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Saprolegnia species in Norwegian salmon hatcheries: field survey identifies *S. diclina* sub-clade IIIB as the dominating taxon

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ABSTRACT: *Saprolegnia* isolates within the recognized clades encompassing the taxa *S. parasitica* and *S. diclina* act as opportunist and aggressive pathogens to both fish and their eggs. They are responsible for significant economic losses in aquaculture, particularly in salmonid hatcheries. However, the identity, distribution and pathogenic significance of involved species often remain unexplored. In this study, 89 *Saprolegnia* isolates were recovered from water, eggs and salmon tissue samples that originated from salmon (*Salmo salar*) hatcheries along the coast of Norway. The cultures were characterized morphologically and molecularly in order to provide an overview of the species composition of *Saprolegnia* spp. present in Norwegian salmon hatcheries. We demonstrate that *S. diclina* clearly dominated and contributed to 79% of the recovered isolates. Parsimony analyses of the nuclear ribosomal internal transcribed spacer (ITS) region split these isolates into 2 strongly supported sub-clades, *S. diclina* sub-clade IIIA and IIIB, where sub-clade IIIB accounted for 66% of all isolates. A minor portion of the isolates constituted other taxa that were either conspecific or showed strong affinity to *S. parasitica, S. ferax, S. hypogyna* and *Scoliolegnia asterophora*. The unique sub-clade IIIB of *S. diclina* was most prevalent in water and salmon eggs, while *S. parasitica* isolates were more frequently isolated from post hatching stages. The study demonstrated that morphological criteria in many cases were insufficient for species delimitation due to lack of sexual structures or incoherent morphological expression of such features within the tested replicates.

KEY WORDS: Aquaculture · *Salmo salar* · Pathogen · Eggs · Oomycetes

INTRODUCTION

Saprolegnia infections are economically burdensome fish diseases occurring mainly throughout the freshwater phase in salmonid aquaculture, but also in wild fish. The causative organisms, species within the oomycete genus *Saprolegnia,* have been subject to research since they were first described by Arderon in 1748 (Hughes 1994). Scientific approaches span from the exploration of new species and pathogen–host relationships (Diéguez-Uribeondo et al. 2007) to the full genome sequencing of *S. parasitica* (van West 2006).

The occurrence of oomycetes in salmonid hatcheries has been investigated in Japan and Spain, but only to a rather limited extent given the nature of the problem (Kitancharoen et al. 1997, Hussein et al. 2001, Fregeneda-Grandes et al. 2007). These studies characterized the *Saprolegnia* species sampled from incubating eggs and fry. However, reports on the diversity of *Saprolegnia* within hatchery water systems are scarce (Olàh & Farkas 1978). As a conse-

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quence, very little is known about the sapro-ecological aspects within water systems of commercial aquaculture facilities and their implications with regard to infection pressure in the different life stages of the fish. It is becoming increasingly clear that the all-encompassing term saprolegniosis is not a single disease, but rather a conglomerate of disease conditions occurring at different life stages, with aetiologies involving different causative species and an array of external factors. Examples of the latter are stress, prior disease and environmental conditions. Knowledge about the pathogen and its ecology within the ecosystem in an aquaculture facility is of importance in order to develop control measures. Even though Norway is a leading country in salmonid aquaculture, very little is known about the occurrence of oomycetes in salmon hatcheries, with regard to both species and quantities. In the present study, we therefore aimed to perform the first extensive survey of the *Saprolegnia* species in Norwegian salmon hatcheries. *Saprolegnia* isolates collected from water systems of 26 salmon hatcheries along the coast of Norway were characterized morphologically, physiologically and molecularly. In addition, *Saprolegnia* spp. from infected eggs and fish were isolated from those hatcheries where such samples were available, to compare with isolates found in water.

MATERIALS AND METHODS

Hatcheries

The 26 hatcheries supplying samples were all commercially run operations with production capacities ranging from 500 000 to 4 000 000 eggs yr^{-1}. Eighteen hatcheries were independent operations selling smolts to other companies, while 8 were an integrated part of a company production chain. None of the hatcheries employed recirculating aquaculture systems, and their water supply came from rivers, lakes, ground water or combinations of these. Moreover, the hatching systems that were employed varied; flow-through hatching trays were most common (16 hatcheries), while hatching cabinets (Comphatch®, Alvestad marin AS) and hatching tanks (Maxiklekk, Namdalsplast AS) were used in 12 and 5 hatcheries, respectively. Salmon eggs (*Salmo salar*) were supplied by the 2 main salmon breeding companies in Norway. Eggs were iodine disinfected before shipment or upon receipt in the hatcheries. Outbreaks of *Saprolegnia* infection were not noted in any of the hatcheries during the sampling period.

Isolation of *Saprolegnia* from hatchery water

Water samples were collected from effluent water after passing through the hatching units with incubating salmon eggs, from a total of 26 salmon hatcheries located along the coastline of Norway. The sampling sites were spread from the southernmost county Agder to the northernmost county Finnmark (see the inset map in Fig. 1 for an overview). For each water sample, 96-well microwell plates (n = 3) were filled with 100 µl of water per well. The plates were incubated in darkness at 20°C and screened for emerging colonies after 24 and 48 h. Emerging *Saprolegnia* colonies were identified and sub-cultured according to Thoen et al. (2010). From the microwell plates, 3 to 6 emerging *Saprolegnia* colonies were chosen randomly for pure culture isolation and further characterization. The selected colonies were transferred aseptically from the microwells by pipetting into Petri dishes containing glucose yeast (GY) broth (Hussein & Hatai 2002) with added streptomycin/ampicillin (200 mg l^{-1}) for propagation at 20 ± 1°C in darkness for 2 to 3 d. To induce zoosporangial formation, a sufficient amount of mycelium was rinsed in filtered, autoclaved aquarium water, cut into smaller tufts and divided into 3 replicate dishes containing the same kind of water. Whole, peeled, autoclaved hemp seeds in Petri dishes with sterile aquarium water were used to culture the isolates for observation of sexual reproduction. The dishes were kept in a refrigerator (5 ± 1°C) and in an incubator (15 ± 1°C) for 8 and 3 wk, respectively, in order to observe development of diagnostic sexual morphological characters. Single spore cultures for long-term storage were produced from zoospores collected from all isolates, and stored at 4°C on hemp seeds in sterilized aquarium water (Stueland et al. 2005).

Isolation of *Saprolegnia* from fish tissues

Saprolegnia strains were isolated from eggs and tissue samples (e.g. gills) or newly hatched larvae from 10 of the hatcheries by transferring specimens with suspected *Saprolegnia* infections to a sterile plastic bottle (sodium thiosulphate-buffered, sterile Sterilin® water sampling bottles, 500 ml volume with rubber stopper and aluminium seal) filled with tap water. The samples were sent via overnight express with cooling and were processed upon arrival at the laboratory. The tissue sample, or mycelium excised from it, was transferred to a GY agar plate with

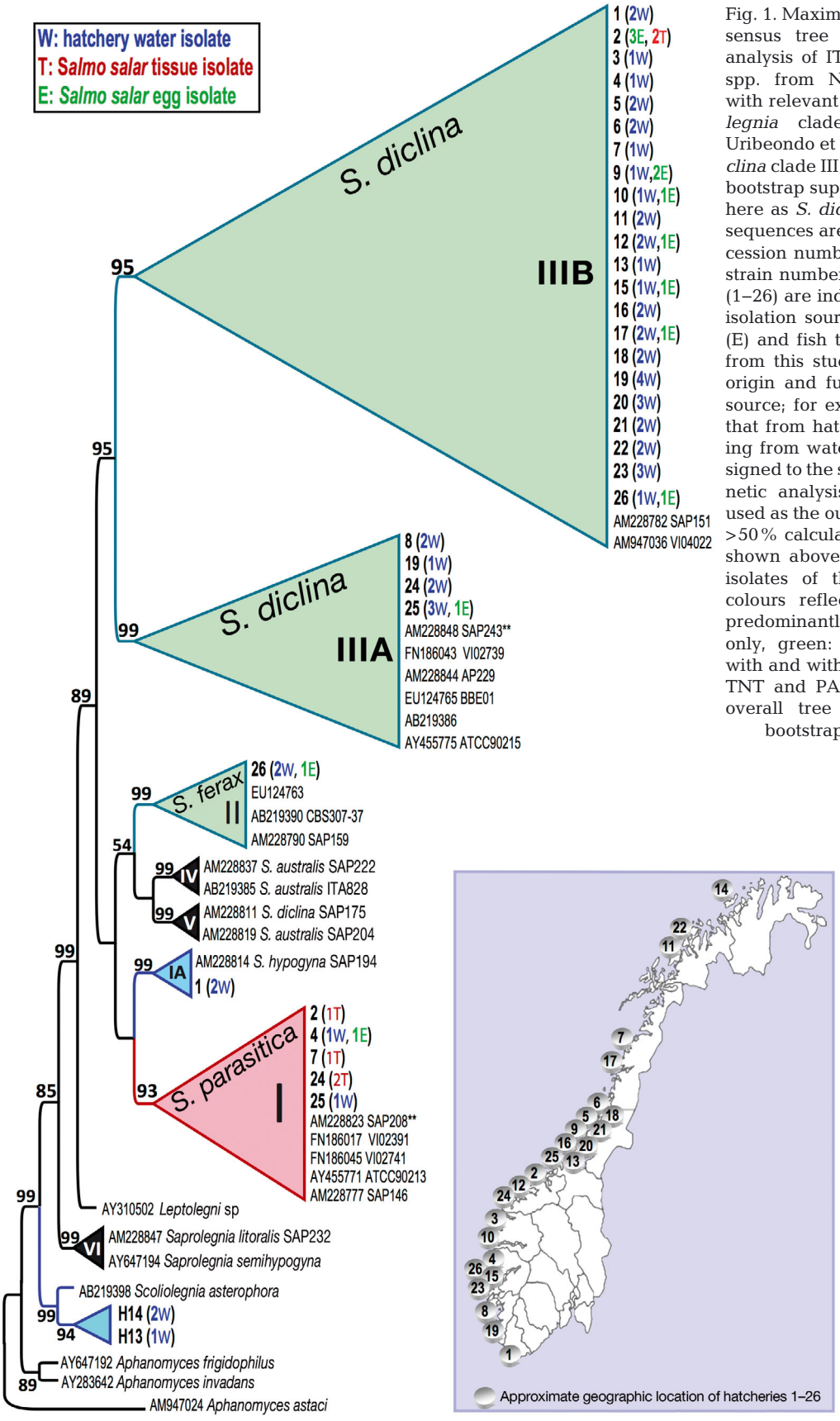

Fig. 1. Maximum parsimony bootstrap consensus tree inferred from MEGA 5.01 analysis of ITS sequences of *Saprolegnia* spp. from Norwegian hatcheries along with relevant reference sequences. *Saprolegnia* clade names follow Diéguez-Uribeondo et al. (2007) except for the *S. diclina* clade III, which was split into 2 highly bootstrap supported sub-clades referred to here as *S. diclina* IIIA and IIIB. Reference sequences are provided with GenBank accession number, species and (if available) strain number. Locations of the hatcheries (1–26) are indicated on the inset map, and isolation sources include water (W), eggs (E) and fish tissues (T). Isolate sequences from this study are assigned to hatchery origin and further connected to isolation source; for example, 25 (3W, 1E) denotes that from hatchery 25, 3 isolates originating from water and 1 from eggs were assigned to the specific clade by the phylogenetic analysis. *Aphanomyces astaci* was used as the outgroup, and bootstrap values >50% calculated from 1000 replicates are shown above branches. Clades including isolates of this study are indicated in colours reflecting isolation source (red: predominantly salmon tissues; blue: water only, green: water and eggs). Analyses with and without gaps in the tree (MEGA, TNT and PAUP) all supported the same overall tree topology with comparable bootstrap values (data not shown)

antibiotics within a sterile glass ring serving as a barrier to the growth of yeasts and potential resistant bacteria. Oomycete mycelium growing through the agar was isolated and maintained as described above. If the above described regime was insufficient to de-contaminate the culture, such isolates were propagated in GY broth with chloramphenicol (50 mg l^{-1}) in successive cycles until they were free from contaminating bacteria, and then maintained as described above. All isolates included in the present study are listed in Table 1 along with accompanying information on origin, isolation source and isolate reference number.

Morphological characterization

Morphological characterization was performed according to Seymour (1970) and Willoughby (1985). Each culture was prepared and examined in triplicate. For diagnostic morphological features of sexual reproduction, each feature was observed a minimum of 5 times per replicate (10 observations if possible). Identification of non-sexual *S. parasitica* was based on observation of long hairs on the secondary cysts, degree of indirect germination and source of isolation (Willoughby 1985, Beakes et al. 1994).

Germination rate in sterilized tap water and germination pattern (direct or indirect) were investigated for a majority of the isolates. Germination rate was examined according to Stueland et al. (2005), except that at least 50 cysts were observed in triplicates. The germination pattern was examined as described by Yuasa et al. (1997) after obtaining secondary cysts according to Fregeneda-Grandes et al. (2007). Duplicates of approximately 100 cysts were observed. Isolates of *S. ferax* and *S. hypogyna* were differentiated by observation of hypogynous antheridia produced in the latter. A number of the morphologically characterized isolates were lost during storage or could not generate satisfactory internal transcribed spacer (ITS) sequences. Such isolates were only included when sequenced and morphological identical isolates from the same hatchery were available (Table 1).

Molecular characterization

In total, 73 single-spore culture isolates of putative *S. diclina*, *S. parasitica*, *S. ferax*, *S. hypogyna* and *Saprolegnia* sp. were subjected to molecular analyses in order to compare and test the morphological determination. Genomic DNA was extracted from ~20 mg mycelia from each isolate using a CTAB miniprep extraction protocol (Gardes & Bruns 1993) according to the modifications described by Vrålstad et al. (2009), with minor adjustments. Briefly, 700 µl of CTAB buffer (20 g l^{-1} CTAB, 1.4 M NaCl, 0.1 M tris-HCl, 20 mM Na_2EDTA) was added to the material in Precellys®MK28 tubes (Bertin Technologies) before grinding, lysation and homogenizing by use of a homogenizer (Precellys®24, Bertin Technologies). The samples were then frozen (–80°C, minimum 10 min), heated (65°C, 5–10 min) and centrifuged (5 min) before proceeding with the DNA extraction procedure described above. From a few culture isolates, DNA was extracted using NucliSENS® easyMAG® (BioMérieux). Mycelia were added to 700 µl NucliSENS easyMAG lysis buffer in Precellys MK28 tubes before grinding and homogenization with Precellys as described above. The samples were then frozen (–80°C, minimum of 10 min), heated (65°C, 5–10 min) and centrifuged (5 min). The supernatants (approximately 650 µl) were transferred to the sample vessels and an additional 350 µl of NucliSENS easyMAG lysis buffer were added to the samples to a total volume of 1 ml. Genomic DNA was then isolated by use of NucliSENS easyMAG following the manufacturer's instructions.

The ITS-region was amplified using the universal primers ITS1 and ITS4 (White et al. 1990). The 25 µl reaction consisted of 1.7 µM of each primer, 2 µl of genomic DNA, PuReTaq Ready-To-Go™ PCR Beads (Amersham Biosciences) and milliQ water. PCR was performed on a DNA Engine Tetrad® Peltier Thermal Cycler (PTC-225, MJ Research) using initial denaturation (95°C, 10 min), 38 cycles of amplification (95°C for 1 min, 55°C for 45 s, 72°C for 1 min) and a final elongation (72°C for 5 min). The PCR amplicons were visualized by gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide, using pUC Mix Marker, 8, ready-to-use (19-1118 bp; Fermentas) for sizing of DNA fragments. PCR products were purified with ExoSAP-IT (Amersham Biosciences) according to the manufacturer's protocol. The products were then sequenced in both directions with their respective primers, using the The BigDye® Terminator v3.1 Ready Reaction mix (Applied Biosystems, Life Technologies). 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 sequencing PCR products were purified with a BigDye® XTerminator Purification Kit

Table 1. Overview of the origin, source and characteristics of the 89 isolates obtained. Origin: region (county) in Norway (North: Finmark, Troms, Nordland; Central: N. Trøndelag, S. Trøndelag; West: Møre og Romsdal, Sogn og Fjordane, Hordaland, Rogaland; South: Agder). VI numbers refer to the isolate ID number in the culture collection of the Norwegian Veterinary Institute. Presence or absence of sexual structures (oogonia and antheridia) is indicated. SGR: spore germination rate (%) in sterile tap water. Morphological and phylogenetic ID indicate identification based on morphological/physiological characters and phylogenetic analyses of ITS-sequences, respectively. The phylogenetic identification is based on ITS-sequence accession numbers from public sequence databases (EMBL/GenBank/DDBJ). Asterisks indicate that morphological characters were identical to sequenced isolates from the same hatchery. ND: not determined; NA: not available; s-c: sub-clade

Hatchery ID	Origin	Culture ID	Source	Sexual	Long cyst hairs	SGR	Morphological ID	Phylogenetic ID
1	Agder	VI 05423	Water	Yes	No	5	*S. hypogyna*	*S. hypogyna*
	Agder	VI 05424	Water	Yes	No	4	*S. hypogyna*	*S. hypogyna*
	Agder	VI 03804	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	Agder	VI 03805	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
2	S. Trøndelag	VI 03788	Eggs	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	S. Trøndelag	VI 03779	Eggs	Yes	No	2	*S. diclina*	*S. diclina* s-c IIIB
	S. Trøndelag	VI 03797	Eggs	Yes	No	2	*S. diclina*	*S. diclina* s-c IIIB
	S. Trøndelag	VI 03800	Gill, broodst.	No	Yes	39	*S. parasitica*	*S. parasitica*
	S. Trøndelag	VI 03796	Gill, broodst.	Yes	No	12	*S. diclina*	*S. diclina* s-c IIIB
	S. Trøndelag	VI 03780	Gill, broodst.	Yes	No	6	*S. diclina*	*S. diclina* s-c IIIB
3	Sogn og Fjordane	VI 03794	Water	Yes	No	1	*S. diclina*	*S. diclina* s-c IIIB
	Sogn og Fjordane	VI 05287	Water	Yes	No	0	*S. diclina**	NA
	Sogn og Fjordane	VI 05288	Water	Yes	No	0	*S. diclina**	NA
	Sogn og Fjordane	VI 05289	Water	Yes	No	ND	*S. diclina**	NA
4	Hordaland	VI 03787	Eggs	No	Yes	42	*S. parasitica*	*S. parasitica*
	Hordaland	VI 03777	Water	No	Yes	39	*S. parasitica*	*S. parasitica*
	Hordaland	VI 03781	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	Hordaland	VI 05446	Water	Yes	No	2	*S. diclina**	NA
5	N. Trøndelag	VI 03782	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	N. Trøndelag	VI 03792	Water	Yes	No	2	*S. diclina*	*S. diclina* s-c IIIB
	N. Trøndelag	VI 05290	Water	Yes	No	0	*S. diclina**	NA
6	N. Trøndelag	VI 03783	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	N. Trøndelag	VI 03784	Water	Yes	No	3	*S. diclina*	*S. diclina* s-c IIIB
7	Nordland	VI 03809	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	Nordland	VI 03810	Fry	No	Yes	56	*S. parasitica*	*S. parasitica*
	Nordland	VI 05284	Eggs	No	Yes	42	*S. parasitica**	NA
	Nordland	VI 05285	Fry	No	Yes	59	*S. parasitica**	NA
8	Rogaland	VI 03776	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIA
	Rogaland	VI 03778	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIA
	Rogaland	VI 05292	Water	Yes	No	0	*S. diclina**	NA
	Rogaland	VI 05293	Water	Yes	No	4	*S. diclina**	NA
9	S. Trøndelag	VI 03807	Eggs	Yes	No	3	*S. diclina*	*S. diclina* s-c IIIB
	S. Trøndelag	VI 03806	Eggs	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	S. Trøndelag	VI 03811	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
10	Sogn og Fjordane	VI 03808	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	Sogn og Fjordane	VI 03790	Eggs	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
11	Troms	VI 03799	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	Troms	VI 03791	Water	Yes	No	2	*S. diclina*	*S. diclina* s-c IIIB
	Troms	VI 05294	Water	Yes	No	1	*S. diclina**	NA
12	Møre og Romsdal	VI 03812	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	Møre og Romsdal	VI 05296	Water	Yes	No	0	*S. diclina**	NA
	Møre og Romsdal	VI 03820	Water	Yes	No	1	*S. diclina*	*S. diclina* s-c IIIB
	Møre og Romsdal	VI 03813	Eggs	Yes	No	1	*S. diclina*	*S. diclina* s-c IIIB
13	S. Trøndelag	VI 05447	Water	Yes	No	0	*S. diclina**	NA
	S. Trøndelag	VI 03814	Water	Yes	No	2	*Saprolegnia* sp.	*Scoliolegnia* sp.
	S. Trøndelag	VI 03815	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
14	Finmark	VI 03785	Water	Yes	No	0	*Saprolegnia* sp.	*Scoliolegnia* sp.
	Finmark	VI 03793	Water	Yes	No	0	*Saprolegnia* sp.	*Scoliolegnia* sp.
	Finmark	VI 05286	Water	Yes	No	2	*Saprolegnia* sp.	NA

(Table continued on next page)

Table 1 (continued)

Hatchery ID	Origin	Culture ID	Source	Sexual	Long cyst hairs	SGR	Morphological ID	Phylogenetic ID
15	Hordaland	VI 03816	Water	Yes	No	4	*S. diclina*	*S. diclina* s-c IIIB
	Hordaland	VI 03817	Eggs	Yes	No	3	*S. diclina*	*S. diclina* s-c IIIB
16	N. Trøndelag	VI 03818	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	N. Trøndelag	VI 03819	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
17	Nordland	VI 03774	Eggs	Yes	No	3	*S. diclina*	*S. diclina* s-c IIIB
	Nordland	VI 03775	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	Nordland	VI 03798	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
18	N. Trøndelag	VI 03801	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	N. Trøndelag	VI 03795	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
19	Rogaland	VI 03786	Water	Yes	No	3	*S. diclina*	*S. diclina* s-c IIIB
	Rogaland	VI 03789	Water	Yes	No	3	*S. diclina*	*S. diclina* s-c IIIB
	Rogaland	VI 03802	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	Rogaland	VI 03803	Water	Yes	No	1	*S. diclina*	*S. diclina* s-c IIIA
20	N. Trøndelag	VI 03821	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	N. Trøndelag	VI 03822	Water	Yes	No	2	*S. diclina*	*S. diclina* s-c IIIB
	N. Trøndelag	VI 03823	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
21	N. Trøndelag	VI 03824	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	N. Trøndelag	VI 03825	Water	No	No	42	*S. diclina*	*S. diclina* s-c IIIB
22	Troms	VI 05299	Water	Yes	No	0	*S. diclina**	NA
	Troms	VI 03826	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	Troms	VI 03827	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
23	Hordaland	VI 03828	Water	Yes	No	1	*S. diclina*	*S. diclina* s-c IIIB
	Hordaland	VI 03829	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	Hordaland	VI 03830	Water	Yes	No	2	*S. diclina*	*S. diclina* s-c IIIB
	Hordaland	VI 05297	Eggs	Yes	No	4	*S. diclina**	NA
24	Møre og Romsdal	VI 05413	Fry	No	Yes	66	*S. parasitica*	*S. parasitica*
	Møre og Romsdal	VI 05414	Fry	Yes	Yes	59	*S. parasitica*	*S. parasitica*
	Møre og Romsdal	VI 05295	Fry	No	Yes	61	*S. parasitica**	NA
	Møre og Romsdal	VI 05416	Water	Yes	No	4	*S. diclina*	*S. diclina* s-c IIIA
	Møre og Romsdal	VI 05417	Water	Yes	No	4	*S. diclina*	*S. diclina* s-c IIIA
25	S. Trøndelag	VI 05418	Eggs	Yes	No	7	*Saprolegnia* sp.	*S. diclina* s-c IIIA
	S. Trøndelag	VI 05419	Water	Yes	No	3	*S. diclina*	*S. diclina* s-c IIIA
	S. Trøndelag	Vi 05420	Water	Yes	No	1	*S. diclina*	*S. diclina* s-c IIIA
	S. Trøndelag	VI 05421	Water	No	No	0	*S. diclina*	*S. diclina* s-c IIIA
	S. Trøndelag	VI 05415	Water	No	Yes	59	*S. parasitica*	*S. diclina* s-c IIIA
26	Hordaland	VI 05425	Water	Yes	No	1	*S. ferax*	*S. ferax*
	Hordaland	VI 05426	Water	Yes	No	2	*S. ferax*	*S. ferax*
	Hordaland	VI 05427	Eggs	Yes	No	4	*S. ferax*	*S. ferax*
	Hordaland	VI 05435	Eggs	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB
	Hordaland	VI 05448	Water	Yes	No	0	*S. diclina*	*S. diclina* s-c IIIB

(Applied Biosystems, Life Technologies) according to the manufacturer's instructions, and subsequently analysed on an ABI PRISM® 3100 - Avant Genetic Analyzer (Applied Biosystems). Assembly and manual editing of the sequence chromatograms was conducted in BioEdit (Hall 1999).

All sequences generated in this study are available from the EMBL/GenBank sequence databases. The ITS sequences were compared to existing sequences in public sequence databases using NCBI nucleotide BLAST (Basic Local Alignment Search Tool: Altschul et al. 1997). Publicly available ITS sequences representing a selection of relevant species within the family Saprolegniaceae including the closest matches obtained through the BLAST searches were aligned in BioEdit along with the sequences of this study using the ClustalW automatic alignment option followed by manual adjustments. *Aphanomyces astaci* (AM947024) was used as the outgroup. The alignment was analysed with MEGA v.5.01 (Tamura et al. 2011) using maximum parsimony phylogeny reconstruction with all sites included and close-neighbour-interchange on random trees as the search method. The phylogeny

was tested with 1000 bootstrap replicates using the same settings. Corresponding analyses excluding gaps were also performed. Additionally, analyses were conducted in TNT version 1.1 (Goloboff et al. 2008) and PAUP* v4.0b10 (Swofford 2003) using maximum parsimony analyses with heuristic searches (1000 random addition sequences) and TBR branch swapping, saving 10 trees per replication. In the TNT analysis, gaps were included as an extra (5th) character, while in the PAUP analysis gaps were treated as missing. Standard bootstrap (Felsenstein 1985) and jackknife (Farris et al. 1996) procedures, the latter performed using 36% deletion, were conducted in TNT with 1000 replicates (traditional search) and with a cut-off value of 50%. In PAUP, bootstrap analysis was performed with 1000 bootstrap replicates using a heuristic search algorithm and 1000 random additions of sequences per bootstrap replicate.

RESULTS

Morphological characterization

In total, 89 pure culture isolates obtained from 26 different hatcheries were characterized morphologically. Four different species were recognized based on morphological and physiological characteristics: *Saprolegnia diclina, S. parasitica, S. hypogyna* and *S. ferax*. Additionally, a group of 15 isolates that could not be assigned with confidence to a species was denoted as *Saprolegnia* sp. All isolates assigned to *S. diclina* were of the presumed saprophytic type according to the morphological characterization. *S. diclina* was the most prevalent species, comprising 70 (79%) of the examined culture isolates (Table 2). Ten isolates (11%) were determined to be *S. parasitica*, 2 isolates (2%) were designated *S. hypogyna*, and 3 isolates (3%) were assigned to the species *S. erax*.

Table 2. Characterized *Saprolegnia* isolates in relation to their source

Isolate	Water	Eggs	Tissue	Total
S. diclina sub–clade IIIA	10	1	0	11
S. diclina sub–clade IIIB	46	11	2	59
S. parasitica	2	2	6	10
S. hypogyna	2	0	0	2
S. ferax	2	1	0	3
Scoliolegnia sp.	4	0	0	4
Total	66	15	8	89

Molecular characterization

The sequence alignment of ITS included 28 publicly available reference sequences of species within the family Saprolegniaceae (*Saprolegnia*, *Leptolegnia*, *Scoliolegnia*, *Aphanomyces*) and 73 sequences of putative *Saprolegnia* spp. generated in this study. The bootstrap consensus tree resulting from the Mega 5 parsimony analysis (Fig. 1) demonstrates that the vast majority (79%) of the sequenced isolates grouped within clade III of the *S. diclina* complex following the clade annotations of Diéguez-Uribeondo et al. (2007). This clade was further split into 2 strongly supported sub-clades, hereafter referred to as *S. diclina* sub-clade IIIA and IIIB (Fig. 1). Only 9 of the isolates (12%) grouped with well annotated reference sequences of *S. diclina*, such as SAP243 (AM228848) within sub-clade IIIA, while 49 of the isolates (67%) grouped in the novel sub-clade IIIB with the previously published isolates VI04022 and SAP151 (AM947036 and AM228782, respectively). These are annotated *Saprolegnia* cf. *ferax* in GenBank, but both ITS sequence similarity and phylogenetic analysis support a closer affinity to *S. diclina* than to *S. ferax*. Seven isolates grouped with the *S. parasitica* sequences and 3 isolates grouped with *Scoliolegnia asterophora* (AB219398) in a 100% bootstrap supported clade, but constituted a unique genotype sharing 98% sequence identity to *S. asterophora*. The analyses conducted with MEGA, PAUP and TNT were largely congruent, with only minor differences in the statistical bootstrap support values for the different clades (data not shown). For a subset of isolates from each clade, sequences from the GH18 domains of chitinase genes generated according to Hochwimmer et al. (2009) supported the ITS-based species identification (data not shown).

Isolated strains related to geography

When extracting isolates from the 3 main geographical salmon producing regions, viz. western, central and northern Norway, species diversity was highest in western Norway, which also had the highest total number of isolates, and lowest in northern Norway, which had the lowest total number of isolates. The frequency of *S. diclina* sub-clade IIIB was high in all regions, but higher in central than in western Norway, albeit at a low significance level ($p = 0.08$; JMP® Software). Species distributions within the geographical regions are summarized graphically in Fig. 2.

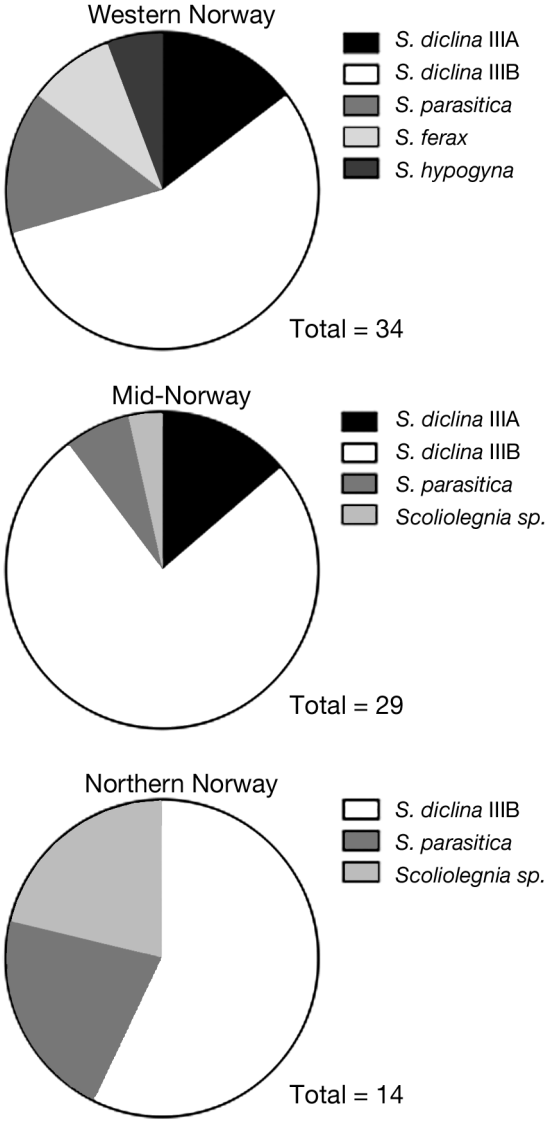

Fig. 2. Regional overview of isolates, showing that *Saprolegnia diclina* sub-clade IIIB is the dominating taxon (>50%) in all regions examined

Isolated strains related to source

A total of 66 isolates originated from water samples, 15 were isolated from eggs, and 8 were isolated from infected tissues of fry and broodfish. Isolates from tissue samples were dominated by *S. parasitica* (6 isolates), while *S. diclina* sub-clade IIIB accounted for the remaining 2 isolates. Isolates originating from eggs comprised 13 *S. diclina* and 2 *S. parasitica* (see Table 2). The isolated species related to their source of isolation are listed in Table 2.

DISCUSSION

The present study is the first extensive survey on the occurrence of *Saprolegnia* species in Norwegian salmon hatcheries. Only 4 different *Saprolegnia* spp. were isolated. The low diversity of *Saprolegnia* spp., with a clear dominance of *S. diclina* sub-clade IIIB in all geographical regions, was surprising given the number of hatcheries and their substantial geographical spread. Both Olàh & Farkas (1978), Wood & Willoughby (1986) and Maestres (1977) found greater numbers of species in single natural water locations. Also, Fregeneda-Grandes et al. (2007) identified 7 different species from 1 brown trout *Salmo trutta* hatchery in the province of Leòn, Spain. Possible explanations for the low species diversity recorded includes that the sampling was performed in the winter when minima in total spore load and species number have been reported previously (Maestres 1977, Langvad 1994). Nevertheless, if the species composition in hatchery water systems reflects the diversity in free water systems, it seems unlikely that 1 specific clade of *Saprolegnia* should be over-represented to the degree observed. We thus believe other explanations must be considered. Recently, Ali et al. (2013) showed that *Saprolegnia* spp. are able to form, live and propagate in biofilms, both experimentally and by analysing natural biofilms from fish tanks. Biofilms are believed to serve as a protective environment and provide nutrition under unfavorable conditions, and was proposed by Ali et al. (2013) as a possible explanation for recurring infections after chemical treatments. We speculate that certain competitive isolates may be able to establish and propagate in biofilm communities, and subsequently dominate spore numbers, if they are favoured by the special environmental conditions inside the plastic water pipes and indoor tanks. The relevance of this hypothesis is increasing with the rapid introduction of recirculating aquaculture systems, where spores presumably are accumulated in the system, resulting in high spore counts and potentially high infection pressures. However, these aspects are poorly explored and more in-depth studies on the ecology of different *Saprolegnia* species, including biofilm formation, in aquaculture water systems could yield knowledge of vital importance for designing combat strategies and should be welcomed.

We demonstrate a clear dominance of isolates assigned to a sub-clade of *S. diclina* type III (Diéguez-Uribeondo et al. 2007), both in hatchery water and infected salmon eggs. More specifically, the well resolved and 99% bootstrap-supported *S. diclina*

sub-clade IIIB dominated. This sub-clade has not been recognized in previous studies, and the only publicly available representatives of the group are erroneously annotated *Saprolegnia* cf. *ferax* (VI04022/AM947036 and SAP151/AM228782). Our study clearly demonstrates, both in terms of morphology and phylogeny, that these isolates are distant from *S. ferax*, and rather are assigned to the IIIB sub-clade of *S. diclina*. The ITS sequences of the 2 sub-clades of the *S. diclina* complex are separated by 10 consistent substitutions and 1 insertion/deletion over 660 base pairs, corresponding to 98% sequence identity. Whether this reflects 2 independent species or simply intraspecific ITS variation within the *S. diclina* type III awaits further molecular analyses of a wider range of genetic markers suitable for oomycete species delimitation. Parsimony analyses of the independent dataset of chitinase genes were largely congruent with the ITS phylogeny and supported 2 different genotypes of the *S. diclina* sub-clade IIIA and IIIB isolates. However, these data were not included due to presently unresolved problems with insufficient sequence accuracy that may relate to the simultaneous amplification and sequencing of the 3 slightly different homologues of chitinase genes CHI1, CHI2 and CHI3 (see Hochwimmer et al. 2009).

The congruence between morphological and molecular identification was generally good, even though a considerable number of strains could not be assigned with certainty to species level with the morphological criteria available. Especially the isolates identified to *S. diclina* displayed heterogeneous morphology in sexual structures, making confident identification difficult. However, morphological and physiological identification was satisfactory to distinguish *S. parasitica* from the other species. Of note, no presumed pathogenic isolates of *S. diclina* type I (Diéguez-Uribeondo et al. 2007) were identified. Also, the molecular analysis revealed the erroneous identification to the genus *Saprolegnia* of 4 isolates of *Scoliolegnia* sp. These expressed what seemed to be a saprolegnoid zoospore release, but could not be assigned to species level from sexual structure morphology.

Finally, since we aimed to obtain a basic overview over a broad geographic range, only a limited number of isolates per hatchery were selected for characterization. It is therefore likely that *Saprolegnia* species with low prevalence have escaped our detection.

None of the hatcheries experienced outbreaks of saprolegniosis in the sampling period of this project, which may relate to higher egg quality and increased knowledge on precautionary actions to reduce the *Saprolegnia* problem (i.e. immediate removal of infected eggs as recommended by Thoen et al. 2010). Consequently, we received only a limited number of egg and tissue samples. The observation that *S. diclina* sub-clade IIIB isolates also dominated infected eggs (73%) seems natural considering the high overall prevalence in the hatchery water systems. It is also in concordance with Thoen et al. (2011), who demonstrated through a challenge experiment that a presumed saprotrophic isolate was the single most pathogenic isolate to salmon eggs. Even though *S. diclina* is often believed to be a saprophytic species, our results may suggest that this species is responsible for a considerable portion of the eggs lost in saprolegniosis in Norway. These observations also correlate well with Fregeneda-Grandes et al. (2007), who reported an over-representation of *S. diclina* among isolates originating from brown trout eggs. In our study, *S. diclina* sub-clade IIIB was somewhat surprisingly isolated from 2 broodfish gill samples. This was likely due to the compromised immune status of the sexually mature fish. On the other hand, presumed pathogenic *S. parasitica* isolates were only found in 2 water samples, but were more frequently isolated from infected salmon tissues than other species. This may reflect the pathogenic potential of this species to post-hatching stages even when present at low prevalence, and corroborates previous findings (Hatai et al. 1990, Hatai & Hoshiai 1992, Fregeneda-Grandes et al. 2007).

In conclusion, our study revealed a limited diversity of *Saprolegnia* species present in Norwegian salmon hatcheries, dominated by saprobiotic *S. diclina* strains which are possibly adapted to the special environmental conditions found in the water systems investigated here. This aspect could be further explored by comparing water samples from the water source (prior to entering the tube system) with hatchery water. Molecular tools were of decisive importance in species determination for a number of isolates that could not be determined by morphology alone, and allowed further delimitation of *S. diclina* isolates into 2 strongly supported sub-clades. The presumed saprotrophic isolates of *S. diclina* type IIIB frequently isolated from salmon eggs may be causative agents in egg saprolegniosis. Future studies should explore this lineage in further detail, with regard to pathogenic potential, niche preferences and species delimitation.

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