Identification and molecular characterization of oomycete isolates from trout farms in Croatia, and their upstream and downstream water environments

Published Version:
Pavic, D., Miljanovic, A., Grbin, D., Sver, L., Vladusic, T., Galuppi, R., et al. (2021). Identification and molecular characterization of oomycete isolates from trout farms in Croatia, and their upstream and downstream water environments. AQUACULTURE, 540, 1-11 [10.1016/j.aquaculture.2021.736652].

Availability:
This version is available at: https://hdl.handle.net/11585/828869 since: 2021-07-27

Published:
DOI: http://doi.org/10.1016/j.aquaculture.2021.736652

(Article begins on next page)

Identification and molecular characterization of oomycete isolates from trout farms in Croatia, and their upstream and downstream water environments

Dora Pavić[a], Anđela Miljanović[a], Dorotea Grbin[a], Lidija Šver[a], Tomislav Vladušić[a], Roberta Galuppi[b], Perla Tedesco[b], Ana Bielen[a,*]

[a] *Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, 10000 Zagreb, Croatia*

[b] *Department of Veterinary Medical Science, Alma Mater Studiorum, University of Bologna, 40064 Ozzano Emilia (BO), Italy*

*Corresponding author: Tel.: +385 98 179 3307. E-mail address: abielen@pbf.hr (Ana Bielen)

Abstract

Oomycetes from the genus *Saprolegnia* are opportunistic pathogens that cause significant losses in salmonid aquaculture. Despite this, studies reporting dominant *Saprolegnia* species in different fish farming facilities, as well as analyses of their spreading to natural environments, are still scarce. In this study, we have for the first time identified oomycete species present in four different trout farms in Croatia. We have collected 220 oomycete isolates, both from affected tissue (46 in total: adult trout - 28, eggs - 13, and alevins - 5) and from water (174 in total: in the fish farm – 78, upstream – 50, and downstream - 46). We have used Bayesian inference to reconstruct phylogenetic relationship among the internal transcribed spacer (ITS) sequences of the collected isolates and referent strains, and determined that the isolates belonged to three different oomycete genera: *Saprolegnia* (64 % of isolates), *Pythium* (35 %), and *Leptolegnia* (1 %). *Saprolegnia* isolates were classified into four species: *S. parasitica* with 53 isolates, *S. australis* - 52, *S. delica* – 25, and *S. ferax* – 11. *Pythium* and *Leptolegnia* isolates couldn't be identified to the species level and probably belong to so far undescribed species since their sequences didn't group with previously described species. Next, isolates from the affected tissue were mostly *S. parasitica* (32), while *S. australis, S. delica,* and *S. ferax* were less common (≤ 4 isolates per species). Furthermore, we used hempseed baits to capture oomycetes from water and positioned them inside the fish farms, as well as upstream (between 55 and 155 m) and downstream (between 95 and 140 m) of the fish farms. According to correspondence analysis, *Saprolegnia* species showed a strong association with fish farms and downstream locations, while upstream locations were associated with *Pythium* species, highlighting a possible role of trout farms as a source of spreading *Saprolegnia* species into the environment.

Keywords: oomycetes, opportunistic pathogens, aquaculture, trout, ITS

1. Introduction

Oomycetes, commonly known as 'water molds', are fungal-like microorganisms that can be parasitic towards a large number of plant and animal host species (Beakes et al., 2012). Today, oomycete-caused disease outbreaks are threatening wild species biodiversity and food security (Fisher et al., 2012; Phillips et al., 2008). Agriculturally important plant-pathogens have traditionally been receiving much attention, but animal pathogens are understudied even though several genera, such as *Saprolegnia* and *Aphanomyces*, cause devastating diseases in freshwater ecosystems (Bruno et al., 2011; Hussein and Hatai, 2002; Kamoun et al., 2015; Phillips et al., 2008; van West, 2006).

Saprolegnia species (*S. parasitica*, *S. australis*, *S. diclina,* and others) are ubiquitous in the freshwater environment and mostly considered as opportunistic secondary pathogens that infect the host in stressful conditions (such as infection by other pathogens, injuries, or adverse environmental conditions in general) (Gozlan et al., 2014; van den Berg et al., 2013). However, some S*. parasitica* strains were reported to be highly virulent and cause primary infections (Neish and Hughes, 1980; Stueland et al., 2005; Thoen et al., 2011; Whisler, 1996; Willoughby and Pickering, 1977). Saprolegniosis is a fish disease that affects all developmental stages – from eggs to juveniles and adults. It is a major problem in many wild and farmed fish species, such as Atlantic salmon, rainbow and brown trout, and also non-salmonid species like perch, eel, and catfish (Bruno et al., 2011; Gozlan et al., 2014). The main symptom of the disease is circular or crescent-shaped, white or grey, cotton-like mycelium developing anywhere on the fish body (Fregeneda Grandes et al., 2001; Hussein et al., 2001; Willoughbay, 1994, 1989). The disease is frequent during the winter when fish are often immunocompromised due to decreased water temperature (Bly et al., 1992; Bly and Clem, 1992).

Saprolegniosis is a serious problem in salmon and trout farms and hatcheries. Massive infections of eggs are common, and entire batches can be lost (Cao et al., 2012; Meyer, 1991; Rach et al., 2005; Thoen et al., 2011; van den Berg et al., 2013). This is a significant problem worldwide, commonly causing yearly economic losses of more than 10 %, and occasionally up to 50 % (Diéguez-Uribeondo et al., 2007; Rezinciuc et al., 2014; van den Berg et al., 2013; van West, 2006). Further, the primary existing disease control measure, malachite green, was banned by the European Union in 2002, due to its carcinogenicity and toxicity. The same fate is expected to befall formalin, leaving very limited control options available (Gozlan et al., 2014; Phillips et al., 2008; Tedesco et al., 2019; van den Berg et al., 2013; van West, 2006), and most likely causing an increase in saprolegniosis outbreaks.

Knowledge about the identity, distribution, and pathogenic significance of *Saprolegnia* species in aquaculture facilities is a necessary prerequisite for the development of efficient control measures. However, until recently little was known about dominant species associated with saprolegniosis outbreaks. This was probably because species were identified only based on the morphology of their sexual structures (Seymour, 1970), which was time-consuming and often unsuccessful (Diéguez-Uribeondo et al., 2007; Fregeneda-Grandes et al., 2007; van den Berg et al., 2013). Molecular diagnostic tools have been recently introduced in the identification of *Saprolegnia* spp., based on the sequence of internal transcribed spacer (ITS) region positioned between rRNA encoding genes (Cao et al., 2012; Diéguez-Uribeondo et al., 2007; Kozubíková-Balcarová et al., 2013; Rezinciuc et al., 2014; Sandoval-Sierra et al., 2014; Sarowar et al., 2019a; Tandel et al., 2020). However, the presence of many misassigned ITS sequences in DNA databases (e.g. GenBank) caused the erroneous classification of many isolates in the culture collections. A recent study on *Saprolegnia* molecular taxonomy resolved this issue and enabled the correct identification of *Saprolegnia* isolates to the species level, without the need for morphological characterization (Sandoval-Sierra et al., 2013). This allowed the recent accurate identification of *Saprolegnia* species in fish farms in Canada (Sarowar et al., 2019a), Chile (Sandoval-Sierra et al. 2014), Spain (Rezinciuc et al., 2014), and elsewhere (Paul et al., 2015; Sakaguchi et al., 2019).

It has been shown that pathogens can be transmitted from the fish farms to wild populations and vice versa (Johansen et al., 2011; Kurath and Winton, 2011; McVicar, 1997). In the context of this study, we were interested in the possible transfer of *Saprolegnia* pathogens from the fish farms to the downstream freshwater environments. Since freshwater aquaculture facilities are often connected with rivers/streams, it is possible for fish to escape or water to drain into the surrounding environment, allowing the transfer of pathogens (Andreou et al., 2012; Garseth et al., 2013; Gozlan et al., 2014; Johansen et al., 2011; Thorstad and Finstad, 2018). However, while transmission of viral and bacterial pathogens from farmed fish to wild populations has been repeatedly reported (Johansen et al., 2011; Johnsen and Jensen, 1994; Raynard et al., 2001; Wallace et al., 2008), knowledge of trout farms as points of spreading of *Saprolegnia* spp. to natural waters is limited (Galuppi et al., 2017).

The aim of this study was to perform the first survey of *Saprolegnia* species in selected trout farms in Croatia. Moreover, we have investigated the correlation between the occurrence of pathogenic *Saprolegnia* species in the fish farms and their incidence in natural waters upstream and downstream of the fish farms.

2. Materials and methods

2.1. Sampling

Sampling of oomycetes was carried out at four aquaculture facilities in Croatia (Fig. 1): three were located in central Croatia, at Gračani (part of Zagreb) (fish farm producing rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792)), Kostanjevac (a village near Zagreb) (fish farm producing brown trout, *Salmo trutta* (Linnaeus, 1758)), and Radovan (a village near Varaždin) (fish farm and hatchery producing *O. mykiss*), while the fourth one, Solin (a town near Split) (fish farm and hatchery producing *O. mykiss*), was located at the Adriatic coast. Sampling was carried out during winter (November, December, and January) in 2018 and 2019 (Table A.1). Conditions on the fish farms at the time of sampling were favorable for trout rearing (Woynarovich et al., 2011): water temperature was between 9 and 12.5 °C, pH between 7.3 and 7.8, and dissolved O_2 between 8.5 and 10.3 mg/L, as measured by a portable multimeter (Hach® Field

Case, Colorado, USA) (Table 1). Hatcheries Radovan and Solin (where alevins and eggs were sampled, respectively) were located near the adult fish rearing basins and were using the same water. The number of diseased fish in all farms was less than 1 %. Oomycetes were isolated from the host (eggs, alevins, and adult trout), as well as from the water in the farm, upstream and downstream.

A total of 75 tissue samples were collected from embryonic (eggs and alevins covered in *Saprolegnia*-like mycelium) and adult specimens (having skin lesions with external signs of *Saprolegnia* spp. mycelium growth) (Table A.1). Affected embryonic stages were available only in Radovan (eggs) and Solin (alevins), while affected adult trout were collected in all fish farms. Affected tissue (lesions) was excised from adult fish, while eggs and alevins with cotton-like mycelia growth, dead at the time of sampling, were taken whole. Tissue samples of adult specimens with no signs of infection (gills and skin) were also analysed, in order to compare the oomycete isolation success and the identity of the obtained isolates with those originating from the affected samples. Samples were collected aseptically, dipped for approximately one second in 96 % ethanol, and rinsed with sterile distilled water, to reduce bacterial contamination which could lead to unsuccessful oomycete isolation. Next, rinsed samples were placed onto glucose-yeast extract agar (GY, g/L: 12 g/L agar, 5 g/L glucose, 1 g/L yeast extract) (Min et al., 1998) supplemented with penicillin G and oxolinic acid in the final concentration of 6 and 10 mg/L, respectively (Alderman and Polglase, 1984). Plates were incubated at 18 °C (Galuppi et al., 2017), and pure cultures were obtained by transferring the growing mycelial tips to fresh plates every three days.

Hempseed baits (homemade 'tea balls') were used to isolate oomycetes from water. Each bait contained seven to ten halves of previously boiled hemp seeds (Seymour, 1970). Baits were placed inside of each farm and also upstream and downstream of their water system, following the protocol recently applied by Galuppi and colleagues (2017). The exception was fish farm Solin where *Saprolegnia* baits were not positioned upstream and downstream of the fish farm due to its specific position near the sea. The number of positioned baits and retrieved hempseeds per location is given in Supplementary Table A.1.

Upstream locations were positioned 55, 155, and 60 m upstream of the Gračani, Kostanjevac, and Radovan fish farms, respectively, while downstream locations were 130, 140, and 95 m downstream. Baits were retrieved after 10 days, yielding in total 289 samples (i.e. hempseeds). Hempseeds (with attached microorganisms from water) were treated as described above for host-associated oomycetes, i.e. they were dipped in ethanol, rinsed with distilled water, and then seeded individually on GY.

2.2. DNA isolation, amplification, and sequencing

DNA extraction was carried out from mycelia grown in liquid GY medium (Min et al., 1998) for two days at 18 °C. Mycelia were washed with sterile distilled water and centrifuged at 10 000 × g for 15 minutes to obtain pellets (app. 30 mg wet weight per sample) that were stored at -20 °C until DNA extraction. DNA was extracted using the NucleoSpin® Microbial DNA kit (Macherey Nagel, Germany), following the provided protocol with slight modifications. Samples were lysed by agitation (medium strength, 20 min) on a Vortex Mixer (Corning, USA), using Macherey Nagel Bead Tubes Type B. DNA was eluted from the column using the initial 100 µL eluate for a second elution to increase DNA yield and concentration.

The ITS region (ITS 1, 5.8S rDNA, and ITS 2) was amplified with universal primers for eukaryotes ITS5 (5' GGAAGTAAAAGTCGTAACAAGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990) under conditions described by Sandoval-Sierra et al. (2013). Shortly, the reaction mixture contained 1 µL of the genomic DNA, 12.5 µL of EmeraldAmp® PCR 2× Master Mix (TAKARA), 0.5 µL of 10 µM of primers and dH_2O to a final volume of 25 µL. Thermal cycling was performed in Alpha Cycler 1 (PCRmax) with the following conditions: 2 min at 95 ◦C for initial denaturation, followed by 35 cycles of 1 min at 95 ◦C (denaturation), 30 sec at 60 ◦C (annealing), and 1 min at 72 ◦C (extension), and 10 min at 72 ◦C as a final extension step. *Saprolegnia parasitica* CBS 233.65 genomic DNA and distilled water were used as positive and negative control, respectively. Obtained amplicons, approximately 600 bp long for *Saprolegnia* spp. and *Leptolegnia* spp., and approximately 900 bp for *Pythium* spp. (Fig. 2 - D), were purified and then sequenced (Sanger sequencing, Microsynth, Austria) using primer ITS4. Chromatograms were analyzed and edited, including the trimming of 5′ and 3′ ends with lower quality of peaks, in GeneStudio. Obtained sequences are deposited in GenBank under accession numbers (Acc. No.) MT555787 – MT556006 (Table A.1).

2.3. Alignment and phylogenetic analyses

Multiple sequence alignment (MSA) of the ITS region of all isolates and selected reference sequences was constructed in MAFFT using default settings (Katoh and Standley, 2013), and edited in SeaView (Gouy et al., 2010) and BioEdit (Hall et al., 2011). Reference sequences from genera *Achlya*, *Aphanomyces*, *Leptolegnia*, *Phytophthora, Pythium*, and *Saprolegnia* were selected based on the available literature on their molecular phylogeny (Lévesque and De Cock, 2004; Rocha et al., 2018; Sandoval-Sierra et al., 2013) and retrieved from NCBI database using the Batch Entrez tool (https://www.ncbi.nlm.nih.gov/sites/batchentrez) (Table A.2). Two separate MSAs were constructed: one comprising the sequences from the order Saprolegniales (genera *Saprolegnia* and *Leptolegnia*) (Supplementary material, Figure B.1), and another with sequences from the order Peronosporales (genus *Pythium*) (Supplementary material, Figure B.2). Final MSA of *Saprolegnia* and *Leptolegnia* sequences contained in total 179 sequences (143 sequences of isolates and 36 reference sequences), while MSA of *Pythium* had 112 sequences (77 sequences of isolates and 35 reference sequences). The phylogenetic relationship among the sequences was reconstructed with the Bayesian inference method using MrBayes software 3.2.7a with 200 000 iterations (Ronquist and Huelsenbeck, 2003). Two simultaneous, independent analysis were run with four Markov chain Monte Carlo (MCMC), one cold and three heated chains with temperature set to 0.5. Every 100 generations were sampled and first 25 % of the samples from the cold chain were discarded as ‘burn-in’. Posterior probability was estimated for the remaining trees. Phylogenetic trees were visualized with Figtree v1.4.4. (http://tree.bio.ed.ac.uk/software/figtree/).

2.4. Species diversity and richness

Biodiversity of oomycete species isolated from tissue samples (adult and embryonic stages) and water samples (upstream - U, fish farm - F and downstream - D) was measured and estimated with Shannon (H, species diversity) and Menhinick's index (D, species richness) (Ludwig and Reynolds, 1988; Menhinick, 1964; Shannon, 1948). Species richness (D), a simple measure referring to a number of species in a sampled location, is calculated as follows:

$$D = \frac{s}{\sqrt{N}}$$

where *s* counts the number of different species present in a sampled location and *N* equals the total number of individual in a sampled location. Diversity index (H) gives information about rarity and commonness of species in a sampled location, and is calculated as follows:

$$\mathrm{H} = -\sum_{i=1}^{R} p_i \ \mathrm{In}\ p_i$$

where proportion of species *i* is relative to the total number of species p_i (Ludwig and Reynolds, 1988).

2.5. Statistical analyses

Associations between oomycete species and sampling location/type of sample were analyzed by correspondence analysis (CA) which provides factor scores (coordinates) for both row and column points of the contingency table. These coordinates provide a solution for summarizing the data set in two-dimension plots, used to visualize graphically the association between the row and column elements in the contingency table (Kassambara, 2017). Dimensions 1 and 2 both indicate the percentage of association between the row and column categories. We have tested the following associations: (i) tissue-associated isolates (from all four fish farms) vs. trout developmental stage (egg, alevin, adult), (ii) tissue-associated isolates (from all tissue types) vs. fish farm (all four fish farms included); (iii) farm water-associated isolates (captured by hempseed baits in the fish farms) vs. fish farm (all four fish farms included), and (iv) water-associated isolates (captured by hempseed baits) vs. sampling location (upstream, fish farm, and downstream, from fish farms Kostanjevac, Radovan and Gračani). Noteworthy, fish farm Solin was excluded from the last analysis, since in this case the *Saprolegnia* baits were not positioned upstream and downstream of the fish farm due to its specific position near the sea.

CA was obtained and plotted using R v. 3.2.0. To compute and interpret CA two R packages were used: i) FactoMineR for the analysis, and ii) factoextra for data visualization. The observed associations were tested using Pearson's χ2-test.

3. Results

3.1. Molecular identification of oomycete isolates from Croatian trout farms

A total of 220 oomycete isolates were cultured, 46 originating from tissue samples, and 174 from hempseed baits (Fig. 2 A - C; Tables 2 and 3; Table A.1). Oomycete detection frequency for both sample types/sampling methods was similar: 61 % for tissue samples (46 samples with oomycete growth out of the total number of 75 samples), and 60 % for hempseed halves (174 out of 289 hempseeds resulted in oomycete growth) (Table A.1). From the gill and tissue samples showing no clinical signs of saprolegniosis, the isolation of oomycete was less successful than from skin lesions, 50 % (i.e. 7 samples out of 14 resulted in oomycete growth) and 60 % (21/35) respectively. Furthermore, only 57 % of the isolates collected from the healthy gills and skin were identified as *Saprolegnia* sp., while 90 % of isolates collected from the skin lesions belonged to the genus *Saprolegnia*.

Morphologically, the isolates could be divided into three groups, as depicted in Fig. 2 A-C. PCR amplification of the ITS region of the isolates yielded DNA fragments of 600 – 900 bp (Fig. 2 - D) that were sequenced and used for species identification. Due to the large number of isolates, two separate phylogenetic trees were constructed, one for *Saprolegnia* and *Leptolegnia* isolates (order Saprolegniales), and another for *Pythium* isolates (order Peronosporales) (Figs. 3 and 4). The obtained grouping of the Saprolegniales sequences showed that the *Saprolegnia* isolates (64 % of the total number of isolates) were mostly *S. parasitica* (53; 24 %) and *S. australis* (52; 24 %), followed by *S. delica* (25; 11 %) and *S. ferax* (11;

5 %) (Fig. 3). Additionally, two *Leptolegnia* isolates (B11L3 and BD25, 1 % of the isolates) were identified, but could not be classified to the species level, since they grouped with another unidentified (*Leptolegnia* sp.) sequence. Thus, these isolates probably belong to a so-far undescribed *Leptolegnia* species.

All collected *Pythium* isolates, comprising 77 isolates or 35 % of the total isolates, grouped within the previously described *Pythium* group B (Lévesque and De Cock, 2004) (Fig. 4). Among them, a majority of 75 isolates formed a well-supported clade within the B2 group, most probably a novel *Pythium* species. Isolate B3S1 showed the highest identity with *P. lutarium* (78.28 %), *P. diclinum* (78.15 %), and *P. marinum* (78.03 %), all from the B2 group, while Z111 belonged to the B1 group and showed the highest identity with *P. vanterpoolii* (71.18 %).

3.2. Oomycete isolates from diseased adult and embryonic trout

Diseased adult fish were collected from all farms yielding a total of 28 isolates, while infected eggs and alevins were available only at Radovan and Solin, yielding 13 and 5 isolates, respectively (Table 2; Table A.1).

The most frequent species was *S. parasitica* (70 %, 32 isolates), while other *Saprolegnia* species (*S. australis*, *S. delica*, and *S. ferax*) were less common (≤ 4 isolates per species). *Saprolegnia parasitica* was isolated both from adult and embryonic samples and was found as dominant species in Radovan, Gračani, and Solin fish farms. Besides genus *Saprolegnia*, two more oomycete genera were isolated, *Leptolegnia* sp. from the adult stage and *Pythium* sp. from both adult and embryonic stages.

Correspondence analysis (CA) was performed to analyze the associations between collected oomycete species and trout developmental stage, as well as between oomycete species and fish farms. No significant association (p = 0.1, Table 4) was found between oomycete species and trout developmental stage (Fig. A.1-A). However, significant differences were observed (p = 0.002, Table 4) between oomycete species isolated from tissue samples and different fish farms (Fig. A.1-B). Mainly, Kostanjevac differed from the other fish farms with *S. australis*, and not *S. parasitica*, being the dominant detected species.

Diversity of tissue-associated oomycete isolates, as estimated by the Shannon index (H), was greatest in Kostanjevac (1.33), followed by Solin (1.15) and Radovan (0.51), while in Gračani only *S. parasitica* was detected (H = 0). However, Solin had greater species richness (1.33) than Kostanjevac (1.27), Radovan (0.8), and Gračani (0.28).

3.3. Oomycete isolates from water

The most prevalent *Saprolegnia* species found in water samples was *S. australis*, followed by *S. delica*, *S. parasitica*, and *S. ferax* (Table 3). Additionally, besides being isolated from tissue samples, one *Leptolegnia* sp. isolate was also found downstream of Radovan. *Pythium* sp. isolates were also found, mostly in upstream locations.

The oomycete species collected from water varied according to the fish farm (Solin, Kostanjevac, Radovan or Gračani; Table 3, Fig. A.1-C) and the sampling location (U, F and D) (Tables 3 and 5; Fig. 5). Generally, dominant *Saprolegnia* species captured from water in different fish farms were in accordance with *Saprolegnia* species detected in diseased animal tissues (Tables 2 and 3, Fig. A.1-B and C). For instance, *S. parasitica* dominated in Solin and *S. australis* in Kostanjevac (Tables 2 and 3). However, in one farm (Gračani), *S. parasitica* was dominantly isolated from tissue samples (Table 2), while *S. australis* was most prevalent among isolates from water (Table 3). Further, the correspondence analysis showed strong association ($p = 2.73 \times 10^{-9}$, Table 4) between the sampling location (U, F, or D) and collected oomycete species (Fig. 5). In this analysis, the first two dimensions explained the 100 % of association that exists between oomycete species and sampling locations, where the first dimension explained 91.9 %, and the second dimension explained 8.1 % of the association. Namely, the presence of pathogenic *Saprolegnia* species was associated with fish farms and downstream locations, while *Pythium* sp. was typically dominant in upstream locations (Fig. 5, Table 5).

Oomycete species richness and diversity were highest downstream of fish farms (0.88 and 1.53, respectively). Upstream and inside the fish farm richness was approximately the same (0.56 and 0.57), but diversity was higher inside the fish farms (1.43) than upstream (0.42).

4. Discussion

We report on the oomycete species identified in selected trout farms in Croatia, with emphasis on *Saprolegnia* spp. that cause saprolegniosis and significant economic losses in aquaculture worldwide (van den Berg et al., 2013; van West, 2006). Importantly, we discuss the possible role of trout farms as points of spreading pathogenic *Saprolegnia* species into the environment.

4.1. Pathogenic oomycete sampling approaches in freshwater ecosystems

We have combined two sampling approaches (tissue and water samples) to get the most insight into pathogenic oomycete species present in selected fish farms and the natural environment. Both methods have advantages and disadvantages and can be complementary when used in combination, as was demonstrated in several previous studies (Galuppi et al., 2017; Rahman and Sarowar, 2016; Rezinciuc et al., 2014; Sarowar et al., 2019b, 2013; Thoen et al., 2015). For instance, since hempseeds attract zoospores/cysts in the water, some non-zoosporic species can go undetected. Also, bacteria in the water can sometimes disable zoospore attachment and germination on the baits (Sarowar et al., 2019b). On the other hand, personnel in the fish farms are often reluctant to provide affected animals (as was also the case during this study). Also, it is sometimes difficult to obtain infected, but still living embryonic stages, since they quickly succumb to the disease. It is therefore hard to know whether the isolated oomycete species was the primary pathogen, or if the initial pathogen was overgrown by a secondary, opportunistic species. In our case, the fact that *S. diclina*, well known for egg infections (Fregeneda-Grandes et al., 2007; Sandoval-Sierra et al., 2014; Thoen et al., 2011; van den Berg et al., 2013), was not isolated from dead eggs, might indicate that sometimes the opportunistic species were cultivated (e.g. when *Pythium* sp. was isolated from the infected egg).

In overall, oomycete detection frequency in our study was equal for both sampling methods, approximately 60 %. However, we have observed some differences in *Saprolegnia* isolates collected from the surface of affected animals (mostly *S. parasitica*) and from farm water (most often *S. australis*). This could be explained by the higher pathogenicity of *S. parasitica* (Gozlan et al., 2014; van den Berg et al., 2013; van West, 2006).

4.2. Diversity of oomycete species associated with trout farms in Croatia

In this study, three genera of oomycetes were identified: *Saprolegnia*, *Leptolegnia* and *Pythium*. The dominant species and the only one that was isolated from all fish farms and all trout developmental stages was *S. parasitica*. Thus, our results confirm its dominance over other *Saprolegnia* species in aquaculture facilities (Hussein and Hatai, 2002; Noga, 1993; Sandoval-Sierra et al., 2014; Sarowar et al., 2019a; van den Berg et al., 2013; van West, 2006). Previous infection trials demonstrated pathogenicity of *S. parasitica* towards eggs (Kitancharoen and Hatai, 1996), fingerlings (Yuasa and Hatai, 1995), and adult salmonids (Stueland et al., 2005). In contrast to our findings, in Chilean salmonid farms *S. parasitica* was detected in adult *Salmo salar* and *O. mykiss*, but not in eggs and alevins (Sandoval-Sierra et al., 2014). This could be explained by the known variations in pathogenicity of *S. parasitica* isolates towards different developmental stages of the host (Stueland et al., 2005; Thoen et al., 2011; Yuasa and Hatai, 1995).

Other *Saprolegnia* species isolated from adult and embryonic stage (alevins) in Croatian trout farms as well as from water, were *S. australis*, *S. delica*, and *S. ferax*. Regarding *S. australis,* this species was only isolated from adult *S. trutta* individuals at fish farm Kostanjevac (two isolates from skin lesions and two from healthy skin), while it was not found on *O. mykiss* in other fish farms (that were dominated by *S. parasitica*). Although *Saprolegnia australis* is mostly regarded as pathogenic towards fish embryonic stages (Fregeneda-Grandes et al., 2007; Rezinciuc et al., 2014; Sandoval-Sierra et al., 2014; Tandel et al., 2020; Thoen et al., 2011), in our study it wasn't isolated from this sample type. This result may be caused by the small sample size (i.e. overall low number of isolates collected from eggs and alevins - 18). An earlier analysis of fish farms in Chile, with higher number of isolates from embryonic stage (122) showed an association between different *Saprolegnia* species and salmonid developmental stage, i.e. *S. australis* was associated with alevins (Sandoval-Sierra et al., 2014). Interestingly, *S. australis* was the most prevalent species found in water, especially in fish farms Kostanjevac and Gračani. This might be explained by the fact that, although *S. australis* zoospores might have been present in the water in high number (and thus easily captured by hempseed baits), infection with *S. australis* rarely occurred because the fish and eggs were healthy (and thus the animals were more often infected by *S. parasitica*, as a more virulent pathogen) (van den Berg et al., 2013; van West, 2006).

Lastly, *S. delica* was the second most isolated *Saprolegnia* species in water samples, and *S. ferax* was also occasionally captured by hempseed baits, while these two species were rarely obtained from tissue samples. Both species have previously been associated with embryonic mortality of fish and amphibians (Blaustein et al., 1994; Cao et al., 2012; Fregeneda-Grandes et al., 2007; Kiesecker et al., 2001) and were also often isolated from water (Rezinciuc et al., 2014; Sarowar et al., 2013). Our results are similar to a recent study done in Chilean salmonid farms, where *S. ferax* and *S. delica* were found both on adult and embryonic stages of salmonid fish without a clear preference for any particular developmental stage (Sandoval-Sierra et al., 2014).

Besides *Saprolegnia* species, two isolates from fish farm Radovan were identified as *Leptolegnia* sp., one from a tissue sample (adult stage - lesion) and one from water downstream of the fish farm. It is possible that these two isolates represent new *Leptolegnia* species, since they were not grouped with any of the known species, *L. caudata* or *L. chapmanii*, parasites of mosquitos (Bisht et al., 1996; Lastra et al., 2004; Montalva et al., 2016; Schimmel and Noblet, 1985). *Leptolegnia* sp. have also been isolated from cladocerans, fish, and amphibian eggs and larvae (Petrisko et al., 2008; Rezinciuc et al., 2014; Wolinska et al., 2009), but so far their pathogenicity has only been proven toward amphibian eggs (Ruthig, 2009). Our results might indicate that some *Leptolegnia* species could be opportunistic fish pathogens, but infection trials are needed to confirm this.

Furthermore, *Pythium* sp. isolates were also found in this study, mostly from water upstream of the fish farms (B2 isolates, and one B1 isolate - Z111 from Radovan), while a small number of B2 isolates were also found on diseased fish and only one B2 isolate on an egg sample. Known *Pythium* species are mostly plant pathogens or saprotrophs, mainly associated with natural and agricultural soils (Rahman and Sarowar, 2016; Robideau et al., 2011; Schroeder et al., 2013). However, *Pythium* sp., including B1 and B2 clades, were also isolated from natural and aquacultural freshwater environments (Nechwatal et al., 2008; Rahman and Sarowar, 2016; Schroeder et al., 2013), and some were suggested to be pathogenic towards freshwater animals (Miura et al., 2010). *Pythium* spp., including members of the clade B, were isolated from the carapace of dead crustaceans (Czeczuga et al., 2002b) and from dead or alive fishes and eggs (Czeczuga, 1996; Czeczuga et al., 2002a). *Pythium flevoense* (belonging to clade B2 and most closely related to isolate B3S1 found on *O. mykiss* from Solin) was reported to be responsible for mass mortality of freshwater fish (ayu larvae), but pathogenicity of isolates was not confirmed by infection trials (Miura et al., 2010). Taking all this into account, most of our isolates are probably plant pathogens or soil saprotrophs that arrived to the fish farms by water routes, as can be presumed from their phylogenetic grouping with saprotrophs and plant pathogens, and the fact that the majority were captured in the water upstream of the fish farms. For instance, isolate Z111 was most closely related to a known plant pathogen *P. vanterpoolii* (clade B1) (Asano et al., 2010; Ichitani et al., 1989; Muse et al., 1974). Also in concordance with this hypothesis, most of the studies that isolated *Pythium* spp. from fish tissue or water samples were conducted in the fish farms or ponds surrounded by agricultural fields, grassland, or forests (Czeczuga et al., 2005, 2002a; Naznin et al., 2017; Rahman and Sarowar, 2016; Sarowar et al., 2019b). This was also the case for three out of four fish farms sampled here (Gračani, Kostanjevac, Radovan).

Finally, our *Pythium* isolates probably belong to a novel, so far undescribed species. To confirm this, a detailed morphological description of the isolates is needed (Tambong et al., 2006), coupled with the analysis of additional molecular markers, besides ITS. Cytochrome c oxidase subunit I (COI) is a mitochondrially encoded gene that is more discriminative at the species level than the ITS region (Schroeder et al., 2013). Using both ITS and COI, rather than only one of them, is recommended for taxonomic identification of *Pythiu*m species (Bala et al., 2010; Robideau et al., 2011), and should be applied in the future studies of *Pythium* isolates associated with fish farms.

4.3. Trout farms enrich the pathogenic oomycetes in the downstream freshwater environment

To elucidate whether trout farms act as reservoirs of pathogenic *Saprolegnia* species that can spread to natural environments, we have collected oomycete isolates upstream, downstream, and inside the farms. Our study is the first one highlighting the spread of *Saprolegnia* species from Croatian fish farms to downstream locations. All *Saprolegnia* species captured by hempseed baits (*S. australis*, *S. delica*, *S. ferax,* and *S. parasitica*) were more abundant in the fish farms (43 isolates) and downstream locations (29) than upstream (5) of the fish farms (Table A.1; Table 5). In comparison, upstream locations were strongly associated with *Pythium* species (45), which were less often captured in the fish farms (11) and downstream (16). Noteworthy, *Saprolegnia* species were not found downstream from one fish farm (Gračani) which indicates that this farm had a smaller negative impact on the downstream environment than Kostanjevac and Radovan, probably due to a well maintained settler tank used in the fish farm Gračani.

Salmonid farms have been previously pinpointed as 'hot spots' of infections for nearby wild populations (Johansen et al., 2011). Fish escaping or water draining from fish farms often leads to the transfer of pathogens to the natural environment, as demonstrated for salmon lice, infectious pancreatic necrosis virus (IPNV), betanodavirus (NV), *Aeromonas salmonicida* subsp*. salmonicida* and other pathogens (Andreou et al., 2012; Garant et al., 2003; Johansen et al., 2011; Munday et al., 2002; Raynard et al., 2001; Thorstad and Finstad, 2018; Wallace et al., 2008). Our study illustrates this effect also for trout farms and *Saprolegnia* pathogens, which is relevant since saprolegniosis causes high annual economic losses in salmonid aquaculture (Hussein and Hatai, 2002; Phillips et al., 2008; van den Berg et al., 2013; van West, 2006) and has a negative impact on wild populations of salmonids and other freshwater fish, as well as other aquatic animals (Blaustein et al., 1994; Fregeneda Grandes et al., 2000; Kiesecker et al., 2001; Neitzel et al., 2004; Pickering and Willoughby, 1982; van West, 2006). For instance, *Saprolegnia* spp. can infect and kill crayfish specimens and it can be pathogenic towards amphibians (salamander adult and frog eggs) (Dieguez-Uribeondo et al., 1994; Gil-Turnes et al., 1989; Hirsch et al., 2008; Kiesecker and Blaustein, 1995; Kozubíková-Balcarová et al., 2013; Krugner-Higby et al., 2010; Lefcort et al., 1997). *Saprolegnia* species that have been introduced to the natural environment via fish restocking caused amphibian mortality (Blaustein et al., 1994; Kiesecker et al., 2001). In the last two decades, many diseases have increased in prevalence and distribution (emerging infectious diseases) (Fisher et al., 2012; Gozlan et al., 2014; van den Berg et al., 2013). Due to negative anthropogenic impacts on the natural environment (e.g. climate change, pollution, the introduction of new species, habitat alteration and degradation) the host species are becoming more vulnerable to various pathogens leading to disease outbreaks and sometimes even to the extinction of whole populations (Fisher et al., 2012; Gozlan et al., 2014). In this context, the transfer of *Saprolegnia* spp. from fish farms to the surrounding environment could lead to increased mortalities in natural populations, and it is crucial to undertake detailed surveys to follow pathogenic *Saprolegnia* spreading and distribution from fish farms to the natural environment, such as this one.

5. Conclusions

Our study highlights the role of trout farms as potential points of release of *Saprolegnia* pathogens to downstream freshwater ecosystems. Further studies are needed to assess the real impact of such pathogen spread, for instance, by sampling multiple points downstream from aquaculture sites coupled with pathogen quantification via molecular techniques, such as quantitative PCR or droplet digital PCR. This could be done by the combination of hempseed baiting (as applied here) and isolation of environmental DNA (eDNA) directly from water, an approach that has been widely used in recent years for detection and monitoring of species of interest (Dougherty et al., 2016; Strand et al., 2011). Also, the knowledge on the *Saprolegnia* spp. pathogenicity for free-living animal species is scarce, and further studies should be performed to assess *Saprolegnia* virulence, especially in combination with other stressors, such as elevated water temperature due to climate change, anthropogenic pollution, and pressure of invasive competing species.

Acknowledgments

This study was performed in the scope of the project "Interactions of freshwater pathogenic oomycetes and the environment" (InteractOomyc, UIP-2017-05-6267), funded by the Croatian Science Foundation. Dora Pavić was partially financed by the Research and Training grant [FEMS-GO-2017-007] awarded by the Federation of European Microbiological Societies (FEMS). Roberta Galuppi and Perla Tedesco developed the methodology for oomycetes sampling within the project ParaFishControl funded by the European Union's Horizon 2020 research and innovation programme under grant agreement No. 634429. This publication reflects the views only of the authors, and the European Commission cannot be held responsible for any use which may be made of the information contained therein.

The authors are thankful to Ivona Mladineo, Dražen Oraić, Hrvoje Ritterman, Luka Ritterman, Emin Teskeredžić, Tomo Vrabac and Mario Zagorec for help with the oomycete sampling, and Martin Sačer and Tamara Vujović for the help in the laboratory work and sequence analysis.

References

Alderman, D.J., Polglase, J.L., 1984. A comparative investigation of the effects of fungicides on *Saprolegnia parasitica* and *Aphanomyces astaci*. Trans. Br. Mycol. Soc. 83, 313–318. https://doi.org/10.1016/S0007-1536(84)80153-9

Andreou, D., Arkush, K.D., Guégan, J.F., Gozlan, R.E., 2012. Introduced pathogens and native freshwater biodiversity: a case study of *Sphaerothecum destruens*. PLoS One 7, e36998. https://doi.org/10.1371/journal.pone.0036998

Asano, T., Senda, M., Suga, H., Kageyama, K., 2010. Development of multiplex PCR to detect five *Pythium* species related to turfgrass diseases. J. Phytopathol. 158, 609–615. https://doi.org/10.1111/j.1439-0434.2009.01660.x

Bala, K., Robideau, G.P., Désaulniers, N., De Cock, A.W.A.M., Lévesque, C.A., 2010. Taxonomy, DNA barcoding and phylogeny of three new species of *Pythium* from Canada. Persoonia Mol. Phylogeny Evol. Fungi 25, 22–31. https://doi.org/10.3767/003158510X524754

Beakes, G.W., Glockling, S.L., Sekimoto, S., 2012. The evolutionary phylogeny of the oomycete "fungi". Protoplasma 249, 3–19. https://doi.org/10.1007/s00709-011-0269-2

Bisht, G., Joshi, C., Khulbe, R., 1996. Watermolds: potential biological control agents of malaria vector *Anopheles culicifacies*. Curr. Sci. 70, 393–395.

Blaustein, A.R., Hokit, D.G., O'Hara, R.K., Holt, R.A., 1994. Pathogenic fungus contributes to amphibian losses in the Pacific Northwest. Biol. Conserv. 67, 251–254. https://doi.org/10.1016/0006-3207(94)90616-5

Bly, J.E., Clem, L.W., 1992. Temperature and teleost immune functions. Fish Shellfish Immunol. 2, 159–171. https://doi.org/10.1016/S1050-4648(05)80056-7

Bly, J.E., Lawson, L.A., Dale, D.J., Szalai, A.J., Durburow, R.M., Clem, L.W., 1992. Winter saprolegniosis in channel catfish. Dis. Aquat. Organ. 13, 155–164. https://doi.org/10.3354/dao013155

Bruno, D., West, V.P., Beakes, G., 2011. *Saprolegnia* and other oomycetes, in: Woo, P.T.K., Bruno, D.W. (Eds.), Fish Diseases and Disorders: Viral, Bacterial and Fungal Infections Vol 3. CABI International, Wallingford, England, pp. 669–720.

Cao, H., Zheng, W., Xu, J., Ou, R., He, S., Yang, X., 2012. Identification of an isolate of *Saprolegnia ferax* as the causal agent of saprolegniosis of Yellow catfish (*Pelteobagrus fulvidraco*) eggs. Vet. Res. Commun. 36, 239–244.

Czeczuga, B., 1996. Species of *Pythium* isolated from eggs of fresh-water fishes. Acta Mycol. 31, 151–161.

Czeczuga, B., Kiziewicz, B., Danilkiewicz, Z., 2002a. Zoosporic fungi growing on the specimens of certain fish species recently introduced to Polish waters. Acta Ichthyol. Piscat. 32, 117–126. https://doi.org/10.3750/aip2002.32.2.02

Czeczuga, B., Kozłowska, M., Godlewska, A., 2002b. Zoosporic aquatic fungi growing on dead specimens of 29 freshwater crustacean species. Limnologica 32, 180–193. https://doi.org/10.1016/S0075-9511(02)80007-X

Czeczuga, B., Mazalska, B., Godlewska, A., Muszynska, E., 2005. Aquatic fungi growing on dead fragments of submerged plants. Limnologica 35, 283–297. https://doi.org/10.1016/j.limno.2005.07.002

Dieguez-Uribeondo, J., Cerenius, L., Soderhall, K., 1994. *Saprolegnia parasitica* and its virulence on three different species of freshwater crayfish. Aquaculture 120, 219–228. https://doi.org/https://doi.org/10.1016/0044-8486(94)90080-9

Diéguez-Uribeondo, J., Fregeneda-Grandes, J.M., Cerenius, L., Pérez-Iniesta, E., Aller-Gancedo, J.M., Tellería, M.T., Söderhäll, K., Martín, M.P., 2007. Re-evaluation of the enigmatic species complex *Saprolegnia diclina-Saprolegnia parasitica* based on morphological, physiological and molecular data. Fungal Genet. Biol. 44, 585–601. https://doi.org/10.1016/j.fgb.2007.02.010

Dougherty, M.M., Larson, E.R., Renshaw, M.A., Gantz, C.A., Egan, S.P., Erickson, D.M., Lodge, D.M., 2016. Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. J. Appl. Ecol. 53, 722–732. https://doi.org/10.1111/1365-2664.12621

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L., Gurr, S.J., 2012. Emerging fungal threats to animal, plant and ecosystem health. Nature 484, 186–194. https://doi.org/10.1038/nature10947

Fregeneda-Grandes, J.M., Rodríguez-Cadenas, F., Aller-Gancedo, J.M., 2007. Fungi isolated from cultured eggs, alevins and broodfish of brown trout in a hatchery affected by saprolegniosis. J. Fish Biol. 71, 510–518. https://doi.org/10.1111/j.1095-8649.2007.01510.x

Fregeneda Grandes, J.M., Fernández Diez, M., Aller Gancedo, J.M., 2000. Ultrastructural analysis of *Saprolegnia* secondary zoospore cyst ornamentation from infected wild brown trout, *Salmo trutta* L., and river water indicates two distinct morphotypes amongst long-spined isolates. J. Fish Dis. 23, 147–160. https://doi.org/10.1046/j.1365-2761.2003.00265.x

Fregeneda Grandes, J.M., Fernández Díez, M., Aller Gancedo, J.M., 2001. Experimental pathogenicity in rainbow trout, *Oncorhynchus mykiss* (Walbaum), of two distinct morphotypes of long-spined *Saprolegnia* isolates obtained from wild brown trout, *Salmo trutta* L., and river water. J. Fish Dis. 24, 351–359. https://doi.org/10.1046/j.1365-2761.2001.00305.x

Galuppi, R., Sandoval-Sierra, J. V., Cainero, M., Menconi, V., Tedesco, P., Gustinelli, A., Diéguez -

Uribeondo, J., 2017. Potenziale transferimento di *Saprolegnia* spp. dall'allevamento all'ambiente selvatico: risultati preliminari, in: XXIII Convegno Nazionale S. I. P. I. - Società Italiana Di Patologia Ittica. https://doi.org/https://www.sipi-online.it/convegni/2017/atti.pdf

Garant, D., Fleming, I.A., Einum, S., Bernatchez, L., 2003. Alternative male life-history tactics as potential vehicles for speeding introgression of farm salmon traits into wild populations. Ecol. Lett. 6, 541–549. https://doi.org/10.1046/j.1461-0248.2003.00462.x

Garseth, Å.H., Ekrem, T., Biering, E., 2013. Phylogenetic evidence of long distance dispersal and transmission of piscine reovirus (PRV) between farmed and wild Atlantic salmon. PLoS One 8, e82202. https://doi.org/10.1371/journal.pone.0082202

Gil-Turnes, M.S., Hay, M.E., Fenical, W., 1989. Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. Science 246, 116–118. https://doi.org/10.1126/science.2781297

Gouy, M., Guindon, S., Gascuel, O., 2010. Sea view version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27, 221–224. https://doi.org/10.1093/molbev/msp259

Gozlan, R.E., Marshall, W., Lilje, O., Jessop, C., Gleason, F.H., Andreou, D., 2014. Current ecological understanding of fungal-like pathogens of fish: what lies beneath? Front. Microbiol. 5, 62. https://doi.org/10.3389/fmicb.2014.00062

Hall, T., Biosciences, I., Carlsbad, C., 2011. BioEdit: An important software for molecular biology. GERF Bull. Biosci. 2, 60–61.

Hirsch, P.E., Nechwatal, J., Fischer, P., 2008. A previously undescribed set of *Saprolegnia* spp. in the invasive spiny-cheek crayfish (*Orconectes limosus*, Rafinesque). Fundam. Appl. Limnol. 172, 161–

165. https://doi.org/10.1127/1863-9135/2008/0172-0161

Hussein, M.M.A., Hatai, K., 2002. Pathogenicity of *Saprolegnia* species associated with outbreaks of salmonid saprolegniosis in Japan. Fish. Sci. 68, 1067–1072. https://doi.org/https://doi.org/10.1046/j.1444-2906.2002.00533.x

Hussein, M.M.A., Hatai, K., Nomura, T., 2001. Saprolegniosis in salmonids and their eggs in Japan. J. Wildl. Dis. 37, 204–207. https://doi.org/10.7589/0090-3558-37.1.204

Ichitani, T., Kang, H., Mine, K., 1989. Materials for *Pythium* flora of Japan (II) *Pythium torulosum* and *P. vanterpoolii* from golfgreens of manilagrass or bentgrass. Bull. Univ. Osaka Prefect. Ser. B 41, 9–19.

Johansen, L.H., Jensen, I., Mikkelsen, H., Bjørn, P.A., Jansen, P.A., Bergh, O., 2011. Disease interaction and pathogens exchange between wild and farmed fish populations with special reference to Norway. Aquaculture 315, 167–186. https://doi.org/10.1016/j.aquaculture.2011.02.014

Johnsen, B.O., Jensen, A.J., 1994. The spread of furunculosis in salmonids in Norwegian rivers. J. Fish Biol. 45, 47–55. https://doi.org/10.1111/j.1095-8649.1994.tb01285.x

Kamoun, S., Furzer, O., Jones, J.D.G., Judelson, H.S., Ali, G.S., Dalio, R.J.D., Roy, S.G., Schena, L., Zambounis, A., Panabières, F., Cahill, D., Ruocco, M., Figueiredo, A., Chen, X.R., Hulvey, J., Stam, R., Lamour, K., Gijzen, M., Tyler, B.M., Grünwald, N.J., Mukhtar, M.S., Tomé, D.F.A., Tör, M., Van Den Ackerveken, G., Mcdowell, J., Daayf, F., Fry, W.E., Lindqvist-Kreuze, H., Meijer, H.J.G., Petre, B., Ristaino, J., Yoshida, K., Birch, P.R.J., Govers, F., 2015. The top 10 oomycete pathogens in molecular plant pathology. Mol. Plant Pathol. 16, 413–434. https://doi.org/10.1111/mpp.12190

Kassambara, A., 2017. Practical guide to principal component methods in R: PCA, M (CA), FAMD, MFA, HCPC, factoextra (Vol. 2). STHDA.

Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol. Biol. Evol. 30, 772–780. https://doi.org/10.1093/molbev/mst010

Kiesecker, J.M., Blaustein, A.R., 1995. Synergism between UV-B radiation and a pathogen magnifies amphibian embryo mortality in nature. Proc. Natl. Acad. Sci. 92, 11049–11052. https://doi.org/10.1073/pnas.92.24.11049

Kiesecker, J.M., Blaustein, A.R., Miller, C.L., 2001. Transfer of a pathogen from fish to amphibians. Conserv. Biol. 15, 1064–1070. https://doi.org/10.1046/j.1523-1739.2001.0150041064.x

Kitancharoen, N., Hatai, K., 1996. Experimental infection of *Saprolegnia* spp. in rainbow trout eggs. Fish Pathol. 31, 49–50. https://doi.org/10.3147/jsfp.31.49

Kozubíková-Balcarová, E., Koukol, O., Martín, M.P., Svoboda, J., Petrusek, A., Diéguez-Uribeondo, J., 2013. The diversity of oomycetes on crayfish: morphological vs. molecular identification of cultures obtained while isolating the crayfish plague pathogen. Fungal Biol. 117, 682–691. https://doi.org/10.1016/j.funbio.2013.07.005

Krugner-Higby, L., Haak, D., Johnson, P.T., Shields, J.D., Jones, W.M. 3rd, Reece, K.S., Meinke, T., Gendron, A., Rusak, J.A., 2010. Ulcerative disease outbreak in crayfish *Orconectes propinquus* linked to *Saprolegnia australis* in big Muskellunge Lake, Wisconsin. Dis. Aquat. Organ. 91, 57–66. https://doi.org/10.3354/dao02237

Kurath, G., Winton, J., 2011. Complex dynamics at the interface between wild and domestic viruses of finfish. Curr. Opin. Virol. 1, 73–80. https://doi.org/10.1016/j.coviro.2011.05.010

Lastra, C.L., Scorsetti, A.C., Marti, G.A., García, J.J., 2004. Host range and specificity of an Argentinean isolate of the aquatic fungus *Leptolegnia chapmanii* (Oomycetes: Saprolegniales), a pathogen of mosquito larvae (Diptera: Culicidae). Mycopathologia 158, 311–315.

https://doi.org/10.1007/s11046-005-0498-z

Lefcort, H., Hancock, K.A., Maur, K.M., Rostal, D.C., 1997. The effects of used motor oil, silt, and the water mold *Saprolegnia parasitica* on the growth and survival of mole salamanders (genus *Ambystoma*). Arch. Environ. Contam. Toxicol. 32, 383–388. https://doi.org/10.1007/s002449900200

Lévesque, C.A., De Cock, A.W., 2004. Molecular phylogeny and taxonomy of the genus *Pythium*. Mycol. Res. 108, 1363–1383. https://doi.org/10.1017/S0953756204001431

Ludwig, J.A., Reynolds, J.F., 1988. Statistical Ecology. A Primer on Methods and Computing. Wiley, New York.

McVicar, A.H., 1997. Interaction of pathogens in aquaculture with wild fish populations. Bull. Eur. Assoc. Fish Pathol. 17, 197–201.

Menhinick, E.F., 1964. A comparison of some species-individuals diversity indices applied to samples of field insects. Ecology 45, 859–861.

Meyer, F.P., 1991. Aquaculture disease and health management. J. Anim. Sci. 69, 4201–4208.

Min, H., Hatai, K., Bai, S., 1998. Some inhibitory effects of chitosan on fish-pathogenic oomycete, *Saprolegnia parasitica*. Fish Pathol. 29, 73–77. https://doi.org/10.3147/jsfp.29.73

Miura, M., Hatai, K., Tojo, M., Wada, S., Kobayashi, S., Okazaki, T., 2010. Visceral mycosis in ayu *Plecoglossus altivelis* larvae caused by *Pythium flevoense*. Fish Pathol. 45, 24–30. https://doi.org/10.3147/jsfp.45.24

Montalva, C., dos Santos, K., Collier, K., Rocha, L.F.N., Fernandes, É.K., Castrillo, L.A., Luz, C., Humber, R.A., 2016. First report of *Leptolegnia chapmanii* (Peronosporomycetes: Saprolegniales) affecting mosquitoes in central Brazil. J. Invertebr. Pathol. 136, 109–116. https://doi.org/10.1016/j.jip.2016.03.012

Munday, B.L., Kwang, J., Moody, N., 2002. Betanodavirus infections of teleost fish: a review. J. Fish Dis. 25, 127–142. https://doi.org/10.1046/j.1365-2761.2002.00350.x

Muse, R.R., Schmitthenner, A.F., Partyka, R.E., 1974. *Pythium* spp. associated with foliar blighting of creeping bentgrass. Phytopathology. https://doi.org/10.1094/phyto-64-252

Naznin, T., Hossain, M.J., Nasrin, T., Hossain, Z., Sarowar, M.N., 2017. Molecular characterization reveals the presence of plant pathogenic *Pythium* spp. around Bangladesh Agricultural University Campus, Mymensingh, Bangladesh. Int. J. Agric. Res. 1, 1–7. https://doi.org/10.3923/ijar.2017.199.205

Nechwatal, J., Wielgoss, A., Mendgen, K., 2008. Diversity, host, and habitat specificity of oomycete communities in declining reed stands (*Phragmites australis*) of a large freshwater lake. Mycol. Res. 112, 689–696. https://doi.org/10.1016/j.mycres.2007.11.015

Neish, G.A., Hughes, G.C., 1980. Diseases of fish, in: Fungal Diseases of Fishes. T.W.F. Publications, Neptune, New Jersey, p. 159.

Neitzel, D.A., Elston, R.A., Abernethy, C.S., 2004. Prevention of prespawning mortality: cause of salmon headburns and cranial lesions. Richland, WA (United States).

Noga, E.J., 1993. Water mold infections of freshwater fish: recent advances. Annu. Rev. Fish Dis. 3, 291–304.

Paul, 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 Biol. 119, 27–43. https://doi.org/10.1016/j.funbio.2014.10.006

Petrisko, J.E., Pearl, C.A., Pilliod, D.S., Sheridan, P.P., Williams, C.F., Peterson, C.R., Bury, R.B., 2008. Saprolegniaceae identified on amphibian eggs throughout the Pacific Northwest, USA, by internal transcribed spacer sequences and phylogenetic analysis. Mycologia 100, 171–180. https://doi.org/10.1080/15572536.2008.11832474

Phillips, A.J., Anderson, V.L., Robertson, E.J., Secombes, C.J., van West, P., 2008. New insights into animal pathogenic oomycetes. Trends Microbiol. 16, 13–19. https://doi.org/10.1016/j.tim.2007.10.013

Pickering, A.D., Willoughby, L.G., 1982. *Saprolegnia* infections of Salmonid fish, in: Roberts, R.J. (Ed.), Microbial Diseases of Fish. Academic Press, London, pp. 271–297.

Rach, J.J., Redman, S., Bast, D., Gaikowski, M.P., 2005. Efficacy of hydrogen peroxide versus formalin treatments to control mortality associated with saprolegniasis on lake trout eggs. N. Am. J. Aquacult. 67, 148–154. https://doi.org/10.1577/a04-062.1

Rahman, K.M., Sarowar, M.N., 2016. Molecular characterisation of oomycetes from fish farm located in Mymensingh sadar during summer. Asian J. Med. Biol. Res. 2, 236–246. https://doi.org/10.3329/ajmbr.v2i2.29066

Raynard, R.S., Murray, A.G., Gregory, A., 2001. Infectious salmon anaemia virus in wild fish from Scotland. Dis. Aquat. Organ. 46, 93–100. https://doi.org/10.3354/dao046093

Rezinciuc, S., Sandoval-Sierra, J.V., Diéguez-Uribeondo, J., 2014. Molecular identification of a bronopol tolerant strain of *Saprolegnia australis* causing egg and fry mortality in farmed brown trout, *Salmo trutta*. Fungal Biol. 118, 591–600. https://doi.org/10.1016/j.funbio.2013.11.011

Robideau, G.P., De Cock, A.W.A.M., Coffey, M.D., Voglmayr, H., Brouwer, H., Bala, K., Chitty, D.W., Désaulniers, N., Eggertson, Q.A., Gachon, C.M.M., Hu, C.H., Küpper, F.C., Rintoul, T.L., Sarhan, E., Verstappen, E.C.P., Zhang, Y., Bonants, P.J.M., Ristaino, J.B., André Lévesque, C., 2011. DNA barcoding of oomycetes with cytochrome c oxidase subunit I and internal transcribed spacer. Mol. Ecol. Resour. 11. https://doi.org/10.1111/j.1755-0998.2011.03041.x

Rocha, S.C., Lopez-Lastra, C.C., Marano, A. V., de Souza, J.I., Rueda-Páramo, M.E., Pires-Zottarelli, C.L., 2018. New phylogenetic insights into Saprolegniales (Oomycota, Straminipila) based upon studies of specimens isolated from Brazil and Argentina. Mycol. Prog. 17, 691–700. https://doi.org/10.1007/s11557-018-1381-x

Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Ruthig, G.R., 2009. Water molds of the genera *Saprolegnia* and *Leptolegnia* are pathogenic to the north American frogs *Rana catesbeiana* and *Pseudacris crucifer*, respectively. Dis. Aquat. Organ. 84, 173–178. https://doi.org/10.3354/dao02042

Sakaguchi, S.O., Ogawa, G., Kasai, H., Shimizu, Y., Kitazato, H., Fujikura, K., Takishita, K., 2019. Molecular identification of water molds (oomycetes) associated with chum salmon eggs from hatcheries in Japan and possible sources of their infection. Aquac. Int. 27, 1739–1749. https://doi.org/10.1007/s10499-019-00427-w

Sandoval-Sierra, J.V., Latif-Eugenin, F., Martín, M.P., Zaror, L., Diéguez-Uribeondo, J., 2014. *Saprolegnia* species affecting the salmonid aquaculture in Chile and their associations with fish developmental stage. Aquaculture 434, 462–469. https://doi.org/10.1016/j.aquaculture.2014.09.005

Sandoval-Sierra, J.V., Martín, M.P., Diéguez-Uribeondo, J., 2013. Species identification in the genus *Saprolegnia* (Oomycetes): Defining DNA-based molecular operational taxonomic units. Fungal Biol. 118, 559–578. https://doi.org/10.1016/j.funbio.2013.10.005

Sarowar, M.N., Cusack, R., Duston, J., 2019a. *Saprolegnia* molecular phylogeny among farmed teleosts in

Nova Scotia, Canada. J. Fish Dis. 42, 1745–1760. https://doi.org/10.1111/jfd.13090

Sarowar, M.N., Hossain, M.J., Nasrin, T., Naznin, T., Hossain, Z., Rahman, M.M., 2019b. Molecular identification of oomycete species affecting aquaculture in Bangladesh. Aquac. Fish. 4, 105–113. https://doi.org/10.1016/j.aaf.2018.12.003

Sarowar, N.M., Van den Berg, H.A., Mclaggan, D., Young, R.M., Van West, P., 2013. *Saprolegnia* strains isolated from river insects and amphipods are broad spectrum pathogens. Fungal Biol. 117, 752–763. https://doi.org/10.1016/j.funbio.2013.09.002

Schimmel, L., Noblet, R., 1985. Host range studies with fungus *Leptolegnia*, a parasite of mosquito larvae (Diptera: Culicidae). J. Med. Entomol. 22, 226–227. https://doi.org/10.1093/jmedent/22.2.226

Schroeder, K.L., Martin, F.N., de Cock, A.W., Lévesque, C.A., Spies, C.F.J., Okubara, P.A., Paulitz, T.C., 2013. Molecular detection and quantification of *Pythium* species: evolving taxonomy, new tools, and challenges. Plant Dis. 97, 4–20. https://doi.org/10.1094/PDIS-03-12-0243-FE

Seymour, R.L., 1970. The genus *Saprolegnia*. Nov. Hedwigia 19, 1–124.

Shannon, C., 1948. A mathematical theory of communication. Bell Syst. Technol. J. 27, 379–423.

Strand, D.A., Holst-Jensen, A., Viljugrein, H., Edvardsen, B., Klaveness, D., Jussila, J., Vrålstad, T., 2011. Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. Dis. Aquat. Organ. 95, 9–17. https://doi.org/10.3354/dao02334

Stueland, S., Hatai, K., Skaar, I., 2005. Morphological and physiological characteristics of *Saprolegnia* spp. strains pathogenic to Atlantic salmon, *Salmo salar* L. J. Fish Dis. 28, 445–453. https://doi.org/10.1111/j.1365-2761.2005.00635.x

Tambong, J.T., De Cock, A.W.A.M., Tinker, N.A., Lévesque, C.A., 2006. Oligonucleotide array for identification and detection of *Pythium* species. Appl. Environ. Microbiol. 72, 2691–2706. https://doi.org/10.1128/AEM.72.4.2691-2706.2006

Tandel, R.S., Dash, P., Aadil, R., Bhat, H., Sharma, P., Kalingapuram, K., Dubey, M., Sarma, D., 2020. Morphological and molecular characterization of *Saprolegnia* spp . from Himalayan snow trout , *Schizothorax richardsonii* : A case study report. Aquaculture 531, 735824. https://doi.org/10.1016/j.aquaculture.2020.735824

Tedesco, P., Fioravanti, M.L., Galuppi, R., 2019. In vitro activity of chemicals and commercial products against *Saprolegnia parasitica* and *Saprolegnia delica* strains. J. Fish Dis. 42, 237–248. https://doi.org/10.1111/jfd.12923

Thoen, E., Evensen, Ø., Skaar, I., 2011. Pathogenicity of *Saprolegnia* spp. to Atlantic salmon, *Salmo salar* L., eggs. J. Fish Dis. 34, 601–608. https://doi.org/10.1111/j.1365-2761.2011.01273.x

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. Dis. Aquat. Organ. 114, 189–198. https://doi.org/10.3354/dao02863

Thorstad, E.B., Finstad, B., 2018. Impacts of salmon lice emanating from salmon farms on wild Atlantic salmon and sea trout. NINA Rep. 1449, 1–22.

van den Berg, A.H., McLaggan, D., Diéguez-Uribeondo, J., van West, P., 2013. The impact of the water moulds *Saprolegnia diclina* and *Saprolegnia parasitica* on natural ecosystems and the aquaculture industry. Fungal Biol. Rev. 27, 33–42. https://doi.org/10.1016/j.fbr.2013.05.001

van West, P., 2006. *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite: new challenges for an old problem. Mycologist 20, 99–104. https://doi.org/10.1016/j.mycol.2006.06.004

Wallace, I.S., Gregory, A., Murray, A.G., Munro, E.S., Raynard, R.S., 2008. Distribution of infectious pancreatic necrosis virus (IPNV) in wild marine fish from Scottish waters with respect to clinically infected aquaculture sites producing Atlantic salmon, *Salmo salar* L. J. Fish Dis. 31, 177–186. https://doi.org/10.1111/j.1365-2761.2007.00886.x

Whisler, H.C., 1996. Identification od *Saprolegnia* spp. pathogenic in Chinook Salmon. Final Report, DE-AC79-90BP02836. Washington, D.C.

White, T.J., Bruns, T., Lee, S.J.W.T., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, in: Innis, M., Gelfand, D., Sninsky, J., White, T. (Eds.), PCR Protocol: A Guide to Methods and Applications. Academic Press Inc, San Diego, CA, pp. 315–322.

Willoughby, L.G., 1994. Fungi and Fish Diseases. Pisces Press, Stirling, Scotland, UK.

Willoughby, L.G., 1989. Continued defence of salmonid fish against *Saprolegnia* fungus, after its establishment. J. Fish Dis. 12, 63–67.

Willoughby, L.G., Pickering, A.D., 1977. Viable Saprolegniaceae spores on the epidermis of the salmonid fish *Salmo trutta* and *Salvelinus alpinus*. Trans. Br. Mycol. Soc. 68, 91–95.

Wolinska, J., Giessler, S., Koerner, H., 2009. Molecular identification and hidden diversity of novel Daphnia parasites from European lakes. Appl. Environ. Microbiol. 75, 7051–7059. https://doi.org/10.1128/AEM.01306-09

Woynarovich, A., Hoitsy, G., Moth-Poulsen, T., 2011. Small-scale rainbow trout farming, FAO fisheries and aquaculture. Food and agriculture organization of the United Nations, Rome.

Yuasa, K., Hatai, K., 1995. Relationship between pathogenicity of *Saprolegnia* spp. isolates to rainbow trout and their biological characteristics. Fish Pathol. 30, 101–106.

Figure captions

Figure 1. Position of studied fish farms in Croatia with coordinates (WGS84 coordinate reference system).

Figure 2. Representatives of the isolates collected at the Croatian trout aquaculture facilities from the genera (A) *Pythium* (isolate Z121), (B) *Leptolegnia* (isolate BD25), and (C) *Saprolegnia* (*S. parasitica* isolate BF1). (D) PCR amplification of the ITS region of the respective isolates with universal primers ITS5 and ITS4. M – SimplyLoad™ Tandem DNA Ladder (Lonza), P – *Pythium* sp. (Z121), L – *Leptolegnia* sp. (BD25), S – *S. parasitica* (BF1), + - positive control (S*. parasitica* CBS 233.65), N - negative control (distilled water).

Figure 3. Phylogenetic analysis of *Saprolegnia* and *Leptolegnia* isolates (in bold) from Croatian trout farms and their upstream and downstream water environments. The phylogenetic tree is based on Bayesian inference analysis of ITS sequences. Bayesian posterior probabilities ≥ 80 % are shown at the nodes. GenBank accession numbers of reference sequences are given in Table A.2, except for two *Leptolegnia* sp. isolates (accession numbers shown in the tree).

Figure 4. Phylogenetic analysis of *Pythium* isolates (in bold) from Croatian trout farms and their upstream and downstream water environments. The phylogenetic tree is based on Bayesian inference analysis of ITS sequences. Bayesian posterior probabilities ≥ 80 % are shown at nodes. Clades A – K are labeled according to the available molecular phylogeny and taxonomy of the genus *Pythium* (Lévesque and De Cock, 2004). GenBank accession numbers of reference sequences are given in Table A.2.

Figure 5. Correspondence analysis biplot displaying the associations of oomycete species isolated from water (hempseed baits) with the sampling location (upstream, fish farm, or downstream). Oomycete species are represented by black points and sampling locations by red arrows. The distance between any species points or sampling location points gives a measure of their similarity (or dissimilarity). Points with

a similar profile are closer on the factor map. Dimensions (Dim) 1 and 2 both indicate the percentage of association between the row and column categories.

Figure 1

	Fish farm	GPS coordinates	
		N	E
🔴	Solin	43° 32′ 14′′	16° 29′ 33′′
🟢	Kostanjevac	45° 43′ 59′′	15° 25′ 18′′
🟣	Radovan	46° 12′ 22′′	16° 15′ 12′′
🔵	Gračani	45° 51′ 24′′	15° 57′ 53′′

Figure 2

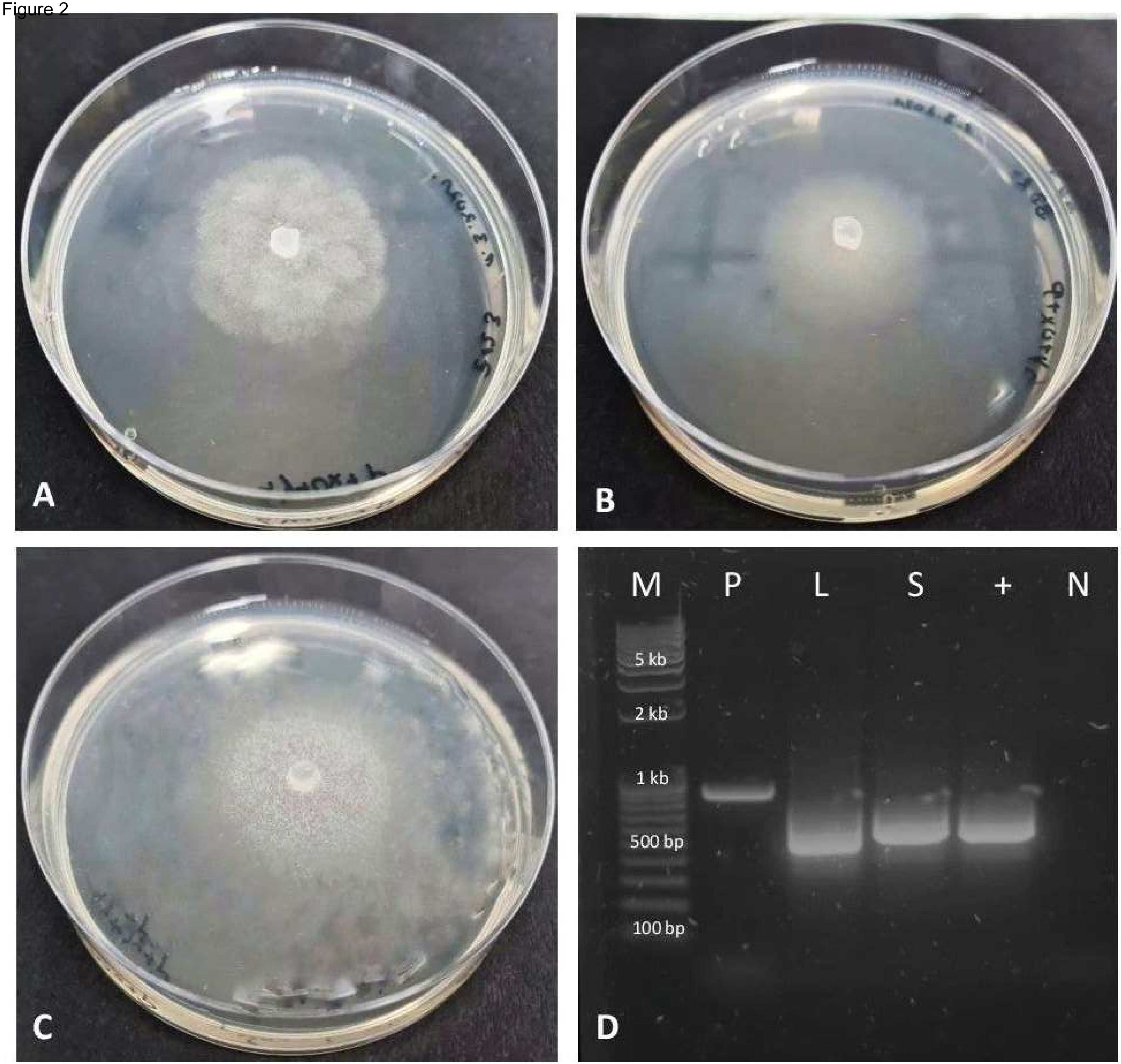
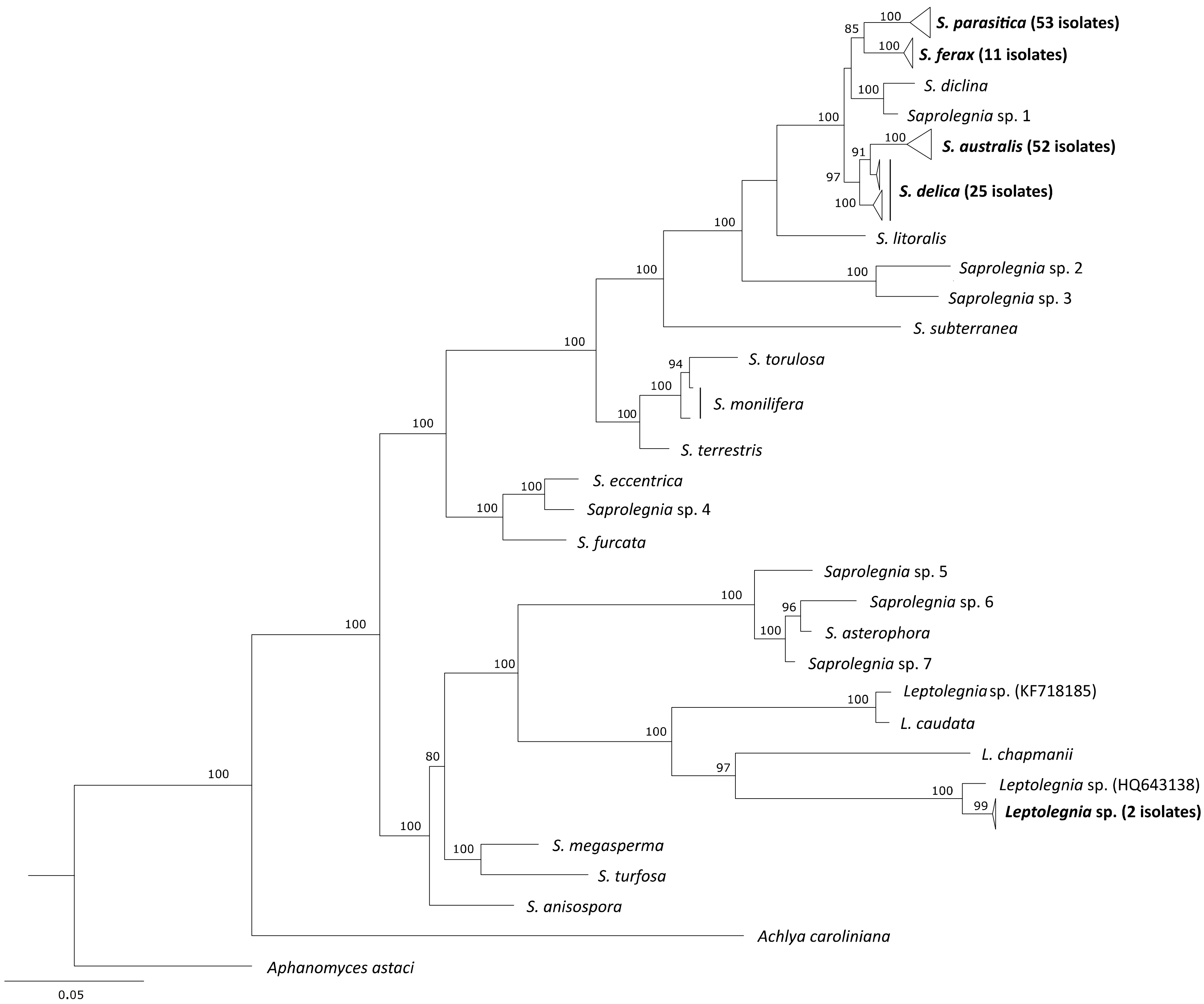
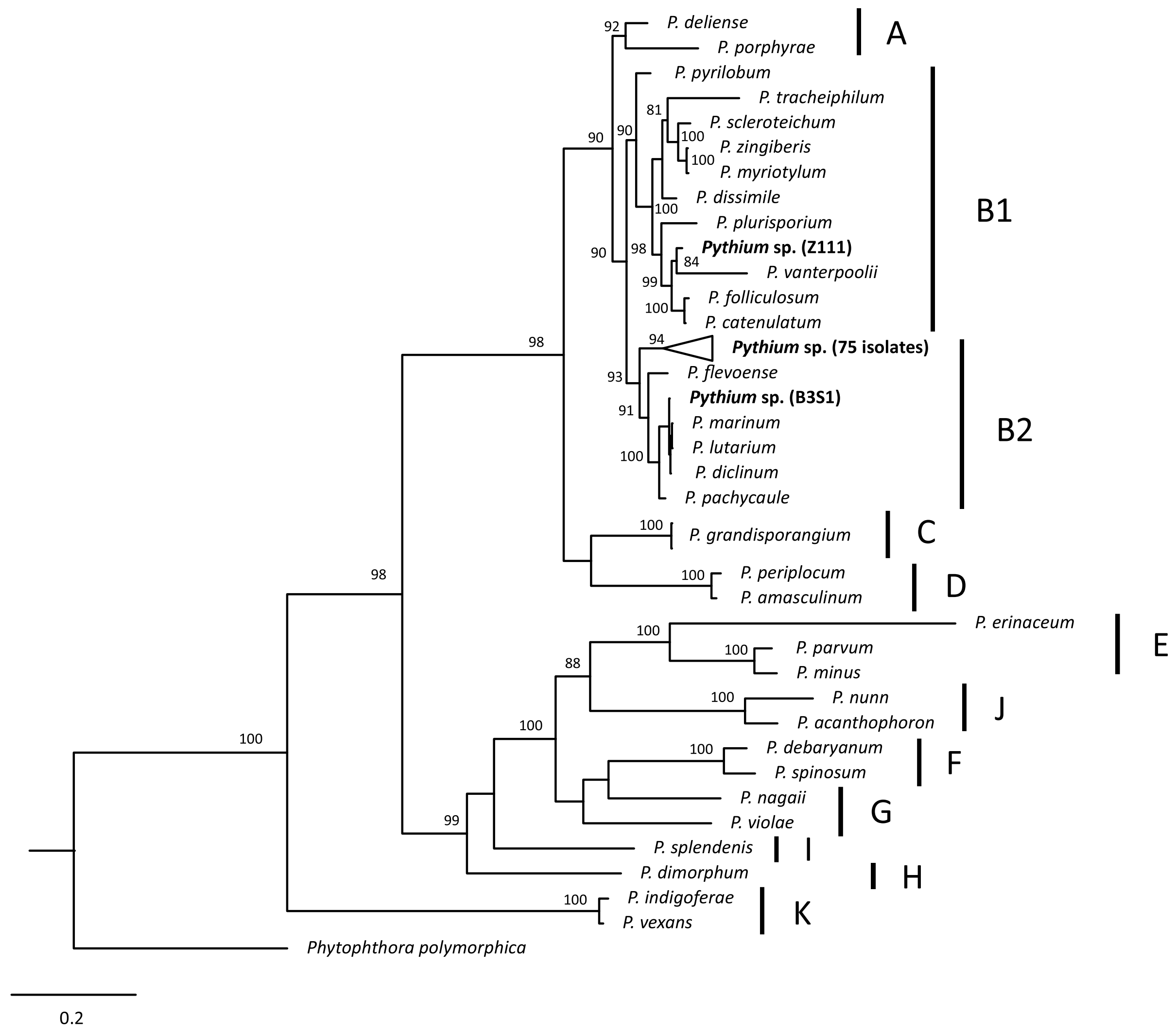


Figure 3



S. parasitica (53 isolates)
S. ferax (11 isolates)
S. diclina
Saprolegnia sp. 1
S. australis (52 isolates)
S. delica (25 isolates)
S. litoralis
Saprolegnia sp. 2
Saprolegnia sp. 3
S. subterranea
S. torulosa
S. monilifera
S. terrestris
S. eccentrica
Saprolegnia sp. 4
S. furcata
Saprolegnia sp. 5
Saprolegnia sp. 6
S. asterophora
Saprolegnia sp. 7
Leptolegnia sp. (KF718185)
L. caudata
L. chapmanii
Leptolegnia sp. (HQ643138)
Leptolegnia sp. (2 isolates)
S. megasperma
S. turfosa
S. anisospora
Achlya caroliniana
Aphanomyces astaci
0.05

Figure 4



P. deliense
P. porphyrae
A
P. pyrilobum
P. tracheiphilum
P. scleroteichum
P. zingiberis
P. myriotylum
P. dissimile
P. plurisporium
Pythium sp. (Z111)
P. vanterpoolii
P. folliculosum
P. catenulatum
B1
Pythium sp. (75 isolates)
P. flevoense
Pythium sp. (B3S1)
P. marinum
P. lutarium
P. diclinum
P. pachycaule
B2
P. grandisporangium
C
P. periplocum
P. amasculinum
D
P. erinaceum
P. parvum
P. minus
E
P. nunn
P. acanthophoron
J
P. debaryanum
P. spinosum
F
P. nagaii
P. violae
G
P. splendenis
I
P. dimorphum
H
P. indigoferae
P. vexans
K
Phytophthora polymorphica
92
90
90
81
100
100
100
98
84
99
100
98
94
93
91
100
100
100
98
100
100
88
100
100
100
100
99
100
0.2

Figure 5

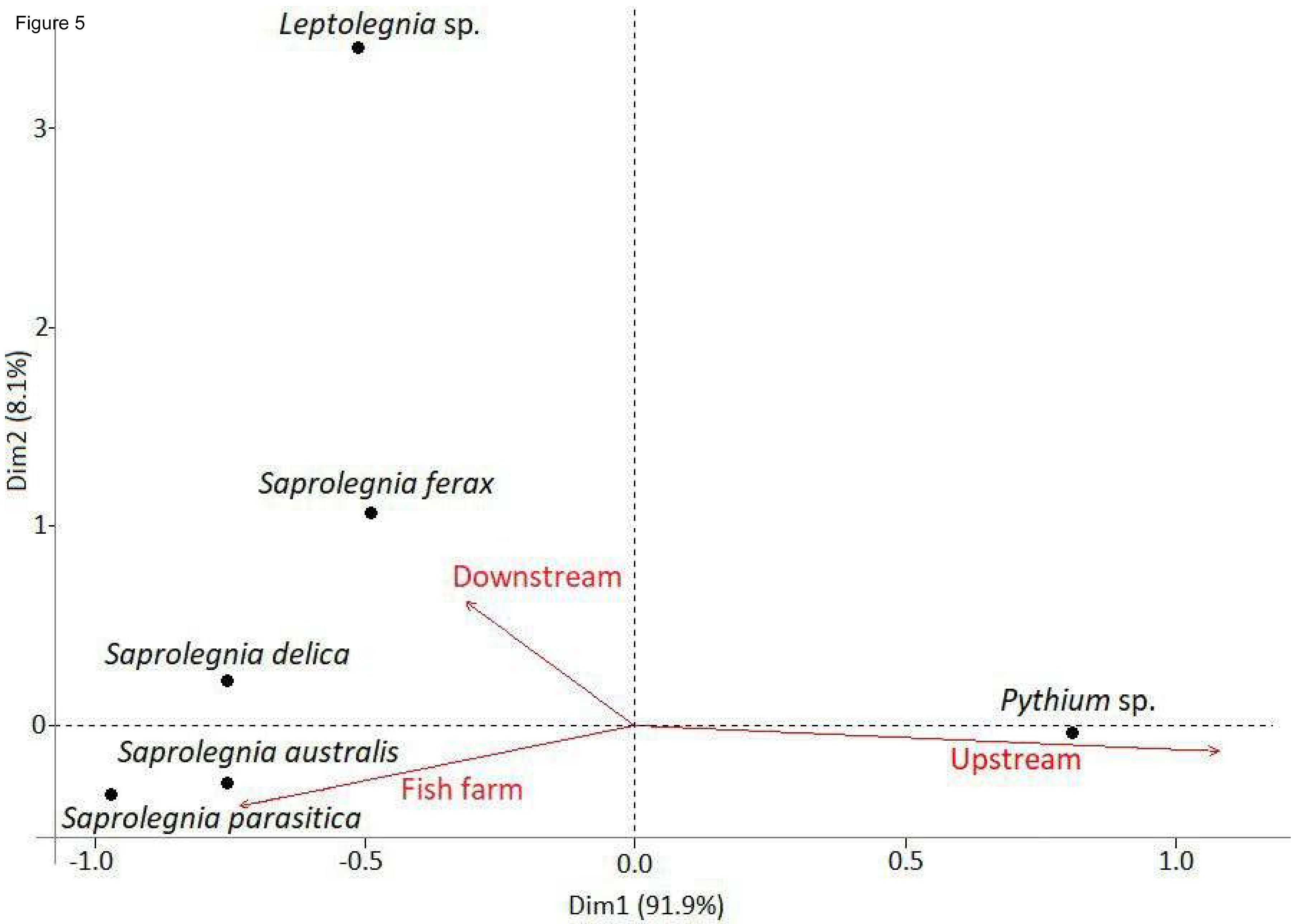

Leptolegnia sp.
Saprolegnia ferax
Saprolegnia delica
Saprolegnia australis
Saprolegnia parasitica
Pythium sp.
Downstream
Upstream
Fish farm
3
2
1
0
-1.0
-0.5
0.0
0.5
1.0
Dim2 (8.1%)
Dim1 (91.9%)

Table 1. Water quality parameters inside the sampled fish farms.

	Solin	Kostanjevac	Radovan	Gračani
conductivity (μS/cm)	523	470	549	393
dissolved oxygen (mg/L)	9.34	8.54	10.25	9.13
pH	7.65	7.84	7.94	7.35
average temperature (°C)	8.3	8.9	12.47	12.5

Table 2. Overview of oomycete isolates obtained from the surface of eggs, alevins, and adult fish with signs of disease at the trout farms in Croatia.

Species	**Adult stage**				**Embryonic stage**		**Total No. (%)**
	Solin	**Kostanjevac**	**Radovan**	**Gračani**	**Radovan (eggs)**	**Solin (alevins)**	
S. australis	0	4	0	0	0	0	**4 (9)**
S. delica	0	2	0	0	0	1	**3 (6)**
S. ferax	0	0	0	0	0	1	**1 (2)**
S. parasitica	2	2	0	13	12	3	**32 (70)**
Leptolegnia sp.	0	0	1	0	0	0	**1 (2)**
Pythium sp.	2	2	0	0	1	0	**5 (11)**
No. of isolates	**4**	**10**	**1**	**13**	**13**	**5**	**46**

Table 3. Overview of oomycete isolates obtained by hempseed baits from the water in the fish farms (F), as well as upstream (U) and downstream (D) locations.

Species	**Solin***	**Kostanjevac**			**Radovan**			**Gračani**			**Total No. (%)**
	F	**U**	**F**	**D**	**U**	**F**	**D**	**U**	**F**	**D**	
S. australis	8	1	12	13	0	0	0	2	12	0	**48 (27)**
S. delica	3	1	10	8	0	0	0	0	0	0	**22 (13)**
S. ferax	1	1	0	0	0	3	5	0	0	0	**10 (6)**
S. parasitica	12	0	1	1	0	3	2	0	2	0	**21 (12)**
Leptolegnia sp.	0	0	0	0	0	0	1	0	0	0	**1 (1)**
Pythium sp.	0	29	0	1	5	10	6	11	1	9	**72 (41)**
No. of isolates	**24**	**32**	**23**	**23**	**5**	**16**	**14**	**13**	**15**	**9**	**174**

***** Hempseed baits were not positioned upstream and downstream of the fish farm Solin due to its specific position near the sea.

Table 4. Chi-square test displaying dependence between row and column categories. X-squared, degrees of freedom (df) and *p* values are indicated. *P* values falling below the critical α = 0.05 are in boldface. Location: U – upstream locations; F – fish farm; D – downstream locations, fish farms: Fish farms: S – Solin; K – Kostanjevac; R – Radovan; G – Gračani.

Pearson's Chi-squared test	*X*-squared	df	p-value
Tissue-associated isolates vs. trout developmental stage	15.984	10	0.1001
Tissue-associated isolates vs. fish farm (S, K, R, G)	56.798	30	**0.002**
Farm water-associated isolates (F) vs. fish farm (S, K, R, G)	107.69	15	**4.48E-16**
Water-associated isolates vs. sampling location (U, F, D)	60.653	10	**2.73E-09**

Table 5. A contingency table displaying the number of oomycete species isolated from water (hempseed baits) on the sampling locations (upstream, fish farm or downstream, from fish farms Kostanjevac, Radovan and Gračani).

Oomycete species	Upstream	Fish farm	Downstream	Total
Leptolegnia sp.	0	0	1	**1**
Pythium sp.	45	11	16	**72**
Saprolegnia australis	3	24	13	**40**
Saprolegnia delica	1	10	8	**19**
Saprolegnia ferax	1	3	5	**9**
Saprolegnia parasitica	0	6	3	**9**
Total	**50**	**54**	**46**	**150**