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

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9 Abstract

10 Oomycetes from the genus Saprolegnia are opportunistic pathogens that cause significant losses in 11 salmonid aquaculture. Despite this, studies reporting dominant Saprolegnia species in different fish 12 farming facilities, as well as analyses of their spreading to natural environments, are still scarce. In this 13 study, we have for the first time identified oomycete species present in four different trout farms in 14 Croatia. We have collected 220 oomycete isolates, both from affected tissue (46 in total: adult trout - 28, 15 eggs - 13, and alevins - 5) and from water (174 in total: in the fish farm - 78, upstream - 50, and 16 downstream - 46). We have used Bayesian inference to reconstruct phylogenetic relationship among the 17 internal transcribed spacer (ITS) sequences of the collected isolates and referent strains, and determined 18 that the isolates belonged to three different oomycete genera: Saprolegnia (64 % of isolates), Pythium (35 19 %), and Leptolegnia (1 %). Saprolegnia isolates were classified into four species: S. parasitica with 53 20 isolates, S. australis - 52, S. delica – 25, and S. ferax – 11. Pythium and Leptolegnia isolates couldn't be 21 identified to the species level and probably belong to so far undescribed species since their sequences 22 didn't group with previously described species. Next, isolates from the affected tissue were mostly S. 23 parasitica (32), while S. australis, S. delica, and S. ferax were less common (\leq 4 isolates per species). 24 Furthermore, we used hempseed baits to capture oomycetes from water and positioned them inside the 25 fish farms, as well as upstream (between 55 and 155 m) and downstream (between 95 and 140 m) of the 26 fish farms. According to correspondence analysis, Saprolegnia species showed a strong association with 27 fish farms and downstream locations, while upstream locations were associated with Pythium species, 28 highlighting a possible role of trout farms as a source of spreading Saprolegnia species into the 29 environment.

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

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31 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, oomycetecaused 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).

39 Saprolegnia species (S. parasitica, S. australis, S. diclina, and others) are ubiquitous in the 40 freshwater environment and mostly considered as opportunistic secondary pathogens that infect the host 41 in stressful conditions (such as infection by other pathogens, injuries, or adverse environmental conditions 42 in general) (Gozlan et al., 2014; van den Berg et al., 2013). However, some S. parasitica strains were 43 reported to be highly virulent and cause primary infections (Neish and Hughes, 1980; Stueland et al., 2005; 44 Thoen et al., 2011; Whisler, 1996; Willoughby and Pickering, 1977). Saprolegniosis is a fish disease that 45 affects all developmental stages – from eggs to juveniles and adults. It is a major problem in many wild 46 and farmed fish species, such as Atlantic salmon, rainbow and brown trout, and also non-salmonid species 47 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 48 49 (Fregeneda Grandes et al., 2001; Hussein et al., 2001; Willoughbay, 1994, 1989). The disease is frequent 50 during the winter when fish are often immunocompromised due to decreased water temperature (Bly et 51 al., 1992; Bly and Clem, 1992).

52 Saprolegniosis is a serious problem in salmon and trout farms and hatcheries. Massive infections 53 of eggs are common, and entire batches can be lost (Cao et al., 2012; Meyer, 1991; Rach et al., 2005; 54 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.

61 Knowledge about the identity, distribution, and pathogenic significance of Saprolegnia species in 62 aquaculture facilities is a necessary prerequisite for the development of efficient control measures. 63 However, until recently little was known about dominant species associated with saprolegniosis 64 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-65 Uribeondo et al., 2007; Fregeneda-Grandes et al., 2007; van den Berg et al., 2013). Molecular diagnostic 66 tools have been recently introduced in the identification of Saprolegnia spp., based on the sequence of 67 68 internal transcribed spacer (ITS) region positioned between rRNA encoding genes (Cao et al., 2012; 69 Diéguez-Uribeondo et al., 2007; Kozubíková-Balcarová et al., 2013; Rezinciuc et al., 2014; Sandoval-Sierra 70 et al., 2014; Sarowar et al., 2019a; Tandel et al., 2020). However, the presence of many misassigned ITS 71 sequences in DNA databases (e.g. GenBank) caused the erroneous classification of many isolates in the 72 culture collections. A recent study on Saprolegnia molecular taxonomy resolved this issue and enabled 73 the correct identification of Saprolegnia isolates to the species level, without the need for morphological 74 characterization (Sandoval-Sierra et al., 2013). This allowed the recent accurate identification of 75 Saprolegnia species in fish farms in Canada (Sarowar et al., 2019a), Chile (Sandoval-Sierra et al. 2014), 76 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,

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79 we were interested in the possible transfer of Saprolegnia pathogens from the fish farms to the 80 downstream freshwater environments. Since freshwater aquaculture facilities are often connected with 81 rivers/streams, it is possible for fish to escape or water to drain into the surrounding environment, 82 allowing the transfer of pathogens (Andreou et al., 2012; Garseth et al., 2013; Gozlan et al., 2014; 83 Johansen et al., 2011; Thorstad and Finstad, 2018). However, while transmission of viral and bacterial 84 pathogens from farmed fish to wild populations has been repeatedly reported (Johansen et al., 2011; 85 Johnsen and Jensen, 1994; Raynard et al., 2001; Wallace et al., 2008), knowledge of trout farms as points 86 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.

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92 2. Materials and methods

93 2.1. Sampling

94 Sampling of oomycetes was carried out at four aquaculture facilities in Croatia (Fig. 1): three were 95 located in central Croatia, at Gračani (part of Zagreb) (fish farm producing rainbow trout, Oncorhynchus 96 mykiss (Walbaum, 1792)), Kostanjevac (a village near Zagreb) (fish farm producing brown trout, Salmo 97 trutta (Linnaeus, 1758)), and Radovan (a village near Varaždin) (fish farm and hatchery producing O. 98 mykiss), while the fourth one, Solin (a town near Split) (fish farm and hatchery producing O. mykiss), was 99 located at the Adriatic coast. Sampling was carried out during winter (November, December, and January) 100 in 2018 and 2019 (Table A.1). Conditions on the fish farms at the time of sampling were favorable for trout 101 rearing (Woynarovich et al., 2011): water temperature was between 9 and 12.5 °C, pH between 7.3 and 102 7.8, and dissolved O_2 between 8.5 and 10.3 mg/L, as measured by a portable multimeter (Hach[®] Field

103 Case, Colorado, USA) (Table 1). Hatcheries Radovan and Solin (where alevins and eggs were sampled, 104 respectively) were located near the adult fish rearing basins and were using the same water. The number 105 of diseased fish in all farms was less than 1 %. Oomycetes were isolated from the host (eggs, alevins, and 106 adult trout), as well as from the water in the farm, upstream and downstream.

107 A total of 75 tissue samples were collected from embryonic (eggs and alevins covered in 108 Saprolegnia-like mycelium) and adult specimens (having skin lesions with external signs of Saprolegnia 109 spp. mycelium growth) (Table A.1). Affected embryonic stages were available only in Radovan (eggs) and 110 Solin (alevins), while affected adult trout were collected in all fish farms. Affected tissue (lesions) was 111 excised from adult fish, while eggs and alevins with cotton-like mycelia growth, dead at the time of 112 sampling, were taken whole. Tissue samples of adult specimens with no signs of infection (gills and skin) 113 were also analysed, in order to compare the oomycete isolation success and the identity of the obtained 114 isolates with those originating from the affected samples. Samples were collected aseptically, dipped for 115 approximately one second in 96 % ethanol, and rinsed with sterile distilled water, to reduce bacterial 116 contamination which could lead to unsuccessful oomycete isolation. Next, rinsed samples were placed 117 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) 118 supplemented with penicillin G and oxolinic acid in the final concentration of 6 and 10 mg/L, respectively 119 (Alderman and Polglase, 1984). Plates were incubated at 18 °C (Galuppi et al., 2017), and pure cultures 120 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.

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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.

140 The ITS region (ITS 1, 5.8S rDNA, and ITS 2) was amplified with universal primers for eukaryotes 141 ITS5 (5' GGAAGTAAAAGTCGTAACAAGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') (White et al., 1990) 142 under conditions described by Sandoval-Sierra et al. (2013). Shortly, the reaction mixture contained 1 µL 143 of the genomic DNA, 12.5 μL of EmeraldAmp[®] PCR 2× Master Mix (TAKARA), 0.5 μL of 10 μM of primers 144 and dH₂O to a final volume of 25 µL. Thermal cycling was performed in Alpha Cycler 1 (PCRmax) with the 145 following conditions: 2 min at 95 °C for initial denaturation, followed by 35 cycles of 1 min at 95 °C 146 (denaturation), 30 sec at 60 °C (annealing), and 1 min at 72 °C (extension), and 10 min at 72 °C as a final 147 extension step. Saprolegnia parasitica CBS 233.65 genomic DNA and distilled water were used as positive 148 and negative control, respectively. Obtained amplicons, approximately 600 bp long for Saprolegnia spp. 149 and Leptolegnia spp., and approximately 900 bp for Pythium spp. (Fig. 2 - D), were purified and then 150 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).

154 **2.3. Alignment and phylogenetic analyses**

155 Multiple sequence alignment (MSA) of the ITS region of all isolates and selected reference 156 sequences was constructed in MAFFT using default settings (Katoh and Standley, 2013), and edited in 157 SeaView (Gouy et al., 2010) and BioEdit (Hall et al., 2011). Reference sequences from genera Achlya, 158 Aphanomyces, Leptolegnia, Phytophthora, Pythium, and Saprolegnia were selected based on the available 159 literature on their molecular phylogeny (Lévesque and De Cock, 2004; Rocha et al., 2018; Sandoval-Sierra 160 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 161 162 comprising the sequences from the order Saprolegniales (genera Saprolegnia and Leptolegnia) 163 (Supplementary material, Figure B.1), and another with sequences from the order Peronosporales (genus 164 Pythium) (Supplementary material, Figure B.2). Final MSA of Saprolegnia and Leptolegnia sequences 165 contained in total 179 sequences (143 sequences of isolates and 36 reference sequences), while MSA of 166 Pythium had 112 sequences (77 sequences of isolates and 35 reference sequences). The phylogenetic 167 relationship among the sequences was reconstructed with the Bayesian inference method using MrBayes 168 software 3.2.7a with 200 000 iterations (Ronquist and Huelsenbeck, 2003). Two simultaneous, 169 independent analysis were run with four Markov chain Monte Carlo (MCMC), one cold and three heated 170 chains with temperature set to 0.5. Every 100 generations were sampled and first 25 % of the samples 171 from the cold chain were discarded as 'burn-in'. Posterior probability was estimated for the remaining 172 trees. Phylogenetic trees were visualized with Figtree v1.4.4. (http://tree.bio.ed.ac.uk/software/figtree/).

173 **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:

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

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:

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

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

185 2.5. Statistical analyses

186 Associations between oomycete species and sampling location/type of sample were analyzed by 187 correspondence analysis (CA) which provides factor scores (coordinates) for both row and column points 188 of the contingency table. These coordinates provide a solution for summarizing the data set in two-189 dimension plots, used to visualize graphically the association between the row and column elements in 190 the contingency table (Kassambara, 2017). Dimensions 1 and 2 both indicate the percentage of association 191 between the row and column categories. We have tested the following associations: (i) tissue-associated 192 isolates (from all four fish farms) vs. trout developmental stage (egg, alevin, adult), (ii) tissue-associated 193 isolates (from all tissue types) vs. fish farm (all four fish farms included); (iii) farm water-associated isolates 194 (captured by hempseed baits in the fish farms) vs. fish farm (all four fish farms included), and (iv) waterassociated isolates (captured by hempseed baits) vs. sampling location (upstream, fish farm, and 195 196 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.

- 202
- 203 **3. Results**

3.1. Molecular identification of oomycete isolates from Croatian trout farms

205 A total of 220 oomycete isolates were cultured, 46 originating from tissue samples, and 174 from 206 hempseed baits (Fig. 2 A - C; Tables 2 and 3; Table A.1). Oomycete detection frequency for both sample 207 types/sampling methods was similar: 61 % for tissue samples (46 samples with oomycete growth out of 208 the total number of 75 samples), and 60 % for hempseed halves (174 out of 289 hempseeds resulted in 209 oomycete growth) (Table A.1). From the gill and tissue samples showing no clinical signs of saprolegniosis, 210 the isolation of oomycete was less successful than from skin lesions, 50 % (i.e. 7 samples out of 14 resulted 211 in oomycete growth) and 60 % (21/35) respectively. Furthermore, only 57 % of the isolates collected from 212 the healthy gills and skin were identified as Saprolegnia sp., while 90 % of isolates collected from the skin 213 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 %).

230 **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 (\leq 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).

250 **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.

255 The oomycete species collected from water varied according to the fish farm (Solin, Kostanjevac, 256 Radovan or Gračani; Table 3, Fig. A.1-C) and the sampling location (U, F and D) (Tables 3 and 5; Fig. 5). 257 Generally, dominant Saprolegnia species captured from water in different fish farms were in accordance 258 with Saprolegnia species detected in diseased animal tissues (Tables 2 and 3, Fig. A.1-B and C). For 259 instance, S. parasitica dominated in Solin and S. australis in Kostanjevac (Tables 2 and 3). However, in one 260 farm (Gračani), S. parasitica was dominantly isolated from tissue samples (Table 2), while S. australis was 261 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 262 263 species (Fig. 5). In this analysis, the first two dimensions explained the 100 % of association that exists 264 between oomycete species and sampling locations, where the first dimension explained 91.9 %, and the 265 second dimension explained 8.1 % of the association. Namely, the presence of pathogenic Saprolegnia 266 species was associated with fish farms and downstream locations, while Pythium sp. was typically 267 dominant in upstream locations (Fig. 5, Table 5).

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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).

271

272 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.

277 4.1. Pathogenic oomycete sampling approaches in freshwater ecosystems

278 We have combined two sampling approaches (tissue and water samples) to get the most insight 279 into pathogenic oomycete species present in selected fish farms and the natural environment. Both 280 methods have advantages and disadvantages and can be complementary when used in combination, as 281 was demonstrated in several previous studies (Galuppi et al., 2017; Rahman and Sarowar, 2016; Rezinciuc 282 et al., 2014; Sarowar et al., 2019b, 2013; Thoen et al., 2015). For instance, since hempseeds attract 283 zoospores/cysts in the water, some non-zoosporic species can go undetected. Also, bacteria in the water 284 can sometimes disable zoospore attachment and germination on the baits (Sarowar et al., 2019b). On the 285 other hand, personnel in the fish farms are often reluctant to provide affected animals (as was also the 286 case during this study). Also, it is sometimes difficult to obtain infected, but still living embryonic stages, 287 since they quickly succumb to the disease. It is therefore hard to know whether the isolated oomycete 288 species was the primary pathogen, or if the initial pathogen was overgrown by a secondary, opportunistic 289 species. In our case, the fact that S. diclina, well known for egg infections (Fregeneda-Grandes et al., 2007; 290 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

299 In this study, three genera of oomycetes were identified: Saprolegnia, Leptolegnia and Pythium. 300 The dominant species and the only one that was isolated from all fish farms and all trout developmental 301 stages was S. parasitica. Thus, our results confirm its dominance over other Saprolegnia species in 302 aquaculture facilities (Hussein and Hatai, 2002; Noga, 1993; Sandoval-Sierra et al., 2014; Sarowar et al., 303 2019a; van den Berg et al., 2013; van West, 2006). Previous infection trials demonstrated pathogenicity 304 of S. parasitica towards eggs (Kitancharoen and Hatai, 1996), fingerlings (Yuasa and Hatai, 1995), and adult 305 salmonids (Stueland et al., 2005). In contrast to our findings, in Chilean salmonid farms S. parasitica was 306 detected in adult Salmo salar and O. mykiss, but not in eggs and alevins (Sandoval-Sierra et al., 2014). This 307 could be explained by the known variations in pathogenicity of S. parasitica isolates towards different 308 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., 315 2020; Thoen et al., 2011), in our study it wasn't isolated from this sample type. This result may be caused 316 by the small sample size (i.e. overall low number of isolates collected from eggs and alevins - 18). An 317 earlier analysis of fish farms in Chile, with higher number of isolates from embryonic stage (122) showed 318 an association between different Saprolegnia species and salmonid developmental stage, i.e. S. australis 319 was associated with alevins (Sandoval-Sierra et al., 2014). Interestingly, S. australis was the most prevalent 320 species found in water, especially in fish farms Kostanjevac and Gračani. This might be explained by the 321 fact that, although S. australis zoospores might have been present in the water in high number (and thus 322 easily captured by hempseed baits), infection with S. australis rarely occurred because the fish and eggs 323 were healthy (and thus the animals were more often infected by S. parasitica, as a more virulent 324 pathogen) (van den Berg et al., 2013; van West, 2006).

325 Lastly, S. delica was the second most isolated Saprolegnia species in water samples, and S. ferax 326 was also occasionally captured by hempseed baits, while these two species were rarely obtained from 327 tissue samples. Both species have previously been associated with embryonic mortality of fish and 328 amphibians (Blaustein et al., 1994; Cao et al., 2012; Fregeneda-Grandes et al., 2007; Kiesecker et al., 2001) 329 and were also often isolated from water (Rezinciuc et al., 2014; Sarowar et al., 2013). Our results are 330 similar to a recent study done in Chilean salmonid farms, where S. ferax and S. delica were found both on 331 adult and embryonic stages of salmonid fish without a clear preference for any particular developmental 332 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.

342 Furthermore, Pythium sp. isolates were also found in this study, mostly from water upstream of 343 the fish farms (B2 isolates, and one B1 isolate - Z111 from Radovan), while a small number of B2 isolates 344 were also found on diseased fish and only one B2 isolate on an egg sample. Known Pythium species are 345 mostly plant pathogens or saprotrophs, mainly associated with natural and agricultural soils (Rahman and 346 Sarowar, 2016; Robideau et al., 2011; Schroeder et al., 2013). However, Pythium sp., including B1 and B2 347 clades, were also isolated from natural and aquacultural freshwater environments (Nechwatal et al., 2008; 348 Rahman and Sarowar, 2016; Schroeder et al., 2013), and some were suggested to be pathogenic towards 349 freshwater animals (Miura et al., 2010). Pythium spp., including members of the clade B, were isolated 350 from the carapace of dead crustaceans (Czeczuga et al., 2002b) and from dead or alive fishes and eggs 351 (Czeczuga, 1996; Czeczuga et al., 2002a). Pythium flevoense (belonging to clade B2 and most closely 352 related to isolate B3S1 found on O. mykiss from Solin) was reported to be responsible for mass mortality 353 of freshwater fish (ayu larvae), but pathogenicity of isolates was not confirmed by infection trials (Miura 354 et al., 2010). Taking all this into account, most of our isolates are probably plant pathogens or soil 355 saprotrophs that arrived to the fish farms by water routes, as can be presumed from their phylogenetic 356 grouping with saprotrophs and plant pathogens, and the fact that the majority were captured in the water 357 upstream of the fish farms. For instance, isolate Z111 was most closely related to a known plant pathogen 358 P. vanterpoolii (clade B1) (Asano et al., 2010; Ichitani et al., 1989; Muse et al., 1974). Also in concordance 359 with this hypothesis, most of the studies that isolated Pythium spp. from fish tissue or water samples were 360 conducted in the fish farms or ponds surrounded by agricultural fields, grassland, or forests (Czeczuga et 361 al., 2005, 2002a; Naznin et al., 2017; Rahman and Sarowar, 2016; Sarowar et al., 2019b). This was also the 362 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 *Pythium* 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

371 To elucidate whether trout farms act as reservoirs of pathogenic Saprolegnia species that can 372 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 373 374 to downstream locations. All Saprolegnia species captured by hempseed baits (S. australis, S. delica, S. 375 ferax, and S. parasitica) were more abundant in the fish farms (43 isolates) and downstream locations (29) 376 than upstream (5) of the fish farms (Table A.1; Table 5). In comparison, upstream locations were strongly 377 associated with Pythium species (45), which were less often captured in the fish farms (11) and 378 downstream (16). Noteworthy, Saprolegnia species were not found downstream from one fish farm 379 (Gračani) which indicates that this farm had a smaller negative impact on the downstream environment 380 than Kostanjevac and Radovan, probably due to a well maintained settler tank used in the fish farm 381 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 387 et al., 2001; Thorstad and Finstad, 2018; Wallace et al., 2008). Our study illustrates this effect also for 388 trout farms and Saprolegnia pathogens, which is relevant since saprolegniosis causes high annual 389 economic losses in salmonid aquaculture (Hussein and Hatai, 2002; Phillips et al., 2008; van den Berg et 390 al., 2013; van West, 2006) and has a negative impact on wild populations of salmonids and other 391 freshwater fish, as well as other aquatic animals (Blaustein et al., 1994; Fregeneda Grandes et al., 2000; 392 Kiesecker et al., 2001; Neitzel et al., 2004; Pickering and Willoughby, 1982; van West, 2006). For instance, 393 Saprolegnia spp. can infect and kill crayfish specimens and it can be pathogenic towards amphibians 394 (salamander adult and frog eggs) (Dieguez-Uribeondo et al., 1994; Gil-Turnes et al., 1989; Hirsch et al., 395 2008; Kiesecker and Blaustein, 1995; Kozubíková-Balcarová et al., 2013; Krugner-Higby et al., 2010; Lefcort 396 et al., 1997). Saprolegnia species that have been introduced to the natural environment via fish restocking 397 caused amphibian mortality (Blaustein et al., 1994; Kiesecker et al., 2001). In the last two decades, many 398 diseases have increased in prevalence and distribution (emerging infectious diseases) (Fisher et al., 2012; 399 Gozlan et al., 2014; van den Berg et al., 2013). Due to negative anthropogenic impacts on the natural 400 environment (e.g. climate change, pollution, the introduction of new species, habitat alteration and 401 degradation) the host species are becoming more vulnerable to various pathogens leading to disease 402 outbreaks and sometimes even to the extinction of whole populations (Fisher et al., 2012; Gozlan et al., 403 2014). In this context, the transfer of Saprolegnia spp. from fish farms to the surrounding environment 404 could lead to increased mortalities in natural populations, and it is crucial to undertake detailed surveys 405 to follow pathogenic Saprolegnia spreading and distribution from fish farms to the natural environment, 406 such as this one.

407 **5. Conclusions**

408 Our study highlights the role of trout farms as potential points of release of *Saprolegnia* pathogens 409 to downstream freshwater ecosystems. Further studies are needed to assess the real impact of such 410 pathogen spread, for instance, by sampling multiple points downstream from aquaculture sites coupled 411 with pathogen quantification via molecular techniques, such as quantitative PCR or droplet digital PCR. 412 This could be done by the combination of hempseed baiting (as applied here) and isolation of 413 environmental DNA (eDNA) directly from water, an approach that has been widely used in recent years 414 for detection and monitoring of species of interest (Dougherty et al., 2016; Strand et al., 2011). Also, the 415 knowledge on the Saprolegnia spp. pathogenicity for free-living animal species is scarce, and further 416 studies should be performed to assess Saprolegnia virulence, especially in combination with other 417 stressors, such as elevated water temperature due to climate change, anthropogenic pollution, and 418 pressure of invasive competing species.

419

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716 Figure captions

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

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 \geq 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 3

0.05



0.2

Figure 4



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

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

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.

		Adult	Embryo	Tatal			
Species	Solin	Kostanjevac	Radovan	Gračani	Radovan (eggs)	Solin (alevins)	No. (%)
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)
<i>Leptolegnia</i> sp.	0	0	1	0	0	0	1 (2)
<i>Pythium</i> 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.

Enocioc	Solin*	Кс	ostanje	vac		Radova	an		Grača	ni	Total
species	F	U	F	D	U	F	D	U	F	D	No. (%)
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)
<i>Leptolegnia</i> 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 $\alpha = 0.05$ 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
<i>Leptolegnia</i> 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