Saprolegniosis in goldfish (*Carassius auratus*) associated with novel strain; molecular characterization and electron scanning

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Running title:- Saprolegniosis in goldfish (*Carassius auratus*)

ABSTRACT

A novel *Saprolegnia parasitica* strain was isolated from moribund and freshly dead goldfish, *Carassius auratus*, fingerlings during mid February 2014. Fish showed extensive hyphal growth on the skin, fins, gills and eyes as if it is warped with cotton. The isolate was identified using the traditional methods through its asexual and sexual organs, followed by 18S rRNA gene sequencing; showing identity 91.6% with *S. parasitica*. Scanning electron microscopy was applied for evaluating the pathogenicity of the retrieved isolate; which proved its high virulence accompanied with the high mortality rate reaching 100%.

Keywords: *Saprolegnia parasitica* new strain, *Carassius auratus*, molecular characterization, Electron microscope.

INTRODUCTION

Ornamental fishes share in the worldwide trade by a value of about US$ 900 million, playing a role in some countries income and occupation (Meshgi, Eslami & Yazdani 2006). Family Cyprinidae is one of the most commercially important and commonly available ornamental fish (Sharma *et al*. 2011). This industry is threatened by losses due to infectious diseases, including fungal diseases. Saprolegnia has been considered as one of the most destructive oomycete pathogen in fish hatcheries and larval stages specially; *S. parasitica*, which account for major losses in aquaculture (Willoghby 1970; Hussein, Hatai. & Nomura 2001; Bruno & Wood, 1999; West, 2006; Van den berg *et al*. 2013). Saprolegniosis is always associated with prolonged low water temperature at early spring, late autumn and winter. Although, saprolegnia-infected fish are easily recognized by the cotton-like, white to grayish patches on the skin and gills (Stueland, Hatai & Skaar 2005), but it is still difficult to reach the full data about the causative strain unless following

both traditional and recent diagnostic tools. The lack of a robust taxonomy in the genus Saprolegnia (Oomycetes) is leading to the presence of incorrectly named isolates in culture collections and of an increasing number of mis-assigned named sequences in DNA databases (Sandoval- Saierra, Martín & Diéguez-Uribeondo 2014). In addition, accurate species delimitation is critical for most biological disciplines. A recently proposed approach to solve species delimitation (taxon diagnosis system) of difficult organisms is the definition based on molecular analyses of rRNA-encoding genes. Analysis of 18S rRNA gene and internal transcribed spacer (ITS) can be used for species identification; furthermore it provides information about new species and their genetic diversity (Paul & Steciow 2004; Steciow, Paul & Bala 2007; Sandoval-Saierra *et al*. 2014). On the same hand, the ultra-structure of the early invasive stages can draw the scenario of the fungus pathogenicity through its phenotypic features including; long boat hooks on the spores and their germination rate/pattern (Thoen, Evensen & Skaar 2011). The aim of this study was to identify the causative agent associated with goldfish fingerlings mortality using both traditional and molecular methods of diagnosis. Scanning microscopic analysis was performed for tracing the pathogenesis during the early infection stages.

MATERIALS AND METHODS

Fingerlings sampling

Moribund and freshly dead Goldfish *Carassius auratus (C. auratus)* fingerlings were obtained from a private ornamental fish farm located at south Cairo, Egypt with history of high mortality during mid February 2014. Freshly dead fingerlings were transported as soon as possible to the wetlab of fish diseases and management, Faculty of Veterinary Medicine, Benha University, Egypt. Moribund fish were kept in glass aquaria at water temperature 16±1˚C for subsequent examination.

Clinical examination

Fish samples were examined for clinical signs and postmortem according to (Schaeperclaus 1992).

Isolation

Isolation of *Saprolegnia* spp from infected fishes was performed according to the method of Stueland *et al*, 2005, with modifications. Parts of the outer surface of the examined fish, covered with cotton wool-like fungal mats, were washed up with distilled water to get rid of solid particles trapped within fungal mats from the surrounding water and cleaned with 70% ethyl alcohol. Samples from the skin lesions were inoculated onto sterile plates of Sabouraud dextrose agar (SDA) (Difco Lab., USA) with chloramphenicol. Culture plates were incubated at 20˚C for 3–5 days with regular daily inspection for any expected fungal growths. Fungal growth was observed and identified according to Willoughby (1985); Hatai, Willoughby & Beakes (1990). In order to stimulate the production of sexual organs, a part of the growing fungus was aseptically transferred into tube containing sterile pierced hemp seeds in sterile tap water, incubated at 20˚C and was observed periodically for up to 2 months Johnson (1956).

Molecular characterization

Genomic DNA was extracted from 200 ul of the isolate cells, using high pure PCR template preparation kit, catalog no. 11796828001, Roche, with modifications. The fungus cells were centrifuged at 3000 *X g* for 5 min. and the supernatant was discarded. The cells were re-suspended in 200 µL PBS, 137 mM NaCl; 2.7 mM KCl; 4.3 mM $Na_2HPO4.7H2O$; 1.4 mM KH_2PO_4 and treated with 10 µL lyticase, 0.5 mg/ml at 37^{o}C for 30 minutes. The cell solution was incubated with 200 µL binding buffer and 40 µL proteinase K for 10 min at 72 ^{o}C. The DNA was purified from crude lysate using sephadex column according to kit instruction manual. The size of the extracted DNA was checked by electrophoresis on a 0.9% agarose gel against a Lambda-HindIII digest marker (New England BioLabs, Hitchin, Hertfordshire, UK) with ethidium bromide staining.

PCR reaction mixture, 50 µL, contained 10X EX taq buffer II (Mg2+ plus), 0.2 lM primer, 400 lM dNTP each, 2.25 U Takara EX-Taq Polymerase (Takara, Japan) and 5–30 ng DNA template. PCR amplification of the 18S rRNA gene, from the purified genomic DNA, was carried out using the primers 5$^{/}$-AAC CTG GTT GAT CCT GCC AGT-3$^{/}$ and 5$^{/}$-TGA TCC TTC TGC AGG TTC ACC TAC-3$^{/.}$ (Borchiellini *et al.* 2001). PCR product was confirmed by running on 1.2 % agarose gel and stained with ethidium bromide. PCR amplicon was eluted from the gel, using QIAquick Gel Extraction Kit, catalog no. 28704 Qiagen. The 18S rRNA gene fragment was analyzed by sequence, using capillary 3500 series genetic analyzer, Applied Biosystems.

The 18S rRNA gene sequence was analyzed by FASTA screening to determine its similarity to known fungus species in the DNA database.

Construction of the phylogenetic tree was done through two bioinformatic processes. In the first processes, the nucleotide sequence of the recovered 18S rRNA gene phylotype and its homologue sequences, from the DNA database, beside out-group sequences, were aligned using the online program ‘‘Clustal Omega’’, http://www.ebi.ac.uk/ Tools/msa/clustalo/. In the second processes, the aligned sequences, including the sequence gaps, were submitted to the MEGA software, V. 6.06, http://www.megasoftware.net/, for drawing the phylogenetic tree. Bootstrap method, provided as a phylogeny test, in the MEGA software, was performed using a number of 500 Bootstrap replications. Phylogenetic tree was constructed by applying the maximum parsimony algorithm. The branching patterns of the constructed tree were in agreement with other compared algorithm, maximum likelihood, in the same MEGA software.

Phylogenetic analyses

Isolate sequence was analyzed by FASTA screening to determine its similarity to known *Saprolegnia* sequences in the DNA database (http://ddbj.nig.ac.jp). The recovered sequences were aligned using CLUSTAL W software (DDBJ, http://clustalw. ddbj.nig.ac.jp). Construction of the phylogenetic tree was done through two bioinformatic processes. In the first processes, the nucleotide sequence of the recovered *Saprolegnia* 16S rRNA gene phylotype and homologue sequences, from the DNA database, beside out-group sequences, were aligned using the online program ‘‘Clustal Omega’’,

http://www.ebi.ac.uk/Tools/msa/clustalo/. In the second processes, the aligned sequences, including the sequence gaps, were submitted to the Molecular Evolutionary Genetic Analyses, MEGA, software version 6.0.6, for drawing the phylogenetic trees. Bootstrap method, provided as a phylogeny test, in the MEGAsoftware, was performed, using a number of 500 Bootstrap replications. Phylogenetic trees were constructed by applying the maximum parsimony and maximum likelihood algorithms, in order to compare both of phylograms.

Scanning Electron Microscopy (SEM)

Preparation of the attached secondary spores

Zoospores production and preparation were performed according to Willoughby, Mcgrory & Pickering (1983). Briefly, hemp seeds, colonized by the fungus, were incubated in sterile tap water at 20°C for 2 days. The water surrounding the seeds contained numerous motile secondary zoospores. Zoospores concentrations were adjusted to be $1x10^4$/L.

Zoospore attachment was performed following El-Feki & Refaat, (2014) with modifications. Two healthy goldfish of an average weight 20.0 ± 0.4 g and average length 8 ± 0.2 cm were collected from private aquarium shop. Pieces of skin were aseptically collected and immersed in spore suspension for 180 min., to allow zoospores attachment. Skin pieces with germinating zoospores were kept for SEM in 5 mL of 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4, and stored at 4 °C for 2 days. Fixed skin samples were rinsed in 0.1M phosphate buffer, re-fixed in 2% osmium tetra-oxide for 12 hours, washed 4-5 times in distilled water for 15 min., and dehydrated in ethanol series using automatic tissue processor (Leica Microsystems, Wetzlar, Germany). The specimens were immersed in t-Butyl alcohol (2-methyl-2-propanol) three times for 10-20 min. at 4˚C. The dehydrated samples were critical point dried using CO_2 critical point dryer (Tousimis Autosamdri-815). Dried specimens were mounted on aluminum stubs and coated with gold/palladium, using sputter coater (SPI- Module). The specimens were examined at 17KV using a scanning electron microscope, JOEL-JSM-5500 LV, Japan.

RESULTS

Clinical and microscopic examination

Gross examination of the freshely dead fingerlings showed extensive hyphal growth on the skin, fins, gills and eyes (Fig. 1, a) , the sabroud dextrose agar (SDA) plate was covered with white dense mycelia growth (data not shown).

The asexual reproduction, of the current fungus isolate, was characterized by presence of branched non septated hyphae, together with masses of mature and immature sporangia (Fig. 1, b). Two months post inoculation on hemp seeds containing tap water, the sexual organs were observed forming terminal oogonia with centric oospores (Fig.1, c). Periodical examination of the culture along three months showed the presence of abundant gammae (Fig. 1, d).

Identification of pathogenic fungal isolate based on 18S rRNA and ITS genes sequences

The PCR product was 1.8 k bp for 18S rRNA gene. The sequence of recovered 18S rRNA gene was

deposited in DNA data base bank under accession number AB985402. The FASTA homology showed that the 18S rRNA gene of the current isolate had 91.6% nucleotide identity with that of *S. parasitica*, strain recorded in Taiwan (acc. no. HQ384412). This result was confirmed by the phylogenetic position of the current isolate, forming monophyletic clade with *S. parasitica*, but with an obvious phylegentic distance (Fig. 2).

Scanning electron microscope (SEM)

The fine structure of the retrieved fungus and its pathogenicity was confirmed through SEM. More than one germinating secondary spore with appresorium like structure appeared as club shape attached to the end of long germinating tube at the site of skin invasion were observed (Fig. 3, A, B). The newly germinating spore showed the presence of hair like structures and presence of globular adhesive materials (pad) (Fig. 3, C) these adhesive materials were progressive and cone shape along the germination tube (Fig. 3, D).

DISCUSSION

S. parasitica is global freshwater pathogenic fungus (van West 2006). As an opportunistic microorganism, the host immunity is crucial in inducing infection; hence suppressed immunity enables its invasion and induction of disease condition. Depending on the history of the disease in this study; an attempt for water exchange to the aquaria, where fish was subjected to sudden decrease in the temperature and handling stress. Eissa et al (2014) observed drastic increase in the plasma cortisol levels post handling and low temperature stress. It is well known that cortisol is the key hormone in fish stress response suppressing the immune system, rendering the host vulnerable to infection (Cortes et al., 2013).

Secondary zoospores have been considered as the infective stage of *S. parasitica*, encysting on the host fish and forming secondary cysts that release the next generation of laterally biflagellate (Robertson et al. 2009). In winter season, the temperature drop below 10 ºC predispose saprolegnia attack through triggering zoospore release (Bly et al. 1992). The infected fish showed cotton like growth on the skin, fins, gills and eyes with mortality reaching 100%. The nearly same results were observed in tilapia, angel fish and carp infected with saprolegnia species (Mortada et al., 2013, Eissa et al., 2013 and Iqbal, Asghar &Rubaba 2012).

For diagnosis of saprolegnia, the steps included; examination of both of asexual and sexual mode of reproduction. The asexual reproduction was characterized by the presence of branched non septated hyphae with sporangia containing zoospores. The obtained results were matched with those described by Daugherty et al., (1998) and Seymour (1970). On the other hand, the sexual organs appeared 2 months post inoculation on hemp seeds containing tap water. Coker (1923) was able to identify saprolegnia species based on the sexually produced oospores, which differ in number, size and shape from species to species. In the present study, the oogonia was embedded with centric oospores matching the findings of Johnson Jr. et al. (2002) and Seymour (1970), implicating the causative agent belonging to Saprolegniales. Gemmae formation is characteristic structure for *S. parasitica* identification which was observed in three months culture. Vega-

Ramiraz *et al*. (2013) noticed that the isolated *S. parasitica* didn't produce sexual organs and instead abundant gemmae was formed; which was persistent for long time.

The molecular evolutionary patterns of Saprolegnia are still not well understood, due in part to the lack of molecular markers suited to resolve the deep phylogeny of this genus (Enrique & Lassaad 2011). The phylogenetic relationship between the current isolate and those of the *S. parasitica* was investigated based on 18S rRNA gene sequencing. The length of amplified 18S rRNA gene in this study was longer than those of recorded in previous studies of (Enrique & Lassaad 2011), increasing the accuracy for identification of the current isolate. The phylogenetic analyses, based on maximum parsimony and maximum likelihood, implicated a new recorded species. The molecular studies have supported the validity of 18 species of *Saprolegnia* and identified 11 potential new species (de la Bastide et al. 2015).

Although most of saprolegnia species are opportunistic pathogens but some strains are pathogenic and cause primary infection to both fish and eggs (Willoughby & Pickering 1977). The mode of zoospore attachment is one of the virulence factors and pathogenicity indicator. Where the mechanism of fungal infection involves; adhesion of the zoospores and germination (Dieguez-Uribeondo et al. 2007). The phenotypic characters of the secondary zoospores of *Saprolegnia* can be used for recognizing its virulence; like hairs which is required for attachment to the host (Beakes 1982; Willoughby 1994). The electron microscope revealed the presence long haird attached spores. Inadditon to the adhesive materials which render the secondary cystospore resistant to detachment (Durso et al. 1993). Approsorium is another important structure was observed while cystospore germination. Money et al. (2004) had described the appressorium a swollen structure formed at the tip of a hypha or a germ tube at the point of contact with the host cell, that facilitates the penetration of the hyphae into the hard cuticle mechanically or through enzymatic activity. Finally, the presented diagnosis approach of *Saprolegnia* species might help setting the basis for a suitable identification of species in this economically important genus and will help to better understand the emergence of pathogenicity of current isolate in the different oomycete groups.

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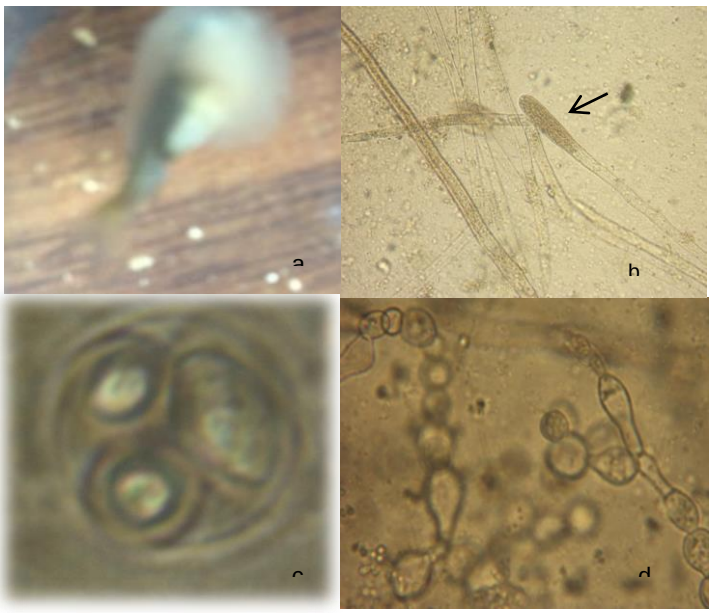

Figure 1(a) goldfish fingerling warped with cotton wool like hyphal mats, (b) Asexual reproduction showing; branched nonseptated hyphae & sporangia filled with large number of spherical sporangiospores (arrow) (c) sexual reproduction showing; oogonia with centric oospores, (d) germination of oospore with formation of Gemmae.

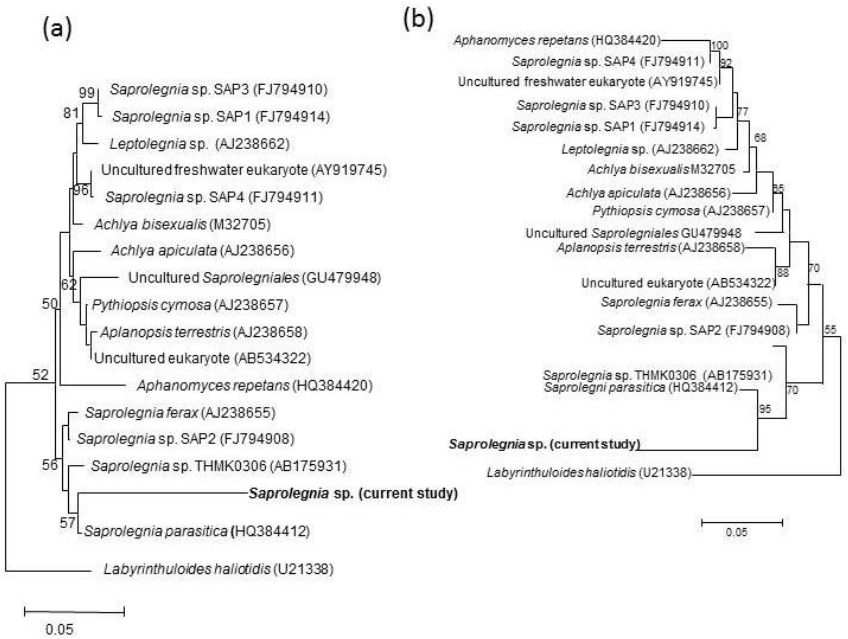

Figure 2 Phylogentic tree of 18 SrRNA sequence of the retrived saprolegnia isolate (accession number in gene bank HQ384412)

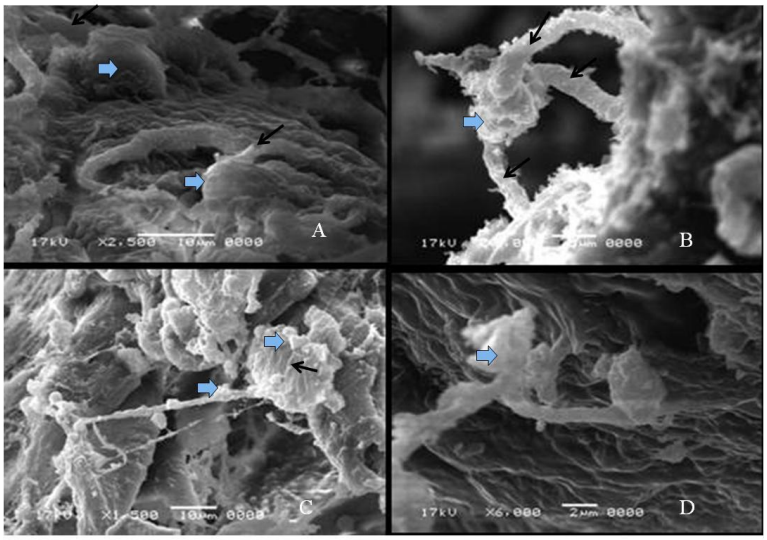

Figure 3 Scanning electron microscope; A,B) a newly germinating secondary zoospores of S. parasitica with germinating tube (black arrows) and appersoria (blue arrows) C) secondary zoospores of S. parasitica with globular adhesive materials around the zoospore (blue arrows) with hair like tuft on the secondary cyst (black arrow) D) deep inclusion of the emerging tubes into the skin, note the accumulation of the adhesive materials around the germinating tube (arrow).

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عترة جديدة لمرض السابروليجنيوسيس فى اسماك الجولد. دراسات جزيئية ومسح الكترونى

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فى هذه الدراسة تم عزل عترة جديدة من فطر السابروليجنيا باراسيتكا والمتسببة فى نفوق اسماك الجولد فى منتصف شهر فبراير مع وجود اعراض من نموات قطنية على الجلد والزعانف والخياشيم والعين كما لو كانت الاسماك ملفوفة بالقطن وقد تم تصنيف هذه العترة من خلال التكاثر الجنسى والاجنسى لها وايضا من خلال التتابع الجينى لـ18S rRNA والذى اظهر تشابه بنسبه ٩١,٦% مع السابروليجنيا باراسيتكا. بالاضافة الى ذلك فقد تم عمل مسح الكترونى لتقييم ضراوة العترة المعزولة والتى اثبتت شدة ضراوته حيث وصلت نسبة النفوق الى ١٠٠%.