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Full Length Article

Detection of *Saprolegnia parasitica* in eggs of angelfish *Pterophyllum scalare* (Cuvier–Valenciennes) with a history of decreased hatchability

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Saprolegnia parasitica;
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Eggs;
Povidone iodine

Abstract Mass mortalities of angelfish eggs accompanied with very low hatchability were reported in a private ornamental fish farm in Egypt. Examined eggs were badly damaged by water mould that was decisively confirmed as *Saprolegnia* species. Presumptive identification of the ten retrieved isolates was initially suggestive of *Saprolegnia* species. Mycological investigations have revealed that only 7 out of 10 isolates were capable of producing sexual stages. Therefore, using molecular tools such as PCR coupled with partial sequencing of inter-transcribed spacer (ITS) gene was one of the most important approaches to distinguish *Saprolegnia parasitica* from other water moulds. The sequences of ITS gene data derived from eight isolates showed 100% similarity with *S. parasitica* ATCC90312 sequence and the remaining two isolates were different in one nucleotide (99.9%). The phylogenetic analysis of ITS genes grouped the ten isolates with other *S. parasitica* in one clad. Further, to control such fungal infection, the efficacy of povidone iodine as surface disinfectant for angelfish and their fertilized eggs were tested. By trial, it was obvious that the obtained post-rinsing results were highly suggestive for the efficacy of povidone iodine as an efficient antifungal disinfectant for both fish and eggs.

1. Introduction

Fungal infections are one of the main factors responsible for mortality and economic losses among the ornamental and food fish farming industries [1–4]. The most commonly identified fungal pathogens of fish are *water molds* (Class *Oomycetes*) of the *Saprolegnia* genus [5,6]. Many literatures have reported Saprolegniosis to be the cause of mass kills among Nile tilapia *Orechromis niloticus* (Linnaeus) eggs, grey mullet *Mugil*

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cephalus (Linnaeus) [7–9], and in many non native fish species such as roach *Rutilus rutilus* (Linnaeus), barramundi *Lates calcarifer* (Bloch), kissing gourami *Helostoma temminckii* (Cuvier–Valenciennes), guppy *Poecilia reticulate* (Peters), swordfish *Xiphias gladius* (Bloch), platyfish *Xiphophorus maculatus* (Günther) [10], sockeye salmon *Oncorhynchus nerka* (Walbaum) [11], eels *Anguilla anguilla* (Linnaeus), channel catfish *Ictalurus punctatus* (Rafinesque) [12] and sturgeon *Acipencer persicus* (Borodin) eggs [2,13]. Mass kills due to Saprolegniosis are particularly catastrophic at lower water temperatures. Thereby, most of *Saprolegnia* associated mortalities are confined to late autumn, winter and early spring seasons [14,15].

These water moulds affect fish eggs by direct adhesion mechanism followed by dynamic penetration into the egg resulting in mass mortality during hatching period due to the direct oxygen withdrawal from the egg surroundings [16–19]. Infections often occur soon after eggs are exposed to water [2,20]. Once established, these fungi can rapidly spread to healthy eggs [6,21]. *Saprolegnia* spp. were traditionally identified based on the morphology of their reproductive structure [22,23]. Recently, molecular tools such as PCR coupled with partial sequencing of inter transcribed spacer (ITS) gene are the most current approaches to distinguish *S. parasitica* from other *Saprolegnia* spp. [24].

Routine application of disinfectants is a commonly used procedure during egg incubation at fish hatcheries worldwide [25,26]. However, most of them have been considered obsolete. Formerly, formalin, formalin – malachite green, malachite green – oxalate solutions were the most potent fish fungicide to be used in a fish hatchery [26–28]. A bunch of literatures has confirmed that malachite green is a potential carcinogen, teratogen and mutagen; hence, it has been banned for usage in aquaculture by FDA [19,29,30]. This ban has necessitated the search for acceptable safe/efficient alternatives to be used instead [22]. Eissa et al. [31] declared that povidone iodine is an efficient/safe disinfectant against bacterial, viral and fungal pathogens affecting both eggs and spawner salmon. In a similar comparative study, Eric et al. [32] concluded that povidone iodine is a potential egg disinfectant in rainbow trout hatcheries.

In Egypt, Saprolegniosis is considered one of the most important causes of mortalities among angelfish *Pterophyllum scalare* (Cuvier–Valenciennes) [33]. However, literatures about *Saprolgenia* infection in spawner angelfish were nil. Thereby, the current study aimed to identify aquatic fungi affecting angelfish eggs through using DNA based phylogenetic technique and some fungal morphological parameters. An ultimate aim was to test the efficacy of povidone iodine to control *Saprolegnia* infections in angelfish eggs under normal hatchery conditions in Egypt.

2. Materials and methods

2.1. Eggs and spawner fish' sampling

On the mid December 2011, we have received an official report indicating the emergence of mass mortalities among spawner angelfish and their eggs with consequent low hatchability at a private ornamental fish farm located at south of Giza province, Egypt. To investigate the problem, 400 eggs and 10 spawner angelfish were obtained from aforementioned ornamental fish farm. During sampling time, average water temperature was 16 ± 0.5 °C.

Eggs were washed several times with sterile distilled water, and then placed in sterile 7.5 × 18.5 cm Whirl-Pak bags (Nasco, Fort Atkinson, WI, USA) (10 eggs per bag). Washed eggs were flooded with double distilled water containing chloramphenicol/gentamycin at a concentration of 100 mg mL^{-1} for 12 h at 18 °C to prevent bacterial contamination. Fish with cotton wool like fungal mats were washed up with double distilled water to get rid of superficial bacterial contaminants and kept on crushed ice during entire process of examination.

2.2. Isolation and purification of Saprolegnia species

Washed eggs (10 eggs/sample) were placed in 7.5 × 18.5 cm Whirl-Pak bags (Nasco, Fort Atkinson, WI, USA) then diluted with Hank's Balanced Salt Solution (HBSS, Sigma Chemical Co., St. Louis, MO, USA) in a ratio of 1 whole egg mixture:2 HBSS (v/v). Eggs–HBSS mixture was stomached for 2 min at high-speed stomacher till mixture got completely homogenized. Aliquots from the homogenate were inoculated into sterile plates of Sabouraud dextrose agar with chloramphenicol and sterile hemp seeds (*Cannabis sativa*) to investigate sexual structure of isolates (SDA, Difco Lab., USA).

Fish with cotton wool like fungal mats were washed up with double distilled water to get rid of superficial bacterial contaminants then loopfuls from the deep cotton wool like mats as well as deep skin lesions were spread onto sterile plates of Sabouraud dextrose agar with chloramphenicol and sterile hemp seeds (SDA, Difco Lab., USA).

Culture plates were incubated at 20°C for 3–5 days with regular daily inspection for any expected fungal growths. Harvested fungal colonies were purified then slide culture technique was adopted on retrieved colonies for initial morphological identification. Fungal spores were fixed with one drop of methyl alcohol and stained with lactophenol cotton blue as described by Willoughby [16]. *S. parasitica* ATCC90213 was used as a reference strain for cultural morphology confirmation as well as positive control during molecular study.

2.3. Morphological studies

Infested hemp seeds for each isolate were placed into six-well culture plate confining sterile freshwater and incubated at 20°C for 21 days. Identification of sexual structure and pattern of germination were made under inverted microscope. Finally, the isolated strains were identified according to the criteria of Coker [34], Seymour [35], Willoughby [36] and Pickering et al. [37].

2.4. DNA extraction

The protocol of DNA extraction was adopted from Moller et al. [38]. Briefly, 10 mg (net weight) of freshly harvested fungal mats were transferred to a 1.5 mL centrifuge tube containing 20 μL Tris–EDTA (TE) buffer (10 mM Tris–HCl, pH 8.0; 10 mM EDTA, pH 8.0) and frozen at −70 °C for 30 min, then grinded into a slurry using Kontes micro-homogenizer with sterilized tips (Fisher Scientific, Hanover Park, IL, USA), then incubated for 60 min. This process was repeated once. The

mycelial slurry was diluted with 400 μL sterile double distilled water. Consecutively, 100 μL of the dilute was suspended in 0.5 mL CTAB digestion buffer [100 mM Tris–HCl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl; 3% CTAB; 2% β-mercaptoethanol; 4% (w/v) PVP (polyvinylpyrrolidone)] and incubated at 65 °C for 2 h. To increase the nucleic acid yield, the DNA was extracted successively with phenol–chloroform–isoamyl alcohol.

2.5. Detection of ITS gene by PCR

The 750 bp of the internal transcribed spacer (ITS) gene was amplified by PCR using two ITS gene primers: 5′-TCCGTAGGTGAACCTGCGG-3′ (ITS1) and 5′-TCCTCCGCTTATTGATATGC-3′ (ITS4). Amplification was done after 35 cycles of denaturation at 94 °C for 0.5 min, annealing at 58 °C for 0.5 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min using a PCR gradient thermal cycler (TC-3000G, Bibby Scientific Ltd., Staffordshire, United Kingdom) [39]. The PCR product was subject to electrophoresis on 1% agarose gel and specific bands were detected under the ultraviolet (UV) trans-illuminator.

2.6. Cloning and sequencing of ITS gene

The purified amplified fragments were cloned into pGEM-T Easy vector® plasmid by T4 ligase (Promega, Madison, WI, USA), and then the cloned plasmid transformed into *Escherichia coli* DH5α by the heat shock method. The transformants clones were screened by colony PCR with the oligonucleotide primers SP6 (5′-ATTTAGGTGACACTATAGAA-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′). The plasmid DNA of clones containing the correct insert segments were then purified using the QIAprep Spin Miniprep Kit (Qiagen, Germantown, MD, USA) to be sequenced [40]. Sequence reactions were then performed using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, CA, USA) with the oligonucleotide primers SP6 and T7. The samples were then loaded into the ABI Prism 310 Genetic Analyzer (Perkin Elmer Applied Biosystems, CA, USA), and the nucleotide sequences were determined. The nucleotide sequences were analyzed using BioEdit version 7.0 [41]. The phylogenetic analysis of partially sequenced (ITS) gene was carried out by the neighbor joining method using MEGA version 5 [42].

2.7. Nucleotide sequence accession number

The nucleotide sequences determined in this study were submitted to GenBank nucleotide sequence database, and the accession numbers were given) Table 1).

2.8. Field trial evaluation of povidone iodine as potent disinfectant

A buffered 1% Betadine solution which contains 10% povidone iodine complex was used in the current experiment (Betadine, Mundipharma AG-Basel, Switzerland). Prior to spawning, rinsing solutions of 60 mg L^{-1} povidone iodine for 30 min as initial dose followed by a maintenance dose of 70 mg L^{-1} for 10 min were applied to angelfish spawners. In hatchery, angelfish eggs were shell-hardened in 80 mg L^{-1} povidone iodine for 30 min as described by Eissa et al. [31]. The tested eggs were observed under light microscope on daily bases for five successive days. Isolation trials from equal number of post rinsing spawner angelfish and their eggs were performed under the same laboratory conditions.

3. Results

3.1. Identification of the retrieved water mould

The hypothetical problem of this manuscript was built on the emerging event of mass mortalities of angelfish eggs in the investigated private ornamental fish farm at Giza, Egypt. The overall assessment of the achieved data confirmed that mortality rates among examined angelfish eggs have approached 70% of the entire egg stock. Reduced hatchability among examined eggs was very noticeable. Whitish opaque eggs (dead eggs) were abundant.

Ten water mould isolates were obtained from the infected eggs. Visual inspection of the cultured SDA plates has exposed the eminent growth of mould colonies. The colonies can be morphologically depicted as cysts of whitish cottony long hairs that quickly shifted to grey then black after 96 h. Microscopically, fungal colonies were characterized by an extensive and dense mycelium. By examination, 3 out of 10 isolates showed the characteristic appearance of branched nonseptated hyphae together with masses of mature and immature sporangia, which is indicative for asexual reproduction. Such sporangia were filled with large number of spherical sporangiospores, which were separated from the basal somatic hyphae by a

Table 1 Key details of retrieved *Saprolegnia parasitica* isolates.

Strain No.	Isolates	Source	Country	Year of isolation	Sexual reproduction *in vitro*	PCR	Accession no.	References
1	EGY111220	Angelfish eggs	Egypt	2011	Attainable	Positive	AB727986	This study
2	EGY111224	Angelfish eggs	Egypt	2011	Unattainable	Positive	AB727987	This study
3	EGY120111	Angelfish eggs	Egypt	2012	Unattainable	Positive	AB727984	This study
4	EGY120114	Angelfish eggs	Egypt	2012	Attainable	Positive	AB727985	This study
5	EGY120115	Angelfish eggs	Egypt	2012	Unattainable	Positive	AB727988	This study
6	EGY120120	Angelfish eggs	Egypt	2012	Attainable	Positive	AB727989	This study
7	EGY120130	Angelfish eggs	Egypt	2012	Attainable	Positive	AB727990	This study
8	EGY120212	Angelfish eggs	Egypt	2012	Attainable	Positive	AB727991	This study
9	EGY120221	Angelfish eggs	Egypt	2012	Attainable	Positive	AB727992	This study
10	EGY120223	Angelfish eggs	Egypt	2012	Attainable	Positive	AB727993	This study

small septum. However, the majority of the retrieved isolates (7/10 isolates) showed identical morphological characteristics of Saprolegnia' sexual reproduction such as terminal oogonia with centric oospores and antheridia (Fig. 1).

3.2. PCR and phylogenetic analysis

A 750 bp fragment was amplified from high-quality DNA extracted from 10 isolates using primers targeting the ITS gene (Fig. 2). The 10 ITS region' sequences achieved from the highest-scoring segment pairs from the BLAST search using the ITS region sequence were from *S. parasitica*. The ITS region sequence from *S. parasitica* ATCC90213 (GenBank Accession No. AY455771) showed 99% identity to the portion of the PCR fragment containing the ITS region sequences. The phylogenetic analysis using neighbor joining method showed that the amplified sequences were grouped with known sequences of *S. parasitica* and separated from other groups belonged to *Saprolegnia hypogyna*, *Saprolegnia diclina* and *Saprolegnia longicaulis* (Fig. 3)

3.3. Field trial evaluation of povidone iodine

The used concentrations of 60 mg L^{-1} povidone iodine for 30 min as initial dose followed by a maintenance dose of 70 mg L^{-1} for 10 min as rinsing solutions for spawner angelfish has proved its disinfectant efficacy against the mould infection. Egg wise, 60 mg L^{-1} for 30 min as an immersion solution for eggs was very efficient to combat mould infection among egg stocks. These conclusive results were confirmed by inability to retrieve the water mould back from the treated angelfish spawners and their eggs. Such disinfection protocol has resulted in an abrupt decline of spawner angelfish and their egg mortalities. Number wise, the mortality rates of incubated eggs were abruptly declined from 70% to 30%, while hatching rate had sharply increased from 10% to 60% within one week post treatment.

4. Discussion

Initial assessment of the mass mortalities which have occurred among spawner angelfish stocks and their egg progeny were very complicated if multifactorial hypothesis was considered. However, by progress of diagnostic investigations through the entire event of mass kills, visual detection of hyphal masses on both spawners and their egg progeny were presumptively suggestive of a water mould infection. Ten pathogenic water moulds were identified as *Saprolegnia* spp. based on morphological characteristics and phylogenetic analysis. Our findings confirm that *Saprolegnia* spp. are the major cause of *Saprolegnia*sis in ornamental fish production [43].

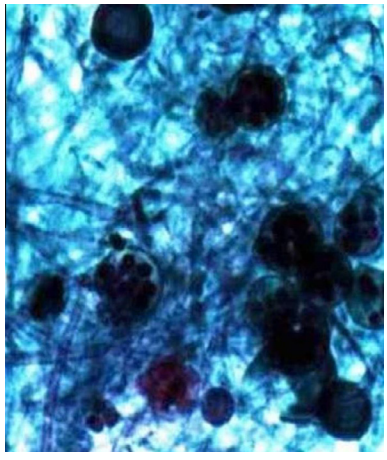

Figure. 1 Sexual reproduction of the *Saprolegnia parasitica* isolate showing mature oogonium with centric oospores.

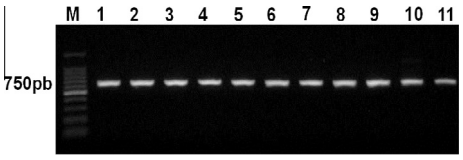

Figure. 2 The amplified fragments of the internal transcribed spacer (ITS) gene extracted from fish eggs isolates ($n = 10$) and the reference strains of *Saprolegnia parasitica* ATCC90213 ($n = 1$) using two ITS gene primers: (ITS1) 5′-TCCGTAGGTGAACCTGCGG-3′ and (ITS4) 5′-TCCTCCGCTTATTGATATGC-3′ Lane M, Marker; lane 1, *S. parasitica* ATCC90213; and lanes 2–11, angelfish eggs isolates of *S. parasitica*.

Identification of *Saprolegnia* spp. is complex and sometimes confusing. However, several typical morphological features involving asexual and sexual reproductive organs serve for classical *Saprolegnia* identification [44]. In the current study, the fact that 30% of the isolates were unable to develop sexual stages even after prolonged incubation period cannot be neglected, particularly after the addition of hemp seeds as biological enhancer. The oogonia of these isolates are either completely absent (30% of the retrieved isolates) or are formed only after a prolonged period of time (70% of the retrieved isolates). Hence, Saprolegnia which were pathogenic to angelfish spawners/egg progeny have been identified as *S. parasitica* according to criteria described by Hatai and Hoshiai [45]. Our data (unattainable sexual reproduction after 21 days incubation for 30% of the isolates) coincide with Ristanović and Miller [46], Hatai and Hoshiai [45] and Hussein et al. [47] who concluded that hemp seed could be of no identification value if the oogonia or antheridia (sexual structures) were not seen within 60 days of incubation at maximum (very long incubation period compared to ours). Therefore, using molecular tools such as PCR coupled with partial sequencing of inter-transcribed spacer (ITS) gene was one of the most important choices to distinguish *S. parasitica* from other water moulds (*Oomycetes*) [39].

Non coding internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal DNA are considered to be the most accepted genetic markers because of their relatively high sequence variability [48] and the availability of primers that would supply sequence data for *Oomycetes* [49]. These non coding regions are located between two coding regions, the 18S and the 28S genes. Another coding region, the 5.8S gene, is found between the ITS1 and ITS2. Thus, genetic sequential analysis of these regions has been adopted to study the intragenic as well as the inter-genic relationships among the 10 retrieved *Saprolegnia* isolates [50]. Further, the phylogenetic analysis based on the ITS rDNA region further confirmed the taxonomic position of the 10 *Saprolegnia* isolates and con-

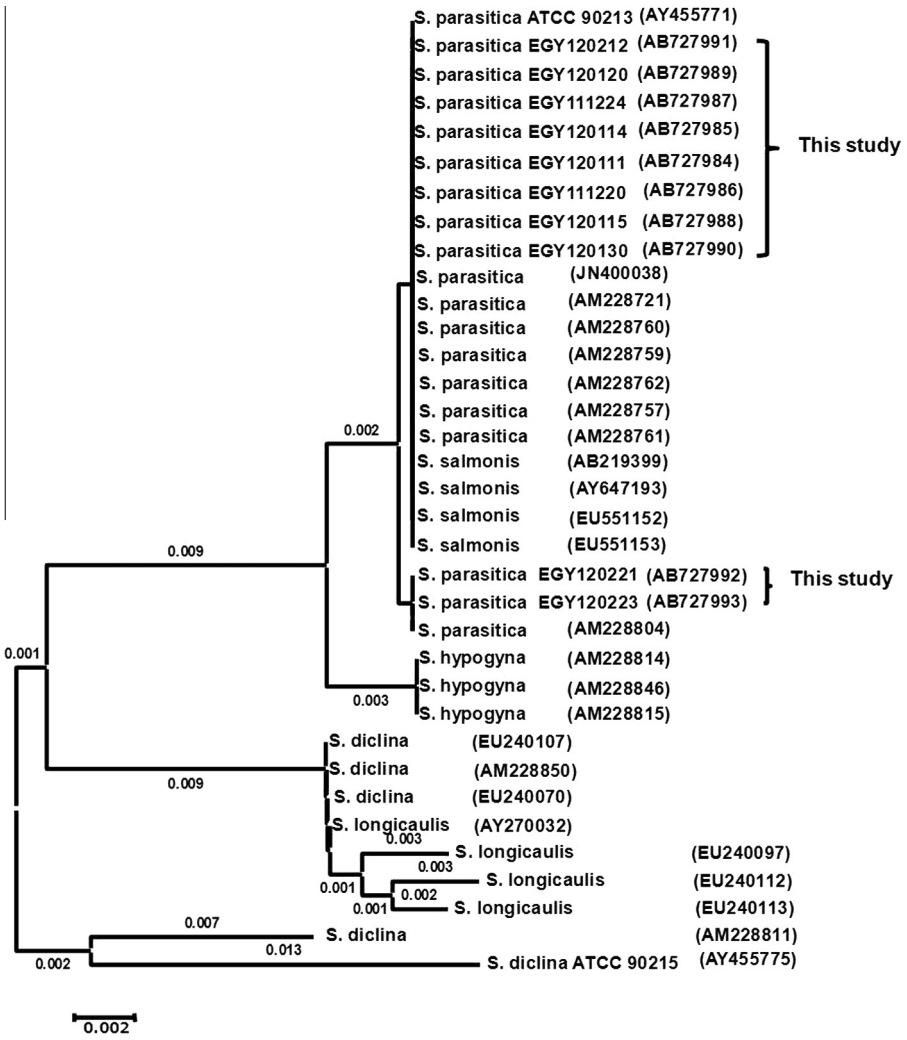

Figure. 3 Phylogenetic tree generated based on the sequence of ITS gene of fish eggs isolates and other ITS genes of *S. parasitica*, *S. diclina*, *S. salmonis*, *S. hypogyna* and *S. longicaulis*. This phylogenetic tree was constructed with the neighbor joining method using MEGA version 5.

firmed its identification as *S. parasitica* which coincides with similar findings by Cao et al. [39].

Based upon the pathogenic mechanism adopted by the water mould, the process of *S. parasitica* invasion to spawner fishes is relatively different from that utilized toward their egg progeny [3]. This hypothesis was confounded on the fact that eggs/yolk sac stage were infected or colonized after death by various *Saprolegnia* saprophytic species; whereas Angelfish spawners were infected by pathogenic *S. parasitica* isolates [3]. Saprophytic *Saprolegnia* spp. are well known for their strict aerobic nature. They grow on the egg shell surface with continuous withdrawal of oxygen from the egg surrounding. This mechanism will ultimately lead to complete exhaustion of oxygen with consequent egg death due to hypoxia [15,51]. On the contrary, *S. parasitica* were capable of invading fish tissues after being attached to the skin by the cilia of the motile zoospores with consequent germination and production of proteolytic enzymes (chemotrypsin like) [15,51].

Numbers of environmental as well as wildlife dynamic factors could be presented as the main predisposing factors behind fish/egg mortalities. In the Greater Cairo/Giza provinces, air temperatures fluctuate swiftly throughout the majority of winter days. This swift fluctuation in air temperatures usually triggers consequent profound fluctuations in water bodies' temperatures. Additionally, the commercial ornamental fish' ponds have a large surface area coupled with shallow depth (1–1.5 m) which constitute another detrimental dynamic factor imposing a dramatic water temperatures fluctuation. Such environmental stresses would initiate a cascade of dynamic bombardments of the physical/innate skin barrier with consequent immunosuppression followed by quick shift of the mould pathogenicity from saprophytic into pathogenic phase [14,15].

Ecologically, the sharp decrease in water temperature enhanced the quick proliferation of *Saprolegnia* free swimming zoospores with consequent attachment to skin/eggs of spawner angelfish [3]. The germination of the mould spores has initiated severe fungal invasion of the skin and muscles. The proteolytic activity of the germinating mould has induced remarkable ulcerative skin lesions [3]. Ultimately, the osmoregulatory failure arising from the massive skin lesions would be the real cause behind the angelfish mortalities [5,6].

Epidemiologically, amphibians (frogs, amphipods) intruding the fish ponds would be another potential source of infection to the cohabitating angelfish. Numerous *Saprolegnia* species are known to cause fatal skin diseases in frog/toad populations. Infected amphibians usually shed the mould spores from their bodies while moving through the fish pond/hatcheries which represent a reliable mechanical transmission route to their neighboring fishes/eggs [52,53].

The selection of the proper disinfectant is an essential decision for the maintenance of healthy stocks of fish and their eggs in intensive aquaculture operations. For decades, malachite green has been known for its effective disinfection power against water moulds including *Saprolgenia* spp. [27]. However, because of its potential teratogenic/mutagenic properties, malachite green has been banned [54,55].

Formalin is another potential disinfectant that has been used on a large scale throughout the past few decades. Such outdated disinfectant is still adopted by some fish hatchery managers as the best multipurpose disinfectant for broodstocks and their egg progeny [54]. However, formalin has the potential of being toxic, where it has been reported to cause total eggs mortality when temperature rises above 25 °C [56]. Numerous disinfectant were further developed and proved to be less efficient and of narrow safety margin. Thus, the search for an efficient and safe disinfectant was a must in modern food/ornamental aquaculture operations.

Based on its high efficacy/safety, iodophors are widely used as the sole disinfection protocol for both broodstocks and eggs at modern fish farms/hatchery facilities. Office of International Epizootics (OIE) mandated that eggs be disinfected in 50 mg L^{-1} iodophors for 30 min at the facility where those eggs are being fertilized. In the current study, a simple treatment protocol using 10% povidone iodine (Betadine®) solution has been efficiently used for both infected angelfish' spawners and their eggs. The used concentrations of 60 mg L^{-1} povidone iodine for 30 min as initial dose followed by a maintenance dose of 70 mg L^{-1} for 10 min as rinsing solutions for spawner' angelfish has proved its disinfectant efficacy against the mould infection. Egg wise, 60 mg L^{-1} for 30 min as an immersion solution for eggs was very efficient to combat mould infection among egg stocks.

The povidone iodine antifungal disinfectant efficacy was confirmed by the subsequent failure to re-isolate the mould back from the treated broodstocks as well as eggs. Such disinfection protocol has resulted in an abrupt decline of spawner angelfish and their egg mortalities. Impressively, the mortality rates of incubated eggs were abruptly declined from 70% to 30%, while hatching rate had sharply increased from 10% to 60% within one week post treatment which explicitly confirms the antifungal efficacy/safety of povidone iodine at the above stated treatment doses. These results are in complete accordance with original studies published by Cipriano et al. [57] and Eissa et al. [31].

Although the internationally approved 10% povidone iodine is considered among the most efficient and safe disinfectants due to its organic nature, its effective concentration was moderately affected by the presence of excessive organic matter and fish mucus which enforced us to continuously replace the rinsing solution every hour of full rinsing . Removal of infested eggs from the troughs or incubation gutter is also important in the control of the mould infection [31].

In brief, the diminished identification power (low percentage of isolates with attainable sexual structures together with longevity of incubation period) of using morphological characteristics based on sexual structure development of regular or hemp seed supplemented media has triggered us to adopt an accurate/swift/specific molecular protocol to fully identify the retrieved isolates. Further, the utilization of an accurate phylogram has enabled us to depict the phylogenetic relationships between our isolates (10 isolates with accession numbers in GenBank data base) and many *Saprolegnia* spp. of regional and worldwide importance. To sum up, the current work reports on the first isolation of *S. parasitica* during an episode of angelfish/egg mortalities at a private Egyptian ornamental fish farm with consequent successful treatment trial of fish/egg progeny using safe/effective concentrations of povidone iodine.

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References

[1] Torto-Alalibo T, Tian M, Gajendran K, Waugh M, van West P, Kamoun S. Expressed sequence tags from the oomycete fish pathogen *Saprolegnia parasitica* reveals putative virulence factors. BMC Microbiol 2005;5:46.

[2] Jalilpoor J, Mosouleh SA, Masoumzadeh M. Fungal flora in *Acipenser persicus* eggs with particular emphasis on *Saprolegnia* sp. (*Oomycetes*) and mortality during mass incubation at the Shahid Behesti hatchery. J Appl Ichthyol 2006;22:265–8.

[3] Fregeneda-Grandes JM, Rodríguez-Cadenas F, Aller-Gancedo JM. Fungi isolated from cultured eggs, alevins and broodfish of brown trout in a hatchery affected by Saprolegniosis. J Fish Biol 2007;71:510–8.

[4] Ali EH. Antifungal activity of sodium chloride on *Saprolegnia diclina* and *Aphanomyces* sp.. Acta Mycol 2009;44:125–38.

[5] Osman HA, Solman WE, Noor El Deen AE, Mohamed LA. Induction of Saprolegniosis in *Oreochromis niloticus* with special reference to its biological control. Global Vet 2008;2:32–7.

[6] Thoen E, Evensen Ø, Skaar I. Pathogenicity of *Saprolegnia* spp. to Atlantic salmon, *Salmo salar* L., eggs. J Fish Dis 2011;34:601–8.

[7] Zaki MS, Fawzi OM, Jackey JE. Pathological and biochemical studies in *Tilapia nilotica* infected with *Saprolegnia parasitica* and treated with potassium permanganate. Am-Euras J Agric Environ Sci 2008;3:677–80.

[8] Noor El- Deen AE, Mona SZ, Razin AM, Shalaby SI. Field study on the use of *Artemisa cina* (Sheih Baladi) and humates (Humpol-Fis) in control of Saprolegniosis in fingerlings of Nile tilapias and *Mugil cephalus* in freshwater fish farms. Life Sci J 2010;7:125–8.

[9] Eli A, Briyai OF, Abowei JF. A review of some fungi infection in African fish *Saprolegnia*sis, dermal mycoses; branchiomyces infections, systemic mycoses and dermocystidium. Asian J Med Sci 2011;3:198–205.

[10] Das SK, Murmu K, Das A, Shakuntala I, Das RK, Ngachan SV, Majhi SK. Studies on the identification and control of pathogen *Saprolegnia* in selected Indian major carp fingerlings at mid hill altitude. J Environ Biol 2012;33:545–9.

[11] Hatai K, Hoshiai GI. Pathogenicity of *Saprolegnia* parasitica coker. In: Mueller GJ, editor. Salmon *Saprolegnia*sis. Portland, Oregon: U.S. Department of Energy, Bonneville Power Administration; 1994. p. 87–98.
[12] Bruno DW, Wood BP. Saprolegnia and other oomycetes. In: Woo PTK, Bruno DW, editors. Fish disease and disorder. Viral, bacterial and fungal infections, vol. 3. Wallingford, Oxon, UK: CABI publishing; 1999. p. 599–659.
[13] Ghiasi M, Khosravi AR, Soltani M, Binaii M, Shokri H, Tootian Z, Rostamibashman M, Ebrahimzademousavi H. Characterization of *Saprolegnia* isolates from Persian sturgeon (*Acipencer persicus*) eggs based on physiological and molecular data. J Med Mycol 2010;20:1–7.
[14] Abou El Atta ME. Saprolegniosis in freshwater cultured tilapia nilotica (*O. niloticus*) and trial for control by using Bafry D 50/500. In: 8th International symposium on tilapia aquaculture, 2008. p. 1408–14.
[15] Bly JE, Lawson LA, Dale DJ, Szalai AJ, Durborow RM, Clem LW. Winter Saprolegniosis in channel catfish. Dis Aquat Organ 1992;13:155–65.
[16] Willoughby LG. Fungi and fish diseases. Stirling, UK: Pisces Press Publication; 1994, pp. 57.
[17] Mousavi H, Soltani M, Khosravi A, Mood S, Hosseinifard M. Isolation and characterization of *Saprolegnia*ceae from rainbow trout (*Oncorhynchus mykiss*) eggs in Iran. J Fish. Aquat Sci 2009;4:330–3.
[18] Czeczuga B, Muszynska E. Aquatic fungi growing on the eggs of various fish families. Acta Hydrobiol 1999;41:235–46.
[19] Forneris G, Bellardib S, Palmegianoc GB, Sarogliad M, Sicuroa B, Gascoe L, Zoccarato I. The use of ozone in trout hatchery to reduce *Saprolegnia*sis incidence. Aquaculture 2003;221:157–66.
[20] Fadaeifard F, Raissay M, Bahrami H, Rahimi E, Najafipoor A. Freshwater fungi isolated from eggs and broodstocks with an emphasis on *Saprolegnia* in rainbow trout farms in west Iran. Afr J Microbiol Res 2011;4:3647–51.
[21] Noga EJ. Fish diseases, diagnosis and treatment. St. Louis, USA: Mosby Publishers; 2000, 367 pp.
[22] van West P. *Saprolegnia parasitica*, an oomycete pathogen with a fishy appetite: new challenges for an old problem. Mycologist 2006;20:99–104.
[23] Diéguez-Uribeondo J, Fregeneda-Grandes JM, Cerenius L, Pérez-Iniesta E, Aller-Gancedo JM, Tellería MT, Söderhäll K, Martín MP. Re-evaluation of the enigmatic species complex *Saprolegnia diclina-S. parasitica* based on morphological, physiological and molecular data. Fungal Genet Biol 2007;44:585–601.
[24] Ke X, Wang J, Gu Z, Li M, Gong X. Morphological and molecular phylogenetic analysis of two *Saprolegnia* sp. (*Oomycetes*) isolated from silver crucian carp and zebra fish. Mycol Res 2009;113:637–44.
[25] Khomvilai C, Kashiwagi M, Yoshioka M. Fungicidal efficacy of sodium hypochorit on a fish- Pathogen oomycetes, *Saprolegnia* from Thailand. Bull Faculty Bioresour Mie Univ 2005;32:39–44.
[26] Niska K, Korkea-aho T, Lindfors E, Kiuru T, Tuomainen M, Taskinen J, Peltonen K. Disappearance of malachite green residues in fry of rainbow trout (*Oncorhynchus mykiss*) after treatment of eggs at the hatching stage. Aquaculture 2009;297:25–30.
[27] Willoughby LG, Roberts RJ. Towards strategic use of fungicides against *Saprolegnia parasitica* in salmonid fish hatcheries. J Fish Dis 1992;15:1–13.
[28] Schreier TM, Rach JJ, Howe GE. Efficacy of formalin, hydrogen peroxide, and sodium chloride on fungal infected rainbow trout eggs. Aquaculture 1996;140:323–31.
[29] Culp SJ, Beland FA, Heflich RH, Benson RW, Blankenship LR, Webb PJ, Mellick PW, Trotter RW, Shelton SD, Greenlees KJ, Manjanatha MG. Mutagenicity and carcinogenicity in relation to DNA adduct formation in rats fed leucomalachite green. Mutat Res 2002;506:55–63.
[30] Gieseker CM, Serfling SG, Reimschuessel R. Formalin treatment to reduce mortality associated with *Saprolegnia parasitica* in rainbow trout, *Oncorhynchus mykiss*. Aquaculture 2006;253:120–9.
[31] Eissa AE, ElSayed EE, Faisal M. Field trial evaluation of povidone iodine as an effective disinfectant for different stages of returning spawners salmon. Life Sci J 2007;96:87–93.
[32] Eric JW, Randall WO, Ronney EA, Anna MF, Matthew B. The Safety and effectiveness of various hydrogen peroxide and iodine treatment regimens for rainbow trout egg disinfection. North Am J Aquacult 2010;72:34–42.
[33] Ahmed LS, Ahmed SM, Ali HS, Kamel YY, El-Allawy TA. Cause of mortality in aquarium fish, angelfish (*Pterophyllum scalare*). Assiut Vet Med J 1990;23:179–87.
[34] Coker WC. The Saprolegniaceae, with notes on other water molds. Chapel Hill, North Carolina: University of North Carolina Press; 1923, 201 pp.
[35] Seymour RL. The genus Sapnoiegnla. Federal Republic of Germany: Verlag von J. Cramer, Lehre; 1970, 124 pp.
[36] Willoughby LG. Saprolegniasis of salmonid fish in Windermere: a critical analysis. J Fish Dis 1978;1:51–67.
[37] Pickering AD, Willoughby LG, Mccrory CB. Fine structure of secondary zoospore cyst cases of Sapnolegnia isolates from infected fish. Trans Br Mycol Soc 1979;72:427–36.
[38] Moller EM, Bahnweg G, Sandermann H, Geiger H. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies and infected plant tissues. Nucleic Acids Res 1992;20:6115–6.
[39] Cao H, Xia W, Zhang S, He S, Wei R, Lu L, Yang X. *Saprolegnia* pathogen from Pengze Crucian Carp (*Carassius auratus* var. Pengze) eggs and its control with traditional Chinese herb. Israeli J Aquacult Bamidgeh 2012;64:1–7.
[40] Abdelsalam M, Chen SC, Yoshida T. Dissemination of streptococcal pyrogenic exotoxin G (*spegg*) with an IS-like element in fish isolates of *Streptococcus dysgalactiae*. FEMS Microbiol Lett 2010;309:105–13.
[41] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 1999;41:95–8.
[42] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731–9.
[43] Bangyeekhun E, Pylkko P, Vennerstrom P, Kuronen H, Cerenius L. Prevalence of a single fish-pathogenic *Saprolegnia sp*. clone in Finland and Sweden. Dis Aquat Organ 2003;53:47–53.
[44] Stueland S, Hatai K, Skaar I. Morphological and physiological characteristics of *Saprolegnia spp*. Strains pathogenic to Atlantic salmon, *Salmo salar* L. J Fish Dis 2005;28:445–53.
[45] Hatai K, Hoshiai GI. Mass mortality in cultured coho salmon (*Oncorhynchus kisutch*) due to Saprolegnia parasitica coker. J Wild life Dis 1992;28:532–6.
[46] Ristanović B, Miller CE. Salinity tolerances and ecological aspects of some fungi collected from fresh-water, estuarine and marine habitats. Mycopathologia 1969;37:273–80.
[47] Hussein M, Hatai K, Nomura T. Saprolegniosis in salmonid and their egg in Japan. J Wild Life Dis 2001;37:204–7.
[48] Irwin JA, Crawford AR, Drenth A. The origins of *Phytophthora* species attacking legumes in Australia. Adv Bot Res 1997;24:431–56.
[49] White TJ, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR Protocols: a guide to methods and applications. San Diego: Academic Press; 1990. p. 315–22.

[50] Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM. A molecular phylogeny of *Phytophthora* and related *Oomycetes*. Fungal Genet Biol 2000;30:17–32.

[51] Abdelaziz MA, Kamel J, Mahmoud A. Seasonal study, histopathological and treatment trial on Saprolegniosis in some fish farms. In: The proceeding of first scientific conference of veterinary Medicine, University of Banha, Moshtohor, 2004. p. 1–4.

[52] Milius S. Fatal skin fungus found in U.S. frogs. Sci News 1998;15:7–8.

[53] Petrisko JE, Pearl CA, Pilliod DS, Sheridan PP, Williams CF, Peterson CR, Bury RB. *Saprolegnia*ceae identified on amphibian eggs throughout the Pacific Northwest, USA, by internal transcribed spacer sequences and phylogenetic analysis. Mycologia 2008;100:171–80.

[54] Marking LL, Rach JJ, Schreier TM. Evaluation of antifungal agents for fish culture. Progressive Fish Culturist 1994;56:225–31.

[55] Fitzpatrick MS, Schreck CB, Chitwood RL. Evaluation of three candidate fungicides for treatment of adult spring chinook salmon. Progress Fish Culturist 1995;57:153–5.

[56] Akpoilih BU, Adebayo OT. Effect of formalin on the hatching rate of eggs and survival of larvae of the African Catish (*Clarias gariepinus*). J Appl Sci Environ Manag 2010;14:31–4.

[57] Cipriano R, Novak BM, Flint DE, Cutting DC. Reappraisal of the federal fish health recommendation for disinfecting eggs of Atlantic Salmon in Iodophor. J Aquat Animal Health 2001;13:320–7.