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Review

Advancements, deficiencies, and future necessities of studying *Saprolegniales*: A semi-quantitative review of 1073 published papers

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ABSTRACT

Research on the order *Saprolegniales* (*Oomycota*) has been an ongoing quest for more than a century. The best studied genera are *Saprolegnia* and *Aphanomyces*, known for their pathogenicity on freshwater animals. In this study, we reviewed 1073 papers and 2803 ITS sequences of *Saprolegniales* to investigate their taxonomy, diversity and potential roles in mainly freshwater ecosytems. We found that, in general, our knowledge on diversity and ecology of *Saprolegniales* is limited. Neither classic taxonomy nor available molecular techniques have been sufficient to delineate genera and species and show their relative distribution in freshwater-associated habitats. Also, we currently lack a comprehensive understanding of their involvement in carbon turnover and food web dynamics. Finally, due to lack of using high-throughput sequencing techniques, it is not clear how and to what extent communities of *Saprolegniales* might differ in freshwater econiches. Therefore, we provide a historical perspective on the establishment of *Saprolegniales*, explain improvements, highlight deficiencies, and finally propose new research avenues for more systematic studies. We conclude that challenges in studying *Saprolegniales* can be removed by

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increasing the practicality of classic taxonomy and applying available molecular toolboxes (multi-gene phylogeny and high-throughput sequencing). Additionally, inclusion of *Saprolegniales* in freshwater carbon cycling should be addressed for their better ecological resolution.

1. Introduction

The order *Saprolegniales* belongs to the phylum *Oomycota* which puts biflagellate heterotrophic organisms together (Beakes *et al.*, 2014). *Saprolegniales* are known as water moulds placed within the Saprolegnialean galaxy and the best-studied freshwater oomycetes by far. They are, inferred from subsequent molecular studies, considered as "*crown oomycetes*" populated in freshwater ecosystems along with *Peronosporales* and *Albuginales* as their terrestrial counterparts (Beakes and Sekimoto, 2009). They have been researched extensively for more than a century due to their devastating impact on fisheries industry and aquatic animal health (Van West, 2006; Rezinciuc *et al.*, 2015). Following the rise of molecular techniques, their phylogenetic affiliation has been dramatically changed, i.e., from being a member of "*Phycomycetes*" (Fitzpatrick, 1930) to be part of the kingdom *Chromista* (Cavalier-Smith, 2010). This was a major turning point in their systematics and phylogeny.

Nevertheless, studying *Saprolegniales* suffers from several flaws. In particular, classic taxonomy of this order has become, to a large extent, obsolete. In fact, the current knowledge of *Saprolegniales*' classic taxonomy has no or very little practical value for successful delineation of many taxa. Similarly, the picture of their diversity has been distorted even after incorporating PCR-based investigations into many studies. Moreover, there has been very little effort applying more powerful molecular-based tools to decode the hidden diversity of *Saprolegniales*. Additionally, due to the high economic importance of aquatic animals' pathogens such as *Saprolegnia* and *Achlya*, ecological implications of *seemingly* unimportant taxa have been largely neglected. In particular, the role of *Saprolegniales* in freshwater carbon cycling as well as food web dynamics is categorically ignored. Thus, we here review the current knowledge, pinpoint challenges, and offer perspectives toward future research avenues on freshwater *Saprolegniales*. The review's structure is graphically displayed in Fig. 1.

2. Material and methods

We conducted a literature review by combining a number of keywords (e.g., *Saprolegniales*, crayfish, saprolegniosis, zoospores, phylogeny of *Saprolegniales*, seasonality, *Aphanomyces*, etc.) to determine main research areas as well as deficiencies in investigating *Saprolegniales*. After obtaining several key papers, a snowball effect raised the number of related papers by using several reference lists as a new source of inquiry. Nearly 1200 papers were downloaded and evaluated carefully by taking abstract, method, result and discussion sections into account. In the end, 1073 papers were deemed suitable through the authors' judgement considering the aim of this study. Even though more relevant papers could have been investigated, they were omitted from the study due to either lack of accessibility of their full manuscripts, using other languages than English, etc.

To conduct a semi-qualitative analysis, these 1073 articles were carefully studied and categorized into four classes based on their content including A) diversity and identification (subclasses: classical taxonomy, PCR-related and phylogenetic studies), B) ecology (seasonality and effects of environmental factors), C) growth and development (sexual reproduction, morphogenesis, and sporogenesis), and D) pathogenicity (in aquatic and terrestrial ecosystems). Thereafter, the Meta-chart website (https://www.meta-chart.com/venn) was used to draw a Venn chart showing the significance as well as potential overlaps between the determined four classes. Additionally, three more items including studied ecosystem, associated-substrates and genera were recorded for each article (see supplementary materials).

Moreover, 2803 valid ITS accession numbers assigned to *Saprolegniales* available on the GenBank (https://www.ncbi.nlm.nih.gov/genbank/) were downloaded, annotated and analysed subsequently to yield a comprehensive phylogenetic overview. Additional information with respect to taxonomy of *Saprolegniales* were gathered from Index Fungorum (https://www.indexfungorum.org/).

3. Advancements

The phylogenetic exclusion of oomycetes (and consequently *Saprolegniales*) from fungi was a turning point as they received more attention and were studied more systematically than fungi. However, reaching such an exclusion has never been easy as the order *Saprolegniales* was subject to constant changes. To improve our knowledge on *Saprolegniales*, it is necessary to evaluate what had happened before and after this exclusion. First, the establishment of *Saprolegniales* will be presented. Additionally, as pathogenicity has been the most common topic, the crayfish plague by *Aphanomyces astaci* will be reviewed as a well-studied case. Although saprolegnionsis and epizootic ulcerative syndrome have also been confirmed to be destructive fish diseases, they aren't included to keep the manuscript to the point.

3.1. *Establishment*

Studies on *Saprolegniales* started in the late 19th century. An overview of all major events in their taxonomic journey is given in Fig. 2. One of the first notions of *Saprolegniales* as fungi started with Massee (1891) who divided fungi into

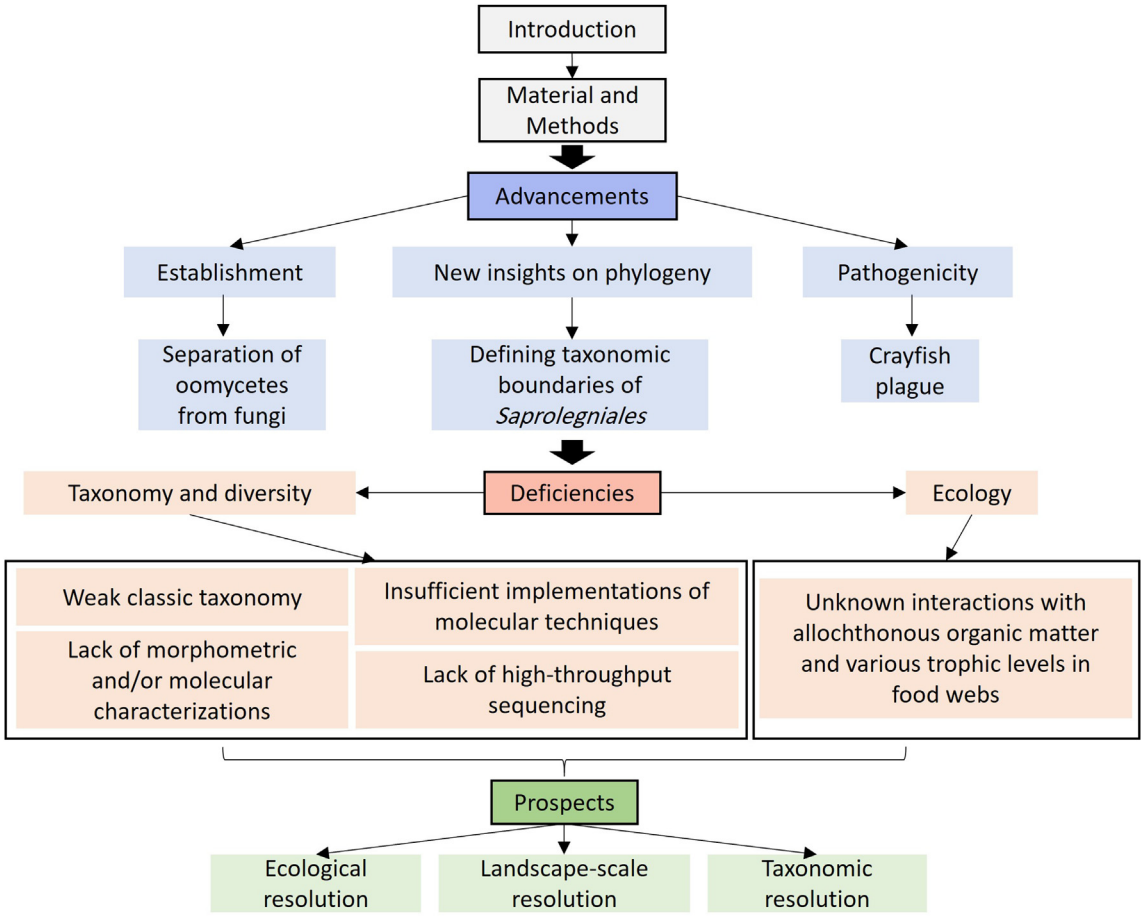

Fig. 1 – Structure of the review paper.

Mycomycota and Phycomycetes. Phycomycetes were also divided into six families including *Saprolegniaceae*. *Saprolegniaceae* (the largest family in *Saprolegniales*) was characterized by biciliate zoogonidia, hyphae with the ability to become zoosporangia, and sexual reproduction by antheridia and oogonia (including *Leptomitus*, *Saprolegnia*, *Pythium*, *Dictyuchus*, *Diplanes*, *Achlya*, and *Monoblepharis*). Although *Saprolegniaceae* was reported as aquatic fungi, there was no explanation regarding their aquatic habitats and/or pathogenicity towards aquatic animals. With some partial modifications, Abney (1912), Coker (1923) and Fitzpatrick (1930) adopted Massee's core systematics framework and expanded the knowledge on *Saprolegniales* with respect to diversity, habitats, lifestyle, and global distribution. For instance, Abney (1912) placed *Saprolegniaceae* together with two other families (*Leptomitaceae* and *Pythiaceae*) in the order *Saprolegniales* with both *Pythiopsis* and *Thraustotheca* as new genera. The descriptions were extended and more accurate, but still contained very few figures and little reference to the aquatic nature of taxa in *Saprolegniales*.

The publication '*The Saprolegniaceae, with notes on other water molds*' by Coker (1923) was a breakthrough compared to previous works, as it exclusively focused on the family *Saprolegniaceae* (Fig. 2). For the first time, a relatively comprehensive dichotomous key to both genera and species of the family had been presented including detailed morphometric descriptions. In addition to the previously described genera (*Aplanes*, *Saprolegnia*, *Achlya*, *Aphanomyces*, *Thraustotheca*, and *Dictyuchus*), according to the key to genera, *Protoachlya*, *Leptolegnia*, *Pythiopsis*, and *Isoachlya* formed new genera in *Saprolegniaceae*. Thereby, *Saprolegnia* was the only genus assigned to be a parasite of aquatic animals such as fish, frog eggs, etc. Coker's superiority over previous works lies in his dedication in generating 67 detailed plates drawing all morphological features of the described taxa. This was a significant improvement to the two and six plates, earlier presented by Massee (1891) and Abney (1912), respectively. Fitzpatrick (1930) had produced another detailed systematic scheme of the family *Saprolegniaceae* and added several new genera including *Calyptralegnia*, *Brevilegnia*, *Geolegnia*, *Plectospira*, and *Isoachlya*. Although these studies have mentioned that *Saprolegniales* are specifically associated with the aquatic environment due to their water-dependent life cycle, they were not placed within the same systematic scheme until Sparrow (1935) who invented the term "*Aquatic Phycomycetes*". Additionally, other features such as their occurrence and cultivation, as well as their sexual and asexual reproduction were discussed. In another major 1200-page document by Sparrow (1960), *Saprolegniales* and seven other orders were considered as phycomycetes (Fig. 2). He had proposed that *Saprolegniales* consists

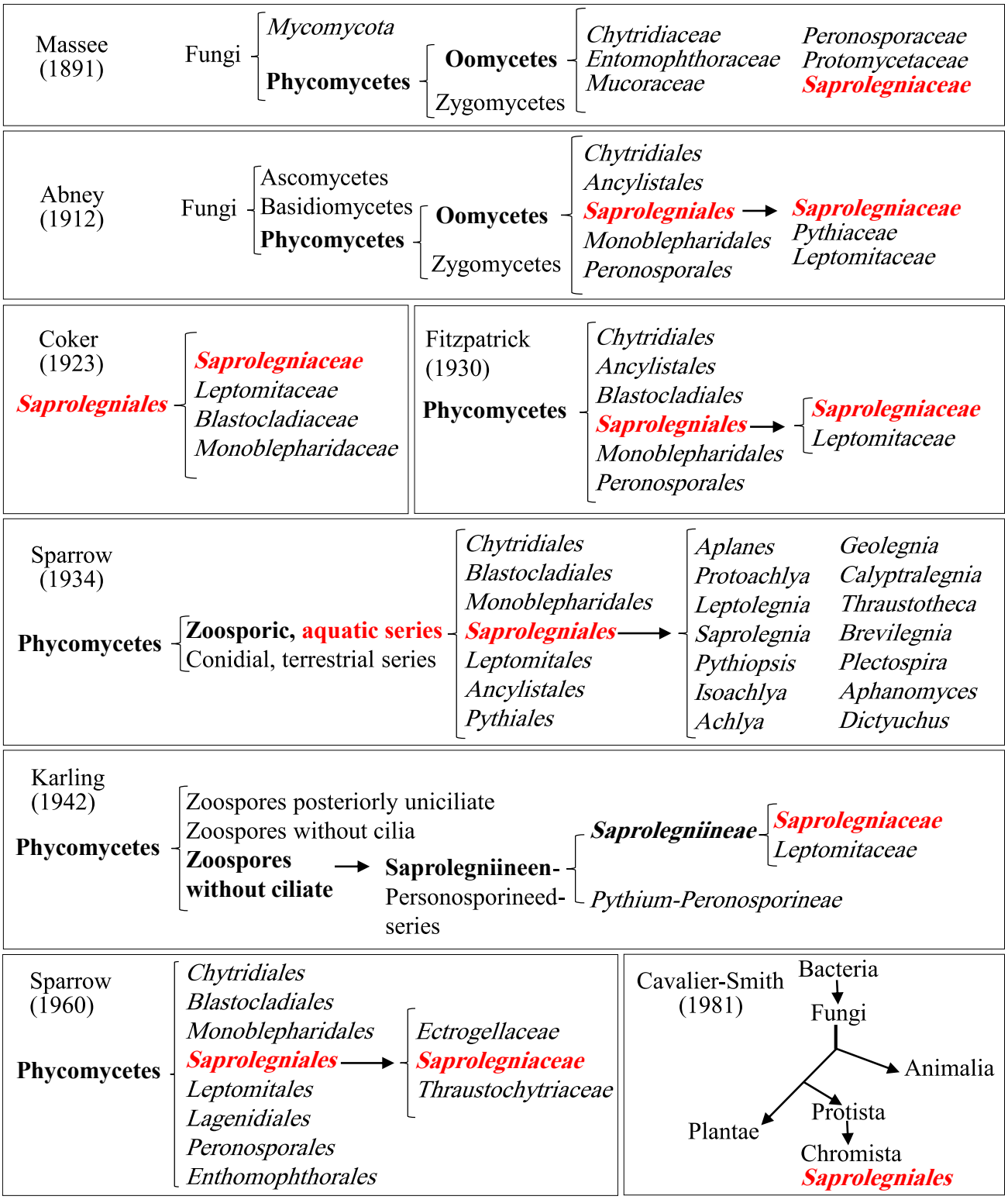

Fig. 2 – A simplified chronological overview of *Saprolegniales* taxonomy based on major studies published from 1891 to 1981.

of three families including *Saprolegniaceae*, *Ectrogellaceae* and *Thraustochytriaceae* (the latter two were assigned to the order for the first time).

Along with exploring the diversity of *Saprolegniales*, researchers have always been speculating about the separation between fungi and oomycetes. For years, all these speculations were based on subtle and inconclusive morphological and eco-physiological traits. In his book, Scherffel (1925) postulated that oomycetes should be separated from *Chytridiales*, *Monoblepharidiales*, and *Blastocladiales* based on the

morphology of their zoospores and the protoplasm, lack of motile male cells, and the presence of cellulose in the cell walls. Additionally, Mez (1929) derived *Saprolegniales* from *Siphonales*, and placed them near *Voucheria* mainly based on zoospore. A detailed review of such ideas can be found in Karling (1942) and Bessey (1942). Additionally, Bartnicki-Garcia (1968) observed that, in contrast to fungi, oomycetes have glucan instead of chitin derivatives as the main cell-wall polymer. Later on, studies mainly conducted by Cavalier-Smith (1981, 1986, 1998) confirmed previous speculations and established the kingdom Chromista (with oomycetes as one of the most diverse lineages) due to its distinctive cytological features. His effort paved the way to conclusively separate oomycetes (including *Saprolegniales*) from fungi.

3.2. *New insights on phylogeny*

Following the application of molecular data (from the late 1990s onward), the order *Saprolegniales* gradually found its distinctive taxonomic placement as one of the most recent evolutionary branches of oomycetes, which are predominantly freshwater saprophytes (Beakes and Sekimoto, 2009). Primarily, Dick *et al.* (1999) and Spencer (2002) rearranged the traditional taxonomy by using sequence data from the SSU (small subunit) rDNA region as well as some morphological characters. For instance, Dick *et al.* (1999) showed the deep phylogenetic divide between *Peronosporomycetidae* and *Saprolegniomycetidae*. They also proposed a novel family of *Saprolegniaceae* which includes the *Leptolegnia* lineage. Also, Spencer (2002) studied the polyphyletic nature of some taxa, introduced several novel combinations, and transferred some taxa within *Achlya sensu lato* to *Newbya* gen. nov. Further rearrangement was suggested by Beakes *et al.* (2014) who used both morphological and LSU rDNA region-based sequence data and divided *Saprolegniales* into three families, i.e. *Saprolegniaceae*, *Verrucalvaceae*, and *Achlyaceae* (the latter was introduced for the first time encompassing *Achlya sensu stricto, Brevilegnia*, *Dictyuchus*, and *Thraustotheca*). In another study, Steciow *et al.* (2014) conducted a multi-gene phylogenetic analysis and considered three basic morphological characters (type (I) and discharge mode (II) of zoospores, as well as morphology and proliferation style of sporangia (III) to better organize *Saprolegniales*. This study is exceptional in the sense that it, for the first time, used a multiple barcode assessment to infer taxonomy and phylogeny of *Saprolegniales*. The study also supports the presence of a novel clade called SAP1 (which we currently lack any morphological description for) as a sister group of *Leptolegnia* lineage. Also, following the discovery of *Newbya* as a novel genus by Beakes *et al.* (2014), Steciow *et al.* (2014) introduced a new combination from this genus called *Newbya dichotoma* sp. nov. Additionally, Beakes and Thines (2017) transferred *Aphanomyces* and *Plectospira* to *Verrucalvaceae*, alongside *Aquastella*, *Pachymetra*, *Sommerstorffia*, and *Verrucalvus*. The most recently, Rocha *et al.* (2018) studied Argentinian strains from 22 species (13 genera) using morphology and molecular data from partial LSU and complete ITS rDNA region and confirmed the introduction of *Achlyaceae* and placing *Verrucalvaceae* into the *Saprolegniales* as suggested by Beakes *et al.* (2014) and Beakes and Thines (2017).

3.3. *Pathogenicity*

The result of a semi-quantitative analysis of 1073 downloaded *Saprolegniales*-related papers is provided in Fig. 3 (see also supplementary materials). It clearly shows that *Saprolegniales* have been mainly investigated as one of the major pathogens in freshwater environments. In fact, the issue of pathogenicity has been evidently the best-studied topic since the late 19th century. We found that approximately 39% of all papers deal with different aspects of pathogenicity (Fig. 3, circles A and B). Parasitism and defence mechanisms adopted by pathogens and hosts, controlling the diseases, host range, epidemiology, and the origin of diseases are the most common topics. Primitively, genera like *Aphanomyces*, *Saprolegnia*, and *Achlya* were frequently isolated from different arthropods, amphibians, fish, etc. Their involvement in pathogenicity was gradually confirmed and then followed by precise terminology, assigning distinct names to different symptoms produced by respective pathogens. So far, crayfish plagues are the most important and common diseases caused by *Aphanomyces astaci*. Therefore, in the following sections, we will explore the relevant knowledge in the field.

3.3.1. *Crayfish plague caused by* Aphanomyces astaci

One of the earliest records of *Aphanomyces* is Shanor and Saslow (1944), who introduced *Aphanomyces* as parasite of crayfish and fish species. Later, Unestam (1969) expanded the topic by focusing on some aspects of the physiology of the crayfish plague fungus *Aphanomyces astaci* and how it has been physiologically adapted to be an aggressive parasite. These studies were followed by investigations on the host–parasite relationship between freshwater crayfish and *A. astaci*. Non-chitinous proteolipid epicuticular membrane of crayfish was introduced as a major defensive barrier of crayfish (Unestam and Weiss, 1970). Also, encapsulation of *A. astaci* structure by blood cells (that produce polyphenoloxidase) followed by melanization were recognized in resistant crayfish species (Unestam and Nylund, 1972). At the same time, studying penetration mechanisms illustrated how *A. astaci* attacks crayfish. In particular, extracellular enzymatic activity (including lipase, chitinase, and protease), cyst and germ tube structure, repeated zoospore emergence, and germination of zoospores were identified as important factors causing diseases of crayfish (Söderhäll and Unestam, 1975; Söderhäll *et al.*, 1978).

In parallel, with more frequent observations of *A. astaci* in distant locations from different European and American crayfish species, geographic distribution and the origin of the disease were highlighted more than before. Anecdotally, it was known that *A. astaci* has being introduced to native crayfish species in Europe by imported American crayfish species since 1860 for improving crayfish production in lakes and farms. Some American crayfish species were used for this purpose more frequently because of their lower susceptibility to the crayfish plague (Unestam, 1969). However, it became evident that these species are vectors of *A. astaci* causing a significant threat toward native European crayfish species (Diéguez-Uribeondo and Soderhall, 1993; Huang *et al.*, 1994). Therefore, the course of studying *A. astaci* shifted toward epidemiological investigations, trying to better understand how diverse

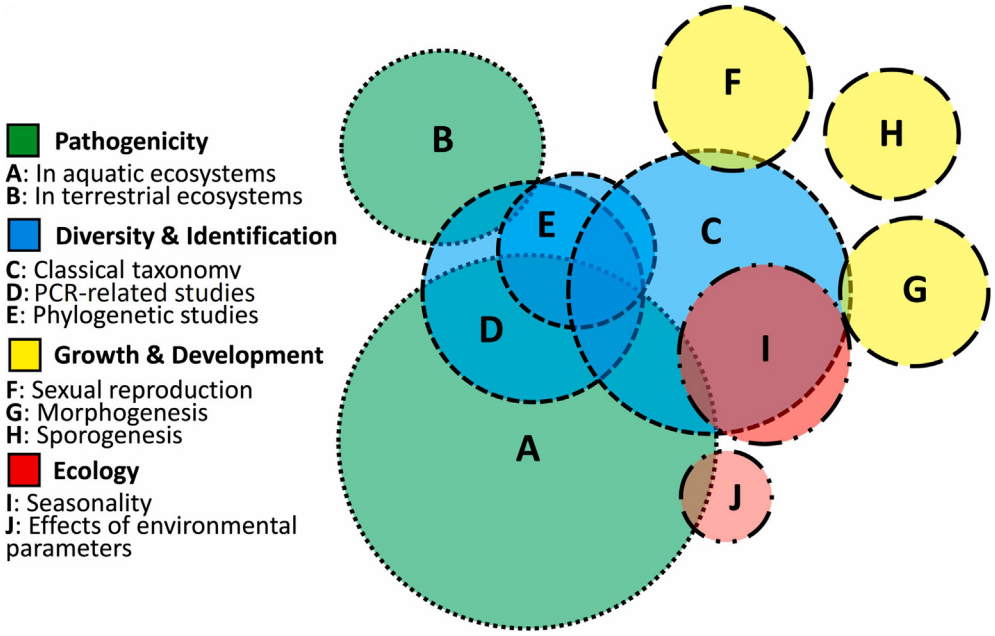

Fig. 3 – Venn diagram showing the relationship between different topical categories (shown in green, blue, yellow, and red) in the field of *Saprolegniales* inferred from 1073 papers published from 1888 to 2021. Each paper was carefully reviewed and accordingly categorized into 1–4 topics. The size of each circle represents the quantity of a given subtopic in relation to all analysed subtopics (by calculating the absolute number of papers assigned to each subtopic). Overlaps were calculated by the absolute number of papers covering 2–4 categories.

distant strains of *A. astaci* are and what threats they pose on native and sensitive populations of crayfish species.

The global distribution of the crayfish plague, which causes life-threatening conditions in different crayfish species in various habitats, could suggest an inter-species diversity of *Aphanomyces astaci*. RAPD-PCR had been previously used in fungi to assess the degree of genetic distance between different strains. Therefore, Huang *et al.* (1994) used this method to study genetic variation between different strains of *A. astaci* and realized that Swedish strains were divided in two sub-specific groups. One group constituted of strains isolated from two crayfish species (*Astacus astacus* and *Pontastacus leptodactylus*). The other group included strains from *A. astacus* a and North American crayfish (*Pacifastacus leniusculus*). The importance of this work is based on presenting the first contribution in understanding genetic diversity of *A. astaci*. Using RAPD-PCR to expand sub-specific groups of *A. astaci* strains was critical since each group was often associated with different ecological, epidemiological, and physiological features. Previously, it was not possible to discriminate *A. astaci* strains isolated from different hosts. However, RAPD-PCR became a sensitive method for the assessing degree of genetic distance between different *A. astaci* strains and the degree of their relatedness.

Based on the same methodology, Diéguez-Uribeondo *et al.* (1995) divided *A. astaci* strains into four genotype groups: 1) *A. astaci* strains isolated from the European crayfish species (*A. astacus* and *P. leptodactylus*), 2) strains isolated from the Californian crayfish species *P. leniusculus*, 3) strains isolated from the Canadian crayfish species *P. leniusculus*, and 4) strains isolated from the American crayfish *Procambarus clarkii* (in South-eastern parts). The grouping was ecologically relevant as it was found that strains in the fourth group are subtropical taxa, while strains in the other three groups usually occur in cold environments. Another genotype group was then added by Kozubíková *et al.* (2011) which included strains isolated from the spiny-cheek crayfish *Faxonius limosus*, a widespread invader in Europe (Ungureanu *et al.*, 2020). Therefore, five distinct *A. astaci* genotype groups were identified according to the RAPD-PCR methodology as follows: group A (known as "As-genotype") isolated from European crayfish species, group B ("PsI") and group C ("PsII") from *P. leniuculus* of Californian and Canadian origin, respectively, and group D ("Pc") and group E ("Or") from *F. limosus* (Svoboda *et al.*, 2017).

Although RAPD-PCR established genotype groups, its limitations, possible reproducibility problems, and the need for axenic cultures in particular, forced researchers to consider other advanced techniques to further explore genotypic diversity of *A. astaci*. Phylogenetic analyses of the chitinase gene showed that *A. astaci* strains can be placed in two lineages (Makkonen *et al.*, 2018): (1) strains from RAPD–PCR groups A, B, C and E and (2) strains from the RAPD–PCR group D. Later on, Grandjean *et al.* (2014) used microsatellite markers for direct genotyping of *A. astaci* from both axenic cultures as well as mixed genome samples. They confirmed that several genotype groups had been carried out by North American crayfish hosts, including the group which was introduced to Europe in the 19th century. With intensity limitations and low quantities of the pathogen, mitochondrial DNA (mtDNA) was introduced as an effective tool to reveal the origin and diversity of *A. astaci* (Makkonen *et al.*, 2018). Accordingly, the

previously reported four genotype groups (Diéguez-Uribeondo *et al.*, 1995) and two lineages (Makkonen *et al.*, 2018) were confirmed by amplifying the mtDNA of ribosomal rnnS and rnnL subunits. The abovementioned progress has made it possible to detect *A. astaci* more effectively in acute disease outbreaks in the wild and track them down to their origins.

4. Deficiencies

Although considerable work has been carried out on *Saprolegniales* taxonomy and diversity, it seems that studies are limited by geographical/methodological-related biases and ambiguous morphometric/molecular characterizations. In addition, the ecological significance of *Saprolegniales* in freshwater ecosystems has been largely overshadowed by their involvement in causing severe diseases in aquatic animals (Fig. 3).

4.1. *Taxonomy and diversity*

Our literature review suggests that more than two-third of all *Saprolegniales*' taxa were introduced exclusively based on morphometric characteristics (before the 1980s). However, it is well-established that morphology-based characterization is not sufficient and should be accompanied with a sequence-based phylogenetic approach. Yet, even after incorporating PCR studies into the realm of oomycetes taxonomy, most of *Saprolegniales*' taxa still remained phylogenetically orphan. In a way, neither classical taxonomy nor PCR-related studies are supporting each other to yield a richer picture of *Saprolegniales*' real diversity. In this chapter we want to explore the reasons why taxonomy and diversity have been overlooked.

4.1.1. *Impracticality of classic taxonomy*

Traditionally, there have been deficiencies in taxonomic studies of *Saprolegniales* at the level of genera as well as species. Firstly, the latest taxonomic dichotomous key (Johnson *et al.*, 2002) is relatively inefficient. The key constitutes 18 genera (*Achyla*, *Aphanomyces*, *Aphanodictyon*, *Aplanopsis*, *Brevilegnia*, *Calyptralegnia*, *Couchia*, *Dictyuchus*, *Geolegnia*, *Leptolegnia*, *Newbya*, *Phragmosporangium*, *Plectospira*, *Protoachlya*, *Pythiopsis*, *Saprolegnia*, *Sommerstorffia*, and *Thraustotheca*). Genera are identified throughout a 18-step dichotomy classification key. The impracticality of the key starts when unstable and sometimes unfeasible features have been considered for separating genera and species.

Sporangia, oogonia, and spores' morphogenesis have been considered main features to delineate different genera. However, these features are ineffective due to a couple of reasons. Sporangia, oogonia, and spores are extremely unsteady with respect to absence/presence and morphogenesis. So, they cannot be investigated as reliable characteristics because the absence/presence criterium has been shown to partly depend on environmental factors and doesn't necessarily reflect the innate features of the studied genera. Although the key has tried to offer precise terminologies, it is impossible to implement them in the lab. For instance, sporangia have been divided to the primary and secondary sporangia, even though their definitions are not distinctive. Or, another sporangium and zoospore-related feature is discharge mode. The discharge mode explains how zoospores are discharged from the sporangia. There are four discharge modes including saprolegnoid, achlyoid, dictyoid, aplanoid, and thraustothecoid which are usually assigned to *Saprolegnia*, *Achlya*, *Dictyuchus*, *Aplanopsis*, and *Thraustotheca*, respectively. Yet, descriptions are vague as the key often mentioned two or even three types of discharge mode for one single species.

The same, as explained above, is true for spores. The primary ones, according to the definition, are pyriform and have two subapical flagella. In contrast, the flagella in secondary spores (reniform planonts) are positioned laterally. Due to small size and huge variations in their shape it is hard even impossible to categorize spores into either primary or secondary categories. Also, too much emphasis is set on sexual organs, even though many strains are sexually sterile (at least at lab conditions), making sexual characterization to a large extent worthless.

The dichotomy classification key for species is even more disordered. According to the Index Fongurum, there are 405 species of *Saprolegniales* reported to date. Although the keys describe all these species in details, descriptions are most of the time either confusing or uncertain. Starting with morphological features, they often don't exclude anything. This is an example from *Saprolegnia* key to species where five different structures are assigned at the same time to describe sporangia of *Saprolegnia subeccentrica* and its renewal methods as it follows: "… *Sporangia abundant in young and old cultures; cylindrical, clavate, or long-fusiform, often curved, or slightly irregular; proliferating internally; or renewed sympodially or in a basipetalous fashion* …". Also, the key is describing morphology of gemmae in *Achlya apiculate* as follows: "… *Gemmae sparse or abundant; fusiform, cylindrical, clavate, globose, infrequently irregular or branched; terminal or intercalary, single or catenulate* …" (Johnson *et al.*, 2002). In addition, superfluous use of relative adverbs such as "predominantly", "rarely", "extremely", "generally", "exclusively", etc. makes distinction more sceptical as it is not clear what proportion they are referring to. In the same way, the range of variation in morphometric features within species is peculiarly very high. As an illustration, in *Achlya androgyna*, the range of variations in sporangia and oogonia' length is 77–988 and 50–508 μm, respectively. Moreover, gemmae which is an important feature in species descriptions, often gets confused as it might refer to sporangia and/or hyphal segments. Also, its absence/presence is very unstable and changes according to culture conditions.

4.1.2. *Lack of morphometric and/or molecular characterizations*

Fungi were traditionally identified based on morphology. However, aspects such as pleomorphism, homoplasy, phenotypic stasis and cryptic speciation within fungal taxa made the morphology-based classification less certain. From 1980's onward, the traditional taxonomy of fungi was accompanied and supported by DNA-based approaches resulting in a better phylogenetic resolution in all taxonomic ranks. Nevertheless, combination of morphology and molecular techniques hasn't worked out for *Saprolegniales* as it did for fungi. To illustrate, from 241 papers dealing with the topic *taxonomy*

(≈22%), only 32 studies applied molecular techniques and phylogenetic construction (Fig. 3D and E). In addition to impracticality of some aspects of morphology-based classification mentioned in 4.1.1, additional flaws render the taxonomy of *Saprolegniales* more challenging.

Firstly, in a large body of studies (≈8%), *Saprolegniales* strains were assigned to different species even though the authors didn't present any morphometric features. Scholars such as Czeczuga, El-Hissy, and some others have been accountable for publicizing this approach. Their work was important in the sense that it showed *Saprolegniales*' great potential in colonizing various organic materials in waterbodies (Czeczuga and Kiziewicz, 1999; Czeczuga and Muszynska, 2001; Czeczuga *et al.*, 2004). However, their publications are misleading and superfluous when taxonomic identification of species is taken into account. In one of their studies, Czeczuga and Muszynska (2001) isolated 593 strains from hairs of animal species floating on diverse water bodies and assigned them to 123 species without presenting any morphometric or physiological features what so ever. It is not clear how strains were grouped to different species as they usually show huge variations with respect to their observable sexual/asexual features. In another similar paper (Czeczuga *et al.*, 2002), although it has been claimed that they have identified 36 *Saprolegniales* taxa from fish species in six Polish waterbodies, no morphometric features have been presented. The same approach have been used in numerous studies, when a large number of strains are identified at the species level, while no morpho- or physiological data are presented, even though PCR-based taxonomy could have been used.

Naturally, weak taxonomic positioning of *Saprolegniales* taxa, as explained above, has also influenced ecological studies. In particular, one study area investigates seasonality of *Saprolegniales* taxa and their fluctuations in relation to environmental parameters in aquatic ecosystems (Fig. 3I and J). The importance of these studies relates to the fact that many of the isolated *Saprolegniales* taxa are responsible for causing severe endemic disease in aquatic animals. Thus, knowledge about their periodicity and occurrence could avoid economical lose in fishery industry. Fig. 3 shows that there is a big overlap between classical taxonomy and seasonality even though taxonomy hasn't been addressed at all. In one study, El-Hissy and Khallil (1991) kept isolating *Saprolegniales* strains per month for almost three years. They claimed that they identified more than 30 species from hundreds of strains but did not provide any morphometric or molecular evidence. Relying solely on the frequency and periodicity of species, they concluded that the highest occurrence of *Saprolegniales* happened in low or moderate temperature months and the lowest in summer months. Therefore, this ecological conclusion (higher/lower presence of *Saprolegniales* in different seasons) is based upon a misleading taxonomic characterization and, thus, not valid. Similar ecological conclusions about occurrence of *Saprolegniales* species (based upon inaccurate taxonomic identitification of strains) in different seasons have been reached by others too (Mer *et al.*, 1981; El-Hissy *et al.*, 1982; de Almeida Nascimento *et al.*, 2011; Muszyńska *et al.*, 2014).

Even further, the number of species isolated per month was used in several other studies to investigate the possible correlation to water chemistry which suffers from the same problem above. Czeczuga *et al.* (2003) determined water chemistry by measuring several parameters such as temperature, pH, COD (chemical oxygen demand), etc. Then, based on cluster analysis, they revealed what parameters are correlated with the number of *Saprolegniales* species in different seasons. Since species identification cannot be confirmed without solid morphometric evidence, ecological studies such as investigating seasonality and relating strains' diversity and distribution to water chemistry will be biased.

4.1.3. *Insufficient implementations of molecular techniques*

As explained above, DNA-based approaches in *Saprolegniales* haven't been as effective and common as in fungi (Põlme *et al.*, 2020). So far, with nearly 3000 sequences deposited in GenBank, ITS is considered the most common region for delineating *Saprolegniales* genera and species. However, the current database is not representative of all *Saprolegniales* due to several biases. Firstly, at the genus level, 79%, 10%, and 5% of all ITS sequences belong to just three genera including *Saprolegnia*, *Aphanomyces*, and *Achlya*, respectively, leaving the rest un-sequenced and hence purely defined (Fig. 4A). One of the main reasons for such a prevalent bias is that the most common genera in GenBank are well-

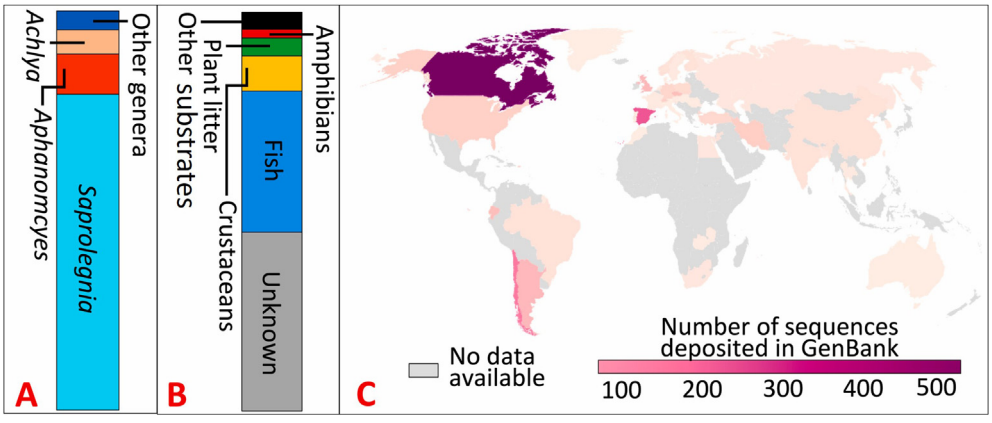

Fig. 4 – Composition of 2803 ITS sequences assigned to *Saprolegniales* submitted to GenBank based on their genus (A), substrate (B) and geographical origin (C).

known aquatic animal pathogens. This is also evident from the substrates used for the isolation of these strains where approximately 46% of all deposited strains are isolated from aquatic animals such as crustaceans, amphibians, and fish (Fig. 4B). According to the ITS annotations, at least 40, 15, and 10 species of fish, amphibians, and crustaceans have been reported as possible hosts of pathogenic *Saprolegniales*, respectively. In contrast, isolating *Saprolegniales* from plant litter has been largely neglected with only 2% of sequences originating from it (Fig. 4B). Therefore, it is not clear how and to what extent plant litter colonizing taxa might be taxonomically, pathogenetically and ecologically different from pathogenic species. Furthermore, the second largest fraction of ITS sequences deposited in the data bases (45%) is assigned without mentioning any hosts and/or substrates (Fig. 3B). It shows that annotating these sequences have not been taken care of properly which makes any ecological conclusions about their origin and lifestyle hard to achieve. Finally, the deposited ITS sequences are geographically biased as most strains have been isolated from North America (Fig. 4C).

The same biases will emerge when we categorized 1073 papers based on the studied genera, ecosystems, hosts, and pathogenicity (Fig. 5). Fig. 5A and B shows that *Achlya*, *Aphanomyces*, and *Saprolegnia* are, again, the most frequent studied genera in freshwater ecosystems. The only genus which is currently associated with both freshwater and terrestrial ecosystems is *Aphanomyces* (Fig. 5B) due to its pathogenicity toward both aquatic animals and terrestrial plants (e.g., *A. euteiches*, *A. cochlioides*, and *A. raphani* as pathogens of pea, sugar beet, and radish, respectively) (Fig. 5C) (Gaulin *et al.*, 2007, 2008). Considering the fact that morphological and molecular characterizations are often inaccurate, it is logical to argue that the current picture of *Saprolegniales*' diversity is misleading. In addition, similar to results from ITS accession numbers, aquatic animals and agricultural plants are the most common hosts/substrates in which *Saprolegniales* have been isolated from (Fig. 5D). In contrast, plant debris-associated *Saprolegniales* have been isolated very infrequently, even though they are recently shown to be important constituents of eukaryotic community in terms of

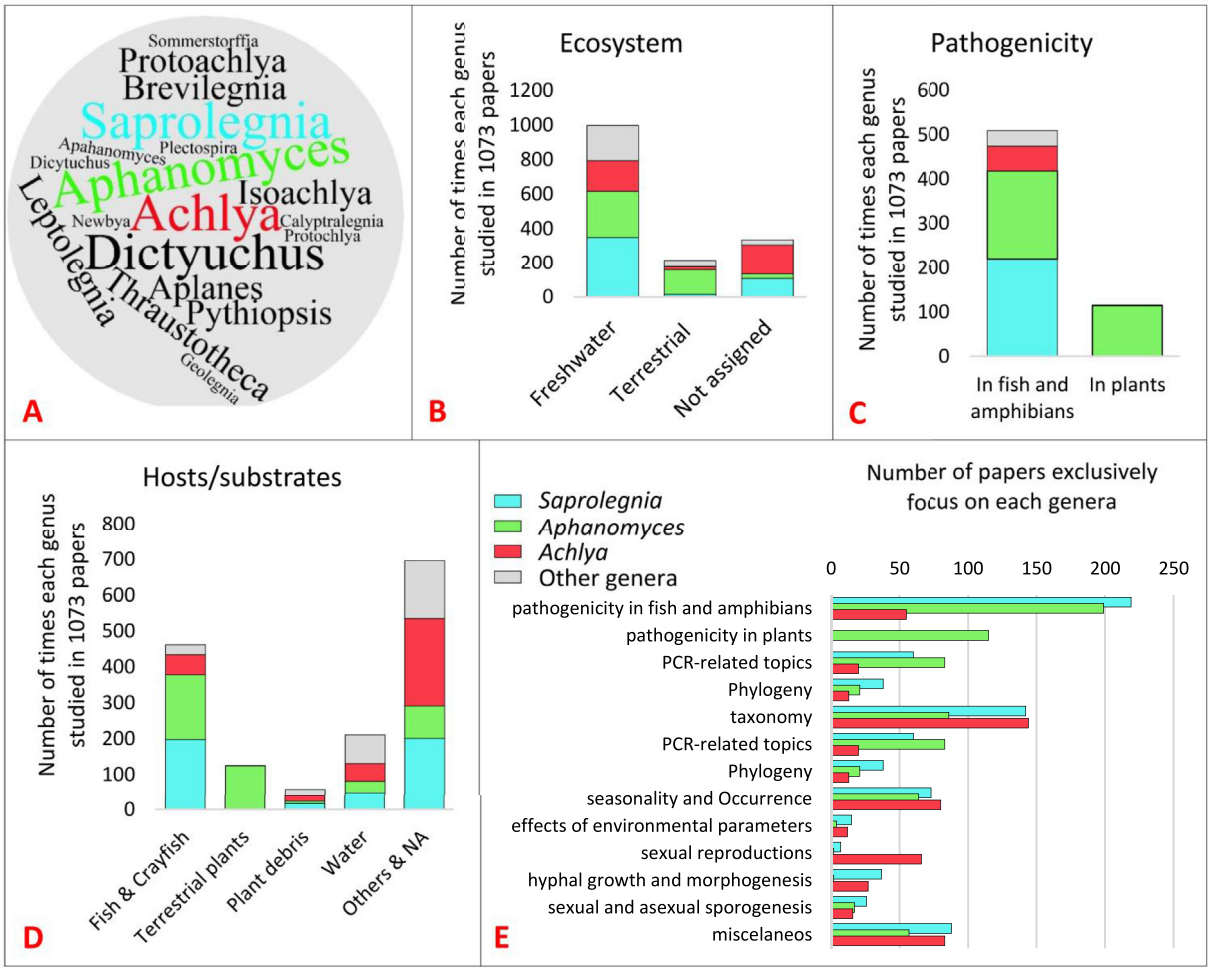

Fig. 5 – *Saprolegniales*-related studies based on genera, ecosystems, pathogenicity, and hosts/substrates (inferred from 1073 papers). (A) Size of the genera reflects the number of times they have been examined by all 1073 papers. (B) (C), and (D) show the composition of *Achlya*, *Aphanomyces*, and *Saprolegnia* based on studied ecosystems, pathogenicity, and hosts/substrates, respectively. (E) Papers that have been exclusively studied *Achlya*, *Aphanomyces*, and *Saprolegnia* based on six specific categories.

organic matter decomposition. Finally, Fig. 5E shows the numbers of papers which have exclusively studied *Saprolegnia*, *Aphanomyces*, or *Achlya* and highlights, once again, the existing biases with respect to pathogenic and ecological aspects of *Saprolegniales*.

A similar biased trend is also observed at species level (Fig. 6). There are currently 405 species of *Saprolegniales* reported in the Index Fungorum database (Fig. 6A). The number of newly described species peaked in 1940 and descended until 2000. However, the validity of at least 290 cases (≈72% of all reported species) must be considered with care because: I) they had been described before the invention of PCR and II) in many cases their morphometric and physiological characters are very similar and not distinctive. Therefore, it might not be surprising that accurate morphometric, physiological, and molecular studies reveal that many of these 290 species have been assigned to different taxa without any convincing special features. Again, this clearly points to a severe divergence between classic and molecular taxonomy (Fig. 3, circles C, D, and E).

The genus *Saprolegnia* is one of the most biased species in *Saprolegniales*. One-third of all ITS sequences are identified as *Saprolegnia parasitica* followed by *S. diclina* (≈9%), *S. ferax* (≈8%), *S. australis* (≈7%), and *S. delica* (≈6%) (Fig. 5B). It could be argued that these species are *indeed* the most universal species, justifying such a disproportionate ITS database. However, this cannot be proven due to the lack of complementary information such as morphometric descriptions. Moreover, 19% of sequences are just assigned to the genus level, making it more challenging to assess the full diversity of the genus. The same is true for *Achlya* and *Aphanomyces* as there is only one species in each genus with a large number of accession numbers and numerous accession numbers not assigned to the species level (Fig. 6B). For example, 27% of all *Achlya* and *Aphanomyces* accession numbers have been assigned to just the genus level. Or, 44% of all *Aphanomyces* accession numbers belong to *A. astaci*. Therefore, whether species such as *S. parasitica* or *A. astaci* really contribute the most to the world-wide diversity of *Saprolegniales* remains currently unknown. Despite these shortcomings, ITS sequences have been vital in constructing phylogenetic relationships among different genera and species of *Saprolegniales*.

Cytochrome c oxidase subunit I (*COX1*) has also been used for molecular identification and phylogeny of *Saprolegniales*, however, the biggest challenge for this region is the very low number of sequences currently deposited in the GenBank. Presently, there are only 126 *COX1* sequences with 52, 46, and 12 accession numbers assigned to *Achlya*, *Aphanomyces*, and *Saprolegnia*, respectively. In many cases, accession numbers have been only assigned to a genus and not a species which could add to the current taxonomic confusion.

4.1.4. *High-throughput approaches to DNA sequencing*

In contrast to fungi, high-throughput approaches (HTA) to understand diversity of oomycetes is still in its infant stage. In one of the earliest studies, Sapkota and Nicolaisen (2015) successfully employed the 454 pyrosequencing method (using primer sets ITS4, ITS6 and ITS7) to optimize the yield of oomycete-derived sequences from a background of soil DNA and showed that *Pythiales* (89%), the genus *Pythium* in particular, is the most dominant lineage. Later, Singer *et al.* (2016) applied Illumina sequencing (V9 region of the SSU rDNA) to characterize the environmental diversity of oomycetes in five oligotrophic peat bogs. They showed that taxa affiliated with unidentified *Saprolegniales* populate natural pools significantly higher than surrounding microhabitats. Interestingly, the most abundant taxa were deep basal lineages which couldn't be classified as *Saprolegniales* or *Peronosporales*. In contrast to Singer *et al.* (2016), Riit *et al.* (2016) used an oomycete-specific ITS primer (ITS-O) and sequenced 20 soil samples from forest nurseries using Illumina Miseq 2 × 300 PE HTS technology. The recovered OTUs affiliated with *Saprolegniales* presented in all samples. Yet, the within composition of *Saprolegniales* taxa in soil remained to be answered as the study did not investigate ranks lower than orders (not included: families, genera, species). Next, Ruiz Gómez *et al.* (2019) studied

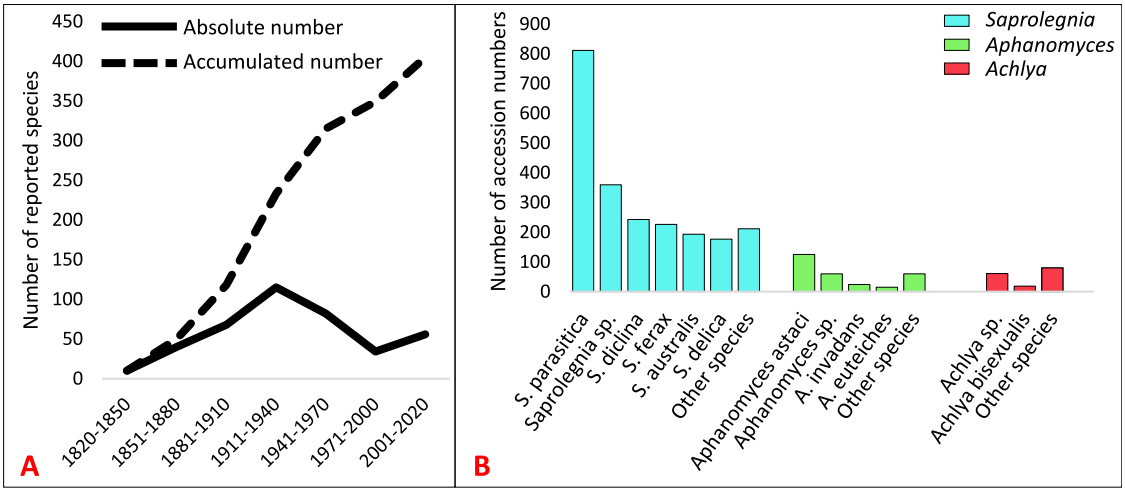

Fig. 6 – Absolute and accumulated number of newly described species of *Saprolegniales* taxa based on the Index Fungorum (A) and a review of ITS accession numbers assigned to *Saprolegnia*, *Aphanomyces*, and *Achlya* in GenBank (B).

oomycete communities in a holm oak declined area (using Illumina MiSeq 2 × 300 bp platform) and showed that infected soils were dominantly populated by *Phytophthora* and *Pythium* (59 and 36% of the whole communities, respectively). Using similar methodology, Sapp *et al.* (2019) proposed cytochrome c oxidase subunit II metabarcoding to determine site-specific distribution of oak rhizosphere-associated oomycetes and found out that members of *Peronosporales* and *Saprolegniales* were the most and least dominant taxa, respectively. Finally, Fiore-Donno and Bonkowski (2021) designed a new pair of oomycete-specific primers based on the V4 region of the 18S rRNA gene and showed that *Peronosporales* (73% of OTUs) dominate grassland and forest landscapes followed by *Saprolegniales* (21%).

So far, almost all studies have focused on terrestrial ecosystems. Despite all shortcomings, using HTAs have enabled researchers to have a fair understanding of entire oomycete communities in soil. In fact, avoiding biases originated from culture-dependent techniques is one of the most important advantages of HTAs. Unfortunately, we still lack such an understanding for oomycete communities in freshwater ecosystems. Traditionally and based on specific culture-dependent methods, researchers have argued that members of *Saprolegniales* especially the pathogenic ones such as *Saprolegnia* and *Aphanomyces* populate freshwater landscapes more than other orders. However, one could argue that the dominance of *Saprolegniales* might be simply the result of ignoring other taxa and/or inefficiency of used methods in recovering them from freshwater environments. In fact, other oomycetes could be also as abundant as *Saprolegniales* but we misinterpret the current knowledge. For instance, *Halophytophthora, Nothophytophthora* and *Phytopythium* are non-*Saprolegniales* members of oomycetes which are recently found in various freshwater ecosystems (Caballol *et al.*, 2021; O'Hanlon *et al.*, 2021; Nam and Choi, 2019). However, since isolating each taxon requires different methodologies, it would be impossible to understand the composition of different taxa in the environment.

4.2. *Ecology*

A very highlighted aspect of *Saprolegniales*' ecological contribution is the establishment of parasitism with fish, crayfish, and amphibian species. However, other potential contributions have been overshadowed and not been taken seriously. In the following section, the interaction of *Saprolegniales* with allochthonous organic matter and trophic levels in food webs will be discussed.

4.2.1. *Interactions with allochthonous organic matter and trophic levels in food webs*

Although most *Saprolegniales* have been isolated from animal sources, it is well-established that they can be also associated to allochthonous organic matter (AOM) (Grossart *et al.*, 2021). AOM is a crucial component of food webs and it greatly contributes to the dissolved organic matter pool, browning (an increase in water colour), humification, increasing extra energetic input to the base of trophic food webs, etc. (Solomon *et al.*, 2015). In nother words, AOM will be firstly degraded and transformed by the microbial communities (fungi and *Saprolegniales* in particular) and then feed food-

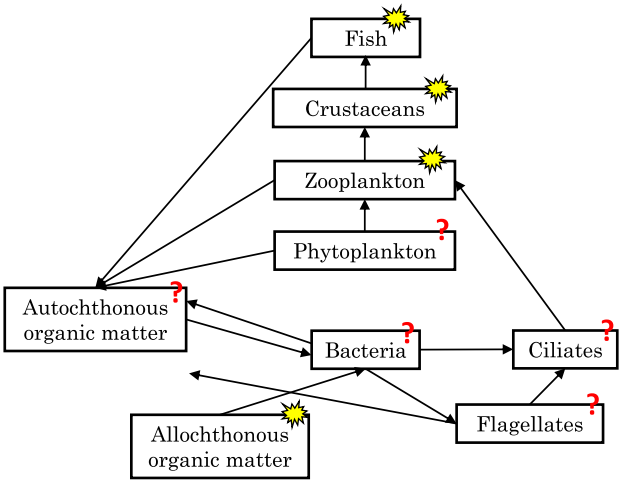

Fig. 7 – The involvement of *Saprolegniales* in freshwater food-webs. Eight-point yellow stars show *Saprolegniales*' possible interactions with various trophic levels. Also, question marks indicate gap of knowledge.

webs accordingly (Fig. 7). However, understanding the association of *Saprolegniales* with AOM has been limited to mainly isolation and taxonomic identification, yet their quality of contribution to the degradation and transformation of AOM has to be studied. In some old studies, *Achlya*, *Aphanomyces*, and *Saprolegnia* were thought to possess cellulolytic and/or chitinolytic activities (Unestam, 1966; Nyhlén and Unestam, 1975; Thompston and Dix, 1985), enabling them to degrade and transform cellulose- and/or chitin-based AOM (Thurman and Thurman, 1985). More recently, Masigol *et al.* (2018, 2019) studied *Dictyuchus* spp. and concluded that although *Saprolegniales* are robust cellulose and chitin degraders, they lack any ligninolytic ability. They argued that there could be an ecological partitioning between fungi and *Saprolegniales* in terms of their interaction with lignin and lignin-like compounds. Another intriguing separation between fungi and *Saprolegniales* by Masigol *et al.* (2019) suggests that humic and humic-like substances suppress *Saprolegniales* mycelial growth, but show no negative impact on fungal growth. The same authors observed the same trend in other genera (Masigol *et al.*, 2020, 2021a, 2023) and concluded that *Saprolegniales* compensate the lack of ligninolytic activity by higher efficiency in utilizing low molecular weight carbon sources.

Earlier, it has been mentioned that *Saprolegniales*, which are pathogenic to large consumers in aquatic environments, have been extensively studied due to their major negative impacts to fishery industries and aquaculture. However, the interactions of *Saprolegniales* with herbivorous consumers (zooplankton) have been largely ignored. These interactions matter because zooplankton are essential regulators of food-webs and any large-scale changes in their biology might lead to a cascade of ecosystem-destabilizing impacts (Balseiro *et al.*, 2022). Despite its importance, the impact of *Saprolegniales* on communities of zooplankton have been addressed in only a few intermittent and small-scale studies. Prowse (1954) and Seymour *et al.* (1984) reported *Aphanomyces daphniae*

sp. nov. as a parasite of the water flea *Daphnia hyaline* and *D. magna*. The same was reported by Wolinska *et al.* (2008, 2009) who showed that *Aphanomyces*, *Leptolegnia*, *Saprolegnia, Scoliolegnia* sp. strains might be important selective pressures in populations of *Daphnia pulex*. Also, occurrence and mortality of other *Aphanomyces* species such as *A. ovidestruens* were found on the copepods *Boeckella dilatate* (Burns, 1980, 1985), *Boekella hamata* (Valois and Burns, 2016), and *Parabroteas sarsi* (Garcia *et al.*, 2020). Water fleas and copepods are two major groups of zooplankton communities which mainly serve as intermediary species in the food chain and transfer energy from planktonic algae (primary producers) to larger invertebrate predators and fish. Therefore, fluctuations in these communities, in our case caused by *Saprolegniales*, might disturb the energy flow and impact organisms at other trophic levels. Interesntingly, the impact of *Saprolegniales* on zooplankton is not always destructive. For example, Ozersky *et al.* (2019) showed that exposure to *Saprolegnia* has a positive effect on the copepod *Epischurella baikalensis*. Additionally, such ecological implications are not limited to daphnids and copepods as *Aquastella* gen. nov. (a recently reported genus of *Saprolegniales*) has been reported to infect three rotifer species (Molloy *et al.*, 2014). Similar to copepods and water fleas, rotifers play an important role in food-webs by, for example, showing efficient predation behavior on protozoans (Arndt, 1993; Gilbert, 2022).

5. Prospect

In this review, we explored approximately a thousand *Saprolegniales*-related papers and highlighted current advancements and deficiencies in the field. At the same time, we pinpointed that diversity and ecology of *Saprolegniales* have been mainly ignored due to the higher importance of their pathogenicity. Better understanding of current knowledge gaps will help us depict the direction for future research.

As for the diversity, conducting more coherent taxonomical research is a matter of great importance. It will be crucial to improve the current sequences of *Saprolegniales* in databases and make them as error-free as possible (Masigol *et al.*, 2021b). One good example (Sandoval-Sierra *et al.*, 2014) is the definition of DNA-based molecular operational taxonomic units for the genus *Saprolegnia*. The authors listed incorrectly strain names in culture collections, determined miss-assigned species names in GenBank, and finally proposed 29 out of 961 ITS sequences (18 species + 11 potential new ones) as reference. The same could be done with other genera such as *Aphanomyces* and *Achlya* as they, similar to *Saprolegnia*, have been annotated incompletely and inaccurately. Generating trustable sequences will pave the way for the next important step which is building curated sequence-based databases. The advantage of such specific databases is that they let you compare new sequence with only reliable well-described and provisional species. This will facilitate further valid phylogenetic studies and avoid any miss-assignment. A good example is the *Phytophthora* database (DB) (Park *et al.*, 2013) which is a web-accessible and searchable format and representative of 138 described and provisional species based upon one to 12 loci.

It is also important to improve the way morphometric features of *Saprolegniales* are currently being used for the taxonomic identification as they are, in many cases, unfeasible and unstable. Recently, a protocol has been proposed by Sandoval-Sierra and Dieguez-Uribeondo (2015) which addressed the abovementioned challenge. They suggested that any novel taxa of *Saprolegnia* must be associated with a holotype preserved by absolute ethanol and/or lyophilization methods so that morphometric features are not altered. This holotype can be later subjected to DNA extractions, making all the resulting sequences as reliable as possible for further phylogenetic investigations. They have also suggested that morphometric features of strains must be analyzed together (not individually) using a linear model analysis for the evaluation of interspecies differences. Since their protocol have been able to characterize and distinguish two *Saprolegnia* species (*Saprolegnia aenigmatica* and *Saprolegnia racemosa*), it could be used for other genera too.

Furthermore, the current consensus implies that single-gene phylogeny is not practical anymore as it doesn't give enough resolution to distinguish close and/or cryptic species in many cases. Therefore, acquisition of living materials belonging to valid accession numbers of ITS sequences and sequencing other barcodes would be a practical approach. Mitochondrial Barcodes such as cytochrome c oxidase subunit 1 (*cox*1), and NADH dehydrogenase subunit 1 (*nadh*1) and nuclear ones such as β-tubulin and heat shock protein 90 which have been already used for the sister group (*Peronosporales*) (Robideau *et al.*, 2011; Scanu *et al.*, 2021) and can be used for *Saprolegniales* as well. For instance, the introduction of the genera *Nothophytophthora* and *Phytopythium* is the result of phylogenetic analyses of both nuclear ITS, LSU, β-tubulin and *HSP90* loci and the mitochondrial genes (Jung *et al.*, 2017; de Cock *et al.*, 2015) as well as in-detailed morphometric characterizations. These newly introduced genera show combined features of previously reported taxa. Therefore, it is highly plausible that with the application of several barcodes, intermediate taxa will emerge from *Saprolegniales* as well.

As for their ecology, although earlier limited studies have suggested parasitic relationship between some members of *Saprolegniales* and various trophic levels of aquatic food-webs, the large-scale impact of such interactions remains unknown. Therefore, in order to go beyond one-to-one interactions, revealing the composition of *Saprolegniales* taxa in their natural environments must be given priority. For instance, it is true that several taxa of *Aphanomyces* are pathogenic toward zooplankton communities in lab settings. However, these taxa might constitute only a very small portion of the entire communities of *Saprolegnia* in natural environments. In fact, such a small portion might be heavily outnumbered by more common taxa and not be able to cause any diseases. Therefore, studying composition of *Saprolegniales* using metabarcoding approaches seems logical as it gives the relative contribution of each genus in entire community. As explained earlier, to this date, no coherent studies have been conducted to explore how *Saprolegniales* are distributed in freshwater environments. Doing so, might suggest that *Aphanomyces*, *Saprolegnia*, and *Achlya* spp. *are not* the most common genera after all (as classic approaches have suggested). However, improvements must be

made (e.g., designing more targeted barcodes) so that metabarcoding approaches can result in a better resolution to relative distribution of taxa, particularly at the genus level.

Only after acquiring more information on the diversity and spatiotemporal distribution of *Saprolegniales*, their large-scale ecological impacts can be addressed coherently. This is another reflection of what we earlier discussed in 4.1.2, stating that the current inaccurate/incomplete picture of diversity will lead to wrong ecological conclusions. Therefore, quantitative measures can be complementary to above-mentioned metabarcoding studies by giving more depth to understanding assemblages of the *Saprolegniales*. Yet, quantitative techniques such as quantitative PCR (qPCR) are not still comprehensive enough to include all genera of *Saprolegniales*. So far, almost all qPCR-related studies have been optimized for only *Saprolegnia* spp. (Rocchi *et al.*, 2017; Korkea-Aho *et al.*, 2022) and *Aphanomyces astaci* (Pavić *et al.*, 2022). So, methods should be adjusted to include all important freshwater taxa so that relative abundance of each taxon could be understood.

Additionally, we discussed earlier that lab-based experiments have shown *Saprolegniales* as active chitino- and cellulolytic degraders and potential pathogens to zooplankton communities. However, this is far from convincing because such interactions happen in a chaotic environment with various biotic and abiotic parameters which could alter the outcomes. In fact, the central question here is that whether *Saprolegniales* can behave the same in natural environments as they do in the lab settings. To address this, we suggest transcriptomics in a meso- or microcosm experiment as it shows how interacting microorganisms in food-webs response to biotic and abiotic environmental stresses. Transcriptomic-based studies have already shown the large-scale impact of *Aphanomyces astaci* and *Saprolegnia parasitica* on their crayfish and fish hosts under various environmental conditions (Boštjančić *et al.*, 2022; Ellison *et al.*, 2018; Gou *et al.*, 2020). We propose an upgraded version of the experiment conducted by Wolinska *et al.* (2009). They suggested specific cultivating conditions for challenging *Daphnia pulex* against several taxa from *Saprolegniales*. Such studies can be the basis of transcriptomic investigations where the molecular mechanisms underlying the interactions of hosts and potential pathogens will be understood. This is how we will be able to more convincingly determine the nature of host-*Saprolegniales* interactions. Ultimately, such notions might lead to understanding the actual contributions *Saprolegniales* can make in freshwater biogeochemical cycles with several major players involved. So far, such contributions have been observed in some members of *Chytridiomycoa* (known as chytids) which, interestingly, share many similarities with *Saprolegniales*, especially in terms of their pathogenic potentials and high motility of zoospores. The recently described phenomenon known as fungal shunt shows how chytrids influence phytoplankton–bacteria interactions and divert the path of photosynthetically derived carbon (Klawonn *et al.*, 2021). Therefore, we speculate that *Saprolegniales* can significantly manipulate microbial-related carbon flow at the base of freshwater food-webs too.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbr.2023.100319.

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