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Review

Comprehensive, classical and molecular characterization methods of Saprolegnia (Oomycota; Stramnipila), an important fungal pathogen of fish

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ABSTRACT

The fish fungal pathogen (Saprolegnia) belonging to heterokonts group, causing Saprolegniosis, received considerable attention among all the fungal pathogens of fish. The pathogen is widely distributed to aquatic habitat and is an economically important member of the class Oomycetes. The genus comprises of opportunistic biotrophic or parasitic pathogens of fish and relevant aquatic organisms. The atrocities of pathogen account for few million dollar losses to the aquaculture production worldwide. It has also remained linked to the cosmopolitan decline in wild fish stocks, amphibian, and crustacean populations. In order to overcome the burden of the disease, there is a crucial need to develop strategies for the effective management of Saprolegniosis. However, in order to manage the disease burden, there is a basic requirement to understand the phylogeny, taxonomy and molecular mechanism involved in the disease progression. With the tremendous advent and escalation in the fungal genome sequencing projects which helped in understanding the novelties of many fungal taxa so far, there is still dearth in the molecular information available on *Saprolegnia* spp. Owing to the scarcity of the cumulative information pool, there arises a need to assemble phylogenetic, taxonomic and molecular data of Saprolegniales along with their transcriptomic data from various databases available, so that the consolidated information be made accessible to the researchers. In the above context, this review shall serve as an important information resource on the overall biological aspects of *Saprolegnia* spp., and a way forward to tackle this pathogen efficiently. Various genomic and transcriptomic studies discussed shall enlighten the understanding of Saprolegnia pathogenesis mechanisms and subsequently benefit in framing strategies to contain the pathogen efficiently.

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1. Introduction

Being key decomposers in ecosystems, the kingdom fungi with more than 2.5 million species represents the supreme and diverse eukaryotic group on the earth (Hawksworth and Lucking, 2017). However, some fungi are pathogenic to many plants and animals including fish (Agrios, 2005). Fatalities due to parasitic, viral and microbial (bacterial and fungal) infections are the biggest threat to the aquaculture, adversely affecting the animal well-being and thereby posing a big challenge to the progress of aquaculture industry (Phillips and Subasinghe, 2008). The fungal pathogens infecting freshwater fish belonging to class Oomycetes are mainly from genera; Saprolegnia, Achylia, Aphanomyces and Branchiomyces (Daugherty *et al.*, 1998). Among the fungal group, Oomycetes (Sub-class Saprolegniomycetidae) evolved as opportunistic fish pathogens which are; saprophytic, necrotrophic or biotrophic parasite (Noga, 1993; Bruno and Poppe, 1996; Judelson, 2012, Judelson, 2017). Disease causing Oomycetes are recognized to be problematic among fish associated fungal groups, causing loss of freshwater fish and fish eggs both in the barren and commercial fish farms (Roberts *et al.*, 1993; Bangyeekhun *et al.*, 2001). Among various fungal pathogens of fish, *Saprolegnia* spp. belonging to the class Oomycetes are economically important infecting many freshwater fish (both ornamental and cultured) (Hatai and Hoshai, 1994; Sosa *et al.*, 2007a, 2007b; Refai *et al.*, 2016). The predisposing factors for the predominance of Saprolegnia infections are mainly aquatic contamination, pollution and congestion. Saprolegniosis has been stated to inflict losses to the tune of billion dollars in Atlantic salmon, rainbow and brown trout, and non-salmonid species including perch, eels and catfish along with several other commercial freshwater fish (Bangyeekhun *et al.*, 2001; Van West, 2006; Bruno *et al.*, 2011). One of the peculiar and undesirable properties of Saprolegnia is its infection on unfertilized eggs; however, the adjacent fertilized eggs may also get infected, thereby, adversely affecting oxygen supply and gaseous exchange to these infected eggs (Refai *et al.*, 2016). The pathogen primarily invades epidermal tissues involving fins or head and spreading over the entire surface of fish body and subsequently the contagion may spread to the internal organs like, liver spleen, kidney and alimentary canal (Van West, 2006) (Fig. 1). Continuous exposure of freshwater fish to Saprolegnia infection has led to the gradual decline in, fish populations, species richness and species diversity (Myers, 1993; Singh, 2002; Romansic *et al.*, 2009).

Owing to the huge economic losses inflicted by Saprolegnia infections, it becomes imperative to have an in-depth study on the ecology, transmission and pathogenesis mechanisms to devise strategies for the effective control and prevention of Saprolegniosis. The constant detection of new emerging mycotic agents which affect the fish swarm in different aquatic environments, with the establishment of dynamic and novel molecular techniques increases the necessity for the expansion of rapid and picture-perfect identification systems and integrative molecular data. Since, delay in the diagnosis and adminstering the therapy leads to a bizarre mortality rates. So far, the focus and efforts to manage the fungal pathogens, by the traditional methods, like use of morphological, physiological and biochemical features for pathogen identification have reformed the field. However, the potential of molecular biology approaches to further refine such studies have not been significantly utilized. Till date substantial progress in the Saprolegnia genomics has been made, by making available the sequences of, the whole genomes, transcriptomes, barcode genes and pathogenic genes which have fairly improved the diagnosis and characterization of *Saprolegnia* spp. This review is an effort to provide comprehensive knowledge about the biology, diversity and distribution, and characterization methods of Saprolegnia group, an opportunistic fish pathogen. Further, the extent to which the genomics and transcriptomics have transformed and revolutionized the Saprolegnia research has been reviewed in detail.

2. Saprolegnia – A fungal pathogen of fish

The pathogen group (Saprolegnia) among Oomycetes received considerable attention; Saprolegnia, a category of heterokonts, order Saprolegniales, family Saprolegniaceae and comprise closely related water molds ubiquitous in freshwater, with the common characteristic feature of producing motile biflagellate spores (Baldauf *et al.*, 2000). The whole ensemble of Oomycetes is taxonomically distributed into three subclasses: Hipidiomycetidae, Peronosporomycetidae and Saprolegniomycetidae. The Oomycetes group which belong to the Saprolegniomycetidae, consists of two orders: Leptomitales and Saprolegniales. They are fungus-like protists, similar to fungi and golden brown algae being the affiliate of the Chromista (Hart and Reynolds, 2002). The saprolegnia pathogen is either associated with dead tissue or live fish and is considered as an opportunistic saprotrophic biotroph (Margulis and Schwartz, 2000; Gijzen and Nurnberger, 2006). Saprolegnia either grow as cotton wool like fungal mats in deep skin lesions on fish which on culturing on artificial media like Potato dextrose agar (PDA) Sabouraud dextrose agar (SDA), and Yeast extract agar (YEA) appear as long hairs with whitish cottony color that quickly shift to grey as cysts are produced (Fig. 2) on incubation for a week or long (Eissa *et al.*, 2013).

Standard morphological benchmarks established on reproductive structures, size, shape, and characters of antheridia, cysts, zoospores, oogonia and oospores, play essential role in taxonomic documentation of *Saprolegnia* spp., to the Oomycetes fungal group (Beakes and Ford, 1983). However, comparisons of internal transcribed spacer (ITS) and cytochrome oxidase (COX I and II) subunit based DNA sequences indicate that with a global distribution they are nearer to brown algae (Dick, 2001; Bouzenzana *et al.*, 2006). In addition, the disparities in esterase isoenzymes patterns (Hatai *et al.*, 1990) and modifications in radial growth rate permit cataloging isolated Saprolegnia species strains in different subgroups (Fregeneda-Grandes *et al.*, 2007).

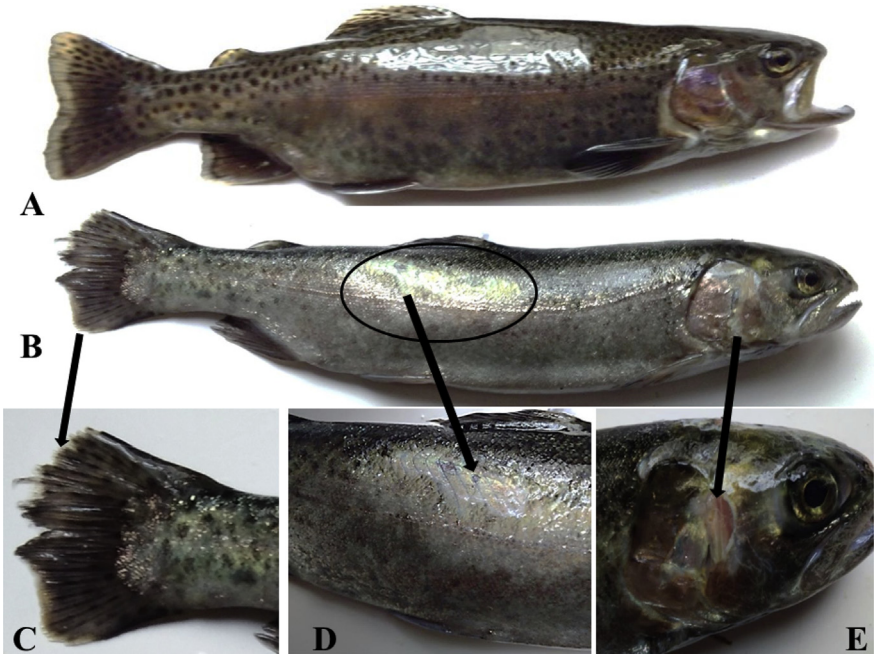

Fig. 1 – Rainbow trout-RBT (*Onchorhynchus mykiss*) infected by fungal pathogen (*Saprolegnia* spp.,). A) A healthy Rainbow trout (*Onchorhynchus mykiss*), B) RBT infected with a fungal pathogen, C) Caudal fin is deteriorated by necrosis and mycelia growth, D) Necrosis and discoloration of the dorsal lateral skin of the fish body, E) Operculum with necrotic lesion and deteriorated underneath cartilage.

3. Historical perspective

The family saprolegniaceae was first introduced by Ledermüller (1760), Wrisberg (1765) and Spallanzani (1777), however, earliest binomial names of fungal group seem to be *Byssus aquatica* by Flora Danica (1780), *Conferva piscium* by Schränk (1789), Dillwyn (1809), and of Lyngbye (1819). The present classification for the Saprolegnia spp., group is (Kingdom: Chromista (Stramenopiles), Phylum: Oomycota, Class: Oomycetes, Order: Saprolegniales, Family: Saprolegniaceae, Genus: Saprolegnia) (Beakes and Ford, 1983). The sequential year of discovery and the respective discoverer(s) of Saprolegniales group are depicted in (Fig. 3).

4. Distribution of *Saprolegnia* spp.

The pathogen group shows cosmopolitan distribution with high abundance towards the northern hemisphere compared to the southern hemisphere. The pathogen is distributed to maximum of the European countries, China, Japan, South and Southeast Asian countries, North and South America and some parts of Australia. It has attained the status of a potential fish-pathogen, confined to cosmopolitan aquatic fresh water habitats (Fig. 4).

5. Morphological features and life cycle of Saprolegnia

The Oomycetes pathogenic species affecting fish hosts are of generalist nature, i-e they are infective agents with widest variety of hosts (Fisher *et al.*, 2012; Zhao *et al.*, 2013). Several features distinguish Oomycetes from other fungi; i.e., unlike the true fungi (haploid or dikaryotic) with flattened mitochondrial cristae, the asexual state of *Saprolegnia* spp. is diploid, the mitochondrion has tubular cristae, nucleus and protoplasm have associated microtubules (Alexopoulos *et al.*, 1997; Kortekamp, 2005). Saprolegnia species have different components on its cell wall, 1–3 glucans, 1–6 glucans like true fungi, but do not encompass chitin, which exists in the cell wall of true fungi. They possess rare septa in the hyphae, resulting in coenocytic hyphae including long filamentous hyphae, with rounded ends containing zoospores; the colonies are white in color (Alexopoulos, *et al.*, 1997; Kortekamp, 2005). Hyphae with rare septate filaments (devoid of cross walls), is a peculiar feature of Oomycetes and hyphal growth helps in the acquisition and colonization of new tissues of a new host (de Hoog *et al.*, 2011). The cell wall molecules of the pathogen can evoke immune response in fish and other organisms (Parra-Laca *et al.*, 2015). The encased long hooked hairy zoospores of *Saprolegnia parasitica* aid in its attachment to the host fish (Van West, 2006). Chemotactic zoospores of some Oomycetes which include that of *Saprolegnia* spp. as well,

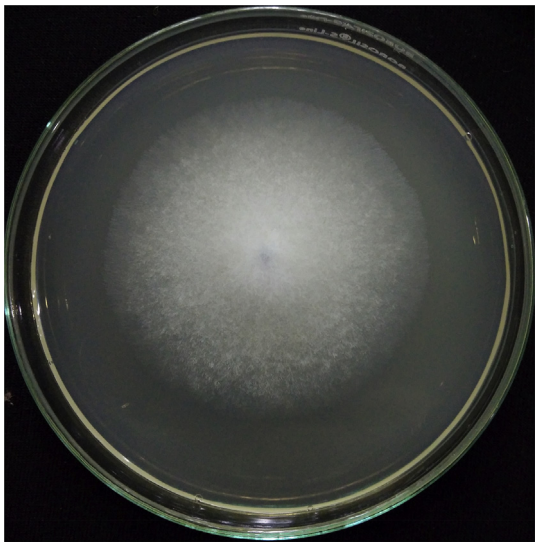

Fig. 2 – Saprolegnia mycelium on Yeast extract agar, fungus isolated from the infected Rainbow trout (*Onchorhynchus mykiss*).

respond to amino acids, aldehyde attractants and carbohydrates (Donaldson and Deacon, 1993).

The pathogen infection and subsequent mycelium penetration into blood vessels, muscles and tissues as revealed by pathological studies are characterized by white or grey patches, apparent of mycelium on skin and fins and other superficial organs of the aquatic organisms (Phillips *et al.*, 2008; Bruno *et al.*, 2011) (Fig. 1). The pathogens either show prolific asexual reproduction (r-strategy) functioning for dispersal and diffusion within the host, owing to the incidence of coenocytic hyphae or less productive sexual (s-strategy). While parasitizing the host fish the saprolegnia pathogen undergoes asexual reproduction (r-strategy) as has only been defined in a few other Oomycetes groups and is responsible for causing saprolegniosis (Andersson and Cerenius, 2002; Robertson *et al.*, 2009; Earle and Hintz, 2014). The Oomycetes infection primarily is contingent on the aptitude of biflagellate zoospores produced at the terminal ends of hyphae which spread to host tissues, except for the species which are incapable to produce swimming cells (Larousse and Galiana, 2017).

In r-strategy, nutrient deficit leads to the induction of sporulation in asexual sporangial hyphal tips apically, subsequently, biflagellate, motile, and primary zoospores are released which in turn lead to primary infection of fish host (Torto-Alalibo *et al.*, 2005; van West, 2006; Robertson *et al.*, 2009). Further, the infection is followed by encystment on host by primary zoospores, and under favorable conditions the encysted primary zoospores germinate with consequent release of secondary zoospores which is the infective stage of *S. parasitica* and many other species of the pathogen group. Secondary zoospores again encyst on the host and develop into secondary cysts, with subsequent release of biflagellate

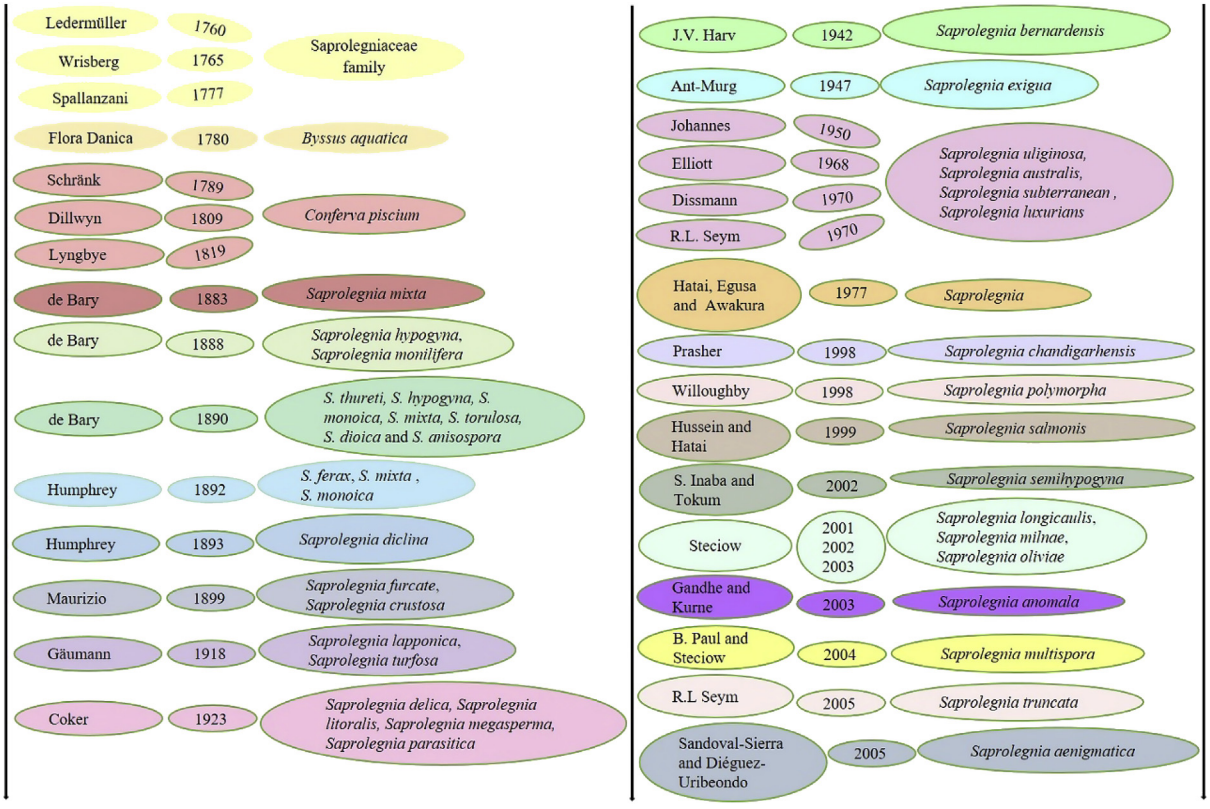

Fig. 3 – The timeline of the discovery of different Saprolegnia species in chronological order.

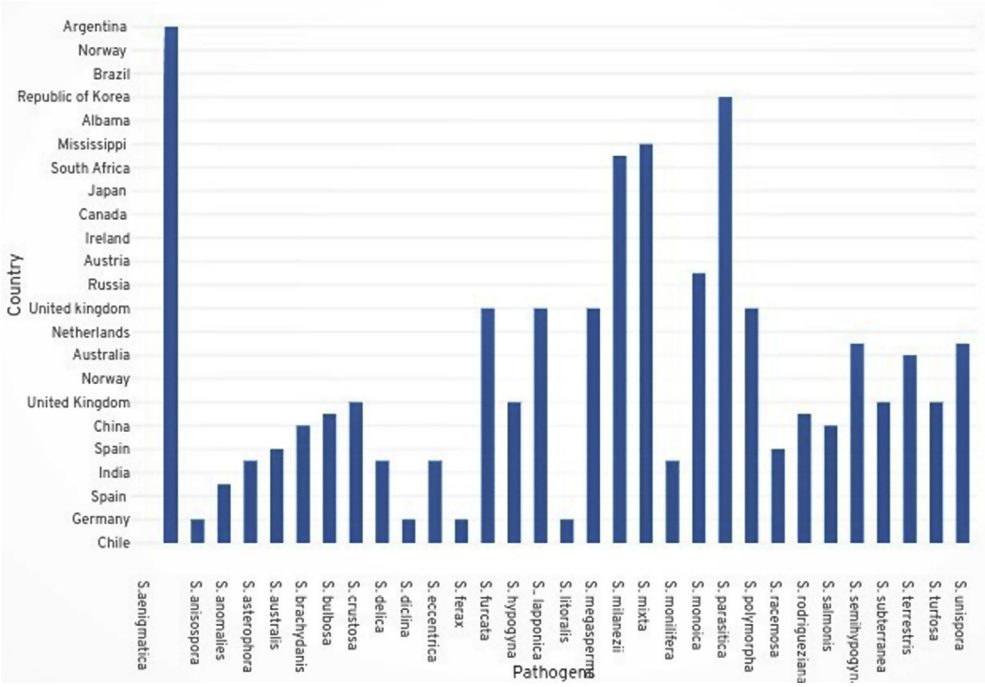

Fig. 4 – Global distribution of *Saprolgenia* spp. according to the Ensemble (http://www.ensembl.org/index.html?redirect=no) EOL (Encyclopedia of life, http://eol.org/), FungiDB (http://fungidb.org/fungidb/) and NCBI (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/) databases.

zoospores (Torto-Alalibo *et al.*, 2005; van West, 2006; Robertson *et al.*, 2009). However, in some members of the Saprolegnia group, the zoospores may be generated repeatedly for up to six generations as in case of *S. parasitica*, by a process known as repeated zoospore emergence (RZE), or polyplanetism (Fig. 5), which is an outcome of non-specific stimuli (physical or mechanical) (Torto-Alalibo *et al.*, 2005; van West 2006; Robertson *et al.*, 2009).

In case of non-infective s-strategists, two dissimilar gametangia; oogonium and antheridium, are developed. Several eggs possessed by large oogonium are fertilized by a smaller antheridium, they grow close together till the reproductive gametangia fuse, haploid nuclei fertilize the eggs, developing diploid oospores (Hernández-Hernández *et al.*, 2003). Whether fertilization occurs within a single strain or between two strains of contrary mating types, the particular pathogens are assigned to homothallic and heterothallic forms of species, respectively.

6. Approaches to study the pathogen

Culture dependent approach

Pathogen culture and microscopy

A number of approaches are practiced to obtain, recognize and classify the pathogen from the site of infection. While acquiring fungal pathogen from fish host lesion or infection site, besides the pathological signs of disease, topography of lesion or necrotic tissue, phenotypic, serological and morphological properties of the fungi are to be taken into consideration (Gozlan and Britton, 2013). The culture dependent approach forms a vital part in appreciative infectivity, etiology of the disease, epidemiology, taxonomic groups, and host-parasite relationships (Gozlan *et al.*, 2014). The conventional techniques and methods of isolating and ascertaining pathogens can though be a time consuming procedure demanding a high level of technical expertise (Gozlan *et al.*, 2014). The fungus can be acquired by swab-culture, direct culture, serial dilution, and streaking or dot culture approach (Declercq *et al.*, 2015). Few of the essential nutrient media used for culturing of Saprolegnia include, Sabouraud dextrose agar (SDA), Rose bengal agar (RBA), Malt extract agar (MEA), Czapek's yeast agar (CYA) and Potato dextrose agar (PDA) (Hussein *et al.*, 2001; Basu *et al.*, 2015). Fungal cultures are purified by sub-culturing and stocks stored at 4–10 °C for future studies (Digamadulla *et al.*, 2016).

Among the culture dependent identification approaches, microscopic examination as used for the identification of many other microbial pathogens has a tremendous scope for Oomycetes identification. The morphological and ultra cell structures of fungi can be visualized by light and electron microscopes, respectively (Songe *et al.*, 2015). The type of specimen to be examined leads to the selection of microscopy type, and the methods of visualizing structures and preparation of specimen differ in both the cases (Songe *et al.*, 2015). In *Saprolegnia* spp. minute reproductive structures are observed by light microscopy as it helps in maximizing the structure in both living and dead samples up to one thousand times. Morphological characteristics of the

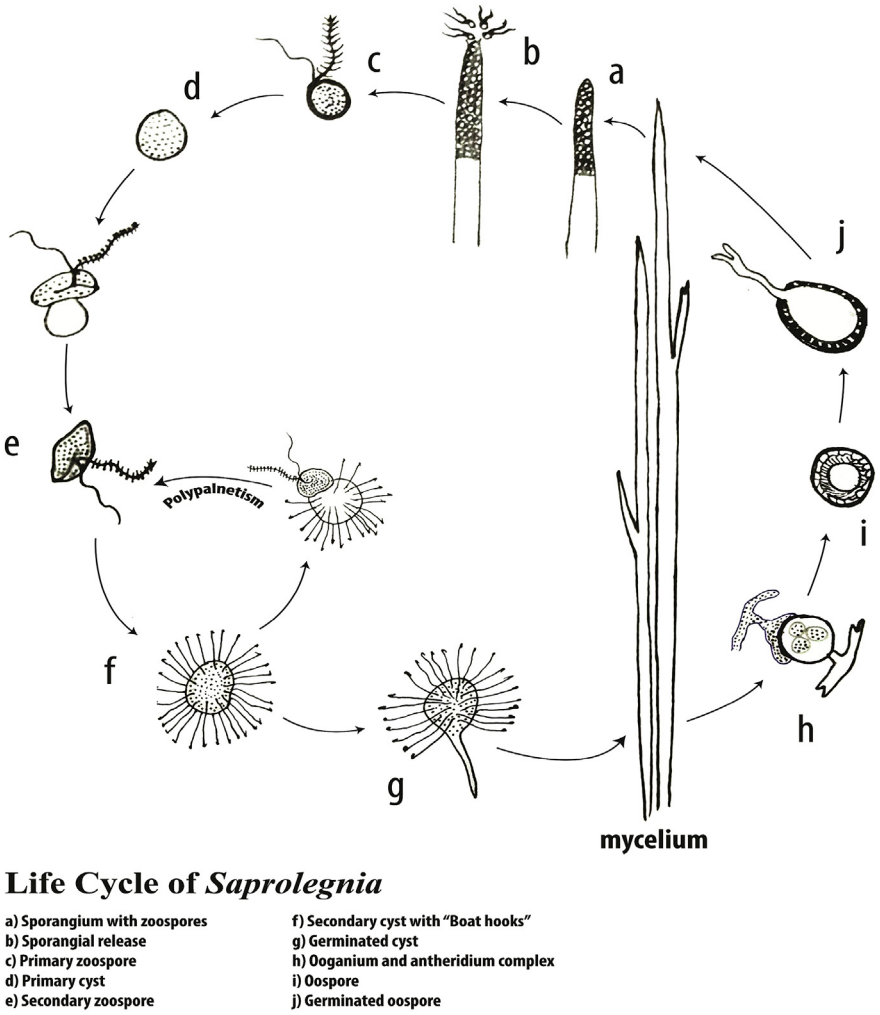

Fig. 5 – Schematic diagram of the life cycle of Saprolegnia. The figure was reproduced and modified with permission from van West (2006).

developing mycelia, ooganium and anthredium can be evaluated by light microscopy in laboratory procedure, starting from the second day upto 1–2-week period of incubation (Songe, *et al.*, 2015). Species level documentation of *Saprolegnia* spp., depends upon the basic morphology of the fungus which include, the main and secondary hyphal branches, septa presence or absence, the position, production and germination of spores, and the size, shape and arrangement of spores, hyphal tip, or a single germinated zoospore cyst, sporangium or oospore (Drenth and Sendall, 2001). However, it is important to observe the non-septate (coenocytes) hyphae which is representative of Oomycetes and saprolegnia in particular.

Histopathological examination of post-fixation tissues infected by *Saprolegnia* spp. is performed by light microscopy. Various stains used for enhancing contrast for the tissue visualization include Heamatoxylin and Eosin (Kiernan, 2008), special stains such as Periodic Acid Schiff (PAS) and Grocott's modification of Gomori's methanamine silver (GMS) stains (Kiernan, 2008) help in demonstrating the presence of hyphae in tissues. Lactophenol cotton blue stain is typical of fungal light microscopic examination, and aids in revealing the morphological structures. The stain helps in examination of sexual structures peculiar to a species, like shape and size of oogonia, antheridia, cysts and oospores, the antheridia position in relation to the oogonium and difference in oospores from oogonia. In order to visualize the finer details of Oomycetes hyphae, Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Scanning Transmission Electron Microscopy (STEM) are used, unlike the light microscopy which uses white light, the source of illumination in all these is a beam of electrons (Songe *et al.*, 2015). In addition to the surface topography, the electron microscopy helps in resolving the ultrastructure of the pathogen and the infected tissues. Other microscopic procedures used to visualize the pathogen and the host tissues on site, include the use of fluorescent *insitu* hybridization (FISH), in which a species-specific fluorescently labelled DNA probe can be exactly spotted and imaged directly without, damaging the host cell, disturbing the cell integrity and subculturing (Liehr, 2017).

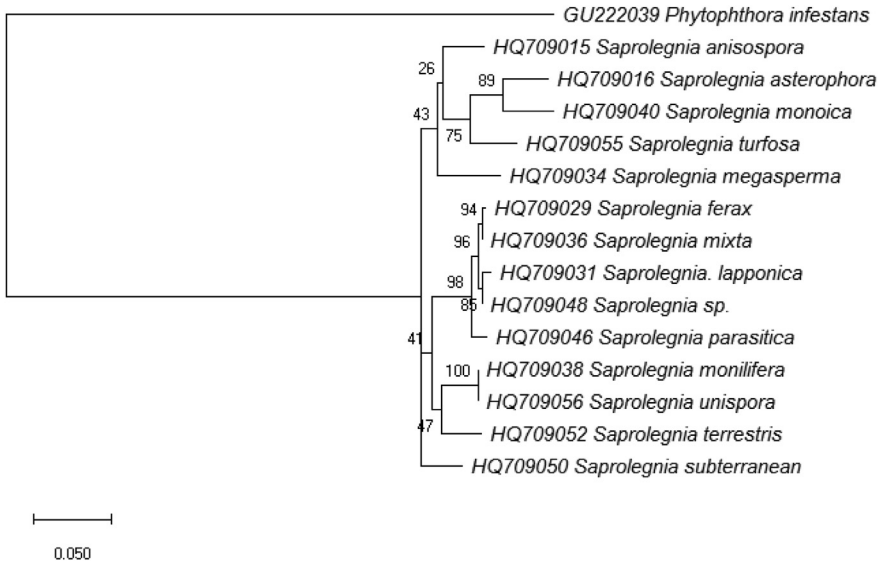

Fig. 6 – Molecular Phylogenetic analysis of Saprolegnia species by Maximum Likelihood method using COX subunit I DNA FASTA sequences. *Using the information COX subunit I FASTA sequences the evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (−3289.58) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 973 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

Molecular approach (Sequencing of rRNA, ITS and other housekeeping genes)

Until the last decade of 20th century, mycologists largely relied on culturing, morphology and physiology of a fungal community for the characterization purposes. However, rapid advancements in the sequencing technology from last two decades has facilitated the characterization of microbes based on molecular features such as the comparison of small subunit ribosomal RNA sequencing, making possible to delineate any unknown isolate to its appropriate genus and or species. However, the genus Saprolegnia owing to its deficient taxonomic information which largely consisted of morphological methods alone is assumed to be mistakenly named in the culture collections. Morphology based identification although still used in combination with other advanced molecular methods, is labor intensive requiring special skills and knowledge to cultivate and is based on microscopic examination to distinguish among fungal species (Robideau et al., 2011).

Of late, matrix-based lucid keys so far developed have significantly improved the speed of identification on morphological and cultural basis (Abad and Coffey, 2008; Ristaino, 2012). The use of small subunit rRNA sequencing has led to many-fold increase in the number of sequences available in public databases which in itself is a double edged sword, because, on one hand it facilitates the sequence comparison and design of robust primers needed for amplification but on the other hand too many sequences available sometimes render the conclusions drawn insignificant. Further, lack of efficient characterization of barcode genes and increasing sequence data accessible in online databases for the fungal pathogen in consideration have stirred a chaos in the phylogeny of the group. In order to decrease the commotion, much of the focus from small and large ribosomal RNA subunit sequencing has been shifted towards multiple barcode gene sequencing analysis e.g. TEF1α, RPβ1, RPβ2, tub2, MCM7, Calmodulin like genes in curated databases (Schoch et al., 2012; Stielow et al., 2015), along with whole genome and comparative genomics for species identification and phylogeny.

Further, mycological genome sequencing approaches have greatly improved several avenues of research on all aspects of Oomycetes biology along with accelerating molecular inheritances and molecular biology. The dawn of bioinformatics has further helped to gain understandings into the fungal genome organization, and thereby changing the focus to a more system-wide understanding of Oomycetes group especially Saprolegnia pathogens. According to consortium for the barcode of life, Internal Transcribed Spacer (ITS) is the actual barcode to differentiate members of Oomycota, however, cytochrome oxidase subunit 1 (COX1) is more reliable to distinguish other closely related species (Robideau et al., 2011). The evidence for the statement can be obtained from recent search of fungal barcodes database. Various fungal genomic sequences analyzed and screened so far as barcodes

Table 1 – Comparative genomics of Oomycetes group depicting the genome size, gene annotations along with the host range and pathogenic nature.

Pathogenic Oomycetes Species	Total Genome Size	Coding vs Non coding genes	Number of			(Host) pathogenic nature	References
			Pseudo genes	Gene transcripts	Putative orthologs		
Albugo candida	45.3 Mb	13,310/NA	235	13,545	NA	(Plants) obligate biotrophs	Links *et al.* (2011)
Albugo laibachii	37 Mb	13,804/171	654	14,629	NA	(Plants) biotrophs	Kemen *et al.* (2011); Links *et al.* (2011)
Aphanomyces astaci	94 Mb	19,119/247	218	26,724	NA	(Animals and plants) pathogens of aquatic animals, but includes pathogens of plants	Gaulin *et al.* (2018)
Hyaloperonospora arabidopsidis	99 Mb	14,321/257	28	14,606	4,922	(Plants) Obligate biotrophs	Baxter *et al.* (2010)
Phytophthora infestans	240 Mb	17,785/1,668	6,183	25,638	5,858	(Plants) hemibiotrophs, severe diseases of dicot plants	Haas *et al.* (2009); Henriquez and Daay, 2010
Phytophthora sojae	95 Mb	26,489/1,631	25	28,145	5,581	(Plants) hemibiotrophs, severe diseases of dicot plants	Tyler *et al.* (2006)
Phytophthora ramorum	65 Mb	15,605/198	152	15,955	5,592	(Plants) hemibiotrophs, severe diseases of dicot plants	Tyler *et al.* (2006)
Pythium ultimum	43 Mb	15,290/536	13	15,871	5,975	(Plants, animals, and microbes), necrotrophs, biotroph, Mostly plant pathogens	Levesque *et al.* (2010); Adhikari *et al.* (2013); Zerillo *et al.* (2013)
Saprolegnia parasitica	53.31 MB	20121/249	42	20,412	19049	(Animals) obligate biotrophs, infect aquatic animals, mainly fish	Jiang *et al.* (2013); Torto-Alalibo *et al.* (2005)
Saprolegnia declina	62.89 MB	17359/74	16	18,319	16893	(Animals) obligate biotrophs, infect aquatic animals, mainly fish	Jiang *et al.* (2013)

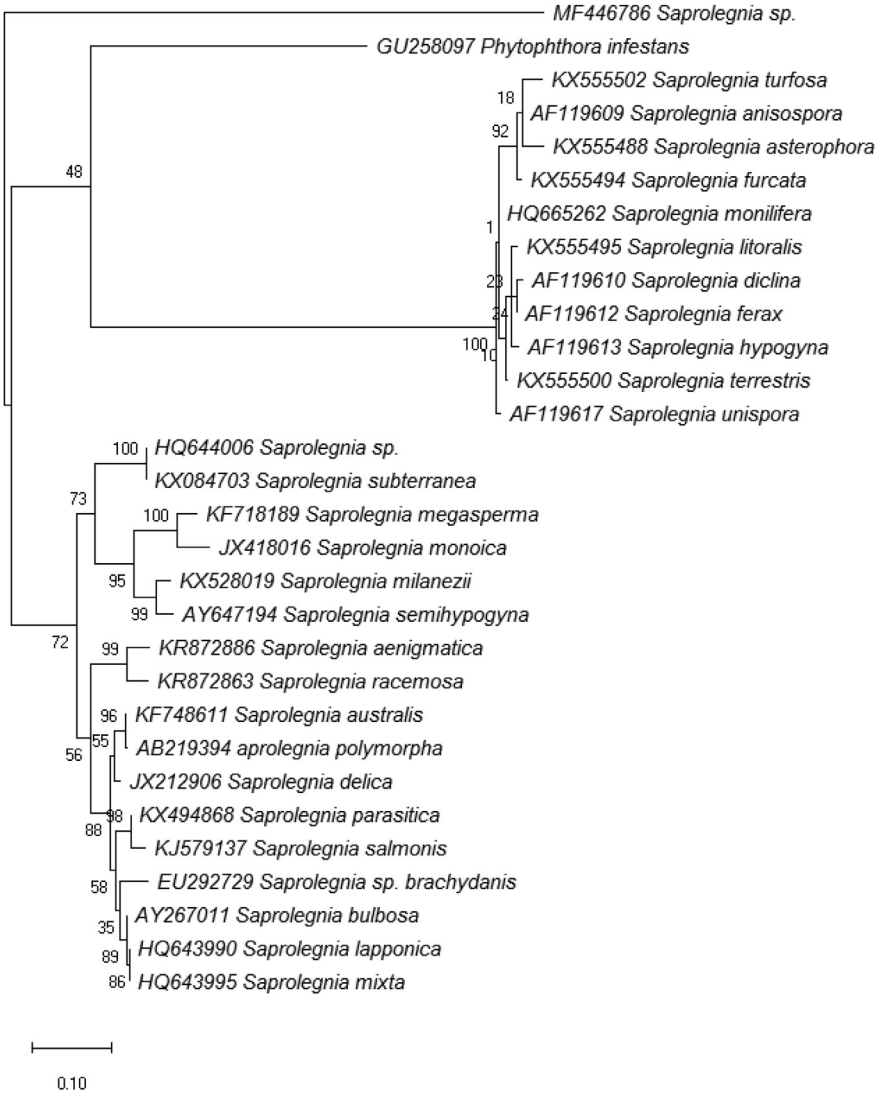

Fig. 7 – Molecular Phylogenetic analysis of Saprolegnia species by Maximum Likelihood method using ITS DNA FASTA sequences. * The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (−6749.22) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1293 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

include; three subunits from the nuclear rRNA cistron which were compared with regions of three representative protein-coding genes (RPB 1, RPB 2, MCM7). However, mitochondrial COX subunit 1 used as the animal barcode (Hebert *et al.*, 2003; Schindel and Miller, 2005) was omitted as a possible marker, because of amplification difficulty as it often contains insufficiently variable large introns (Schoch *et al.*, 2012). The regions, ITS1 and ITS2 compared to the 18S, exhibit more variability hence are more efficient for the identification of fungi having inter and intraspecific variations (Schoch *et al.*, 2012). The 28S rRNA subunit possess higher species resolution in few early diverging lineages of Oomycetes and the Ascomycetes yeasts, ITS based phylogeny is otherwise superior for the group (Schoch *et al.*, 2012). However, the nuclear 18S rRNA small ribosomal subunit gives poor species-level resolution in fungi as well as in Oomycetes. The potentiality of ITS as a universal barcode for Oomycota has been proved by a number of studies carried out on *Saprolegnia* spp. which include, the examination of 961 sequences of ITS by Sandoval-Sierra and Diéguez-Uribeondo, 2015. The study validated a total of 18 Saprolegnia species leading to the documentation of 11 new species as potential pathogens (Sandoval-Sierra and Diéguez-Uribeondo, 2015). Based on Molecular Operational Taxonomic Units (MOTUs)-a taxon

diagnosis system, 29 MOTUs of the Saprolegnia fungal group were found to be in accordance with the ITS sequence data of species, based on this information, the incorrectly named culture collection isolates were renamed.

The study further revealed that the diversity of the Saprolegnia group is many folds larger than indicated by direct culture methods. Besides this, high-throughput sequencing methodology for expressed transcripts and genomic DNA has boosted genetics in the previous decade. An unlimited upsurge in sequencing projects and sequencing databases, led to potential reduction in wet-lab workflow complexity, costs, and the expansion of read length (Voelkerding, *et al.*, 2009). The comprehensive molecular information for the identified species of Saprolegnia is given in Table S1 (Supplementary data) and has been procured from NCBI (https://www.ncbi.nlm.nih.gov/nuccore) and BOLDSYSTEMS (http://boldsystems.org/index.php/TaxbrowserTaxonpage?taxid=23736). The data related to whole Saprolegnia group enlisted in Table S1 has been used as reference to acquire FASTA sequences from GenBank, which were analyzed using MEGA 7 or X (Kumar *et al.*, 2018) and Clustal W programs. Furthermore, Comparison of ITS FASTA sequences and Cox subunit I FASTA sequences stood as a tool for devising phylogenetic tree with *Phytopthora infestans* as out-group (Figs 6 and 7).

Culture independent approach (Metagenomics)

Metagenomics, the study of microbes by culture independent approach is a unique and rapidly developing research area of molecular biology, transforming the field of microbiology, by facilitating the study of microbial communities by cultivation independent assessment (Simon and Daniel, 2011). The advancements in the area of metagenomics such as comparative metagenomics, metatranscriptomics, and metaproteomics have further revolutionized the microbiological research by providing insights into the comparative microbiome analysis, gene expression patterns and proteome analysis of different ecosystems without the necessity for culturing them (Sjöling et al., 2006; Chistoserdova, 2010). Further, the beginning of high-throughput sequencing techniques, bioinformatics tools, affordable investigation methods, web-bases and data set comparisons, facilitate the extensive analysis of microbes and environmental microbial assemblages.

Recently an amplification of universal barcodes dependent advanced metagenomic approach has been developed among whole genome by nested PCR with a new set of genus-specific primers for the study of Oomycetes group in natural ecosystems (Scibetta *et al.*, 2012). So far, the detection of Oomycetes pathogens is largely carried out by basic serological and molecular approaches; however, after the advent of next-generation sequencing (NGS), it is possible to target numerous signature sequences of pathogens in the whole infected metagenome (Espindola *et al.*, 2015). Electronic probe Diagnostic Nucleic acid Analysis (EDNA), a recently developed approach making use of electronic probes has the potential to simplify the detection of various plant and animal pathogens including those belonging to oomycetes in replicated metagenome (Espindola *et al.*, 2015).

The Metagenomic analysis of Oomycete communities in previous studies revealed great diversity, for example, field pea rhizosphere on the Canadian prairies revealed the presence of 105 Operational Taxonomic Units (OTUs), among which 45 OTUs were identified to species level and 16 Oomycetes up to genus levels (Taheri *et al.*, 2017). The analysis revealed Pythium to be the predominant genus with *Pythium heterothallicum* being the most predominant species. *Aphanomyces euteiches*, a pea root pathogen was present at maximum study sites but at very low numbers (Taheri *et al.*, 2017). Amplicon sequence dependent isolation and detection of cultivation-resistant Oomycetes, from roots of winter rye revealed the presence of dominant Oomycetes which include, *Pythium volutum*, *Pythium* sp. F86, an unidentified clade, and *Lagena radicicola*, (Bakker *et al.*, 2017). The usage of such technologies can facilitate the study of epidemiology and microbiota composition in both healthy and diseased states of fish (Larousse and Galiana, 2017).

Genomic comparison with related Oomycetes pathogen groups

The Oomycetes genome size is variable, which in case of *Achlya laibachii* is 37 Mb (Kemen *et al.*, 2011) and 260 Mb in *Phytopthora infestans*, a pathogen causing Potato late blight (Haas *et al.*, 2009). However, the genome size of *Saprolegnia* spp., the fresh water fish pathogens range from 53.31 MB for *S. parasitica* to 62.89 MB for *S. diclina*. The genome size of *S. monoica* was estimated at 51 Mb based on CHEF gel electrophoresis (Mort-Bontemps and Fevre, 1995). The genome sizes difference in certain taxa is mainly contributed by the presence of transposable elements. Repetitive DNA content is among the peculiarities of almost all eukaryote genomes and Oomycetes genomes are no exception to this feature. Like other eukaryotic genomes, the prevalence of protein encoding genes varies among Oomycetes species.

Initial analyses focusing merely on the abundance of protein-coding genes in the plant pathogens, *Phytopthora ramorum* and *Phytopthora sojae* identified large gene families that were not evident in their non-pathogenic relatives such as diatoms (Tyler *et al.*, 2006; Judelson, 2012). There is divergence in protein sequences of *S. parasitica* compared to *Phytophthora* sequences, a plant pathogenic Oomycete. Based on the sequence alignments of 18 conserved proteins, *S. parasitica* showed 77 % identity with three *Phytophthora* species while as the amino acid identity among the *Phytophthora* spp. was as high as 93 % (Torto-Alalibo *et al.*, 2005). The differences in the genome sizes for major genera of pathogenic Oomycetes group along with the Saprolegnia genera are provided in the Table 1.

As of June 2018, more than 40 pathogenic fungal genomes have been sequenced, which include both phytopathogenic and animal pathogenic, with more than 300 sequencing projects being in progress (Genomes Online database, http://www.genomesonline.org/). With the arrival of robust and premium sequencing technologies, there has been a dramatic increase in the number of whole genome sequencing projects. The arrival of new technologies such as Next Generation Sequencing (NGS) has reformed the field of molecular genomics in general and genome sequencing in particular. The use of NGS has been extended to fungal pathogens and the whole genomes of a few of the Oomycetes are characterized to some extent. Among Oomycetes, the first whole genome sequenced organisms were two hemi-biotrophic phytopathogen, *P. ramorum* and *P. sojae*, which became available in the year 2004,

shortly followed by that of *P. infestans* in 2006 (Tyler *et al.*, 2006). Accessibility to the whole genome sequence data has provided the necessary impetus to study the clinical implications and pathogenic determinants of various human, animal and plant pathogens. According to the information available at Fungi DB (http://fungidb.org/fungidb/), so far the genomes of only two Saprolegnia species have been annotated, curated and identified. The two species are *S. parasitica* strain CBS 223.65 (http://fungidb.org/fungi db/app/record/organism/NCBI-TAXON_695850), with whole genome size 53.31 MB and *S. diclina* Strain VS20, (http://fungidb.org/fungidb/app/record/organism/NCBI TAXON_1156394), having genome size of 62.89 MB. The total number of genes characterized for *S. parasitica* and *S. diclina* are 20,435 and 17,448, respectively, with both possessing several ortholog genes, 19,049 for *S. parasitica* and 16,893 for *S. diclina*. *S. parasitica* nuclear genome comprises of several Protein Coding Genes (PCG) (20,121), with protein encoding pseudogenes (25) along with tRNA genes (224) and tRNA pseudogenes (42), rRNA encoding with nano pseudogenes (23) in number, the total pseudogenes characterized are (67) (Tyler *et al.*, 2006). The parasitic pathogenic species consists of multiple nuclear chromosomes along with a single mitochondrial chromosome. However, *S. diclina* possess PCG of 17,359, with total pseudogenes being 16.

Saprolegnia transcriptomic analysis

Genomic analysis at ITS level, COX I and II for the documentation of *Saprolegnia* species is not enough to disclose the infective part of the pathogen. Hence, the transcriptomic and secretome exploration are of elementary requirement to recognize the pathogencity determinants of the group. So far, a number of pathogenic strains were used for the transcriptomic and secretome analysis among the *Saprolegnia* species. The contrivances of fundamental pathogencity in *S. parasitica* and other biotrophic pathogenic Oomycetes is not explicated yet, but a crucial need is felt there (Torto-Alalibo *et al.*, 2005). Furthermore, the transcriptome data available, including transcriptomics in the form of microarrays (Haas *et al.*, 2009; Judelson, 2012), RNA-Seq (Levesque *et al.*, 2010; Links *et al.*, 2011; Savory *et al.*, 2012; Jiang *et al.*, 2013), proteomics (Savidor *et al.*, 2008) and phospho-proteomics is of fundamental importance to reveal infective potential.

First insight into the transcriptomics of *S. parasitica* by Torto-Alalibo *et al.*(2005) revealed an informative and promising transcriptomics data to gain access to the pathogenic potential. A total of 1,510 expressed sequence tags (ESTs) were obtained from mycelial cDNA library of *S. parasitica* consisting of 5,25,944 bp, which were assembled into 1,279 consensus sequences (Torto-Alalibo *et al.*, 2005). The pathogen possesses wealthiest ranges of proteases (270) among eukaryotes, an important characteristic features for pathogenic adaptability. The transcriptomic level study revealed the occurrence of an adhesive extracellular matrix based on immunolocalization studies and is an essential evidence for pathogencity. The various gene families analyzed and sequenced in the transcriptome of *S. parasitica* which are crucial as an infective arsenal include more than 25 genes. Complementary DNA (cDNAs) obtained from *S. parasitica*, which encode secreted proteins that function as virulence factors, comprise of cellulose binding domain proteins, glycosyl hydrolases, elicit and elicit like proteins, polygalacturonases, proteases, PAN/Apple module proteins, CBEL, Ricin, kinisin, heamolysin E, as well as cysteine and serine protease inhibitors.

S. parasitica further possess the large kinosome consisting of 543 kinases, 10 % of which are expressed upon infection. The transcriptomic investigation revealed the presence of a large set of immune responsive genes in four different cell lines (RTG-2, RTGill, RTL and RTS11) of rainbow trout (de Bruijn *et al.*, 2012). All the four cell lines showed the induction of proinflammatory cytokine transcripts including that of IL-8, IL-11, IL-1β1 and TNF-a2, additionally, the innate defenses, the acute phase serum amyloid A, and C-type lectin CD209a and CD209b were also reported to express in the cell lines (de Bruijn *et al.*, 2012). Furthermore, upregulation of certain antimicrobial peptides (AMPs) like Hepcidins and cathelicidin 1 (rtCATH1) and 2 (rtCATH2) was reported in rainbow trout on exposure to Saprolegnia infection. Other genes sequenced and analyzed include, the genes encoding ABC transporters, kinases, extracellular peptidases or tissue material degrading hydrolases (Tyler *et al.*, 2006; Haas *et al.*, 2009; Schornack *et al.*, 2010). The two essential genes of group recently described include genes encoding RXLR and Crinkler proteins, the Oomycetes-specific effector proteins that translocate into host cell cytoplasm. So far, RXLR effectors have been mainly identified within Peronosporales and some of them, e.g. RXLR29 has been reported to be involved in the suppression of host defense in *Hyaloperonospora arabidopsidis* (Cabral *et al.*, 2011). In contrast to RXLR, Crinklers an effector pathogenic gene group are evolutionarily older in origin and experimentally tested however, seem to activate cell death (Schornack *et al.*, 2010). *S. parasitica* contains an expanded repertoire of peptidases, in particular peptidases of the C1 type, eukaryotic kinases, several of which have predicted membrane domains and therefore might be cell surface receptors involved in signaling (Jiang *et al.*, 2013).

Other methods for fungal characterization

Essential diagnosis, characterization of pathogen to species or strain level, modeling of treatment, surveillance of a disease and implementing a disease management strategy require the direct detection of pathogens, which is problematic to accomplish from ulcerated or necrotic tissue. For that matter, certain effective and latest techniques are utilized in the characterization of fungi, including the development of direct detection assays. However, the ability of the fungal pathogens to flourish in multiple species complexes makes the direct detection assays challenging. Moreover, onsite visualization of fungal pathogens in the infected tissues is possible with the progress in the discovery of species-specific fluorescent probes or by Fluorescence *insitu* hybridization (FISH) (Gozlan *et al.*, 2014). Biofilm formation an advanced and robust method for *insitu* pathogen characterization, is not limited to the bacterial world only, but rather expanded to fungal pathogens as well (Fanning and Mitchell, 2012; Borghi *et al.*, 2015). Biofilm development by fungal phyto and biotrophic pathogens recently evidenced and recognized, offered a possibility and

provided the scientific framework for scheming and adjusting methods and impressions established by biofilm exploration that might be applied in integrated management and conservation practices and strategies (Villa *et al.*, 2017). The concept offers a possibility that it can be extended to fungal Oomycetes in order to investigate the pathogenic strategies of the group on site.

7. Conclusions and future perspective

Fungal identification and systematics is still largely based on the morphological criteria, and Oomycetes pathogenic fungi are also largely identified based on their phenotypes. Numerous alternative methods have been developed, including the physiological and serological ones. Although some of these are very useful for identifying poorly differentiated fungi, they are only complementary tools of morphological identification in most cases. Molecular biology techniques, especially the analysis of rRNA, ITS, COX I, II sequences along with other (housekeeping genes) barcode sequences of genome, are currently used for reliable taxonomic studies, which enable a more natural classification system to be established. Although enormous efforts are being put in by mycologist to elucidate the molecular sequencing based phylogeny of *Saprolegnia* spp., the constant discovery of new mycotic agents in different fish hosts belonging to the genus Saprolegnia need rapid and accurate identification systems. There is an immense need for collection of taxonomic data (both phenotypic and molecular) from all species of the group and to make it available at first instance in order to help researchers working on them have a comparative understanding of the similarities and dissimilarities. This review fills up the current knowledge gaps related to fungal diseases caused by *Saprolegnia* spp. and adds knowledge to the existing scientific literature and provides a baseline data on the distribution and taxonomic status of *Saprolegnia* spp. in different ecosystems. Further, the sophisticated molecular, transcriptomic and proteomic tools and data available can be used to refine the phylogenetic status, pathogenecity-related genes and the control of disease progression of *Saprolegnia* spp. as the potential of these technologies have not yet been fully extended to the aquaculture research. Therefore, the modern techniques related to genome editing and gene silencing or mutageneses systems such as RNAi or CRISPR/Cas are urgently required to functionally characterize the pathogencity related genes of Saprolegnia group and subsequently devise the strategies for the control of saprolegniosis. We anticipate that detailed molecular studies of the host–pathogen interaction will ultimately provide new targets for the development of novel therapeutics.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbr.2018.12.001.

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