Taxonomical and functional diversity of *Saprolegniales* in Anzali lagoon, Iran

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Abstract

Studies on the diversity, distribution and ecological role of *Saprolegniales* (*Oomycota*) in freshwater ecosystems are currently receiving attention due to a greater understanding of their role in carbon cycling in various aquatic ecosystems. In this study, we characterized several *Saprolegniales* species isolated from Anzali lagoon, Gilan province, Iran, using morphological and molecular methods. Four species of *Saprolegnia* were identified, including *S. anisospora* and *S. diclina* as first reports for Iran. Evaluation of the ligno-, cellulo- and chitinolytic activities were also measured using plate assay methods. Most of the *Saprolegniales* isolates were obtained in autumn and nearly 50% of the strains showed chitinolytic and cellulolytic activities. However, only a few *Saprolegniales* strains showed lignolytic activities. This study has important implications for better understanding the ecological niche of oomycetes, and to differentiate them from morphologically similar but functional different aquatic fungi in freshwater ecosystems.

Key words – *Achlya*, *Saprolegnia*, aquatic ecosystems, carbon cycling, polymer degradation, *Saprolegniaceae*

Introduction

Saprolegniales, as a monophyletic order, belong to the phylum *Oomycota*. It includes biflagellate heterotrophic microorganisms that have eucarpic mycelial and coenocytic thalli of unlimited growth. They produce asexual (sporangia) and sexual (gametangia) structures delimited by septa. *Saprolegniales* are mainly predominantly freshwater saprophytes of plant and animal debris (Beakes and Sekimoto 2009). This order contains three families; *Achlyaceae* (four genera), *Saprolegniaceae* (11 genera) and *Verrucalvaceae* (7 genera) (Beakes et al. 2014; Molloy et al. 2014; Beakes and Thines 2017; Rocha et al. 2018). Among these, *Achlya*, *Brevilegnia*, *Dictyuchus*, *Leptolegnia*, *Plectospira*, *Saprolegnia* and *Thraustotheca* have been commonly reported to inhabit freshwater ecosystems (Czeczuga et al. 2005; Mousavi et al. 2009; Marano et al. 2011).

Saprolegniales species have been recently receiving increased attention due to their wide distribution, ubiquitous occurrence (Liu and Volz 1976; Kiziewicz and Kurzątkowska 2004; Nascimento et al. 2011), their devastating fish pathogenicity in aquaculture and fish farms and responsibility for massive decline of natural salmonid populations (Griffiths et al. 2003; Van West 2006; Romansic et al. 2009; Van Den Berg et al. 2013). Aside from their pathogenicity, many authors have also investigated relative frequencies of *Saprolegniales* throughout different seasons in relation to physico-chemical features of the respective freshwater ecosystems (El-Hissy and Khallil 1991; Czeczuga et al. 2003; Paliwal et al. 2009). Although these oomycetes are generally isolated from plant debris, their involvement in organic matter degradation in freshwater ecosystems remains less clear.

Species identification of *Saprolegniales* is largely based on morphological features (Coker 1923; Seymour 1970; Johnson et al. 2002). However, this identification is perplexing due to several reasons. First of all, in many cases, morphological and morphometric characters are vague and variable. Secondly, more determinative features like sexual structures are not always produced *in vitro*. Also, lack of type species and accurate description make it even harder to define specific species (Sandoval-Sierra et al. 2015, 2014). Recently, sequencing of the ribosomal internal transcribed spacer (ITS) has been applied to create a phylogenetic framework within which to address issues of morphological and taxonomic ambiguity (Steciow et al. 2014). Whether complementary molecular targets, in addition to

the *de facto* ITS region, would improve this approach or are even necessary is still open to debate (Robideau et al. 2011).

In this study, we investigated the diversity and seasonality of various strains of *Saprolegniales* isolated from Anzali lagoon, Iran. In total, we obtained 511 isolates from three locations during 2017 and studied their seasonality. From these, 23 isolates were randomly selected representing different sampling time points and locations and identified using morphological and phylogenetic analyses. In addition to their taxonomy, we tested the hypothesis raised by Masigol et al. (2019) that fungi and *Saprolegniales* differ in their affinity for polymeric dissolved organic matter (DOM) and consequently in their involvement in aquatic DOM degradation and cycling. To this, we evaluated the ligno-, cellulo- and chitinolytic activities of the selected strains. Our results have important implications for understanding the different roles of fungi and *Saprolegniales* in aquatic ecosystems.

Materials & Methods

Sampling site

Anzali lagoon is situated at the Caspian Sea near Bandar-e Anzali, in the northern Iranian province of Gilan. The lagoon divides Bandar-e Anzali into two parts, and is home to both the Selke Wildlife Refuge and the Siahkesheem Marsh. Three sampling sites as representatives of the main habitats in Anzali lagoon were selected:1) river entrance, 2) shallow water habitat and 3) urban habitat (Fig. 1).

Seasonal distribution of *Saprolegniales* and isolation

Throughout 2017, 511 *Saprolegniales* isolates were isolated using the methods described earlier by Coker (1923) and Seymour (1970). In brief, samples of decaying leaves of the dominant local vegetation collected from the three sampling locations were brought to the mycology laboratory of the University of Guilan in separate sterile polyethylene bags. Leaves were cut into approximately ten equal pieces (0.5×0.5 cm). After washing with distilled water, they were incubated at 20-25°C in sterilized plates containing 10 mL sterile distilled water with 20 sterilized hemp seed halves (*Cannabis sativa* L.) (Middleton 1943). Temperature and pH of surface water were continuously recorded immediately after collecting decaying leaves. Three replicates were considered for each location. The average number of colonized hemp seed halves from 10 Petri dishes was used to estimate the abundance of *Saprolegniales* throughout the year. The presence of *Saprolegniales* was

84 confirmed by observing at least one of the general features of oomycetes such as oogonia, sporangia,
85 large and aseptate mycelia and motile zoospores.
86 After three to five days, a piece of mycelia from the colonized hemp seed halves was transferred to a
87 fresh CMA-PARP medium (CMA-PARP; 40 g/L ground corn meal, 0.5 g/L ampicillin, 0.01
88 rifampicin, 0.2 g/L Delvocidsalt and 0.1 g/L Pentachloronitrobenzene (PCNB), 15 g/L agar)
89 (Kannwischer and Mitchell 1981). This step was repeated three to five times to achieve bacterial free
90 (axenic) cultures. A single hypha was transferred to cornmeal agar (CMA; 40 g/L ground corn meal,
91 15 g/L agar) (Seymour and Fuller 1987). The hyphal-tip technique was conducted three to five times
92 to obtain a pure culture in CMA. The specimens of these new strains were then deposited in the
93 Fungal Herbarium of the Iranian Research Institute of Plant Protection, Iran.
94 **Characterization of morphological features**
95 Asexual and sexual structures of isolates were characterized and measured in liquid (water) cultures
96 (n=30). To investigate strains failing to produce any sexual structures, several treatments were used.
97 The nutrition treatments included (1) reciprocal culturing of all strains with one another and
98 *Trichoderma* sp. (Brasier et al. 1978) on CMA, (2) hemp seed agar (HSA; 60 g/L ground hemp seeds,
99 15 g/L agar) (Hendrix 1964), (3) soybean agar (SA; 100g/L ground soybean seeds, 15 g/L agar)
100 (Savage et al. 1968), (4) rape seed extract agar (REA; 100g/L ground rape seeds, 15 g/L) (Satour
101 1967), (5) carrot juice agar (CJA; 250 g/L boiled carrot extract, 20 g/L agar) (Ershad 1971), (6)
102 mPmTG (2, 0.4, 0.4 and 12 g/L glucose, tryptone, peptonized milk and agar, respectively) (Moreau
103 and Moreau 1936b), (7) immersing colonized CMA in glycerin (4%) (Moreau and Moreau 1936a) and
104 (8) culturing the isolates in Petri dishes containing 10 boiled hemp seeds in distilled lake water and
105 distilled water (50/50). The temperature treatments also included culturing the isolates in 5, 10, 15,
106 20, and 25°C in Petri dishes containing ten boiled hemp seeds in distilled lake water and distilled
107 water (50/50).
108 **DNA extraction and PCR**
109 The DNA extraction was conducted based on a slightly modified protocol of Montero-Pau et al.
110 (2008). Briefly, 100 µL of alkaline lysis buffer (25 mM NaOH, 0.2 mM disodium EDTA, pH 8.0)
111 was aliquoted into 1.5 mL tubes. Malt extract broth (MEB; 17 g/L malt extract) (Galloway and

Burgess 1937) was used for growth of isolates. Mycelial mass was then transferred to the tube and centrifuged for 30 minutes at 9000 rpm. The tube was incubated at 95°C for 30 minutes and then cooled on ice for five minutes. Finally, 100 µL of neutralizing solution (40 mM Tris-HCl, pH 5.0) was added to the tubes. The final solution was vortexed and kept at -20°C. Nuclear ITS region was amplified in a Flexible PCR Thermocycler (Analytikjena, Germany) using ITS1/ITS4 (White et al. 1990) primers. Thermocycler program for amplification of the ITS region was: 94°C for 2 min for initial denaturation followed by 32 cycles of 94°C for 15 s, 53°C for 15 s, 72°C for 30 s, and a final extension at 72°C for 5 min (White et al. 1990). The resulting sequences were quality controlled using the Bioedit software (Hall et al. 2011) and submitted to GenBank (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov) database (for accession numbers see Table1).

Phylogenetic analysis

Molecular taxonomical assignment of the isolates was performed by comparing sequences against the NCBI's GenBank sequence database using the BLASTN search method. We selected and downloaded sequences of cultured isolates for performing phylogenetic analyses. The ITS sequences were aligned by the ClustalW algorithm (Thompson et al. 1994) and the phylogenetic tree was obtained using MEGA version 7 by the Maximum Likelihood (ML) method based on the Tamura-Nei model (Kumar et al. 2016).

Screening for lignolytic, cellulolytic and chitinolytic activities

- Lignolytic assay

Mycelia from the edge of 7-15 days cultures were transferred into 6-well plates containing the cultivation medium proposed by Rojas-Jimenez et al. (2017) (0.94 g KH_2PO_4, 1.9 g K_2HPO_4, 1.6 g KCl, 1.43 g NaCl, 0.15 g NH_4Cl, 0.037 g $MgSO_4$, 0.1 g yeast extract, 10 g malt extract, 15 g/L agar, pH 7.0) and mPmTG agar medium amended with one of the following substrates: (1) 0.1% wt/vol 2,20-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS), (2) 0.02 and 0.005% wt/vol Bromocresol Green (BG), (3) 0.02 and 0.005% wt/vol Congo Red (CR), (4) 0.02 and 0.005% wt/vol Malachite Green (MG) (5) 0.02 and 0.005% wt/vol Phenol Red (PhR) (6) 0.02 and 0.005% wt/vol PolyR-478 (PR) (pH 5 + 7) (7) 0.02 and 0.005% wt/vol Remazol Brilliant Blue (RBBR), and (8) 0.02 and 0.005% wt/vol Toluidine Blue (TB) (Pointing 1999; Swamy and Ramsay

1999; Moreira et al. 2000; Novotny et al. 2001; Gill et al. 2002; Rojas-Jimenez et al. 2017). The concentration of different dyes in previous experiments is highly variable. Therefore, two different concentrations were used to ensure that the applied concentration does not impact on the growth or the enzymatic activities of the tested strains. The capacity of each strain to produce lignolytic activity was determined by decolorization of the aforementioned substrates in the area around the mycelia or as a colour change of the media after three weeks. We evaluated 1-33, 33-66, and 66-100% decolorization of the medium in the Petri dishes as weak, medium, and strong activity, respectively.

- Cellulolytic assay

The same media as used for evaluation of lignolytic activities were amended with the following enzymatic carbon sources to investigate cellulolytic and pectolytic activities: (9) 7.5 g carboxymethylcellulose (CMC), (10) 7.5 g Avicel (AVL) and (11) 5g D-cellobiose (DCB) (Wood and Bhat 1988; Pointing 1999; Yoon et al. 2007; Jo et al. 2010). After three weeks of incubation, Congo Red (1 mg ml^{-1}) was amended to the medium and incubated at room temperature for 15 min. Subsequently, the medium was rinsed with distilled water, and 30 mL of 1 M NaCl added. Degradation of CMC, Avicel, and D-cellobiose was confirmed by a transparent appearance of the medium (and mycelia) (Teather and Wood 1982; Pointing 1999).

- Chitinolytic assay

The method proposed by Agrawal and Kotasthane (2012) was used to evaluate the chitinolytic properties of the isolated strains. Crab shell flakes were ground in a mortar and sieved through the top piece of a 130 mm two-piece polypropylene Buchner filter. Twenty grams of the sieved crab shell flakes were then treated with 150 mL of ~12M concentrated HCl which was added gently and continuously stirred for 45 minutes under a chemical fume hood. The final mixture was passed through eight layers of cheese cloth to remove large chitin chunks. The product was treated with two litres of cold distilled water and incubated overnight under static conditions at 4°C. Sufficient amount of tap water was then passed through the product until the pH of the product reached 7.0. The final product was squeezed between coffee paper and then sterilized by autoclaving at standard temperature and pressure (STP) (15 psi, 20 minutes, 121°C) (Murthy and Bleakley 2012). The chitinase detection medium consisted of a basal medium comprising (per litre) 0.3 g of $MgSO_4.7H_2O$, 3.0 g of

168 $(NH_4)_2SO_4$, 2.0 g of KH_2PO_4, 1.0 g of citric acid monohydrate, 15 g of agar, 200 µL of Tween-80, 4.5
169 g of colloidal chitin (CC) and 0.15 g of Bromocresol Purple; the pH was adjusted to 4.7, and the
170 neutralized medium autoclaved.
171 For each isolate, the experimental procedures mentioned above were repeated twice with three
172 replicates each. When a positive result was observed, this was confirmed in a third experiment using
173 tissue culture plates (TPP®) with the medium described above. We observed no conflicting results
174 from any of the three experimental instances, for each of the tested isolates and substrates. Strains
175 already tested by Rojas-Jimenez et al. (2017) were considered as positive controls.
176 **Statistical analyses**
177 We assessed whether there was a significant impact of month or broadly season at each of the three
178 locations on the frequency of *Saprolegniales* colonisation of hemp leaves, using a two-way ANOVA.
179 The relative contribution of temperature and pH to any spatio-temporal trends were assessed using
180 Spearman's Rank correlations. A propensity of individual genera or taxa to metabolise invidual
181 substrates was assessed using a two-way ANOVA. All statistical analyses were conducted using
182 GraphPad Prism version 8.1. (GraphPad Software, CA, USA).
183 **Results**
184 **Relative abundance of *Saprolegniales* isolates**
185 From all the oomycetes isolated from the three locations sampled along the year, 511 out of 720
186 (~71%) were assigned to the order *Saprolegniales.* The relative abundance of *Saprolegniales* was
187 higher at cold temperatures (autumn, winter and spring seasons) than in summer. The highest
188 temperatures were recorded in Jul. 23th-Aug. 22th (in average~33°C) and the lowest in Jun. 21th-Feb.
189 19th (in average~3°C). The number of isolates from river entrance, shallow lake and urban habitats
190 was negatively correlated with temperature (R^2= 0.7233, 0.5047 and 0.7623, respectively). However,
191 pH was constant and no correlation was observed for river entrance, shallow lake and urban habitats
192 (R^2 = 2E-05, 0.0037 and 0.0684, respectively). Of the 720 hemp seeds, only 209 were not colonized
193 by *Saprolegniales* isolates (~29%). These were either colonized by other microorganisms such as
194 fungi and protists, or remained intact. Co-colonization of *Saprolegniales* isolates and other unwanted
195 subjects or organisms was not counted as a positive result (Table 2).

196 **Morphological and molecular identification**

197 Of the selected isolates, 19 belonged to the genus *Saprolegnia* and four to the genus *Achlya*. Four
198 isolates of *Achlya* failed to produce sexual structures under any circumstances and thus were
199 considered as *Achlya* spp. (Isolates JT2W9-1, F962-15, O962-13 and O963-13). Morphology-based
200 taxonomy was confirmed by phylogenetic analysis of ITS sequences of nrDNA inferred from
201 maximum likelihood method.

202 ***Saprolegnia anisospora*** (Pringsheim) de BaryBot. Zeitung (Berlin) 41:56. 1883 (Fig. 2a-c)

203 Mycelium dense; main hyphae branched, hyaline to dark, with 16–46 µm (average 26 µm) width.
204 Sporangia very abundant, mainly fusiform, straight, sometimes curved, renewed in cymose fashion,
205 80–405 ×18–50 µm (average 220×26 µm). They discharged spores and behaved as saprolegnoids.
206 Cysts 9–13 µm in diameter (average 10 µm). Gemmae absent. Oogonia terminal, always spherical,
207 always immature, 78–107 µm in diameter. Oogonial wall smooth. Oogonial stalks 1-3 times the
208 diameter of oogonium, slender, slightly irregular and unbranched. Oospores never produced. No
209 specific pattern was observed for any of our strains on CMA.

210 **Material examined:** strains MDL5-1 and MDL14-1, on rotten leaves, Anzali lagoon, Anzali, Guilan,
211 Iran, 10-8-2017, H. Masigol; GenBank Acc. No: ITS – MK911009 and MK911010.

212 ***Saprolegnia diclina*** Humphrey Trans. Amer. Phil. Soc. (N.S.) 17:109, pl. 17 (Fig. 2d-f)

213 Mycelium sparingly to moderately branched, 17–42 µm (average 35 µm) in width. Sporangia
214 abundant, cylindrical, always straight, renewed internally, 120–976 ×18–69 µm (average 460×52 µm).
215 They discharged spores and behaved as saprolegnoids. Cysts 8–11 µm in diameter (average 9 µm).
216 Gemmae spherical, 66–110 µm in diameter, terminal, sometimes catenulate. Oogonia terminal,
217 spherical, obpyriform, 75–105 µm in diameter. Oogonial wall smooth. Oogonial stalk 1–3 times the
218 diameter of the oogonium, slender and unbranched. Oospores centric, spherical, 6–26 per oogonium
219 and 14–28 µm in diameter. Antheridia abundant, diclinous and androgynous. No specific pattern
220 observed for any of our isolates on CMA.

221 **Material examined:** isolates JSL25-2, FSL9, JSL24-3, JSW5-1, JSW17 and FSW19, on rotten
222 leaves, Anzali lagoon, Anzali, Guilan, Iran, 10-8-2017, H. Masigol; GenBank Acc. No: ITS –
223 MK911019, MK911018, MK911016, MK911015, MK911014 and MK911013.

Saprolegnia ferax (Grith.) Thuret Ann. Sci. Nat. Bot. 14:229 et spp. pl. 22. 1850 (Fig. 2g-i)

Mycelium dense; main hyphae highly branched, hyaline to dark, 15–62 µm (average 44 µm) in width. Sporangia abundant, cylindrical, rarely fusiform, always straight, renewed sympodially, 60–490 ×22–78 µm (average 352×51 µm). They discharged spores and behaved as saprolegnoids. Cysts 7–11 µm in diameter (average 10 µm). Gemmae were overabundant, extremely irregular, terminal and intercalary. Oogonia terminal, sometimes intercalary, spherical, obpyriform, spherical, 76–99 µm in diameter and sometimes with irregular shapes. Oogonial smooth. Oogonial stalk 1–3 times the oogonium diameter, slender and unbranched. Oospores centric, spherical, 2–48 per oogonium, 15–63 µm in diameter. Antheridia very rare, when present diclinous, 1–5 per oogonia. No specific pattern observed for any of isolates on CMA.

Material examined: isolates JT1L3, JT1W7, JT117, JT1L2, JT1W3-1, JTL6-1 and JT2W6-1, on rotten leaves, Anzali lagoon, Anzali, Guilan, Iran, 10-8-2017, H. Masigol; GenBank Acc. No: ITS – MK911003, MK911002, MK911004, MK911005, MK911006, MK911007 and MK911008.

Saprolegnia parasitica Coker *emend.* Kanouse, Mycologia 24:447, pls. 13. 1932 (Fig. 2j and l-m)

Mycelium moderately branched, with 18–56 µm (average 39 µm) width. Sporangia cylindrical, renewed internally, 150–460 ×20–66 µm (average 325×52 µm). They discharged spores and behaved as saprolegnoid. Cysts 10–12 µm in diameter (average 11 µm). Gemmae absent in water cultures, when present they were terminal, spherical and single. Oogonia mainly lateral, sometimes terminal, 86–110 µm in diameter, very rarely catenulate. Oogonial wall smooth. Oogonial stalk very short, 0.4-0.9 times the oogonium diameter, slender, unbranched and sometimes absent. Oospores centric, spherical, 8–32 per oogonium and 12–29 µm in diameter. Antheridia diclinous. No specific pattern observed for any of our isolates on CMA.

Material examined: isolates DSL1 and FSL17, on rotten leaves, Anzali lagoon, Anzali, Guilan, Iran, 10-8-2017, H. Masigol; GenBank Acc. No: ITS – MK911020 and MK911017.

Phylogenetic analyses

The phylogenetic classification of ITS sequences (694 bp) of the different genera belonging to *Saprolegniaceae* (*Saprolegniales*, *Oomycota*), inferred from both maximum likelihood method was consistent with the morphology-based taxonomy. Moreover, we found for most morphological

identified species some reliable sequences with high similarity (99-100%), so that these sequences clustered with our identified species in the same clade (Fig. 3).

Screening for lignolytic, cellulolytic, pectolytic and chitinolytic activities

Of all tested isolates, 61% showed chitinolytic activities. In addition, 52, 43, and 48% of all tested isolates showed cellulolytic activities in the medium amended with avicel (AVL), carboxymethylcellulose (CMC) and D-cellobiose (DCB), respectively. In contrast, no significant lignolytic activities were observed. Decolorization of dyes was detected only in the medium amended with Bromocresol Green (BG) and Toluidine Blue (TB) (39 and 8% of isolates, respectively). In some cases, isolates failed to grow, especially in the medium amended with Phenol Red (phR). Also, in media amended with Congo Red, adsorption by mycelia was observed. Thus, it was not considered as a proof for lignolytic activity (table 3).

Discussion

With this study we sought to evaluate both the spatio-temporal occurrence of *Saprolegniales* species within the brackish Anzali lagoon and to describe their morphological, phylogenetic, and physiological diversity. Previously, *Dictyuchus* Leitgeb genus (*Stramenopila*, *Oomycetes*) had been reported from Anzali lagoon (Masigol et al. 2018). We did not observe an influence of sampling site on the isolation of the taxa, although that isolation rate was slightly lower at the river mouth site in summer and thus may reflect some local seasonal effects. Generally, we observed a much lower abundance of *Saprolegiales* isolates in summer, when both precipitation and terrestrial input was lowest. This is in agreement with the 25 existing case studies (e.g., Czeczuga et al. 2003; Paliwal and Sati 2009) which show a similar pattern and variation in abundance. High temperature periods are the least favourable for *Saprolegniales* (Fig. 4), although this should be considered alongside other potentially covering aspects. For instance, the diversity of *Saprolegniales* isolates has been correlated with water hardness (Czeczuga et al. 2003) as well as Mg^{2+}, SO_4^{2-} and Ca^{2+}concentrations (Czeczuga et al. 2002). We should also consider that the highest impact of pollution in Anzali lagoon occurs during the summer season (Fallah and Zamani-Ahmadmahmoodi 2017) associated with a high discharge of agricultural waste and increase in fish breeding activities. Khatib and Khodaparast

(2010) noted that the higher water temperature and declining water volume created ideal conditions for bacteria in the summer, which might compete with oomycetes for organic matter.

Previous attempts to fully elucidate the impact of anthropogenic pollution on the microbial ecology of Anzali Lagoon have largely overlooked the role of fungi and oomycetes. Understandably, integration of mycological approaches into aquatic ecology is difficult, with vague morphological features contributing to the inability to distinguish between species *in situ*. This is particularly true for *Saprolegniales* taxa for which species identification is complex and often lacks a conclusive and consistent morphological form (Hulvey et al. 2007; Steciow et al. 2014). With this study, by assessing the number of isolated colonies we were able to better assess the occurrence and distribution of these organisms in the environment. However, this approach is still limited in that it is both time-consuming and not all *Saprolegniales* may be readily cultured.

Using a combination of morphological and molecular approaches we were able to confirm the occurrence of four *Saprolegnia* spp., of which *Saprolegnia anisospora* and *S. diclina* have never been reported in Iran. For the most part, *Saprolegnia* spp. could be differentiated using exclusively molecular approaches. *Saprolegnia anisospora*, confirmed by molecular analyses, was consistent with the morphological description albeit lacking the typical production of oospores in culture. Differentiation of *S. diclina* and *S. parasitica* and *S. aenigmatica* was only possible using molecular approaches due to a high number of shared morphological features (Johnson et al. 2002, Diéguez-Uribeondo et al. 2007, Sandoval-Sierra and Diégues-Uribeond 2015). Phylogenetic analysis generally suggested that sequences of *Achyla* spp. were similar to sequences of *Achlya bisexualis, A. flagellata* and *A. orion*. Clear approaches toward species identification are critical since many species such as *S. diclina* (Hussein et al. 2013), *S. ferax* (Cao et al. 2012) and *S. parasitica* (Griffiths et al. 2003) are considered pathogens and may devastate fish and amphibians' populations.

As our awareness of inland waters increases over time, we are gaining an increased appreciation for their role as key players in the global carbon cycle (Tranvik et al. 2018). Inland waters, through the activity of aquatic bacteria and fungi, are involved in remineralising large proportions of terrestrial organic matter into greenhouse gases. In this context, understanding the relative contribution of bacteria and fungi, of which fungi are better equipped to break down both dissolved and particulate

polymeric organic matter using adverse array of extracellular enzymes, is critical (Grinhut et al. 2011; Zahmatkesh et al. 2016; Collado 2018). However, although aquatic *Saprolegniales* are generally isolated from floated plant and animal debris, their involvement in organic matter degradation in various freshwater ecosystems has been largely ignored. In their study on seven *Saprolegniales* isolates from skin of living crayfish, Unestam (1966) showed a lack of cellulolytic activity by *Aphanomyces* spp., *Pythium* spp. and *Saprolegnia* spp. interestingly, all isolates exhibited chitinolytic activity (Unestam 1966; Nyhlen and Unestam 1975). In contrast, Thompson and Dix (1985) showed moderate to strong cellulolytic activity tested in 27 *Saprolegniales* taxa including *Achlya* spp. and *Saprolegnia* spp.. Whether the capacity of oomycetes to utilize more complex polymers, including lignin, remains unclear. Although our strains can fairly represent *Achlya* and *Saprolegnia*, isolation of less common genera will be necessary to have a better understanding of their enzymatic affinities.

In this study, capacity to degrade lignin was not evident with none of the isolates exhibiting laccase activity, and fewer than 25% of the isolates exhibiting any peroxidase activity (table 3). This agrees with a previous study where the potential for lignin degradation amongst *Dictyuchus* spp. and *Achlya* spp. isolates was essentially absent when compared to filamentous fungi isolated from the same environment (Masigol et al. 2019) and elsewhere (Abdel-Raheem and Shearer 2002; Junghanns et al. 2008; Simonis et al. 2008). The lack of any significant lignolytic activity amongst the aquatic *Saprolegniales* indicates that biopolymer degradation is specific and may be limited to just chitin and cellulose, in contrast with the broader specificity of fungi. Saprophytic *Saprolegiales* exhibiting chitinolytic and cellulolytic activity, as indicated by our study, may be more critical in the remineralisation of chitin-based particulate organic matter. This is supported by a close association of *Saprolegniales* with crustacean carapaces (Czeczuga et al. 1999; Czeczuga et al. 2002), feathers of wild and domestic bird species (Czeczuga et al. 2004), the benthic amphipod *Diporeia* spp. (Kiziewicz and Nalepa 2008) and the seeds of plants (Kiziewicz 2005) where chitin comprises a primary component of the biomass. Whilst the presence of an organism possessing chitinolytic enzymes on chitin rich substrates does not immediately prove its involvement in chitin processing, we feel it warrants additional investigation. In this study we isolated oomycetes directly from plant debris occurring in freshwater systems, despite their inability to utilise the predominantly lignin-based

335 substrate. Steinberg et al. (2003) and Meinelt et al. (2007) argue that plant-derived humic acids, which
336 occur at high abundance at the terrestrial-aquatic interface, inhibit the growth of some oomycetes.
337 Therefore, their ecological role in this niche remains uncertain, although we speculate that they may
338 form, at minimal, a commensal relationship with filamentous fungi, to utilise both the labile (chitin,
339 cellulose) and more refractory (lignin) components on this substrate (Lennon et al. 2013; Solomon et
340 al. 2015).
341 In conclusion, it is important to complement traditional morphology-based taxonomy with molecular-
342 based taxonomy but including several markers. It will be also essential to include other techniques
343 such as metabarcoding the have a better impression of the relative abundance of this group respect
344 other eukaryotes. To our knowledge, most of the studies performed in various freshwaters lack precise
345 taxonomy and hence are greatly impacting ecological interpretations. We observed clear seasonal
346 dynamics in the occurrence of *Saprolegniales* in Anzali lagoon with a decline in summer linked to
347 both increased water temperature and high levels of anthropogenic pollution. We confirmed that
348 *Saprolegniales* isolates lack the broad substrate specificity of fungi, rather exhibiting specific activity
349 towards cellulose or chitin-based substrates. Whether predominantly lignin-based plant-derived
350 substrates are an energy source, or simply a transport vector, for aquatic oomycetes remains unclear
351 and should be further tested. Evaluating the fate of allochthonous carbon in aquatic and global C
352 cycles should better consider the occurrence and impact of oomycetes, particularly for substrates
353 where chitin is particularly dominant. We should better evaluate the interactions between oomycetes
354 and fungi and bacteria both in competition for nutrients and carbon as well as for potential commensal
355 and synergistic impacts on carbon cycling.
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Fig.1 Sampling locations (1: river entrance, 2: shallow lake habitat and 3: urban habitat) for isolation of *Saprolegniales* taxa from Anzali lagoon, Iran during 2017

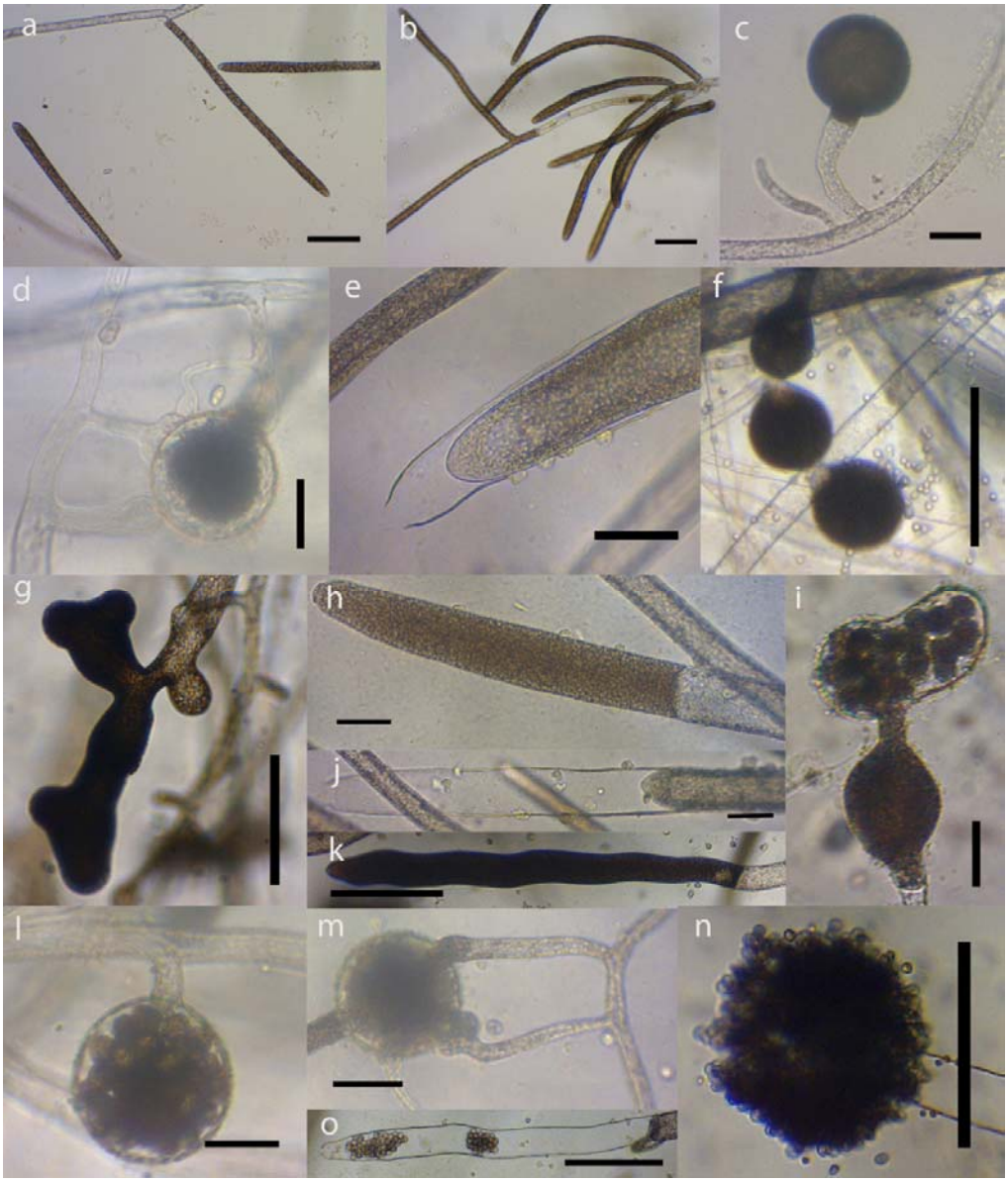
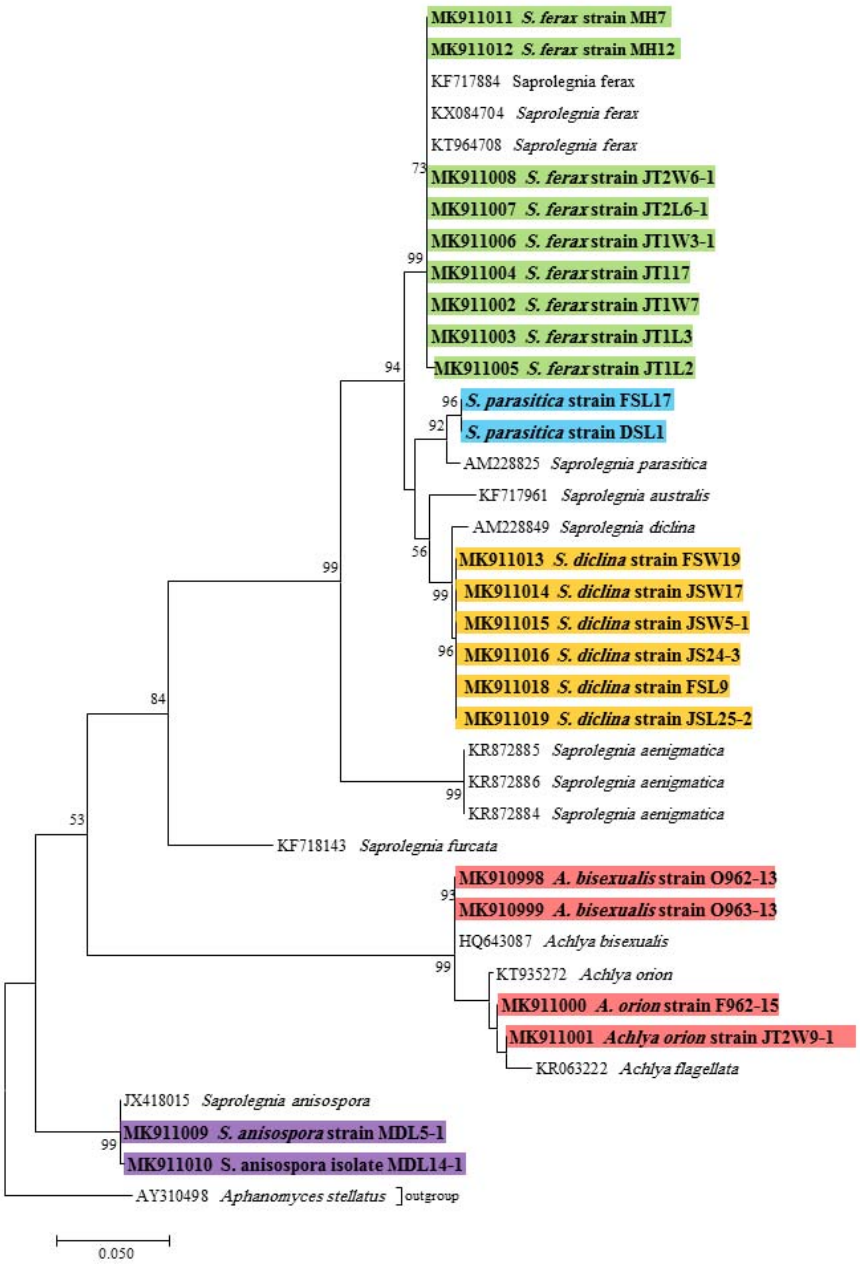


Fig. 2 General morphological characteristics of selected isolates used in this study observed in water culture at room temperature; (a-c) fusiform straight and sporangia (a), cymose fashion renewal of sporangia (b) and terminal immature oogonia with stalk (c) of *Saprolegnia anisospora;* (d-f) diclinous and androgynous antheridia (d), internal renewal of sporangia and catenulate spherical gemma(f) of *S. diclina*; (g-i) an extremely irregular gemma (g), cylindrical sporangia (h) and an oogonium with irregular shape (i) of *S. ferax*; (j and l-m) internal renewal of sporangia (j), lateral oogonia and short stalk (l), diclinous antheridia (m) of *S. parasitica*, (k) sporangia, (o) empty sporangia and (n) spore clump of *Achlya*spp. (bar=50 µm, except for k, n and o which are 200 µm)



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611 **Fig. 3** Phylogenetic tree of the family *Saprolegniaceae* (*Saprolegniales*, *Oomycota*). The analysis was

612 performed based on alignment of the ITS1-5.8S-ITS2 region (694bp) using the maximum likelihood

613 method from isolates in this study (bold and colored) and valid sequences from GenBank. Numbers

614 next to the branches shows bootstraps values ≥ 50%.*Aphanomyces stellatus* was considered as

615 outgroup

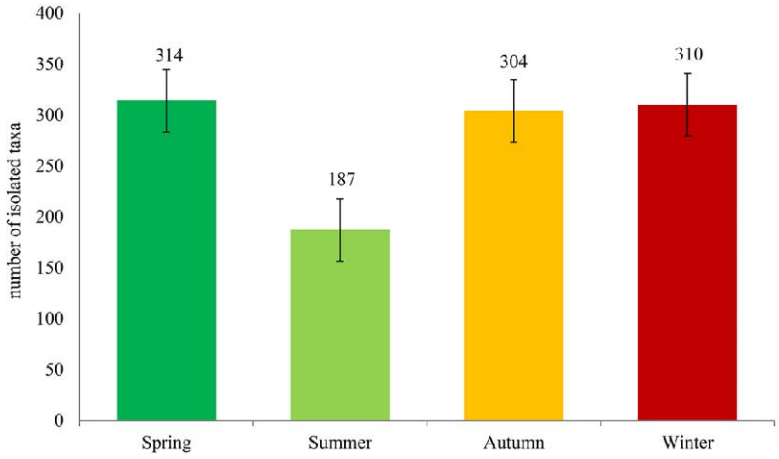

Fig. 4 Seasonal occurrence of *Saprolegniales* isolates reported by various researchers obtained from 25 case studies (1958-2015)

633 **Table 1** *Saprolegniales* isolates obtained from three coordinates Anzali lagoon (Rasht County, Iran)
634 during 2017 and their GenBank accession numbers (ITS region).

Species	Isolates	Isolation time	Acc. numbers
A. bisexualis	O962-13	Dec.	MK910998
	O963-13	Sep.	MK910999
A. orion	F962-15	Feb.	MK911000
S. anisospora	MDL5-1	May	MK911009
	MDL14-1	Aug.	MK911010
S. diclina	FSW19	Jan.	MK911013
	JSW17	Jun.	MK911014
	JSW5-1	Apr.	MK911015
	JSL24-3	Aug.	MK911016
	FSL9	May	MK911018
	JSL25-2	Jun.	MK911019
	JT1L2	Jul.	MK911005
	JT1W3-1	Dec.	MK911006
	JT1L3	Apr.	MK911003
	JT1W7	Jul.	MK911002
S. ferax	JT117	Feb.	MK911004
	JT2L6-1	Sep.	MK911007
	JT2W6-1	Jan.	MK911008
	MH7	Oct.	MK911011
	MH12	Mar.	MK911012
S. parasitica	FSL17	Nov.	MK911017
	DSL1	Oct.	MK911020
Achlya sp.	JT2W9-1	Mar.	MK911001

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637 **Table 2** Number and percentage of colonized hemp seed halves per Petri dish isolated from three
638 sampling sites in Anzali lagoon, Iran throughout 2017.

		averaged number of colonized hemp seed halves per Petri dish[a]											
		Location 1				Location 2				Location 3			
Seasons	**Months**	**R1[b]**	**R2**	**R3**	**%**	**R1**	**R2**	**R3**	**%**	**R1**	**R2**	**R3**	**%**
	Mar. 21- Apr. 20	17[c]	18	19	92.5	18	17	19	90.0	16	17	17	85.0
Spring	Apr. 21- May 21	13	13	13	65.0	13	13	11	60.0	12	13	14	67.5
	May 22- Jun. 21	11	12	11	57.5	12	12	13	62.5	11	10	9	47.5
	Jun. 22- Jul. 22	10	9	9	45.0	13	14	14	70.0	13	14	13	67.5
Summer	Jul. 23- Aug. 22	5	4	5	22.5	4	3	4	17.5	5	5	6	27.5
	Aug. 23- Sep. 22	9	9	9	45.0	12	14	12	65.0	13	14	14	70.0
	Sep. 23- Oct. 22	16	17	17	85.0	18	18	17	87.5	17	19	18	92.5
Autumn	Oct. 23- Nov. 21	17	18	17	87.5	17	16	16	80.0	19	20	20	100.0
	Nov. 22- Dec. 21	19	20	20	100.0	19	19	20	97.5	17	18	19	92.5
	Dec. 22- Jan. 20	16	15	15	75.0	15	14	13	67.5	17	19	19	95.0
Winter	Jan. 21- Feb. 19	17	17	17	85.0	16	16	15	77.5	18	17	19	90.0
	Feb. 20- Mar. 20	18	19	20	97.5	17	17	16	82.5	20	19	18	92.5

639 [a] Each Petri dish contains 20 hemp seed halves. [b]Replications [c]Averaged number of colonized hemp seed halves
640 from 10 Petri dishes.
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Table 3 Results of experimental screening for lignolytic, cellulolytic, and chitinolytic activities of all *Saprolegniales* isolates isolated from Anzali lagoon, Rasht, Iran.

Isolates	Taxa	AVL[a]	CMC[a]	DCB[a]	ABTS[b]	BG[b]	CR[b]	PhR[b]	PR[b]	RBBR[b]	TB[b]	CC[c]
O962-13	*A. bisexualis*											
O963-13	*A. bisexualis*											
F962-15	*A. orion*											
MDL5-1	*S. anisospora*											
MDL14-1	*S. anisospora*											
FSW19	*S. diclina*											
JSW17	*S. diclina*											
JSW5-1	*S. diclina*											
JSL24-3	*S. diclina*											
FSL9	*S. diclina*											
JSL25-2	*S. diclina*											
JT1L2	*S. diclina*											
JT1W3-1	*S. diclina*											
JT1L3	*S. diclina*											
JT1W7	*S. diclina*											
JT117	*S. ferax*											
JT2L6-1	*S. ferax*											
JT2W6-1	*S. ferax*											
MH7	*S. ferax*											
MH12	*S. ferax*											
FSL17	*S. parasitica*											
DSL1	*S. parasitica*											
JT2W9-1	*Achlya sp.*											

[a]Three carbon sources as indicators of cellulolytic activities, carboxymethilcellulose (CMC), Avicel (AVL), D-cellobiose (DCB), as indicators of Endo-1,4-β-glucanase, Cellobiohydrolase and β-Glucosidase

[b]Dyes as indicators of lignolytic activities, 2,20-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) (as specific indicator of Laccase activity), Bromocresol Green (BG), Congo Red (CR), malachite green (MG),Phenol Red (PhR), PolyR-478 (PR, Remazol Brilliant Blue (RBBR), and (8) Toluidine Blue (TB)

[c]Colloidal chitin (CC) as indicator of chitinolytic activities

White color = no activity, pale green = weak, green = medium, and dark green = strong activity, red = no growth, pale red = adsorption