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Saprolegnia infections in Finnish fish farms, identification of disease agents

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Elinkeino-, liikenne- ja ympäristökeskus

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BACKGROUND

Saprolegnia is a genus of water oomycetes that occurs in freshwater, producing characteristic white or grey fibrous hyphae. Current taxonomy defines *Saprolegnia* as a genus of the heterokonts in the order Saprolegniales, class Oomycota.

The disease caused by *Saprolegnia* sp, has produced significant losses in Finnish and global salmonid aquaculture since the ban of malachite green. No effective compound is available for the moment, making the treatment of this disease problematic.

It is known that the genus *Saprolegnia* consists of at least 18 + 11 different species (Sandoval-Sierra *et al.* 2014). The species of primary importance for the infection of fish is *Saprolegnia parasitica* (De la Bastide *et al*. 2015). Also other species are pathogenic for fish, like *S. diclina, S. ferax, S. hypogyna, S. delica*, which also are implicated in infecting fish eggs and fry. In a recent study, it was observed that *S. diclina* sub-clade III clearly dominated in Norwegian salmon hatcheries (Thoen *et al*. 2015). The present disease situation in Finnish fish farms is rather unknown, if one *Saprolegnia* species is dominating or if a number of different species or subgroups are involved in the disease outbreaks.

Traditionally, the identification of *Saprolegnia spp* has been based on morphology of sexual stuctures and zoospores (Seymour 1970, Willoughby 1985). In the present study we intended to do a pilot study, examining the potential presence of different *Saprolegnia* spp in different Finnish fish farms and different life stages of the fish, by sequencing the ITS region of *Saprolegnia* DNA and compare it to previously sequenced *Saprolegnia* species in a data base. The use of nrDNA ITS sequences has shown to be a useful resource for taxonomic and phylogenetic studies (Diéguez-Uribeondo et al. 2007).

MATERIAL & METHODS

Isolation of *Saprolegnia* from infected fish

Potential *Saprolegnia* samples were collected from infected farmed (and one wild) fish, yolk sac fry, and eggs from 20 different locations in Finland. Infected fish were

transported alive on ice or frozen to laboratories in Kuopio, Oulu and Turku, where *Saprolegnia* samples where isolated. Upon arrival, the fish were inspected for signs of *Saprolegnia* infection, and lesions and infected areas on eyes, fins, gills, skin and tail were swabbed to obtain *Saprolegnia* samples. Sometimes a part of a fish fin was directly placed on the isolation agar. Infected eggs were rolled on the agar surface using sterile forceps. The swabs were inoculated on PG-1 agar (Unestam, 1965) containing antibiotics (Alderman & Polglase, 1986): 10 µg/ml of ampicillin (AMP, Sigma-Aldrich, A-9518) and oxolinic acid (OA, Santa Cruz Biotechnology, sc-212488). The agar plates were incubated at 20 °C, and the primary cultures subsequently checked for bacterial contamination under a microscope. Potential *Saprolegnia* samples were collected from white fish (*Coregonus lavaretus*), rainbow trout (*Oncorhychus mykiss*), salmon (*Salmo salar*), sea trout (*Salmo trutta trutta)*, lake trout (*Salmo trutta lacustris*) and brook trout (*Salvelinus alpinus*). Potential *Saprolegnia* samples collected at laboratories in Kuopio and Oulu were sent to Turku for further analysis.

Purifing *Saprolegnia* samples from bacteria

In order to purify the *Saprolegnia* cultures from potential bacterial contaminants, the cultures were re-cultivated in succession every two to three days on nutrient-free (NF) agar plates, containing 1 % agar in mQ-H_2O with the addition of 10 µg/ml of AMP and OA, until no visible bacterial growth was detected under a microscope.

Isolation of *Saprolegnia* monocultures

Isolation of *Saprolegnia* mono-cultures from single zoospores was done from agar cultures on NF or PG-1 agar plates. Single germinating zoospore was isolated for each *Saprolegnia* strain and re-cultivated on separate PG-1 agar plates at 20 °C. The plates were checked to confirm the successful re-cultivation and growth after 24 h.

Preservation of *Saprolegnia* monocultures

For the long-term preservation of the *Saprolegnia* isolates hemp seed cultures were produced. Four sterile hemp seeds of roughly equal size were placed on PG-1 agar plates cultured with an isolate from a single zoospore. The plates were incubated for 4-6 days at 20 °C, until the hyphae had reached the edge of the plate and had covered the seeds.

Two different methods were applied for the long-term preservation of the *Saprolegnia* monocultures.

In the first method, sterile tweezers were used to transfer one hemp seed from each monoculture to a designated tube containing PG-1 agar. The tubes were then incubated at 20 °C until the agar surface was sufficiently covered with growing hyphae. Mineral or paraffin oil was then added to the tubes, covering the agar and submerging the growing culture. The tubes were stored at 6 °C.

For the second preservation method, the remaining three hemp seed from each monoculture were transferred to 1 ml of sterile tap water in a 48-well micro well plate. The plates were sealed and stored at 6 °C (Stueland et al., 2005).

DNA sample preparation

A DNA sample for each monoculture was produced from the same *Saprolegnia* culture from where the hemp seeds had been taken. Using the top of a sterile glass Pasteur pipette, the agar from an undisturbed part of the culture was punctured and the pellet was transferred to a tube with Glucose Yeast Extract broth (GYB) and incubated at 20 °C for 3 to 7 days. The broth culture containing the hyphae was then collected in its entirety into a sterile 1.5 ml microcentrifuge tube, and stored at –20 °C until the DNA extraction was done.

The hyphae were washed with 1 ml of sterilized lake water (FSLW) by centrifugation at 14 000 x g, after which the DNA was extracted using the MasterPure™ Gram Positive DNA Extraction Kit (Lucigen, MGP04100) following the the protocol for Gram Positive DNA Purification. The purified DNA was stored at 6 °C after extraction.

Amplification of internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (nrDNA)

The universal primers for eukaryotes nu-SSU-1766 (ITS5) (forward), and nu-LSU-0041 (ITS4) (reverse) were used to amplify the ITS1, 5.8S, and ITS2 region (White et al 1990).

For each PCR sample (20 µl): 12,2 µl sterile mQ-H2O; 4 µl 5X Phire Reaction Buffer (Thermo Scientific, F-524); 1 µl of each forward and reverse primers (Table x); 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 were used.

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 1 min.

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

ITS nrDNA sequencing

The amplified PCR products were sequenced at the DNA Sequencing and Genomics Laboratory at University of Helsinki.

The obtained sequences were then subject to a BLASTn (NCBI) query search at (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome), and then aligned and compared with *Saprolegnia ssp*. sequences deposited in GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/). Multiple alignments and phylogenetic analyses were performed using the Mega X software (https://www.megasoftware.net/).

RESULTS

During this pilot project, infected fish, yolk sac fry, and eggs were examined from 19 different fish farms in Finland. Also one wild land locked salmon was examined. The majority of the examined farmed fish were rainbow trout (33%), followed by white fish

(31%). Other species were Atlantic salmon (16%), lake trout (15%), sea trout (3%), land locked salmon (2%), and brook trout (0.8%) (Table 1). Eggs were received from two farms and yolk sac fry from one farm (Table 2). Both eggs and fry were rainbow trout.

A total of 163 pure culture isolates were obtained, of these, 139 isolates were from farmed fish, 18 isolates were from eggs, 5 isolates from yolk sac fry and 1 isolate from the wild fish. Of the 139 isolates from fish 113 were sequenced and identified by ITS nrDNA sequencing. BLASTn (NCBI) query gave the following result (Table 1): 104 of the sequenced 113 isolates were identified as different *Saprolegnia* spp (92%), 97 of these 104 *Saprolegnia* spp. (93%) were identified as *Saprolegnia parasitica/salmonis* (subsequently indicated as *Saprolegnia parasitica*). This means that 86% of affected fish was infected with *Saprolegnia parasitica*. Also, the isolate from the wild fish was identified as *Saprolegnia parasitica*. The other occasional *Saprolegnia* spp (7 isolates) included *S. australis, S. diclina, S. torulosa, Saprolegnia* sp*.,* and *Leptolegnia* sp. 9 isolates were identified as different *Fungus* and *Phoma* spp.

Of the 18 isolates from rainbow trout egg (Table 2) 8 were identified as *Saprolegnia diclina*, 2 as *Saprolegnia hypogyna*, the rest as different *Saprolegnia* sp*., Leptolegnia*, and 2 isolates as *Pythium*. *Pythium* is a genus of parasitic oomycotes.

Of the 5 isolates from yolk sac fry, two were identified as *Saprolegnia parasitica* (Table 2.), the rest as *S. ferax, Saprolegnia* sp*,* and *Leptolegnia*.

The BLAST examination also showed that slightly different sequences of *S. parasitica* were obtained, but all of them are considered to belong to the same species *S. parasitica*.

Table 1. Number of examined samples from different fish farms, number of examined fish, total number of isolates, sequenced isolates, and number of *Saprolegnia parasitica/salmonis*

Fish	Number of farms	Number of fish	Number of isolates	Sequenced isolates	Saprolegnia parasitica/salmonis
Rainbow trout	7	42	42	37	30
Sea trout	2	4	4	4	3
Lake trout	4	19	19	13	12
White fish	6	40	48	40	35
Atlantic salmon	3	20	22	15	15
Land locked salmon	2	3	3	3	1
Brook trout	1	1	1	1	1
		129	139	113	97

Table 2. Number of examined egg and yolk sac fry samples (number of farms), number of isolates, sequenced isolates, and number of *Saprolegnia* spp

	Number of farms	Number of isolates	Sequenced isolates	Saprolegnia diclina	Saprolegnia hypogyna	Saprolegnia parasitica
Eggs	2	18	18	8	2	
Yolk sac fry	1	5	5			2

DISCUSSION & CONCLUSIONS

This pilot project describes for the first time which *Saprolegnia* spp are present in Finnish fish farms, affecting the farmed fish in different life stages. This study showed that a clear majority (86%) of the *Saprolegnia* species infecting the adult fish is *Saprolegnia parasitica*. *S. parasitica* could be found in all examined fish species. The main *Saprolegnia* spp. infecting eggs were *S. diclina*, while the number of yolk sac fry were too few to draw any firm conclusions, but there is a tendency that *S.parasitica* is the main pathogen.

Our results also indicate that different strains/clones of *S. parasitica* might be present among the analyzed material. In the sequence data base different clones/strains can sometimes be obtained, for example *S. salmonis* was previously considered to be a separate species, but this strain is now included in the species *S. parasitica*. It is thus important to analyze the heterogeneity of the now obtained Finnish *S. parasitica*

isolates, in order to determine potential dominating clones. This kind of analysis can be done using Multi Locus Sequence Typing (MLST), recently described in Switzerland (Ravasi *et al.* 2018).

In accordance with our results, it has previously been shown in Norway (Thoen *et al*. 2015) that a majority (79%) of the examined isolates from water, and salmon eggs from hatcheries was *Saprolegnia diclina*, while *S. parasitica* were isolated from post hatching stages.

The results from the present study will be very important for the next step of the *Saprolegnia* project. Now that we know that a certain species (*S. parasitica*) is present in the majority of the farms and disease outbreaks, further attempts can be focused on identifying the source or favorable life conditions of this species. Also, additional studies on association between this species and bacterial co-infective bacterial agents are worth addressing, using preferably qPCR (and species specific DNA primers) based on the results obtained in the present study. 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.

Since the transfer and disease development of *Saprolegnia* infections are dependent on spore production, future research could also be focused on the biology of these spores, and how should the transfer and attachment of these be prevented.

REFERENCES

Alderman D. J. & Polglase J. L. 1986. *Aphanomyces astaci*: isolation and culture. Journal of Fish Diseases 9: 367-379.

De la Bastide P.Y., Leung W.L. & Hintz W.E. 2015. Species composition of the genus *Saprolegnia* in fin fish aquaculture environments, as determined by nucleotide sequence analysis of the nuclear rDNA ITS regions. Fungal Biology 119: 27-43.

Diéguez-Uribeondo J., Fregeneda-Grandes J.M., Cerenius L., Pérez-Iniesta E., Aller-Gancedo J.M., Telleria M.T., Söderhäll K & Martin M.P. 2007. Re-evaluation of the enigmatic species complex *Saprolegnia diclina-Saprolegnia parasitica* based on

morphological, physiological and molecular data. Fungal Genetics and Biology 44: 585-601.

Ravasi, D., De Respinis S. & Wahli T. 2018. Multilocus sequence typing reveal clonality in *Saprolegnia parasitica* outbreaks. J. Fish Dis. 00:1–13. https://doi.org/10.1111/jfd.12869

Sandoval-Sierra J.V., Martín M.P. & Diéguez-Uribeondo J. 2014. Species identification in the genus *Saprolegnia* (Oomycetes): Defining DNA based molecular operational taxonomic units. Fungal Biology 118: 559-578.

Seymour R.L. 1970. The genus *Saprolegnia*. Verlag von j. Cramer, Lehre.

Stueland S., Hatai K. & Skaar I. 2005. Morphological and physiological characteristics of *Saprolegnia* spp. strains pathogenic to Atlantic salmon *Salmo salar* L. Journal of Fish Diseases 28: 445-453.

Thoen E., Vrålstad T., Rolén E., Kristensen R., Evensen Ø. & Skaar I. 2015. *Saprolegnia* species in Norwegian salmon hatcheries: field survey identifies *S. diclina* sub-clade IIIB as the dominating taxon. Diseases of Aquatic Organisms 114: 189-198.

Unestam T. 1965. Studies on the crayfish plague fungus *Aphanomyces astaci*. I. Some factors affecting growth *in vitro*. Physiologia Plantarum 18: 483-505.

White T. J., Bruns T., Lee S. & Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR Protocols: a Guide to Methods and Applications (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds): 315–322. San Diego: Academic Press.

Willoughby L.G. 1985. Rapid preliminary screening of *Saprolegnia* on fish. Journal of Fish Diseases 8: 473-476.