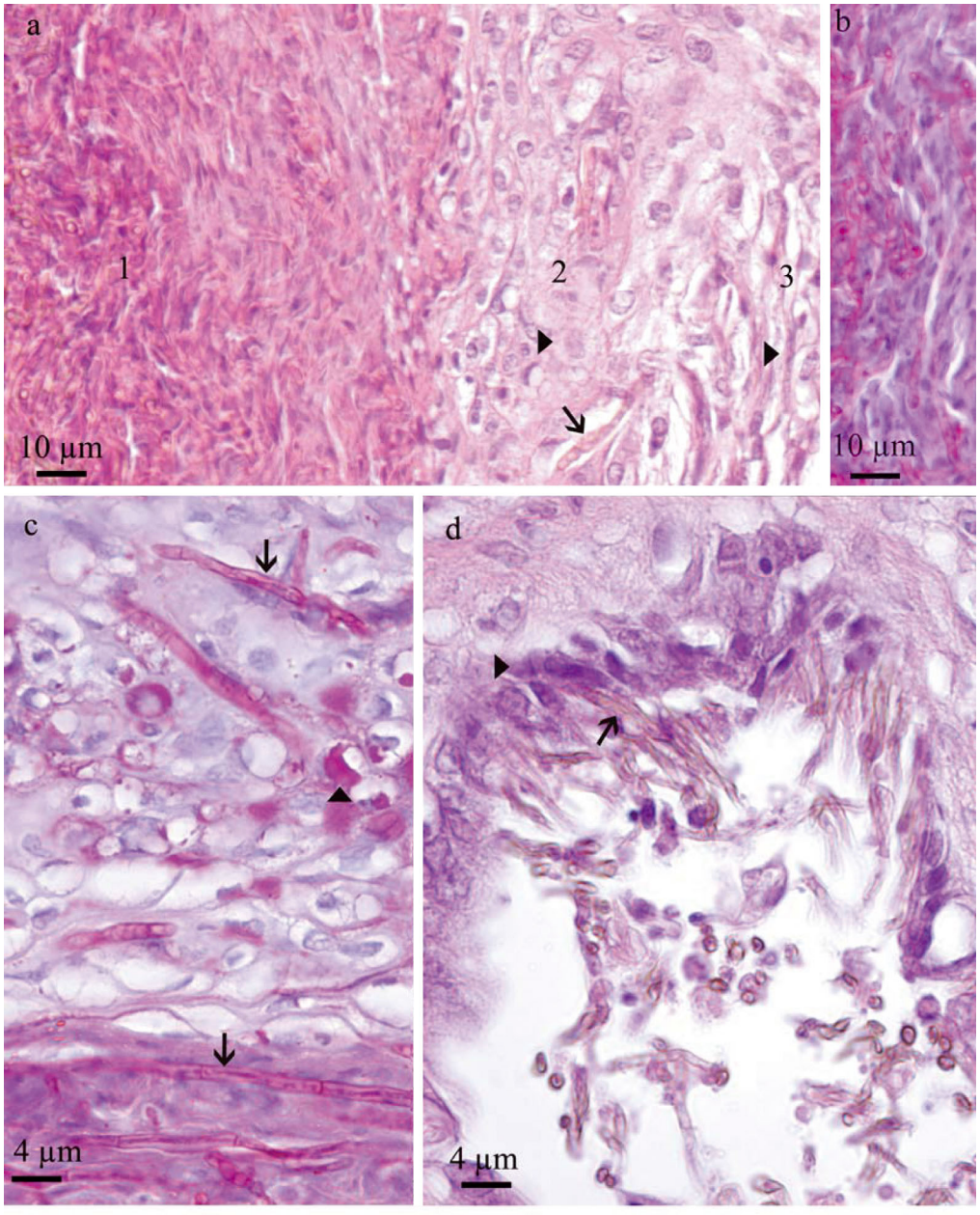

Fig. 2. *Gadus morhua*. Atlantic cod infected with *Exophiala angulospora*. Histological sections of (a–b) kidney interstitium, (c) liver, and (d) a kidney tubule or duct, stained with (a,d) H&E and (b,c) periodic acid-Schiff (PAS). (a) Layer 1; note the high number of hyphae and dead cells (1). Many macrophage-like cells with blastic nuclei (left arrowhead) and and a single hyphae (arrow) are seen in layer 2. Flattened fibrocyte-like cells are seen in layer 3 (right arrowhead). (b) PAS-positive hyphae in layer 1. (c) Some hyphae are seen in layer 1 (lower arrow) and layer 2 (upper arrow). Note branched and septate structure, and some material of unknown nature in putative inflammatory cells staining homogenously for PAS (arrowhead). (d) Many hyphae (arrow) apparently invading from the lumen into the epithelium of a kidney tubule or duct (arrowhead)

The maximum likelihood tree with results of both maximum likelihood and Bayesian inference is shown in Fig. 4. Our isolates of *E. angulospora* form a monophyletic group with *C. coronata* and other isolates of *E. angulospora* from fish, drinking water, human, soil, and plant substrates (100% Bayesian Posterior Probability [BPP] / 97% Bootstrap Support [BS]). The *E. angulospora* / *C. coronata* clade is well resolved from all other *Exophiala* and *Capronia* species, including those previously reported as pathogens of fish: *E. salmonis, E. pisciphila, E. xenobiotica* and a currently unnamed species *Exophiala* sp. 7 in Fig. 4.

DISCUSSION

This is to our knowledge the first documentation of systemic mycosis in Atlantic cod caused by *Exophiala angulospora*. The fungal species, identified by using morphological characteristics and ITS sequence analyses, was isolated in pure culture from numerous foci of diseased fish. Since no other agents were detected and the morphology of fungus in sections was consistent with the observed cultures, there is little doubt that the inflammations were associated with the isolated fungus. As demonstrated by our repeated isolation of *E. angulospora* from foci of diseased fish, the species is easily cultured, and can readily be identified based on morphology by its characteristic more or less triangular conidia. Our phylogenetic analyses show that *E. angulospora* is genetically distinct from all other species of *Exophiala* that so far have been associated with disease in fish, and can be reliably identified using molecular means and the ITS gene region for fungal barcoding. Since our isolates of *E. angulospora* from dis-

Table 2. Cultivation results from water and feed samples given in colony forming units (CFU) of fungi per 100 ml and 1 g, respectively. Colonies of filamentous fungi were, when possible, identified to genus level. No attempts were made to identify dematiaceous fungi to genus level, but it was verified that these did not include any *Exophiala* spp. Yeasts were not identified any further. LOD: limit of detection

Sample	Result	Fungal group
Water superficial	100 CFU 100 ml^{-1}	*Penicillium simplicissimum*
Water deep	5 CFU 100 ml^{-1}	*Penicillium* spp.
Water deep	1 CFU 100 ml^{-1}	Dematiaceous fungi
Water deep	1 CFU 100 ml^{-1}	Other yeasts
Inlet water	14 CFU 100 ml^{-1}	*Penicillium* spp.
Inlet water	2 CFU 100 ml^{-1}	*Cladiosporium* spp.
Inlet water	1 CFU 100 ml^{-1}	Dematiaceous fungi
Feed stored outside	Below LOD	Filamentous fungi and yeast
Feed stored inside	2300 CFU g^{-1}	*Penicillium* spp.
Feed stored inside	110 000 CFU g^{-1}	Yeast

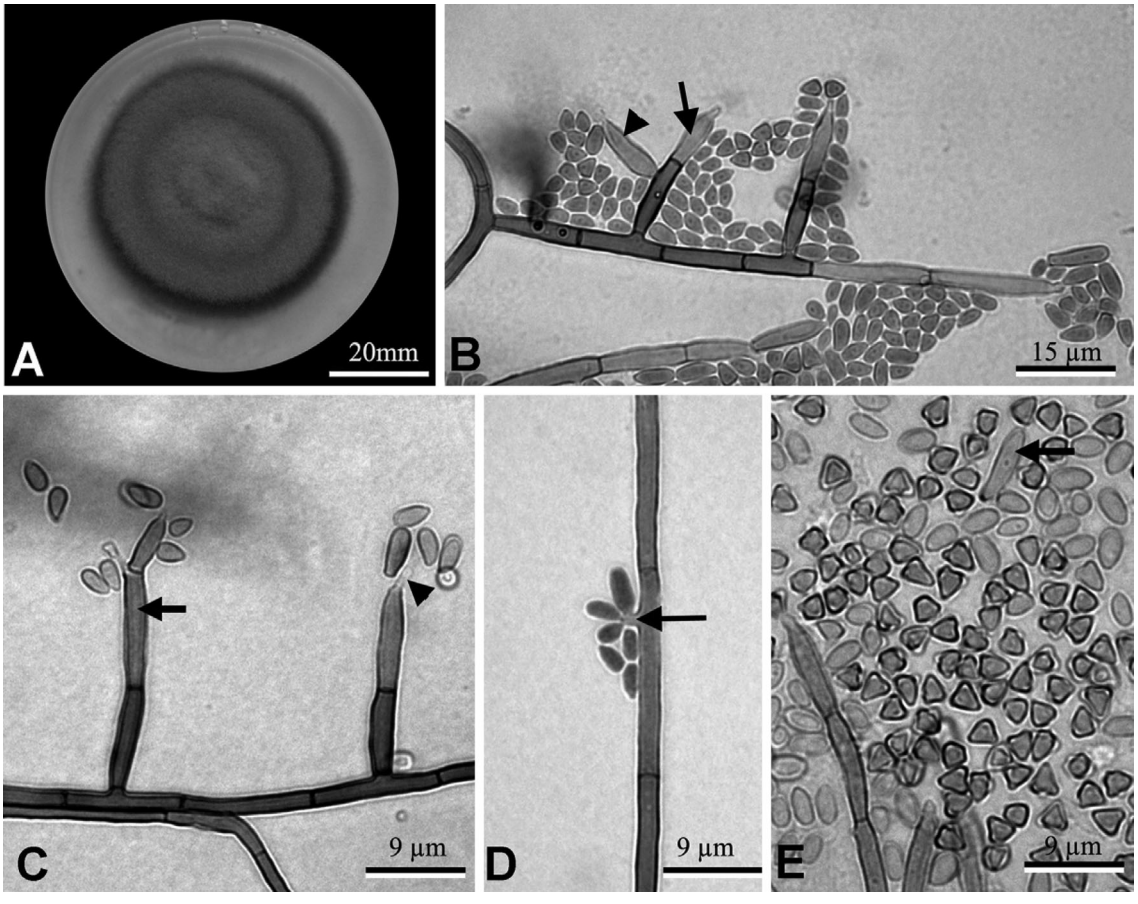

Fig. 3. Morphology of *Exophiala angulospora* isolated from diseased Atlantic cod. (A) Gross colony morphology. (B) Branched, septate conidiophore bearing 4 paler, beaked conidiogenous cells occurring in terminal (arrow) and lateral (arrowhead) positions. (C) Unbranched conidiophores with terminal conidiogenous cells. Arrow indicates an intercalary conidiogenous cell. Arrowhead indicates beak of conidiogenous cell. (D) Vegetative hyphae with conidiogenous peg (arrow). (E) Conidia. Note the diversity of conidium shape and size, ranging from short ellipsoid to more-or-less triangular to long ellipsoid (arrow)

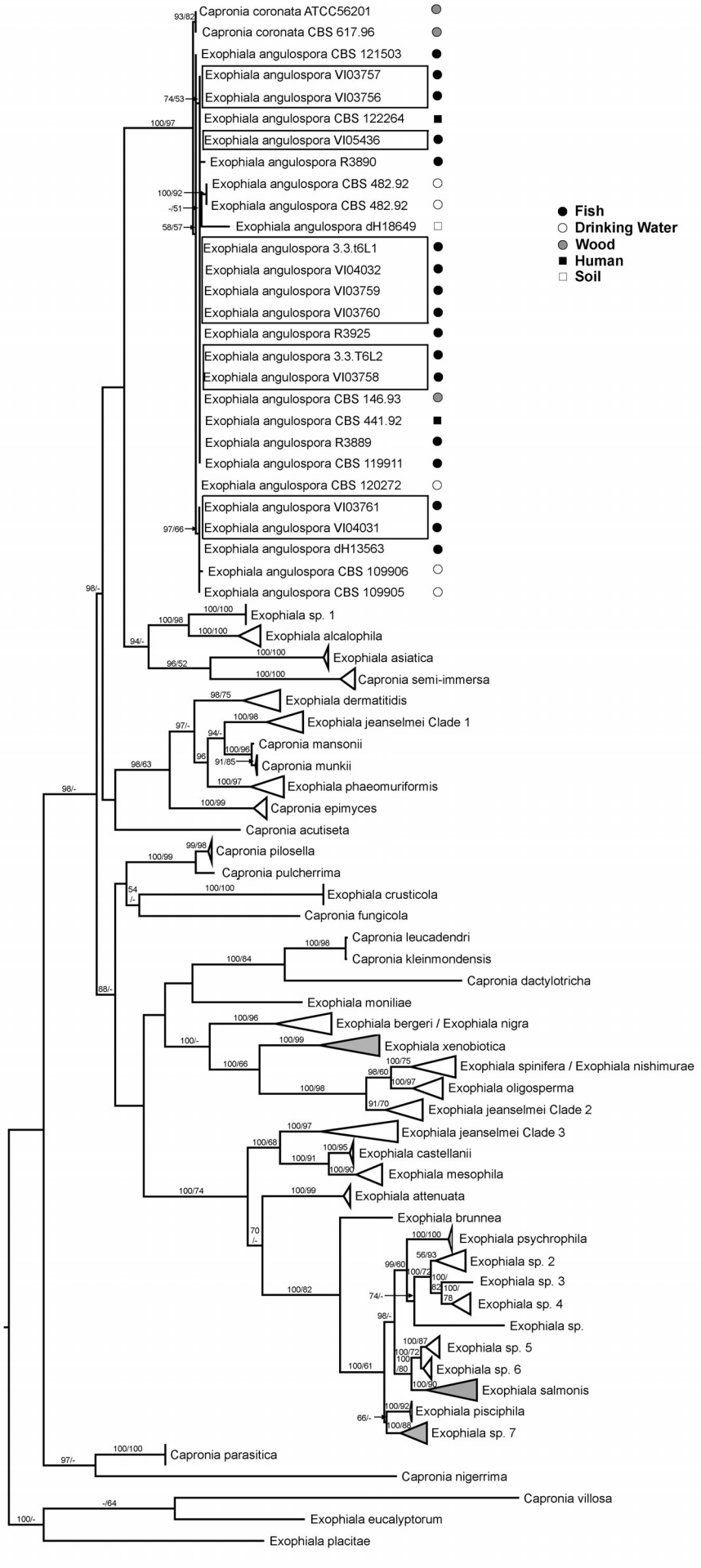
Capronia coronata ATCC56201
Capronia coronata CBS 617.96
Exophiala angulospora CBS 121503
Exophiala angulospora VI03757
Exophiala angulospora VI03756
Exophiala angulospora CBS 122264
Exophiala angulospora VI05436
Exophiala angulospora R3890
Exophiala angulospora CBS 482.92
Exophiala angulospora CBS 482.92
Exophiala angulospora dH18649
Exophiala angulospora 3.3.t6L1
Exophiala angulospora VI04032
Exophiala angulospora VI03759
Exophiala angulospora VI03760
Exophiala angulospora R3925
Exophiala angulospora 3.3.T6L2
Exophiala angulospora VI03758
Exophiala angulospora CBS 146.93
Exophiala angulospora CBS 441.92
Exophiala angulospora R3889
Exophiala angulospora CBS 119911
Exophiala angulospora CBS 120272
Exophiala angulospora VI03761
Exophiala angulospora VI04031
Exophiala angulospora dH13563
Exophiala angulospora CBS 109906
Exophiala angulospora CBS 109905
Fish
Drinking Water
Wood
Human
Soil
Exophiala sp. 1
Exophiala alcalophila
Exophiala asiatica
Capronia semi-immersa
Exophiala dermatitidis
Exophiala jeanselmei Clade 1
Capronia mansonii
Capronia munkii
Exophiala phaeomuriformis
Capronia epimyces
Capronia acutiseta
Capronia pilosella
Capronia pulcherrima
Exophiala crusticola
Capronia fungicola
Capronia leucadendri
Capronia kleinmondensis
Capronia dactylotricha
Exophiala moniliae
Exophiala bergeri / Exophiala nigra
Exophiala xenobiotica
Exophiala spinifera / Exophiala nishimurae
Exophiala oligosperma
Exophiala jeanselmei Clade 2
Exophiala jeanselmei Clade 3
Exophiala castellanii
Exophiala mesophila
Exophiala attenuata
Exophiala brunnea
Exophiala psychrophila
Exophiala sp. 2
Exophiala sp. 3
Exophiala sp. 4
Exophiala sp.
Exophiala sp. 5
Exophiala sp. 6
Exophiala salmonis
Exophiala pisciphila
Exophiala sp. 7
Capronia parasitica
Capronia nigerrima
Capronia villosa
Exophiala eucalyptorum
Exophiala placitae

Fig. 4. Maximum likelihood tree (–lnL 13330.46) inferred from analysis of ITS sequences showing the placement of *Exophiala angulospora* among members of *Exophiala* and *Capronia*. *C. villosa, E. eucalyptorum,* and *E. placitae* serve as outgroup taxa. Strain names are indicated after species of *E. angulospora,* and accession numbers or strain numbers for sequences used in this analysis are available in Table S1 in the supplement at www.int-res.com/articles/suppl/d096p209_supp.pdf. Bootstrap values >50% calculated from 1000 replicates and Bayesian posterior probabilities >50 are given above the branches as Bayesian posterior probability/bootstrap proportion. Gaps (–) indicate a collapsed node in an analysis. Clades previously reported as opportunistic pathogens of fish are marked in grey. Sequences derived from isolates in this study are indicated by boxes. The substrate from which each strain of *E. angulospora* was isolated is indicated by the symbols after the species and strain names

eased fish cannot be separated from isolates obtained from other substrates such as wood and water, at least not with the currently used genetic marker, it is likely that *E. angulospora* is an opportunistic pathogen not restricted to a pathogenic life history.

Species of *Exophiala* are known to produce a variety of extracellular enzymes, including ureases, gelatinases, and lipases (De Souza et al. 2008, Corbellini et al. 2009). Effects of extracellular enzymes on fish host cells may explain why dead cells were observed in layer 1 of the foci where abundant fungal hyphae were present, but not in the layers containing few hyphae. By comparison, in weedy sea dragons infected with *E. angulospora,* the predominant pathological change was described as necrosis (Nyaoke et al. 2009). Hence, differences in pathological manifestations may reflect different host responses, rather than variability in the fungus. This is further supported by the phylogenetic analyses where isolates of *E. angulospora* from different hosts did not form distinct, well-supported subgroupings. However, it is also well known that both morphology and ITS poorly resolve species in fast-evolving lineages of ascomycetes including plant and animal pathogenic species (see Balajee et al. 2009). It remains therefore to test the validity of this hypothesis by multi-locus sequence analyses of genetic markers that have proved more variable than ITS for many important groups of ascomycetes, e.g. EF-1α (translation elongation factor 1α), β-TUB (β-tubulin), and CAM (calmodulin), and RPB2 (RNA polymerase II second largest subunit; Balajee et al. 2009).

A granulomatous inflammation, with distinct layers of inflammatory cells, as seen in the present study, has not been previously reported in *Exophila angulospora* infections of fish. However, it has been reported from captive cod infected with an *Exophila*-like fungus (Blazer & Wolke 1979) and in cod suffering from bacterial diseases (Magnadottir et al. 2002, Gudmundsdottir et al. 2006). It is unclear if the homogenous PAS-positive material observed in areas of foci originates from the fungi (i.e. represents phagocytosed fungal parts). Similar homogenously PAS-stained material was also observed in the control foci caused by atypical *Aeromonas salmonicida,* indicating that at least some of the PAS-positive material may originate from the inflammatory cells.

The production of melanin has been considered an important virulence factor of fungi, including *Exophiala* species, and mutant albino strains of *E. dermatitidis* have been shown to have lower virulence than wildtype melanized strains (De Hoog et al. 2000). More specifically, melanin has been shown to play a crucial role in allowing *E. dermatitidis* to survive oxidative bursts within the phagolysosome of mammalian neutrophils that could otherwise protect the host from this pathogen (Schnitzler et al. 1999). The growth of the darkly pigmented fungus in the present study was clearly invasive with ineffective restriction by the inflammatory response. The success of *E. angulospora* in overcoming host defence to infection may therefore partly be attributed to its melanized hyphae that have been documented to exert larger turgor-derived forces at their apices than non-melanized cells (Brush & Money 1999). Given the large amounts of fungal hyphae observed in the internal organs, and the absence of pigmented host cells, we conclude that the discoloration of the fishes' internal organs can be attributed to fungal pigments. The dark discoloration of skin caudal to foci is similar to that observed in fish infected with *Myxobolus cerebralis* (Speare & Salvatore 2006), and can probably be explained by lesions affecting spinal nerves innervating skin chromatophores (Whitear 1952).

To the best of our knowledge, there are no established upper values of acceptable amounts of *Penicillium simplicissimum* in indoor marine aquaria. However, we assume that the amounts of *P. simplicissimum* detected in the superficial water samples exceeds what can be expected in similar facilities, and may therefore suggest reduced water quality. Further, it cannot be excluded that the *Penicillium* levels in the water may have negatively impacted the immune system of the fish, and, combined with the additional stress of being transferred and maintained in an indoor tank, predisposed them to opportunistic infection by *Exophiala angulospora*. Although *Exophiala* infections have been reported in increasing numbers in healthy humans (Li et al. 2011), they are traditionally reported in immunocompromised individuals (Sughayer et al. 1991). The source of *E. angulospora* inoculum responsible for this observed mycotic outbreak in Atlantic cod was not resolved in the present study. It is not clear if *E. angu-*

lospora was absent in our analyses of feed and water or not detected due to biases in the culturing methods. However, we cannot rule out the possibility that the fish were infected with *E. angulospora* prior to their arrival at the indoor tanks, given the extensive and chronic changes in some individuals. *E. angulospora* and *Capronia coronata*, the putative sexual stage of the fungus judged from the ITS sequence similarity, have been reported from water, decorticated wood, human skin and nail, hydrocarbon polluted soils, as well as diseased fish (Müller et al. 1987, S. de Hoog pers. comm.), suggesting the fungus is widely distributed and the source of infection may be difficult or impossible to pinpoint.

The route by which *Exophiala angulospora* infected the fish remains unknown. However, the hyphae apparently growing from tubuli lumina into the epithelium could indicate an ascending infection to the kidney. This could also be supported by the fact that the kidneys were infected in all diseased fish.

Based on the pathological, cultural, and molecular evidence presented in the present study, we conclude that *Exophiala angulospora* caused the observed chronic multifocal granulomatous inflammation in internal organs of cod, resulting in severe disease and mortality. Further studies should include challenge experiments of *E. angulospora* on cod and salmonids to investigate and compare the portal of entry, pathogenesis, and host response.

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