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RESEARCH ARTICLE

Identification and genetic characterization of *Saprolegnia parasitica*, isolated from farmed and wild fish in Finland

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Abstract

Oomycete infections in farmed fish are one of the most significant disease issues in salmonid aquaculture worldwide. In the present study, *Saprolegnia* spp. in different farmed fish species in Finland were identified, and the molecular epidemiology of especially *Saprolegnia parasitica* was examined. We analysed tissue samples from suspected oomycete-infected salmonids of different life stages from a number of fish farms, as well as three wild salmonids. From collected oomycete isolates, the ITS1, 5.8S and ITS2 genomic regions were amplified, analysed phylogenetically and compared with corresponding sequences deposited in GenBank. Of the sequenced isolates, 91% were identified as *S. parasitica*. Isolates of yolk sac fry were identified as different *Saprolegnia* spp. Among the isolates from rainbow trout eggs *Saprolegnia diclina* dominated. In order to determine potential dominating clones among the *S. parasitica*, isolates were analysed using Multi Locus Sequence Typing (MLST). The results showed that one main clone contained the majority of the isolates. The MLST analysis showed four main sequence types (ST1–ST4) and 13 unique STs. This suggests that the *Saprolegnia* infections in farmed fish in Finland are not caused by different strains originating in the farm environment. Instead, one main clone of *S. parasitica* is present in Finnish fish farms.

KEYWORDS
farmed fish, MLST, oomycetes, *Saprolegnia parasitica*

1 | INTRODUCTION

Saprolegnia has worldwide been involved in mass mortalities of fish, both in aquaculture and in nature (van den Berg et al., 2013). Mortality caused by various *Saprolegnia* species has resulted in significant losses in fish farms in Finland and globally since the ban of malachite green (in the European Union in 2000) which was an efficient treatment of saprolegniosis. Compounds against *Saprolegnia* available at the moment are scarce and low in efficacy, making the treatment of this disease problematic.

Saprolegnia is a genus within the class of oomycetes, commonly found in fresh water. Current taxonomy identify *Saprolegnia* as a genus of the family Saprolegniaceae in the order Saprolegniales, class Oomycetes. Oomycetes are mycelium-forming organisms that resemble fungi but are more closely related to brown algae and diatoms (Baldauf et al., 2000). Some of the oomycetes, among

them *Saprolegnia* sp., are significant fish pathogens (Roberts, 2012). However, it has been shown that there is a significant difference in pathogenicity between different species of *Saprolegnia* (e.g. Fregeneda-Grandes et al., 2001). From a diagnostic point of view it is therefore of importance to identify the *Saprolegnia* strains involved in disease outbreaks. To determine whether the infection is caused by a specific strain, it is useful to gain more knowledge on infection strategies and host/agent relationships.

Saprolegnia causes saprolegniosis, involving infections of the skin and gills of fish, forming a characteristic white or grey cotton wool like mesh. *Saprolegnia* multiplies on the fish by branching hyphae and spreads into the water mainly via zoospores (Roberts, 2012). The infection usually starts on the head or on the fins and then spreads through branching mycelium in circles or curvilinear patterns throughout the body. In worst cases, the infection kills cells and destroys the skin, which becomes completely damaged. Finally, the infection spreads further into muscle tissue and blood vessels, consequently causing advanced infections that are incurable. *Saprolegnia parasitica* has been found to possess the largest number of proteases found in any eukaryotic organism, some of which can suppress the immune responses of the fish (Jiang et al., 2013). Since there is currently no effective cure for the advanced disease, fish die from haemodilution caused by osmotic collapse due to large skin losses. There is no evidence that *Saprolegnia* causes poisoning or other systemic disease (Roberts, 2012). Also yolk sac fry and eggs can be infected (Roberts, 2012).

Saprolegnia outbreaks are mainly associated with poor water quality and stress (Neish, 1977). Frequently, outbreaks occur when the water temperature drops, partly because of the decline in fish immune responses and reduced mucus production, and partly because *Saprolegnia* activity appears to increase in colder waters (Bly et al., 1992). In addition, also high-water temperature stress may induce *Saprolegnia* invasion (Roberts, 2012).

Traditionally, the identification of *Saprolegnia* spp. has been based on morphology of sexual structures and zoospores (Stueland et al., 2005; Willoughby, 1985). Based on the morphology it was considered that the genus *Saprolegnia* consists of 17–19 (Johnson Jr et al., 2002; Seymour, 1970) different species. Subsequently, molecular methods, like DNA sequencing have been used for species identification. Analyses of internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA) sequences are useful means for taxonomic and phylogenetic studies (Diéguez-Uribeondo et al., 2007). Sandoval-Sierra, Martín, and Diéguez-Uribeondo (2014) identified 29 DNA-based species of *Saprolegnia*. According to the same authors, DNA databases like GenBank have a number of miss-assigned names of DNA sequences (Sandoval-Sierra & Diéguez-Uribendeondo, 2015; Sandoval-Sierra, Martín, & Diéguez-Uribeondo, 2014). Using the ITS nrDNA they found, that *Saprolegnia salmonis* and *Saprolegnia hypogyna* do not differ genetically from isolates of *Saprolegnia parasitica*, and these seem to be specimens of the same taxon.

The species of primary importance for the infection of fish is *Saprolegnia parasitica* (De la Bastide et al., 2015). In addition, other species are pathogenic for fish, like *S. diclina*, *S. ferax*, and *S. delica*, which are implicated in infecting fish eggs and fry (Sandoval-Sierra, Latif-Eugenin, et al., 2014). In an earlier study, it was observed that *S. diclina* clearly dominated in Norwegian salmon hatcheries (Thoen et al., 2015). The epidemiological situation of oomycete infections in Finnish fish farms has been sparsely studied; it is not clear if one *Saprolegnia* species is dominating or if several different species or strains are involved in the disease outbreaks. To date, in Finland and Sweden, there is one previous study where a *Saprolegnia* sp clone was characterized using both morphological and genetical methods (Bangyeekhun et al., 2003).

The aim of this study was to identify *Saprolegnia* spp. present in different farmed fish species in Finland, by phylogenetic analysis of the ITS region of collected samples of oomycete-infected fish. In order to determine molecular epidemiology and potential dominating clones, the heterogeneity of the *Saprolegnia* samples was analysed with Multi Locus Sequence Typing (MLST). To obtain a representative overview of the *Saprolegnia* types in Finnish fish farms, we analysed samples from oomycete-infected fish of different life stage from a number of hatcheries and fish farms. In addition, three wild fish with oomycete infection were investigated.

2 | MATERIALS AND METHODS

2.1 | Sampling from infected fish

Tissue samples were collected from oomycete-infected fish from 21 different locations in Finland (19 fish farms and 2 sites in nature), the majority of samples came from salmonid farms in the eastern and northern parts of the country. The samples from the wild fish came from Lake Kallavesi in Eastern Finland and from Torne River, the border river between Finland and Sweden. Farmed fish included rainbow trout (*Oncorhynchus mykiss*) (33%), whitefish (*Coregonus lavaretus*) (29%), Atlantic salmon (*Salmo salar*) (17%), trout (*Salmo trutta*, *Salmo trutta lacustris*, *Salmo trutta trutta*) (18%), landlocked salmon (*Salmo salar sebago*) (2%) and brook trout (*Salvelinus fontinalis*) (1%). The wild fish included two Atlantic salmon and one lake trout (Table 1). Samples were also collected from rainbow trout and landlocked salmon yolk sac fry and from rainbow trout eggs (Table 2). Infected fish were transported to the laboratory, where samples were collected. Upon arrival, the fish and yolk sac fry were inspected for signs of oomycete infection, and infected areas on eyes, fins, gills, skin and tail were swabbed or excised and rinsed with 70% ethanol and MilliQ water. The swabs or excised piece of infected tissue were inoculated on peptone-glucose (PG-1) agar (Unestam, 1965) containing 10 μg/mL of ampicillin (AMP, Sigma-Aldrich, A-9518) and 10 μg/mL oxolinic acid (OA, Santa Cruz Biotechnology, sc-212,488) (Alderman & Polglase, 1986). Individual eggs were placed on a PG-1 agar plate. The agar plates were incubated at 20°C and inspected for growth of oomycete hyphae. In order to purify the oomycete cultures from potential bacterial contaminants, the cultures were re-cultivated in succession every 2–3 days on nutrient-free (NF) agar

plates, a nutrient-poor medium, containing 1% agar in mQ-H_2O and 10 μg/mL of AMP and 10 μg/mL OA.

2.2 | Single-spore isolation of oomycete cultures

Preparation of single-zoospore monocultures of the oomycete isolates was done by culturing the samples on NF or PG-1 agar plates. The bacteria-free oomycete cultures were punctured with the top of a sterile glass Pasteur pipette and the resulting agar culture pellets (Ø about 5 mm) were transferred into separate wells in a 24-well microtiter plate containing 1 mL of sterile glucose yeast extract broth (GYB) (1% glucose, 0.25% yeast extract, and 10 μg/mL of AMP and 10 μg/mL OA). The plates containing the pellets were incubated overnight at 20°C. The broth was then removed, and the pellet was washed twice with filtered and autoclaved lake water (FALW), and then left submerged in 1 mL of FALW overnight at 20°C. The wells were examined under a microscope for the presence of zoospores. Any wells lacking zoospores were incubated in new FALW at 20°C for a further 24 h, after which the isolation process was completed. From each well, 100 μL of zoospore-containing suspension was 10-fold diluted (up to 1:1000) until an adequate concentration of zoospores was reached for single-spore isolation. The dilutions were then pipetted in drops (10 μL) onto PG-1 agar plates and incubated at room temperature overnight.

After incubation, the PG-1 plates were examined under a stereo microscope to detect the presence of single germinating zoospores, of which one was isolated and re-cultivated on PG-1 agar plates. The plates were checked to confirm the successful re-cultivation and growth of hyphae after 24 h. Isolated oomycete cultures were used for DNA isolation, sequencing and subsequent identification and characterization.

The monocultures were preserved on hemp seed cultures in sterile glass tubes covered with sterile mineral or paraffin oil, or in 1 mL of sterile tap water in a 96-well microtiter plate. The tubes and sealed plates were stored at 6°C, to keep oomycete monocultures viable for up to 16 months (Stueland et al., 2005).

2.3 | DNA extraction

The agar of the monoculture with hyphae was punctured using the top of a sterile glass Pasteur pipette, and the resulting pellet was transferred to a tube containing GYB and incubated at 20°C for 3–7 days. The broth culture containing hyphae was then collected in its entirety into a sterile 1.5 mL microcentrifuge tube and stored at −20°C until the DNA extraction could proceed.

The oomycete hyphae were washed with 1 mL FALW by centrifugation at 14,000×*g*, and the DNA was extracted using the MasterPure™ Gram Positive DNA Extraction Kit (Lucigen, MGP04100) according to the Gram-Positive DNA Purification Protocol. For the lysozyme incubation step of the purification process, the samples were incubated over night at 37°C. The purified DNA was used for amplification of the ITS region of nrDNA and subsequent identification of the isolates.

2.4 | Amplification and sequencing of internal transcribed spacer regions

For amplification of the ITS1, 5.8S and ITS2 genomic regions of the isolates, the universal primers for eukaryotes nu-SSU-1766 (ITS5) (forward) and nu-LSU-0041 (ITS4) (reverse) were used (White et al., 1990).

Each PCR reaction (20 μL) contained: 12.2 μL sterile mQ-H_2O; 4 μL 5× Phire Reaction Buffer (Thermo Scientific, F-524); 1 μL of each forward and reverse primers; 0.4 μL dNTP Mix (Thermo Scientific or Fermentas, #R0192); 0.4 μL Phire Hot Start II DNA Polymerase (Thermo Scientific, F-122S); and 1 μL purified DNA. Amplifications were performed on an Artik Thermal Cycler (Finnzymes) with initial denaturation at 98°C for 30 s, followed by 35 cycles of 98°C for 5 s, 56°C for 5 s, 72°C for 10 s and a final extension at 72°C for 60 s.

The PCR products were visualized by gel electrophoresis on 1% agarose gel stained with Midori Green Advanced DNA Stain (Nippon Genetics Europe, MG 04).

The amplified PCR products were sequenced from both ends using BigDye v3.1 chemistry and ABI3130xl capillary sequencer (Thermo Scientific).

2.5 | Identification of oomycete isolates and phylogenetic analysis

For identification of the isolates, the obtained ITS nrDNA sequences were subjected to a BLASTn (NCBI) query search and compared with sequences deposited in GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/). To confirm identification at species level, isolates identified to Saprolegniaceae family, were further subjected to phylogenetic analysis.

The ITS nrDNA sequences of the isolates collected in this study and reference sequences of closely related species were merged to construct a phylogenetic tree. Reference sequences were retrieved from GenBank, and the correct identification of the reference sequences used was confirmed and revised according to Sandoval-Sierra, Martín, and Diéguez-Uribeondo (2014). The reference sequences used are listed as follows with Sandoval-Sierra, Martín, and Diéguez-Uribeondo (2014) species name and accession numbers in parentheses: *S. australis* SAP0218 (AM228833), *S. australis* SAP0212 (AM228827.1), *S. diclina* SAP0229/ATCC5685 (KF717744.1), *S. delica* SAP0353 (KF718021), *S. delica* SAP1524 (KF718043), *S. ferax* SAP0157 (KF717883), *S. ferax* SAP1234 (KF717954), *Saprolegnia* sp. (FN186033.1), *Saprolegnia* sp. SAP708 (KF386721.1), *Saprolegnia* sp. SAP0433 (KF718050.1), *S. torulosa* SAP0856 (KF718125), *S. torulosa* SAP0161 (MK850043.1), *Leptolegnia* sp. SAP0772 (KF718185.1), *Saprolegnia* sp. SAP1563 (KF748556.1), *Saprolegnia* sp. SAP1570 (KF748562.1). *Saprolegnia* sp. SAP1165 (KF718174.1), *Saprolegnia* sp. SAP1536 (KF718176.1), *S. parasitica* SAP0171 (AM228804),

Fish species	Contributing farms	Fish	Isolates	Sequenced isolates	Saprolegnia parasitica
Rainbow trout	7	42	42	38	30
Sea trout	2	4	4	4	4
Lake trout	3	19‡	19	13	13
Whitefish	6	36	44	36	35
Atlantic salmon	3	22‡‡	24	17	17
Landlocked salmon	2	2	2	2	2
Brook trout	1	1	1	1	0
Total number		126	136	111	101

TABLE 1 Examined fish species, number of farms that contributed with samples, number of examined fish, total number of oomycete isolates, number of ITS-sequenced isolates, and number of *Saprolegnia parasitica* isolates, ‡ includes one wild lake trout, and ‡‡ two wild Atlantic salmon.

TABLE 2 Rainbow trout egg, rainbow trout and landlocked salmon yolk sac fry, number of farms that contributed with samples, total number of oomycete isolates, number of ITS sequenced isolates, and number of *Saprolegnia diclina* and *Saprolegnia parasitica* isolates.

	Contributing farms	Isolates	Sequenced isolates	*Saprolegnia diclina*	*Saprolegnia parasitica*
Egg	2	18	18	8	2
Yolk sac fry	2	7	7	0	2

TABLE 3 Primers used in the MLST scheme.

Gene	Product	Primer 5′ – 3′	Expected fragment length with overhangs in PCR (bp)
ALTS1	Alanyl-tRNA Synthetase	Fwd CTACTTCCAGCAGCAYGAGCA Rev GACAAGGTTCCARAGCTCC	634
COX1	Cytochrome c oxidase Subunit 1	Fwd ACCTGGAAATCAAATTT TTATGGG Rev ATCACCTCCACCTGAAGGATCA	626
GLUT	Glutaminase	Fwd GGAGCGGCAGTCCATCAATC Rev CGTCGACGGTGCACATGGAG	619
NAD1	NADH dehydrogenase Subunit 1	Fwd CCTAATGTTGTAGGTAC TTTTGG Rev GAAACTAATTCAGCTTCAGCTT	610
RPB2	RNA polymerase II Subunit B	Fwd TCCAAAAGTGCGTCGACGC Rev CGACACTTCGGCGTCAATGT	590
SHMT	Serine hydroxy-methyltranferase	Fwd CAAGCCGCTCAAGGAGAC Rev CGTGTCRTAGTCGATCAAGC	563
TUBB	Beta tubulin	Fwd CCAGCTCGTCGAAAACGC Rev CTTGAACATCTCCTGGATCG	517

Note: Specific primers were modified from Ravasi et al., 2018 with the addition of Illumina TruSeq overhangs: Overhang added to all the forward primers: 5′ ACACTCTTTCCCTACACGACGCTCTTCCGATCT- specific forward primer. Overhang added to all the reverse primers: 5′ GTGACTGGAGT TCAGACGTGTGCTCTTCCGATCT- specific reverse primer.

S. parasitica SAP0196 (AM228815), *S. parasitica* SAP0107 (AM228744). *Pythium insidiosum* isolate 65 (AY151157.1) was used as a root in the phylogenetic analysis. The sequences were aligned using ClustalW in Molecular Evolutionary Genetics Analysis (MEGA X) (Kumar et al., 2018). Maximum Likelihood (ML) method with Kimura-2 parameter model (Kimura, 1980) was used to analyse the sequences. The tree was built using 1000 bootstrap replications and the Saprolegnicaeae isolates were assigned to species based on molecular clustering with reference sequences.

2.6 | Multi-locus sequence typing scheme and settings

The isolates that were assigned as *Saprolegnia parasitica* were further analysed with a MLST scheme using sequence fragments of seven housekeeping genes (Ravasi et al., 2018) in order to distinguish clones. *S. parasitica* isolates obtained from the eggs were not included in this analysis. The genes used in the PCR amplifications were: ALTS1 (alanyl-tRNA synthetase), COX1 (cytochrome c

oxidase subunit 1), GLUT (glutaminase), NAD1 (NADH dehydrogenase subunit 1), RPB2 (RNA polymerase II subunit B), SHMT (serine hydroxymethyltransferase) and TUBB (beta tubulin). The amplified gene fragments were tuned for paired-end sequencing using Illumina MiSeq. The primer sequences with the attached overhang adapter sequences (for the region to be targeted) used are listed in Table 3.

Each PCR reaction (20 μL) contained: 12.4 μL sterile mQ-H_2O; 4 μL 5× Phusion HF Buffer (Thermo Scientific); 1 μL of each forward and reverse primers; 0.4 μL dNTP Mix (Thermo Scientific or Fermentas); 0.2 μL Phusion Hot Start II DNA Polymerase (Thermo Scientific); and 1 μL purified DNA. Amplifications were performed with initial denaturation at 98°C for 30 s, followed by 34 cycles of 98°C for 10 s, 62°C for 20 s, 72°C for 20 s and a final extension at 72°C for 5 min.

After PCR unused primers and nucleotides were removed by adding Exonuclease I (14 U) and FastAP Thermosensitive Alkaline Phosphatase (0.25 U) (Thermo Scientific) to the reactions, followed by incubation 30 min at 37°C and 5 min at 95°C to inactivate the enzymes.

A second PCR step was performed to introduce full-length adapters and selected indexes for Illumina sequencing. The indexes were selected using Barcosel (Somervuo et al., 2018).

The PCR reaction was performed using 9 pmol of respective index primers and 1–3 μL template from the first PCR reaction as described above except that the reaction volume was 50 μL, elongation time 1 min and cycles 18. The obtained PCR products were pooled, purified using MagSi-NGSPREP Plus magnetic beads (Magtivio) according to the manufacturer's protocol and checked on Fragment Analyser (Agilent Technologies). The pool was paired-end sequenced using an Illumina MiSeq and 300 cycle kit v3 (Illumina). Cutadapt (Martin, 2011) was used for adapter and quality trimming. The obtained paired-end reads were merged using FLASH (Magoč & Salzberg, 2011).

2.7 | MLST analysis

Polymorphic nucleotide sites were identified within multiple alignments for each locus separately in the R-environment (R Core Team et al., 2021) using the match-function comparing each row (as nucleotide sequence) against all other sequences in the data set. Each unique allelic variant of the six genes studied was assigned a different arbitrary number. Subsequently, haploid sequence types (ST) were assigned to each unique combination of alleles. Samples with missing sequences for one or several of the genes studied were discarded from the analysis. In total, 93 of 98 samples were included in downstream analysis. The ST-data was used as input for creating a minimum spanning tree using the full eBURST analysis in Phyloviz v. 2.0 (Francisco et al., 2012) predicting the most parsimonious patterns of descent of all isolates in each clonal complex (Feil et al., 2004). All isolates included in a ST were identical at the studied loci. Isolates assigned to STs with a link were single locus variants (SLVs) while unlinked isolates differ at ≥2 loci.

3 | RESULTS

3.1 | Oomycete isolates

A total of 136 oomycete isolates were obtained from farmed and wild adult fish. In addition, 18 isolates were obtained from eggs, and seven isolates from yolk sac fry. Of the total 161 isolates from adult fish, yolk sac fry and eggs together, 111 isolates from adult fish and all of the isolates from yolk sac fry and eggs were sequenced and identified by their ITS nrDNA sequence (Tables 1 and 2).

BLASTn (NCBI) query showed that different *Saprolegnia* species were present in 107 (96%) of the examined adult fish. Of the sequenced isolates, the wild fish isolates included, 101 (91%) were identified as *Saprolegnia parasitica* (Table 1). Other oomycete species included *Saprolegnia australis* (one isolate), *S. diclina* (one isolate), *S. torulosa* (one isolate) and different *Saprolegnia* sp (two isolates). Four isolates were identified as different *Fungus* or *Phoma* spp.

The seven isolates from yolk sac fry (Table 2) were identified as *Saprolegnia parasitica* (two isolates), *S. ferax* (two isolates) and *Saprolegnia* sp (three isolates).

Of the 18 isolates from rainbow trout eggs (Table 2), eight were identified as *Saprolegnia diclina*, two as *S. parasitica*, five isolates as different *Saprolegnia* sp, one isolate as *Leptolegnia* sp and two isolates as *Pythium* sp.

3.2 | Phylogenetic analysis

The taxonomy of the ITS nrDNA sequence of the examined isolates were confirmed with a Maximum Likelihood (ML) tree (Figure 1). The phylogenetic analysis included 123 of the collected sequences and 21 related reference sequences. Of all the isolates in this study, 100 isolates were assigned as *S. parasitica* with 98% bootstrap support. Other members of the family Saprolegniaceae identified included, *S. australis* (one isolate), *S. diclina* (9 isolates), *S. ferax* (2 isolates), *S. torulosa* (one isolate) and *Leptolegnia* sp (one isolate). Ten of the isolates could not be assigned as any known *Saprolegnia* sp., but these were assigned as reference sequences currently named *Saprolegnia* sp. in two different tree branches (Figure 1).

3.3 | MLST

The sequences for the gene ALTS1 for all examined isolates contained one intron in the forward sequence and two introns in the revers sequence. As a consequence of this a sequence of 100 base pairs were missing and the forward and reverse sequences could not be aligned, and this gene was therefore excluded from the analysis.

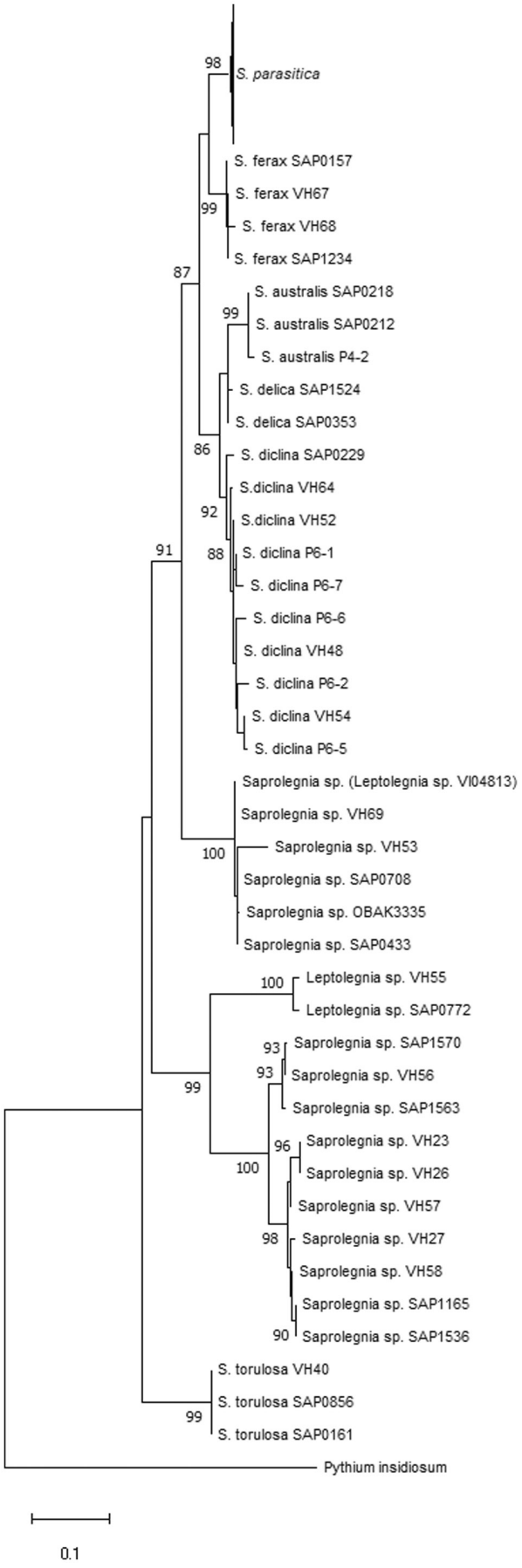

FIGURE 1 Phylogenetic tree of ITS nrDNA sequences from *Saprolegnicaeae* isolates obtained from Finnish fish farms affected by saprolegniosis and reference sequences. Reference sequences were retrieved as closely related sequences from GenBank and confirmed as saprolegnia sequences according to Sandoval-Sierra, Martín, and Diéguez-Uribeondo (2014). The tree was constructed using Maximum Likelihood method. The branch lengths in the tree are measured as the number of substitutions per site. The numbers beside the branches represent the bootstrap value (>80).

In total 93 *Saprolegnia* isolates were included in the analysis (Table 4). The minimum spanning tree visualizes the genetic relatedness between isolates and host species (Figure 2) and their geographic location (Figure 3), resulting in four main sequence types (ST1–ST4, $n = 69$) and 13 unique STs (differ in the sequence of at least one gene out of the examined six) that were observed only once in this study. Additionally, some STs included 2–4 isolates. In general, most isolates were included in the main clonal complex, CC-ST1 ($n = 89$), suggesting they are closely related. In addition, four isolates formed singletons, not associated with the CC-ST1.

A clear association between fish species and ST could not be observed (Figure 2). However, the two isolates from rainbow trout fry were identical and formed a clearly separate ST (ST7) from the CC-ST1.

Neither could a clear association between geographical location and ST (Figure 3) be detected.

Of the total number of fish, 35% were infected with ST1 (37% of examined whitefish, 43% of rainbow trout, and 42% of lake trout). Of examined Atlantic salmon 33% were infected with ST2, while 16% of the total number of fish were infected with ST2, 15% with ST3, 8% with ST4, 4% with ST5, 3% with ST6 and 2% with ST8. Of the three isolates from the wild fish, the lake trout isolate belonged to ST1 and the two Atlantic salmon isolates belonged to ST6. The two rainbow trout yolk sac fry isolates that were assigned as *S. parasitica* had a unique ST (ST7) not related to the main clonal complex.

4 | DISCUSSION

Despite the increasing presence of saprolegniosis in Finnish fish farms, causing devastating infections in the fish populations, the specific pathogen suspected to cause this disease has not previously been studied in detail.

In this study we describe for the first time which oomycete species are present in farmed fish in Finland and in different life stages of the fish. Furthermore, we reveal the molecular epidemiology of the most abundant *Saprolegnia* species causing infections. By sequencing the ITS nrDNA region of the examined isolates and from the results from the phylogenetic analysis, we show that *Saprolegnia parasitica* infected the majority of the investigated salmonids (Table 1 and Figure 1). Other *Saprolegnia* species, isolated from the infected fish, included *S. australis*, *S. diclina*, *S. torulosa*, *S. ferax* and *Saprolegnia* sp. The affected salmonids included Atlantic salmon, lake trout, landlocked salmon, rainbow trout, sea trout and whitefish,

TABLE 4 In total 21 different sequence types (STs) were identified from Finnish fish farms.

Sample	ST	COX1	GLUT	NAD	RPB2	SHMT	TUBB	Region	Host
P12-3	1	1	3	3	4	3	4	IS	Lake trout
P12-8	1	1	3	3	4	3	4	IS	Lake trout
P13-3	1	1	3	3	4	3	4	PS	Lake trout
P13-6	1	1	3	3	4	3	4	PS	Lake trout
VH28	1	1	3	3	4	3	4	WILD	Lake trout
P10-2	1	1	3	3	4	3	4	IS	Rainbow trout
P10-3	1	1	3	3	4	3	4	IS	Rainbow trout
P10-7	1	1	3	3	4	3	4	IS	Rainbow trout
VH15	1	1	3	3	4	3	4	IS	Rainbow trout
VH30	1	1	3	3	4	3	4	IS	Rainbow trout
VH31	1	1	3	3	4	3	4	IS	Rainbow trout
VH46	1	1	3	3	4	3	4	IS	Rainbow trout
VH4	1	1	3	3	4	3	4	IS	Rainbow trout
VH63	1	1	3	3	4	3	4	IS	Rainbow trout
VH78	1	1	3	3	4	3	4	IS	Rainbow trout
VH79	1	1	3	3	4	3	4	IS	Rainbow trout
P4-6	1	1	3	3	4	3	4	LS	Rainbow trout
P17-2A	1	1	3	3	4	3	4	PS	Atlantic salmon
P23-4	1	1	3	3	4	3	4	PS	Atlantic salmon
P23-6	1	1	3	3	4	3	4	PS	Atlantic salmon
OBAK3255	1	1	3	3	4	3	4	PS	Sea trout
P2-7A	1	1	3	3	4	3	4	ES	Whitefish
VH13	1	1	3	3	4	3	4	IS	Whitefish
VH82	1	1	3	3	4	3	4	IS	Whitefish
VH86	1	1	3	3	4	3	4	IS	Whitefish
P4-1	1	1	3	3	4	3	4	LS	Whitefish
P16-1A	1	1	3	3	4	3	4	PS	Whitefish
P16-1B	1	1	3	3	4	3	4	PS	Whitefish
P24-1	1	1	3	3	4	3	4	PS	Whitefish
P24-3	1	1	3	3	4	3	4	PS	Whitefish
P24-4	1	1	3	3	4	3	4	PS	Whitefish
P24-9	1	1	3	3	4	3	4	PS	Whitefish
P13-5	2	1	3	3	4	3	2	PS	Lake trout
OBAK1080	2	1	3	3	4	3	2	PS	Landlocked salmon
VH29	2	1	3	3	4	3	2	IS	Rainbow trout
VH5	2	1	3	3	4	3	2	IS	Rainbow trout
VH62	2	1	3	3	4	3	2	IS	Rainbow trout
P17-3	2	1	3	3	4	3	2	PS	Atlantic salmon
P17-4	2	1	3	3	4	3	2	PS	Atlantic salmon
P23-10	2	1	3	3	4	3	2	PS	Atlantic salmon
P23-2	2	1	3	3	4	3	2	PS	Atlantic salmon
P23-5	2	1	3	3	4	3	2	PS	Atlantic salmon
P2-7C	2	1	3	3	4	3	2	ES	Whitefish
P2-7E	2	1	3	3	4	3	2	ES	Whitefish
P11-5	2	1	3	3	4	3	2	IS	Whitefish

(Continues)

TABLE 4 (Continued)

Sample	ST	COX1	GLUT	NAD	RPB2	SHMT	TUBB	Region	Host
P11-6	2	1	3	3	4	3	2	IS	Whitefish
VH12	2	1	3	3	4	3	2	IS	Whitefish
P13-4	3	1	2	3	4	3	4	PS	Lake trout
VH10	3	1	2	3	4	3	4	IS	Landlocked salmon
P10-4	3	1	2	3	4	3	4	IS	Rainbow trout
VH16	3	1	2	3	4	3	4	IS	Rainbow trout
VH61	3	1	2	3	4	3	4	IS	Rainbow trout
VH80	3	1	2	3	4	3	4	IS	Rainbow trout
P17-6	3	1	2	3	4	3	4	PS	Atlantic salmon
VH11	3	1	2	3	4	3	4	IS	Whitefish
VH83	3	1	2	3	4	3	4	IS	Whitefish
VH85	3	1	2	3	4	3	4	IS	Whitefish
VH87	3	1	2	3	4	3	4	IS	Whitefish
P4-5	3	1	2	3	4	3	4	LS	Whitefish
P16-8	3	1	2	3	4	3	4	PS	Whitefish
P24-10	3	2	3	4	3	4	3	PS	Whitefish
P12-6	4	1	2	3	4	3	2	IS	Lake trout
VH45	4	1	2	3	4	3	2	IS	Lake trout
VH3	4	1	2	3	4	3	2	IS	Rainbow trout
VH44	4	1	2	3	4	3	2	IS	Rainbow trout
VH59	4	1	2	3	4	3	2	IS	Rainbow trout
VH60	4	1	2	3	4	3	2	IS	Rainbow trout
P17-7	4	1	2	3	4	3	2	PS	Atlantic salmon
P16-3	4	1	2	3	4	3	2	PS	Whitefish
P12-4	5	1	3	3	5	3	4	IS	Lake trout
VH39	5	1	3	3	5	3	4	IS	Rainbow trout
P23-7	5	1	3	3	5	3	4	PS	Atlantic salmon
P24-6	5	1	3	3	5	3	4	PS	Whitefish
P10-12	6	1	2	3	3	3	4	IS	Rainbow trout
VH181	6	1	2	3	3	3	4	WILD	Atlantic salmon
VH182	6	1	2	3	3	3	4	WILD	Atlantic salmon
VH70	7	2	6	1	7	7	5	IS	Rainbow trout, fry
VH71	7	2	6	1	7	7	5	IS	Rainbow trout, fry
P16-2A	8	1	2	3	4	2	2	PS	Whitefish
P24-2	8	1	2	3	4	2	2	PS	Whitefish
P10-10	9	1	3	3	2	3	4	IS	Rainbow trout
OBAK993	10	1	3	3	3	3	4	PS	Atlantic salmon
P10-1	11	1	3	3	4	4	4	IS	Rainbow trout
P11-2	12	1	3	3	4	3	8	IS	Whitefish
P12-1	13	1	2	3	4	3	8	IS	Lake trout
P12-7	14	1	3	3	4	3	7	IS	Lake trout
P17-1	15	1	2	3	4	3	6	PS	Atlantic salmon
VH81	16	1	7	4	4	2	2	IS	Rainbow trout
OBAK1057	17	1	3	3	5	2	4	PS	Sea trout
OBAK1817	18	1	2	3	5	3	3	PS	Sea trout

TABLE 4 (Continued)

Sample	ST	COX1	GLUT	NAD	RPB2	SHMT	TUBB	Region	Host
P24-8	19	1	2	3	5	3	4	PS	Whitefish
OBAK1056	20	1	2	3	2	3	2	PS	Sea trout
P11-4	21	3	4	3	4	5	4	IS	Whitefish

Note: They are presented here for each sample separately and with information about the host and geographic area (IS = Eastern Finland, PS = Northern Finland, LS = Western Finland, ES = Southern Finland).

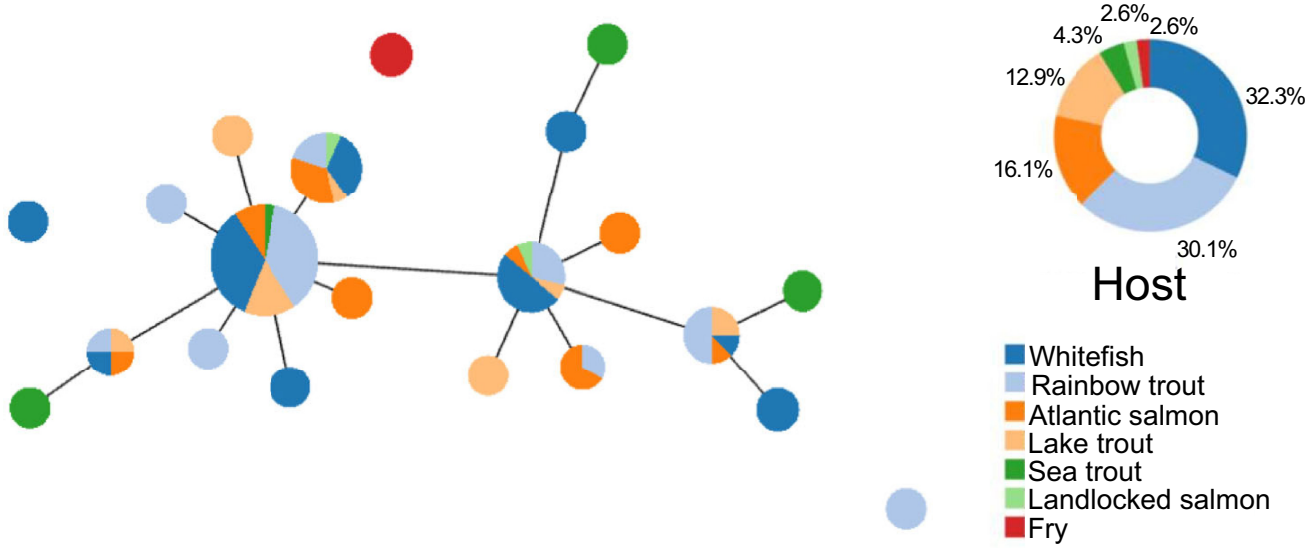

FIGURE 2 Sequence types (1–21) of *S. parasitica* isolates from different fish farms coloured by fish species from which they were isolated. The size of each node represents the number of samples included in the ST. The percent of the different fish species are given in the upper right corner.

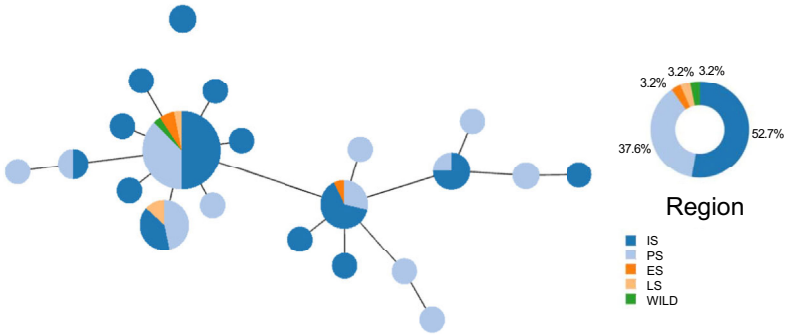

FIGURE 3 Sequence types of *S. parasitica* samples from different fish farms coloured by geographical location. IS = Eastern Finland, PS = Northern Finland, LS = Western Finland, ES = Southern Finland, green colour = natural samples. The size of each node represents the number of samples included in the ST.

suggesting that saprolegniosis can affect any salmonid. *S. parasitica* has also been reported isolated from infected fish in previous studies worldwide (De la Bastide et al., 2015; Diéguez-Uribeondo et al., 2007; Sandoval-Sierra, Latif-Eugenin, et al., 2014; Sarowar et al., 2019), verifying the importance of this pathogen as the most common oomycete species causing saprolegniosis. The fact that the three wild adult fish from Kallavesi and Torne River were infected with *S. parasitica* indicates that this pathogen also is an agent causing disease in wild salmonids in Finland.

Rainbow trout eggs were received from two fish farms, both in eastern Finland. *S. diclina* was the most frequent finding (44% of the eggs) from both farms (Table 2). Our results are supported by previous studies showing that *S. diclina* is frequently isolated from fish eggs and yolk sac fry (Diéguez-Uribeondo et al., 2007;

Fregeneda-Grandes et al., 2007; Sandoval-Sierra, Latif-Eugenin, et al., 2014; Thoen et al., 2015). Interestingly, in a study of De la Bastide et al. (2015), *S. diclina* was not found in fish eggs.

Our results showed that yolk sac fry were infected with different *Saprolegnia* species (*S. parasitica*, *S. ferax* and *Saprolegnia*. sp), and the most abundant oomycete affecting eggs were *S. diclina*. This may be because fish and eggs have a completely different surface, the skin of the fish is mucous while the outer layer of eggs have a thick membrane. Furthermore, *S. parasitica* and *S. diclina* have different infection strategies when infecting eggs (Songe et al., 2016). Interestingly, we found a clear genetical difference between *S. parasitica* isolates from yolk sac fry and adult fish, both in the ITS sequences and in the MLST analyses. The results suggest that the infections of adult fish are a separate problem compared to infections in hatcheries and that a *Saprolegnia* infection in a hatchery is not generally transmitted to adult fish.

In other words, the *Saprolegnia* species/strains infecting the salmonids in Finnish fish farms appears to be related to the developmental stage of the fish. This finding supports earlier studies conducted in Norway and Chile (Sandoval-Sierra, Latif-Eugenin, et al., 2014; Thoen et al., 2015), that also found an association between *Saprolegnia* species infecting fish and fish developmental stage. According to Stueland et al. (2005), *S. diclina* and *S. ferax* have low pathogeny to adult salmonids, which is in accordance with the present study, as we isolated these two species only from eggs and yolk sac fry, respectively. However, the small number of examined yolk sac fry originated from only two fish farms, implies that further studies are needed to support these results indicating different genetical strains affecting eggs and yolk sac fry compared to older fish.

Since the phylogenetic analysis indicated that the ITS nrDNA sequences were not variable enough to analyse the genetic differences between *S. parasitica* isolates collected from Finland, it remained unclear whether the same genotype of *S. parasitica* was involved in all disease outbreaks in Finnish fish farms, in different fish species and different geographical regions. Therefore, we used MLST to further investigate the genetic differences among the *S. parasitica* isolates to reveal the molecular epidemiology of this species in Finland. The isolates obtained from eggs were excluded from these analyses. MLST was initially developed to characterize the presence of genetically different types within a bacterial species, but this method has also previously been used to study oomycetes (Ravasi et al., 2018). The technique compares nucleotide polymorphisms within five to seven gene regions, traditionally housekeeping genes (Maiden et al., 1998).

For the MLST scheme, we chose seven housekeeping genes according to the method developed by Ravasi et al. (2018). However, the sequence for the housekeeping gene ALTS1 contained three introns and had to be excluded from the analyses. This was not the case in the previous study, using the same primers, suggesting that the Finnish isolates are related to each other, but different from those in Switzerland as presented by Ravasi et al. (2018).

The MLST scheme with the six remaining housekeeping genes identified one main clone containing the majority of the isolates, which thus are related to each other. This suggests that the *S. parasitica* infections in farmed fish in Finland are not caused by genetically different strains originating in the farm environment. Instead, one main clone of *S. parasitica* is present in most farms and this clone probably spreads among farms, for example with fish transports.

The main clone contains four different related main genotypes or subclones (ST1–4) and 15 related genotypes that differed in one of the housekeeping genes. All the main genotypes (STs) were found in all fish species in this study. However, the results show some indication that specific fish species are infected by the same specific main genotype, but the number of samples for some fish species were too small to be tested statistically. In addition, all main genotypes were found in every region in Finland, indicating a relatively low diversity in the *S. parasitica* populations across the country.

This result is in accordance with a previous study, showing that one *Saprolegnia* sp clone, analysed with RAPD-PCR, was most prevalent in Finland and Sweden (Bangyeekhun et al., 2003). However, in a previous MLST study of *S. parasitica* isolates in Switzerland, using the same genes as in this study, the main part of the examined isolates belonged to two clonal complexes (CC) and a number of isolates not directly related (more than one allele difference) to these CCs were observed (Ravasi et al., 2018). These results indicate that the *S. parasitica* strains infecting fish in Switzerland are more diverse than those infecting fish in Finland, although the majority of the disease outbreaks were reported to be related to only one genotype (Ravasi et al., 2018).

The *S. parasitica* isolate obtained from the wild lake trout belonged to ST1 and those from the two Atlantic salmon belonged to ST6. This shows that the main genotype (CC-ST1) is present also in wild fish, suggesting that wild fish can serve as vectors for spreading *S. parasitica* between farms or that wild fish can serve as a source for the pathogen. The other way around is also possible, the infection can be transmitted from farmed fish to wild fish.

The *S. parasitica* isolates obtained from yolk sac fry diverged in five of the six genes and the MLST analyses placed these isolates in an ST (ST7) completely separate from the CC-ST1. The divergence in the genes was considerable, possibly indicating that the *S. parasitica* yolk sac fry variant does not infect adult fish. Unfortunately, these isolates were obtained from only one fish farm and cannot be considered as representative for yolk sac fry in general. Additional studies of *Saprolegnia* in yolk sac fry are thus needed.

Knowing that a certain clone of *S. parasitica* is present in the majority of the farms and disease outbreaks, further attempts can be focused on identifying the source or favourable life cycle and environmental conditions of this clone. Such knowledge is important to eventually develop strategies to prevent and control the spreading and transfer of this economically important disease. Also, additional studies on the association between *S. parasitica* and co-infective bacterial agents are worth addressing, preferably using qPCR and species-specific DNA primers based on the results obtained in the present study (Korkea-aho et al., 2022). The presence of the significant *Saprolegnia* species in biofilms of fish farm tanks, tubes and equipment might also be necessary to examine in the future, using the results obtained in the present study as a baseline.

4.1 | Conclusion

The main part of saprolegniosis outbreaks in Finnish fish farms are closely associated with *S. parasitica*. This indicates that the disease is widespread over the whole country, in different salmonid fish species, both in farmed and wild fish populations. Our results also show that a specific clone of *S. parasitica* is associated with the main part of the disease outbreaks, and a weak association between fish species and ST was observed. We also found clear genetic differences between isolates from infections in adult fish compared to yolk sac fry and eggs. *Saprolegnia parasitica* infections were dominating adult fish, while the main part of the investigated eggs were infected with *S. diclina*. However, the number of samples originating from eggs, fry and wild fish was limited in this study and further research is needed to clarify the role of the different species and genotypes of *S. parasitica* in different life stages of fish and if saprolegniosis can spread from farmed to wild fish, and vice versa.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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