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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, as well as *Achlya* strains, which were closely related to *A. bisexualis*, *A. debaryana* and *A. intricata*. Evaluation of the ligno-, cellulo- and chitinolytic activities was performed 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 functionally different aquatic fungi in freshwater ecosystems.

Keywords *Achlya* · *Saprolegnia* · Aquatic ecosystems · Carbon cycling · Polymer degradation · *Saprolegniaceae* · *Achlyaceae*

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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). Amongst 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 and Sati 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 such as 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. 2014, 2015). 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 and 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 ten Petri dishes was used to estimate the abundance of *Saprolegniales* throughout the year. The presence of *Saprolegniales* was confirmed by observing at least one of the general

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

features of oomycetes such as oogonia, sporangia, large and aseptate mycelia and motile zoospores.

After 3–5 days, a piece of mycelia from the colonized hemp seed halves was transferred to a fresh CMA-PARP medium (Kannwischer and Mitchell 1981). This step was repeated three to five times to achieve bacterial-free (axenic) cultures. A single hypha was transferred to cornmeal agar (CMA) medium (Seymour and Fuller 1987). The hyphal-tip technique was conducted three to five times to obtain a pure culture in CMA. The specimens of these new strains were then deposited in the Fungal Herbarium of the Iranian Research Institute of Plant Protection, Iran.

Identification and phylogenetic analyses

Morphological identification

Asexual and sexual structures of isolates were characterized and measured in liquid (water) cultures (n = 30). To investigate strains failing to produce any sexual structures, several treatments were used. The nutrition treatments included reciprocal culturing of all strains with one another and *Trichoderma* sp. (Brasier et al. 1978) on CMA, hemp seed agar (HSA) (Hendrix 1964), soybean agar (SA) (Savage et al. 1968), rape seed extract agar (REA) (Satour 1967), carrot juice agar (CJA) (Ershad 1971), mPmTG (Moreau and Moreau 1936a), immersing colonized CMA in glycerine (4%) (Moreau and Moreau 1936b) and culturing the isolates in Petri dishes containing ten boiled hemp seeds in distilled lake water and distilled water (50/50). The temperature treatments also included culturing the isolates in 5, 10, 15, 20, and 25 °C in Petri dishes containing ten boiled hemp seeds in distilled lake water and distilled water (50/50).

DNA extraction and PCR

The DNA extraction of the isolates was conducted based on a slightly modified protocol of Montero-Pau et al. (2008). Briefly, 100 µL of alkaline lysis buffer

(25 mM NaOH, 0.2 mM disodium EDTA, pH 8.0) was aliquoted into 1.5-mL tubes. Malt extract broth (MEB) (Galloway and Burgess 1962) was used for the growth of isolates. Mycelial mass was then transferred to the tube and centrifuged for 30 min at 9000 rpm. The tube was incubated at 95 °C for 30 min and then cooled on ice for 5 min. 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 primers according to cited amplification conditions (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.

Phylogenetic analyses

Sequence alignment was conducted by Clustal X (Thompson et al. 1994) with subsequent visual adjustment. Partition homogeneity tests were conducted on ITS gene alignment by PAUP * 4.0a136 (Swofford 2002) using 100 replicates and the heuristic general search option. *Aphanomyces stellatus* was chosen as an outgroup. Bayesian inference analyses on ITS were carried out with MrBayes 3.1 (Ronquist and Huelsenbeck 2003) in order to reconstruct the phylogenetic trees, imposing a general time-reversible (GTR) substitution model with gamma (*G*) and proportion of invariable site (*I*) parameters to accommodate variable rates across sites. We determined the best nucleotide substitution model (Nylander 2004) using MrModelTest 2.3. Two independent runs of Markov chain Monte Carlo (MCMC) using four chains were run over 1,000,000 generations. Trees were saved each 1000 generations, resulting in 10 001 trees. Burn-in was set at 5% generations. Maximum likelihood estimation was carried out using PHYLIP DNAML (Felsenstein 1993) in order to conduct phylogenetic comparison. The robustness of the maximum likelihood trees was estimated by 1000 bootstraps.

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) and mPmTG agar medium amended with one of the following substrates: 0.1% wt/vol 2,20-Azino-*bis* 3-ethylbenzothiazoline-6-sulphonic acid diammonium salt (ABTS), 0.02 and 0.005% wt/vol Bromocresol Green (BG), 0.02 and 0.005% wt/vol Congo Red (CR), 0.02 and 0.005% wt/vol Phenol Red (PhR), 0.02 and 0.005% wt/vol PolyR-478 (PR) (pH 5 + 7), 0.02 and 0.005% wt/vol Remazol Brilliant Blue (RBBR), and 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 decolourization of the aforementioned substrates in the area around the mycelia or as a colour change in the media after 3 weeks. We evaluated 1–33, 33–66, and 66–100% decolourization of the medium in the Petri dishes as weak, medium and strong activities, 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: 7.5 g carboxymethylcellulose (CMC), 7.5 g Avicel (AVL) and 5 g D-cellobiose (DCB) (Wood and Bhat 1988; Pointing 1999; Yoon et al. 2007; Jo et al. 2010). After 3 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 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 was then treated with 150 mL of ~ 12 M concentrated HCl which was added gently and continuously stirred for 45 min 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 min, 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 $(NH_4)_2SO_4$, 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 g of colloidal chitin (CC) and 0.15 g of Bromocresol Purple; the pH was adjusted to 4.7, and the neutralized medium autoclaved.

Statistical analyses

We assessed whether there was a significant impact of month or season broadly on each of the three locations on the frequency of *Saprolegniales* colonization of hemp leaves, using a two-way ANOVA. The relative contribution of temperature and pH to any spatio-temporal trends was assessed using Spearman's Rank correlations. A propensity of individual genera or taxa to metabolize individual substrates was assessed using a two-way ANOVA. All statistical analyses were conducted using GraphPad Prism version 8.1 (GraphPad Software, CA, USA).

Results

Relative abundance of *Saprolegniales* isolates

From all oomycetes isolated from the three locations sampled along the year, 511 out of 720 (~ 71%) were assigned to the order *Saprolegniales*. The relative abundance of *Saprolegniales* was higher at cold temperatures (autumn, winter and spring seasons) than in summer. The highest temperatures were recorded in 23 July–22 August (in average ~ 33 °C) and the lowest in 21 June–19 February (in average ~ 3 °C). The number of isolates from river entrance, shallow lake and urban habitats was negatively correlated with temperature (R^2 = 0.7233, 0.5047 and 0.7623, respectively). However, pH was constant and no correlation was observed for river entrance, shallow lake and urban habitats (R^2 = 2*E*−05, 0.0037 and 0.0684, respectively). Of the 720 hemp seeds, only 209 were not colonized by *Saprolegniales* isolates (~ 29%). These were either colonized by other microorganisms such as fungi and protists, or remained intact. Co-colonization of *Saprolegniales* isolates and other unwanted subjects or organisms was not counted as a positive result (Table 1).

Morphological identification

Of the selected isolates, 19 belonged to the genus *Saprolegnia* (*S. anisospora*, S. *diclina*, *S. ferax* and *S. parasitica*) (Fig. 2a–j, l–m) and four to the genus *Achlya* (Fig. 2k, n, o). Four isolates of *Achlya* failed to produce sexual structures under any circumstances and thus were considered as *Achlya* spp. Morphology-based taxonomy of *Saprolegnia* strains was confirmed by phylogenetic analysis of ITS sequences of nrDNA inferred from Bayesian and maximum likelihood methods.

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

Mycelium dense; main hyphae branched, hyaline to dark, with 16–46 µm (average 26 µm) width. Sporangia very abundant, mainly fusiform, straight, sometimes curved, renewed in cymose fashion, 80–405 × 18–50 µm (average 220 × 26 µm). They discharged spores and behaved as saprolegnoids. Cysts 9–13 µm in diameter (average 10 µm). Gemmae absent. Oogonia terminal, always spherical, always immature, 78–107 µm in diameter. Oogonial wall smooth. Oogonial stalks 1–3 times the diameter of oogonium, slender, slightly irregular and

Table 1 Number and percentage of colonized hemp seed halves per Petri dish isolated from three 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	*R*1[b]	*R*2	*R*3	%	*R*1	*R*2	*R*3	%	*R*1	*R*2	*R*3	%
Spring	Mar. 21–Apr. 20	17[c]	18	19	92.5	18	17	19	90.0	16	17	17	85.0
	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
Summer	Jun. 22–Jul. 22	10	9	9	45.0	13	14	14	70.0	13	14	13	67.5
	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
Autumn	Sep. 23–Oct. 22	16	17	17	85.0	18	18	17	87.5	17	19	18	92.5
	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
Winter	Dec. 22–Jan. 20	16	15	15	75.0	15	14	13	67.5	17	19	19	95.0
	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

[a]Each Petri dish contains 20 hemp seed halves

[b]Replications

[c]Averaged number of colonized hemp seed halves from ten Petri dishes

unbranched. Oospores never produced. No specific pattern was observed for any of our strains on CMA.

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

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

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

Material examined Isolates JSL25-2, FSL9, JSL24-3, JSW5-1, JSW17 and FSW19, on rotten leaves, Anzali lagoon, Anzali, Guilan, Iran, 10-8-2017, H. Masigol; GenBank Acc. No: ITS-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.

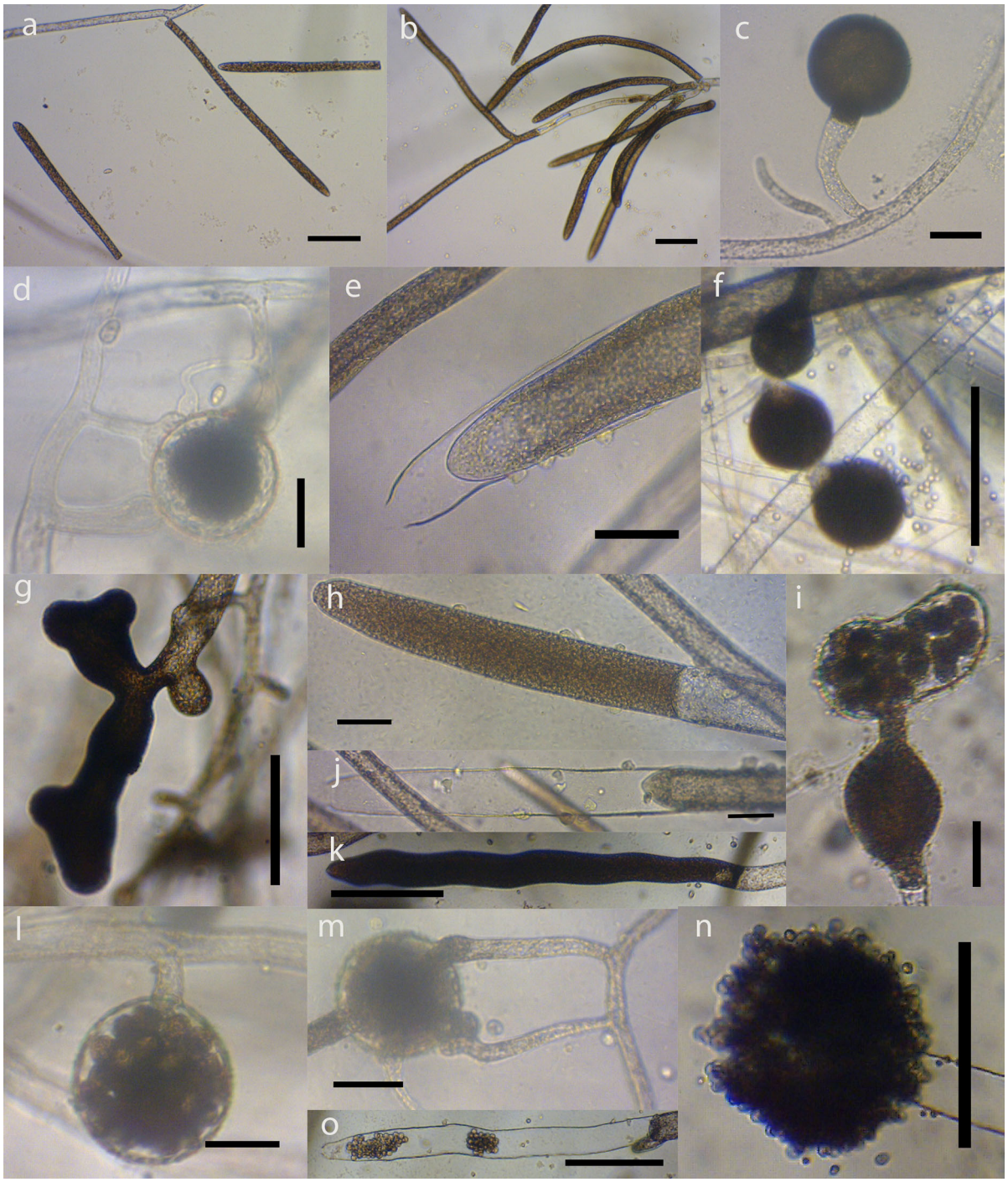

Fig. 2 General morphological characteristics of selected isolates used in this study observed in water culture at room temperature; (**a**–**c**) fusiform straight and sporangia (**a**), cymose fashion renewal of sporangia (**b**) and terminal immature oogonia with stalk (**c**) of *Saprolegnia anisospora*; (**d**–**f**) diclinous and androgynous antheridia (**d**), internal renewal of sporangia (**e**) 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 (**n**), spore clump of *Achlya* spp. and (**o**) empty sporangia (bar = 50 μm, except for **k**, **n** and **o** which are 200 μm)

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 saprolegnoids. 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 of *Saprolegnia* genus inferred from both Bayesian and maximum likelihood methods 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). In addition, O963-13 and O962-13 *Achlya* strains were closely related with *Achlya bisexualis* and F962-15 and JT2W9-1 *Achlya* strains with *A. intricata* and *A. debaryana*.

Fig. 3 Phylogenetic relationships of *Saprolegniales* from Anzali lagoon, Iran; relationships amongst 34 *Achlya* (left tree) and 29 *Saprolegnia* (right tree) taxa based on Bayesian analysis of internal transcribed spacers 1 and 2 and 5.8 S gene of rDNA sequences. Numbers next to the branches represent posterior probability (left values) based on Bayesian analysis and bootstrap support (right values) based on maximum likelihood analysis

Screening for lignolytic, cellulolytic 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. Decolourization 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 2).

Discussion

With this study, we sought both to evaluate the spatio-temporal occurrence of *Saprolegniales* species within the Anzali lagoon and to describe their morphological, phylogenetic and physiological diversity. We were able to confirm the occurrence of four *Saprolegnia* spp., of which *Saprolegnia anisospora* and *S. diclina* have never been reported from Iran. Phylogenetic analysis also suggested that sequences of *Achyla* spp. were similar to sequences of *Achlya bisexualis, A. debaryana* and *A. Achlya intricata*. Previously, *Dictyuchus* Leitgeb and *Brevilegnia* Coker and Couch genera had been reported from Anzali lagoon (Masigol et al. 2017, 2018).

Generally, we observed a much lower abundance of *Saprolegniales* isolates in summer, when both precipitation and terrestrial input were lowest. This is in

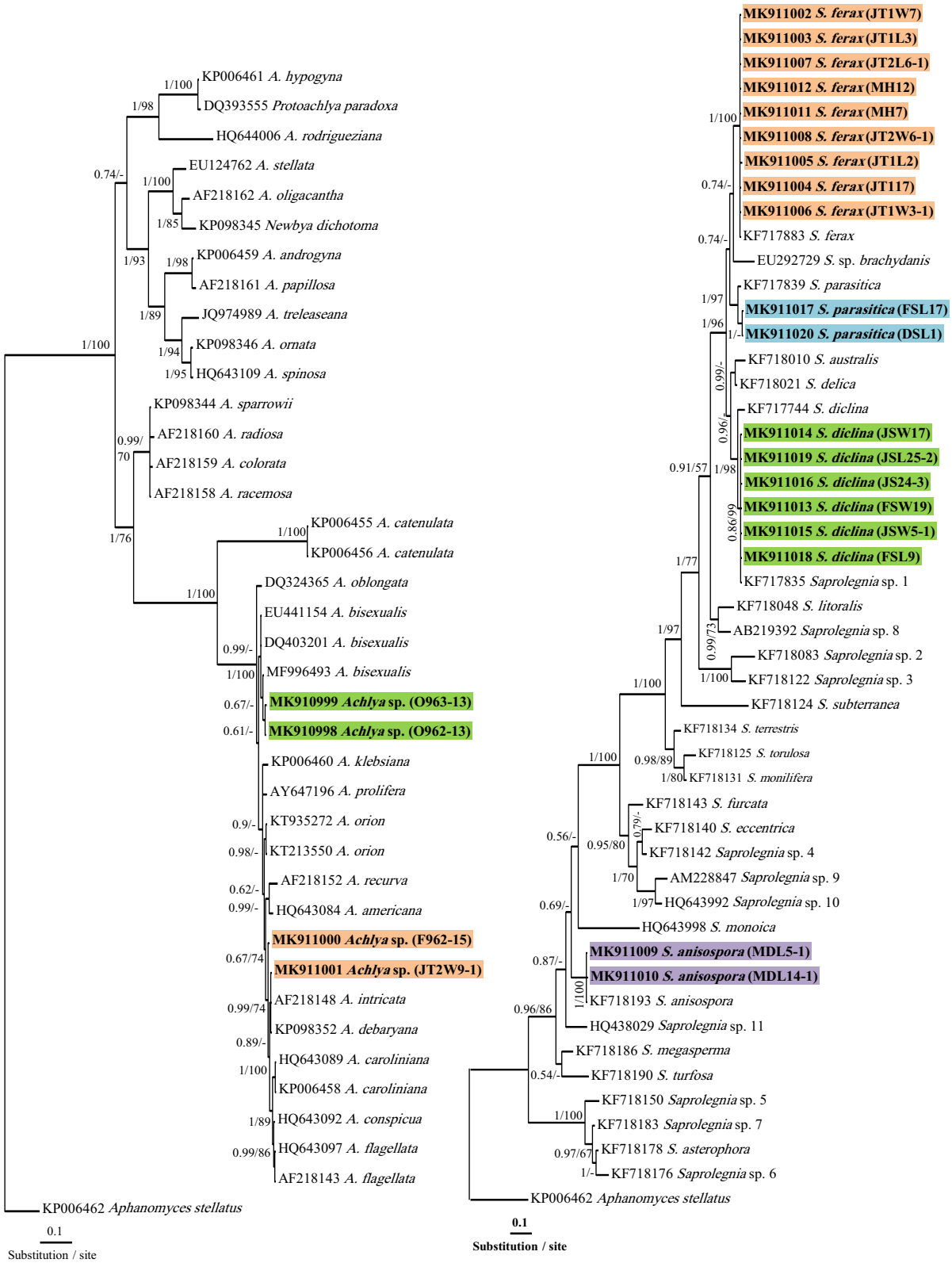
KP006461 A. hypogyna
DQ393555 Protoachlya paradoxa
HQ644006 A. rodrigueziana
EU124762 A. stellata
AF218162 A. oligacantha
KP098345 Newbya dichotoma
KP006459 A. androgyna
AF218161 A. papillosa
JQ974989 A. treleaseana
KP098346 A. ornata
HQ643109 A. spinosa
KP098344 A. sparrowii
AF218160 A. radiosa
AF218159 A. colorata
AF218158 A. racemosa
KP006455 A. catenulata
KP006456 A. catenulata
DQ324365 A. oblongata
EU441154 A. bisexualis
DQ403201 A. bisexualis
MF996493 A. bisexualis
MK910999 Achlya sp. (O963-13)
MK910998 Achlya sp. (O962-13)
KP006460 A. klebsiana
AY647196 A. prolifera
KT935272 A. orion
KT213550 A. orion
AF218152 A. recurva
HQ643084 A. americana
MK911000 Achlya sp. (F962-15)
MK911001 Achlya sp. (JT2W9-1)
AF218148 A. intricata
KP098352 A. debaryana
HQ643089 A. caroliniana
KP006458 A. caroliniana
HQ643092 A. conspicua
HQ643097 A. flagellata
AF218143 A. flagellata
KP006462 Aphanomyces stellatus
0.1
Substitution / site
MK911002 S. ferax (JT1W7)
MK911003 S. ferax (JT1L3)
MK911007 S. ferax (JT2L6-1)
MK911012 S. ferax (MH12)
MK911011 S. ferax (MH7)
MK911008 S. ferax (JT2W6-1)
MK911005 S. ferax (JT1L2)
MK911004 S. ferax (JT117)
MK911006 S. ferax (JT1W3-1)
KF717883 S. ferax
EU292729 S. sp. brachydanis
KF717839 S. parasitica
MK911017 S. parasitica (FSL17)
MK911020 S. parasitica (DSL1)
KF718010 S. australis
KF718021 S. delica
KF717744 S. diclina
MK911014 S. diclina (JSW17)
MK911019 S. diclina (JSL25-2)
MK911016 S. diclina (JS24-3)
MK911013 S. diclina (FSW19)
MK911015 S. diclina (JSW5-1)
MK911018 S. diclina (FSL9)
KF717835 Saprolegnia sp. 1
KF718048 S. litoralis
AB219392 Saprolegnia sp. 8
KF718083 Saprolegnia sp. 2
KF718122 Saprolegnia sp. 3
KF718124 S. subterranea
KF718134 S. terrestris
KF718125 S. torulosa
KF718131 S. monilifera
KF718143 S. furcata
KF718140 S. eccentrica
KF718142 Saprolegnia sp. 4
AM228847 Saprolegnia sp. 9
HQ643992 Saprolegnia sp. 10
HQ643998 S. monoica
MK911009 S. anisospora (MDL5-1)
MK911010 S. anisospora (MDL14-1)
KF718193 S. anisospora
HQ438029 Saprolegnia sp. 11
KF718186 S. megasperma
KF718190 S. turfosa
KF718150 Saprolegnia sp. 5
KF718183 Saprolegnia sp. 7
KF718178 S. asterophora
KF718176 Saprolegnia sp. 6
KP006462 Aphanomyces stellatus
0.1
Substitution / site

Table 2 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	*Achlya* sp.											
O963-13	*Achlya* sp.											
F962-15	*Achlya* sp.											
JT2W9-1	*Achlya* sp.											
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*											

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

[a]Three carbon sources as indicators of cellulolytic activities, carboxymethylcellulose (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-sulphonic acid diammonium salt (ABTS) (as specific indicator of Laccase activity), Bromocresol Green (BG), Congo Red (CR), Phenol Red (PhR), PolyR-478 (PR), Remazol Brilliant Blue (RBBR) and Toluidine Blue (TB) (as specific indicator of Peroxidase activity)

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

agreement with the 25 existing case studies which show a similar pattern and variation in abundance (e.g. Czeczuga et al. 2003; Paliwal and Sati 2009), 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.

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 remineralizing 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, is critical (Grinhut et al. 2011; Zahmatkesh et al. 2016; Collado et al. 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. 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, none of the isolates showed laccase activity. In addition, no peroxidase activity was observed in more than 60% of the isolates (Table 2). 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 to the broader specificity of fungi. Saprophytic *Saprolegniales* exhibiting chitinolytic and cellulolytic activity, as indicated by our study, may be more critical in the remineralization of chitin-based particulate organic matter. This is supported by a close association of *Saprolegniales* with crustacean carapaces (Czeczuga et al. 1999, 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 conclusion, it is important to complement traditional morphology-based taxonomy with molecular-based taxonomy but including several markers. It will be also essential to include other techniques such as metabarcoding to have a better impression of the relative abundance of this group with respect to other eukaryotes. To our knowledge, most of the studies performed in various freshwaters lack precise taxonomy and hence are greatly impacting ecological interpretations. We observed clear seasonal dynamics in the occurrence of *Saprolegniales* in Anzali lagoon with a decline in summer linked to both increased water temperature and high levels of anthropogenic pollution. We confirmed that *Saprolegniales* isolates lack the broad substrate specificity of fungi, rather exhibiting specific activity towards cellulose or chitin-based substrates. Whether predominantly lignin-based plant-derived substrates are an energy source, or simply a transport vector, for aquatic oomycetes remains unclear and should be further tested. Evaluating the fate of allochthonous carbon in aquatic and global C cycles should better consider the occurrence and impact of oomycetes, particularly for substrates where chitin is particularly dominant. We should better evaluate the interactions between oomycetes and fungi and bacteria both in competition for nutrients and carbon and for potential commensal and synergistic impacts on carbon cycling.

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