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Note

Infection of *Achlya* sp. associated with dermal ulceration and mortality in an endemic snakehead fish *Channa aurantimaculata*

Jayasimhan Praveenraj[1, 2], Uma Arumugam[2, 3] and Gnanadesikan Rebecca[3]

[1]ICAR-Central Island Agricultural Research Institute, Port Blair - 744 101, Andaman and Nicobar Islands, India
[2]Department of Aquatic Animal Health Management, Dr. M. G. R. Fisheries College and Research Institute, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Ponneri - 601 204, Tamil Nadu, India
[3]State Referral Laboratory for Aquatic Animal Health, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Madhavaram Campus, Chennai - 600 051, Tamil Nadu, India

Abstract

The present work reports an infection by *Achlya* sp., causing severe dermal ulceration and mortality in the endemic snakehead fish *Channa aurantimaculata*. *Achlya* sp. associated with the infection was isolated on potato dextrose agar (PDA) and confirmed by light microscopy following lactophenol cotton blue staining. Further confirmation was achieved through molecular characterisation of the internal transcribed spacer (ITS) gene. Additionally, bacterial strains associated with the ulcers, such as *Vibrio cholera*, *Tatumella punctata* and *Aeromonas* sp. were also isolated and characterised by sequencing the 16SrDNA gene. This communication constitutes the first report of *Achlya* sp. associated with serious mortality in the endemic ornamental snakehead fish of India.

*Correspondence e-mail:
Praveenraj.j@icar.gov.in

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The class Oomycota, commonly known as water molds are important opportunistic pathogens that multiply on hosts that are physically injured, stressed or already infected. *Achlya* genus of Oomycota consists of a number of species that are parasitic on fish (Neish and Hughes, 1980). In the early 90's, the genus *Saprolegnia* was believed to be the primary cause of major fungal infections in fish and fish eggs (Scott and O'Bier, 1961; Scott and O'Warren, 1964). However, Srivastava and Srivastava (1978) demonstrated that *Achlya* sp. is highly pathogenic to traumatised *Puntius* sp. and *Colisa* sp. Subsequently, Srivastava (1978) reported *Achlya orion* causing mass mortality in the fishes of Ramgarh Lake, Gorakhpur, India. *Achlya klebsiana* causing haemorrhage was reported from Myanmar in cultured *Channa striata* (Kitancharoen *et al*., 1995). Lau *et al.* (2018) reported *Achlya oblongata* infection associated with mortality in freshwater reared Asian seabass *Lates calcarifer* for the first time from Malaysia. Peyghan *et al.* (2019) reported a case of *Achlya* sp. causing infection in *Astronotus ocellatus* from Iran for the first time. The present study reports *Achlya* sp. associated with severe dermal ulceration and mortality, isolated and reported from the endemic snakehead fish *Channa aurantimaculata* brought from Assam, north-eastern India. The pathogenic *Achlya* sp. as well as certain bacterial strains were isolated from the dermal ulcer of infected snakehead fish which were characterised and reported herein as potential pathogens affecting ornamental snakehead fish.

Incidence of fungal infections associated with severe dermal ulceration and mortality were observed in the snakehead fish *Channa aurantimaculata* brought from Assam, north-eastern India and kept at a private ornamental fish export unit located in Chennai, India, during 2019. *C. aurantimaculata* (n=12) weighing 22-70 g, with dermal ulcerations were carefully

packed in a polythene bag and transported live to the laboratory for further investigations. The infected fish were kept in a glass aquarium for observation of clinical signs as well as for further investigations.

Smears were prepared from the ulcers of the fish, stained with lactophenol cotton blue and observed under a light microscope. Swabs were collected aseptically from the ulcers of the fish and inoculated directly in potato dextrose agar medium supplemented with Ampicillin and incubated at 15-20°C for 24 h, incubated and monitored for fungal growth. Swabs were taken from the ulcer of one infected fish aseptically and inoculated on tryptic soy agar (TSA agar) plates. These plates were incubated overnight and observed for bacterial growth. Individual colonies from TSA plates were picked and streaked on Thiosulfate citrate bile salts sucrose (TCBS) as well as *Aeromonas* isolation agar plates and the colonies developed were purified by repeated straking for isolation of pure cultures.

One of the infected fish was euthanised using clove oil and the muscle tissue with ulcer was dissected out and fixed in 10% neutral buffered formalin. The fixed tissues were processed for histopathology following the protocols given by Roberts (2012). Briefly, the fixed tissues were dehydrated in ascending series of alcohol, embedded in paraffin, sectioned (5 µm), stained with hematoxylin and eosin (H&E) and examined under a microscope (EVOS, Thermo Scientific).

Infectivity studies of the fungal isolate were conducted in tilapia *Orechromis mossambicus*. A total of forty healthy fish with an average weight of 3.1-7.0 g were kept for seven days in aquaria (10 l capacity) with continuous aeration and fed with artificial feed for acclimatisation. The experimental aquarium tanks were set up in triplicates, aerated continuously and temperature was maintained between 25-26ºC. Group I was given 0.2 ml of fungal inoculum prepared following Singhal *et al.* (1987). Group II was given freshly harvested fungal mycelia inoculated in the rearing water with heavy aeration. In Group III, small patches of scales were removed from the experimental fish, and the rearing water was inoculated with freshly harvested fungal mycelia. Group IV having 10 fish injected with same volume of sterile water, served as control. The course of infection and mortality was monitored in the challenged fishes and moribund fishes were sampled for re-isolation of the fungus.

Fungal mycelia were collected aseptically from the inoculated plate using sterile loop and washed in sterile distilled water. About 20 mg of the mycelia were used for extracting the DNA using Qiagen Blood and Tissue kit following manufacturer's protocol. DNA amplification of the internal transcribed spacer (ITS) of the oomycete was carried out using universal primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) that target conserved regions in the 18S and 28S rRNA genes (White *et al.,* 1990). The PCR reaction mixture contained 24 µl of nuclease free water, 20 µl of master mix, 2 µl each of forward and reverse primers as well as 2 µl of genomic DNA leading to a final volume of 50 µl reaction mixture. The amplification was carried at an initial denaturation of 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The final extension was at 72°C for 5 min. The PCR products were resolved on a 1.5% agarose gel containing 0.5 µg ml^{-1} ethidium bromide in 1x Tris-borate-EDTA (TBE) buffer. The amplified PCR products were sequenced using ITS primers in ABI 3500 DNA analyser (Eurofins Pvt. Ltd., Bengaluru, India). The homology of the generated sequence was analysed using the Basic Local Alignment Search Tool (BLAST) program in the National Centre for Biotechnology Information GenBank nucleotide collection. Additional sequences of *Achlya* spp. were downloaded from NCBI GenBank (see Appendix 1) to construct the maximum likelihood tree using the freeware MEGA X (Kumar *et al.,* 2016). Similarly DNA was extracted from pure bacterial isolates using DNAzol agent following manufacturer's protocol. Molecular identification of the bacterial isolates was carried out by PCR amplification of 16srDNA gene following the primers and protocol of Weisburg *et al.* (1991). Sequencing of the amplified PCR products and analysis were carried out as described above for the identification of the fungal isolates.

All the snakehead fish (*C. aurantimaculata*) had severe deep ulceration exposing muscle and haemorrhages with heavy tufts of fungal growth in the inter-orbital, snout and nape regions of the head (Fig. 1). The infected fish exhibited anorexia, sudden flashing movements and restlessness. There was 100% mortality recorded for the infected

Fig. 1. Ulceration in the flank, head, opercular region and snout associated with *Achlya* sp. infection in *C. aurantimaculata*

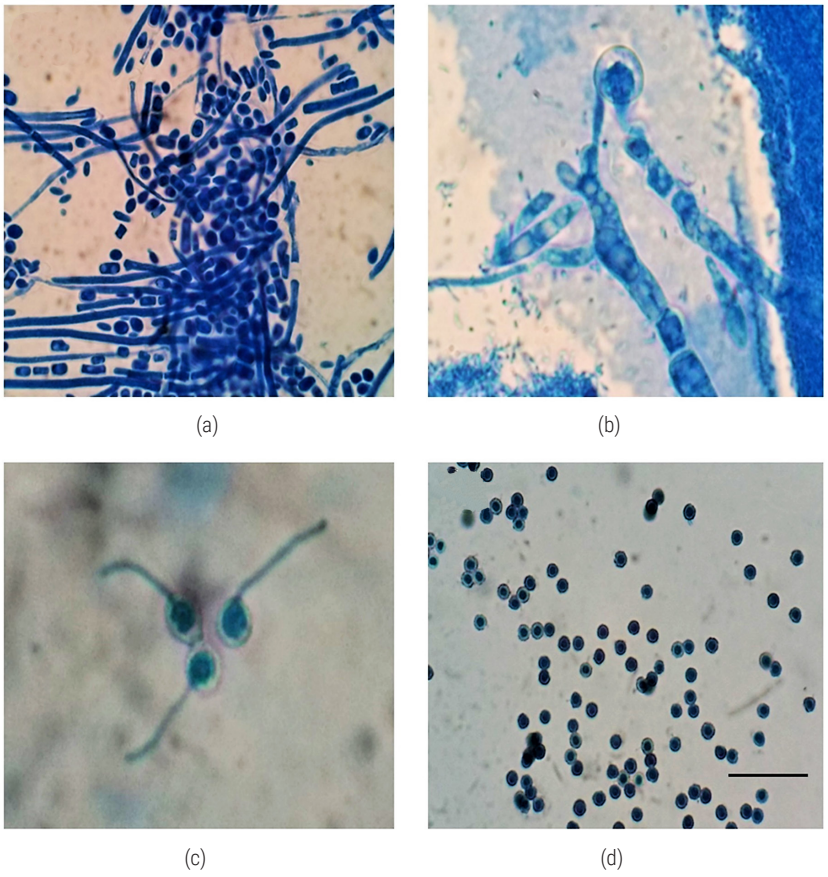
fishes over a period of one week. Fungal growth was observed after 24 h on the isolation media as whitish tufts, which revealed numerous mycelia with lactophenol cotton blue staining (Fig. 2a). Light microscopy of the fungal hyphae revealed oogonia, maturing zoospores and many individual zoospores typical of *Achlya* sp. (Fig. 2 b, c, d). Sterile swab taken from the ulcers of infected fish produced white colonies on TSA agar, yellow colonies on TCBS agar and green colonies on *Aeromonas* isolation agar. Histology of the *Achlya* infected muscle tissue revealed granuloma, degeneration of the muscle tissue, haemorrhages, necrosis and growth of fungal hyphae in the muscle tissue. Presence of numerous melanin pigments were also observed around the necrotic area (Fig. 3).

All the experimental fish infected with *Achlya* sp. showed 100% mortality. The injected group (Group 1) showed slow mortality with no signs or symptoms after 4-5 days post-injection. Group II showed 100% mortality after 48 h, with gills having heavy fungal growth. Group III showed fungal growth on the areas where scale has been removed and had slow mortality over a period of one week.

The identity of the oomycete was confirmed following PCR analysis and sequencing of the ITS region of the 18S and 28S rRNA genes, which amplified a region of ~800 bp (Fig. 4) and sequencing of the amplified product yielded a final product size of 780 bp. The NCBI BLAST analysis revealed it to be an unidentified *Achlya* sp. with 97% homology with an *Achlya* sp. (KJ511774-76) isolated from

Fig. 3. Histopathological changes in the muscle tissue of *C. aurantimaculata* (H&E). Black arrow shows granuloma (Scale bar 400 µm at 40X magnification)

Oreochromis niloticus from Thailand. The maximum likelihood tree generated in the present study reveals the *Achlya* sp. isolated from infected snakehead fishes to be distinct and forming sister clade with *Achlya crenulata* (Fig. 5). The sequence was submitted to the NCBI GenBank under the accession number MH571985. Similarly,



(a) (b) (c) (d)

Fig. 2. Light microscopy of *Achlya* sp. following staining with lactophenol cotton blue. (a) Mycelia; (b, c) Oogonia; (d) Zoospores separated from the zoosporangium (Scale bar 100 µm at 100X magnification)

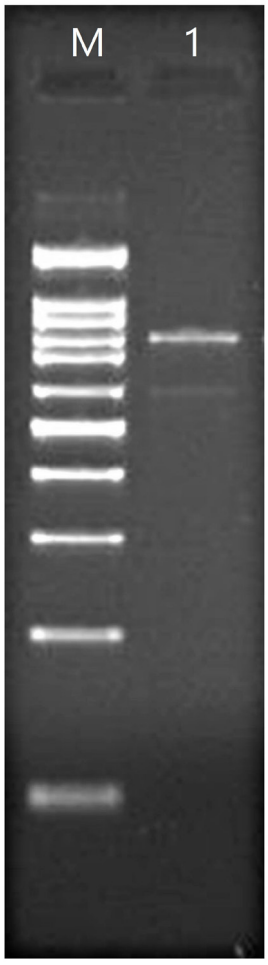


Fig. 4. PCR amplification of the ITS region of *Achlya* sp. Lane M: 1000 bp ladder; Lane 1: 800 bp amplicon of ITS gene of *Achlya* sp.

the molecular confirmation of the bacterial isolates revealed the species to be *Vibrio cholera*, *Tatumella punctata*, *Aeromonas* sp., which were submitted in the NCBI GenBank under the accession number MK590411, MK848419 and MK848415, respectively.

Fishes of the family Channidae popularly known as snakeheads are important food fish and also the colourful ones possess ornamental value (Praveenraj *et al*., 2019a, b). Srivastava and Srivastava (1978) first reported *Achlya orion* as one of the important fish pathogen. Subsequently, Srivastava (1978) reported *Achlya orion* infection in 11 species of freshwater fishes *viz, Puntius sophore, P. conchonius, P. ticto, P. sarana, Colisa lalia, Chanda ranga, Channa punctatus, Channa marulius, Labeo rohita, Anabas testudineus* and *Notopterus notopterus*. Saraswathi *et al.* (2015) isolated five species of *Achlya viz, A. prolifera, A. americana, A. klebsiana, A. flagellata* and *A. hypogyana* infecting *Catla catla, Clarias batrachus, Cirrhinus mrigala, Labeo rohita, Mystus cavasius* and *C. striata.* Their study indicated that *C. striata* was more prone to infection by *Achlya* sp. as all the samples showed the presence of *A. hypogyna, A. prolifera* and *A. americana.* Very recently, Lone *et al.* (2018) reported *A. americana* and *A. klebsiana* infecting *C. punctata* and *A. prolifera* in *C. striata* from Bhopal, India.

The present report of *Achlya* sp. infection causing dermal ulceration and mortality in *C. aurantimaculata* is a serious threat to Indian aquarium industry involved in trading of ornamental snakeheads. The infectivity study revealed the *Achlya* sp. to be highly pathogenic, causing potential outbreaks in confined system leading to mass mortality, which is also supported by the findings of Panchai *et al*. (2015), who experimentally infected Nile tilapia fry with six different isolates of *Achlya* sp.

Histopathological analysis revealed the presence of granuloma, degeneration of the muscle tissue, haemorrhages, necrosis and growth of fungal hyphae in the muscle fibres providing evidence that mortality may have mainly occurred due to loss of body fluid and osmoregulatory failure. Further, the bacterial isolates like

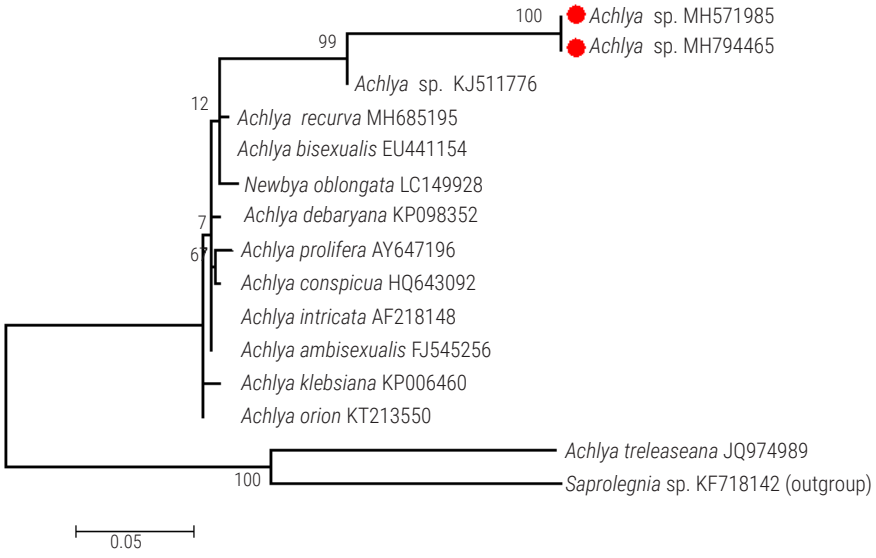


Fig. 5. Phylogenetic position of *Achlya* sp. inferred from maximum likelihood tree. GenBank ID MH571985 and MH794465 denotes *Achlya* sp. isolated from *C. aurantimaculata*

Vibrio cholera, *Tatumella punctata* and *Aeromonas* sp. isolated from the ulcers are normal microflora of aquatic habitats and opportunistic pathogens in fish host, which could have contributed to mixed infection in *C. aurantimaculata*. We conclude that the present *Achlya* sp. in the endemic snakehead fish might have spread from Assam, India, since majority of the ornamental snakehead fish for aquarium trade are from north-eastern India and were reportedly collected by the locals using bamboo traps. The trapped snakeheads undergo physical damage in the snout region, which paves an easy route for fungal attack, eventually leading to development of ulcers and secondary infections.

Further studies on the morphological characteristics and pathogenicity of *Achlya* sp. needs to be undertaken to establish its taxonomic identity. The finding constitutes the first report of serious ulceration in endemic snakehead fish caused by *Achlya* sp. and this is the first report form India on *Achlya* sp. infection in *C. aurantimaculata*.

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