



Taxonomic note on a rare fish infecting freshwater mould *Achlya ambisexualis* Raper 1939 (Achlyaceae) isolated from Chandraprabha dam, Uttar Pradesh, India

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ABSTRACT

Achlya spp. are oomycetous water moulds, responsible for freshwater fish diseases causing great economic losses. An *Achlya* sp. implicated in significant fungal infections of both live and dead fish as well as their eggs, has been isolated from the water and soil samples collected from Chandraprabha dam (Chandauli District, Uttar Pradesh, India) employing standard baiting method. Based on morphological characterisation, the strain was identified as *Achlya ambisexualis* Raper 1939 (Saprolegniales, Oomycetes). It is a dioecious species, characterised by the presence of an achlyoid type of spore dehiscence from both primary and secondary sporangia, differentiated by its oospheres predominantly maturing into eccentric oospores, generally 1-18 per oogonium and gemmae cylindrical in both antheridial and oogonial mycelia. In India, this species was recorded from a single collection in the past but lack proper description and illustrations. The present study describes and illustrates this species for the first time in India and hoped to be beneficial for ichthyopathologists and researchers as *A. ambisexualis* is known as a necrotroph or parasite of fishes and their eggs.

Keywords: *Achlya ambisexualis*, *Achlyaceae*, Chandraprabha dam, Oomycetes, Saprolegniasis, Stramenopiles, Taxonomy

Introduction

Achlya is an important genus of the family *Achlyaceae* (Saprolegniales, Oomycota), under phylum Stramenopiles (also called Heterokonta) (Dick, 2001; Johnson *et al.*, 2002; Beakes *et al.*, 2014). This genus is easily recognised by its profusely branched, coenocytic mycelium which gives rise to long, cylindrical and usually terminal zoosporangia that discharge their primary aplanospores from the sporangium to form a ball of spores (Johnson *et al.*, 2002). Apart from difference in a few morphological features, the genus mostly exhibits similar properties and living conditions as the genus *Saprolegnia*. These fungal-like organisms with a zoosporic stage are often referred to as oomycetes or “water moulds” that are phylogenetically distinct from true fungi and are closer to brown algae, diatoms and plants (Alexopoulos *et al.*, 1996; Guarro *et al.*, 1999; Baldauf *et al.*, 2000; Paul and Steciow, 2008). *Achlya* has approximately 50 valid species (Johnson *et al.*, 2002; El Androusse *et al.*, 2006; Paul and Steciow, 2008; Kirk *et al.*, 2008; Jesus *et al.*, 2015). The most included species in this genus probably are obligate saprotrophs which usually grow saprophytically serving

simply as organic decomposers of animal and plant debris in freshwater and soil ecosystems worldwide (El-Hissy and Khallil, 1991; Czczuga *et al.*, 2002; Kiziewicz and Nalepa, 2008; Mazurkiewicz-Zapałowicz *et al.*, 2008). However, under favourable conditions, *Achlya* spp. can be facultative parasites on various freshwater animals and their eggs and many members are responsible for economically important diseases affecting farmed and wild populations of aquatic animals. Several studies have reported *Achlya* species from infected *Channa striatus* (Kitancharoen *et al.*, 1995), tilapia fish and eggs (El-Sharouny and Badran, 1995; Hanjavanit *et al.*, 2013; Mortada *et al.*, 2013), salmonid eggs (Kitancharoen *et al.*, 1997; Kitancharoen and Hatai, 1998), *Oncorhynchus mykiss* eggs (Kales *et al.*, 2007; Shahbazian *et al.*, 2010), *Cyprinus carpio* eggs (Chukanhom and Hatai, 2004), *Ambystoma maculatum* (Gomez-Mestre *et al.*, 2006), *Oreochromis niloticus* (Panchai *et al.*, 2007; Hussein *et al.*, 2013; Panchai *et al.*, 2014; Panchai *et al.*, 2015; Panchai *et al.*, 2016), *Salmo trutta* eggs (Czczuga *et al.*, 2005), *Pangasianodon gigas* (Abking *et al.*, 2012), *Mystus cavasius* and other species (Chauhan *et al.*, 2013), *Clarias gariepinus* (Hunjavanit *et al.*, 2012), *Tachysurus [Pelteobagrus] fulvidraco* eggs

(Cao *et al.*, 2013), *Coregonus albula*, *Cottus poecilopus* and *S. trutta* (Kiziewicz, 2004), eggs and fry of *P. hypophthalmus* (Duc *et al.*, 2016) and *Mugil cephalus* (Sosa *et al.*, 2007).

Members of the genus *Achlya* cause saprolegniasis, an infectious fungal disease and are responsible for widespread devastating fish infections in aquaculture, fish farms, hobby fish tanks and endemic to all freshwater habitats around the world (Johnson *et al.*, 2002; Willoughby, 2003). Thus, this disease causes serious losses in fish farms and hatcheries and is considered as one of the aquatic disease implicated in mass mortalities of cultured and wild fish in many countries (Singhal *et al.*, 1987; Jeney and Jeney, 1995; Bruno and Wood, 1999). As such, they are responsible for major economic loss in aquaculture industry worldwide accounting for approximately 30% of the global fish production for consumption (Murray and Peeler 2005; van West 2006; Fregeneda-Grandes *et al.*, 2007; Phillips *et al.*, 2008). India is home to more than 10% of the global fish diversity and presently, ranks second in the world after China in total fish production. The estimated fish production of the country was 4.88 million t comprising 3.418 million t from marine fisheries and 1.3 million t from inland fisheries in the year 2014 (Ayyappan, 2014). In India, a variety of freshwater and marine ornamental fishes are available and fungal infection (Saprolegniasis) affecting them is very common. The most species within the genera *Saprolegnia*, *Achlya* and *Aphanomyces* of order Saproleniales are the major players which infect and cause diseases in fishes (Willoughby, 1994; Dick, 2001; Kales *et al.*, 2007; Fuangsawat *et al.*, 2011; Van den berg *et al.*, 2013; Gozlan *et al.*, 2014). Among these, *Saprolegnia* and *Achlya* were the most virulent parasites of freshwater fish in India (Sati, 1991). Therefore, investigations on members of these genera became topic of increasing interest, especially to those who focus on the identification and characterisation of pathogenic species in these genera.

During the aquatic fungal diversity survey of the Chandraprabha dam (Chandauli District, Uttar Pradesh, India), a distinct *Achlya* sp. was encountered, marked by presence of eccentric oospores and dioecious thalli. On further investigation, the identity of the species was determined to be *Achlya ambisexualis* Raper 1939, a known necrotroph or parasite of fishes, originally reported from only one collection made in Bengaluru, Karnataka, India (Nolard-Tintigner, 1973; 1974). To our knowledge, this *Achlya* sp. has not been reported since its original publication in India which lacks proper description and illustrations. Hence, the objective of this study was to contribute to the knowledge of *Achlya* spp. in India giving

emphasis on taxonomical/morphological characteristics. The current study was undertaken on snake skin bait to evaluate this species using morphological characters and to develop a key for its identification.

Materials and methods

Isolation and identification

Parasitism of fish is of common occurrence in Chandraprabha dam, Chandauli District, Uttar Pradesh, India (24°55'59.9"N;83°10'47.6"E) especially during winter months. An aquatic fungal diversity survey was under taken in Chandraprabha dam during February 2015 to January 2016. Water and soil samples for fungal isolation were collected at random in separate sterile polyethylene bags and taken to the laboratory. The samples were processed by standard baiting technique using baited snake keratin (Seymour, 1970; Seymour and Fuller, 1987). Each sample was processed in triplicates, which were introduced in separate petri dishes and flooded with 40 ml of sterile deionised water. The baits were placed in triplicate culture dishes and incubated at 20°C for 7 days. The baits were periodically examined under a microscope for about a week and when growth was observed on the baits, the colonised baits were washed in sterile distilled water and transferred to peptone yeast extract glucose (PYG) agar supplemented with penicillin G and streptomycin sulfate (300 ppm each) to obtain an axenic culture and maintained at 25°C. After incubation and growth, the cultures were made unifungal by dissecting out a block of agar (5 mm in dia) from the advancing edge of the 5 day old colony and placing in a petri dish containing sterile distilled water. The baits were then placed near the agar block. In a few days when hyphae grew, baits became noticeable and they were transplanted onto new PYG agar plates and subcultured until pure cultures were obtained. The pure cultures were transferred to fresh PYG agar plates every month and preserved on autoclaved hemp seeds at 4-8°C for long time storage.

A distinct *Achlya* sp. that was encountered during the study was subjected to detailed investigations on morphological traits using their vegetative and reproductive characteristics on keratin baits under a light microscope. Identification of the isolate was done on the basis of vegetative organs (shape and size of the hyphae), asexual structures (shape and size of zoosporangium and spores, their formation, patterns of discharge and germination) and reproductive organs (production and structure of the oogonium, antheridium and oospores) using the monographs and descriptions of Johnson (1956), Seymour (1970), Muhsin *et al.* (1984), Willoughby (1994), Johnson *et al.* (2002), Vega-Ramirez *et al.* (2013) and other relevant taxonomic literatures containing original

descriptions of taxa. Observations and measurements were recorded and photographed with a light microscope (Dewinter microscope). Permanent mounts of the isolate was prepared by fixing in formalin-acetic-alcohol and mounting in lactophenol (Johnson, 1956; Willoughby, 1994). The isolate was deposited at the Laboratory of Mycopathology and Microbial Technology, Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi, India with the accession no. 178.

Results and discussion

Description of the species

Achlya ambisexualis Raper, *Amer. J. Bot.*, 26: 639. 1939 (Fig. 1 a-f).

Morphology

The vegetative thallus consisted of coenocytic, stout and extensively dense mycelia (Fig. 1 a, b). The aseptate and hyaline hyphae emerged from the snake skin were single or catenulate stout or delicate, branched, straight, usually broadest about 10-14 μm wide and thick-walled at the base but become progressively narrow and thin walled towards the apices. The septa were formed in the hyphae only to delimit the reproductive organs, asexual

sporangia or gemmae and sexual oogonia and antheridia. Gemmae abundant and cylindrical, arise from the hyphae either terminally or intercalary and usually with dense cytoplasmic contents, spherical, pyriform, occasionally irregular, single or catenulate, often germinating to produce sporangia or functioning as oogonia. Asexual reproduction by zoosporangia. Zoosporangia abundant, generally cylindrical or clavate or fusiform, renewed sympodially, 18-44 and 210-500 μm in dia, slightly curved, sometimes hyphoid to irregular, separated from the hypha by a septum. Spores monomorphic in both thalli; discharge and behaviour achlyoid; primary spore cysts 9-13 μm in dia with smooth walls and two subapical flagella; spore cluster persisting at exit orifice immediately upon emergence or disintegrating in part (Fig. 1c). Encysted spores globose and showed direct germination. Sexual reproduction by differentiated oogonia. Oogonia formed abundantly often single with 56-84 μm in dia, lateral or less commonly terminal or rarely intercalary, mostly spherical or obpyriform; oogonial wall smooth and slightly pitted; stalk short to long, slender, straight or curved, tapering towards the base and mostly unbranched with oogonial wall pitted under the region of antheridial cell attachment (Fig. 1d). Oospores always maturing, 1-18 in number,

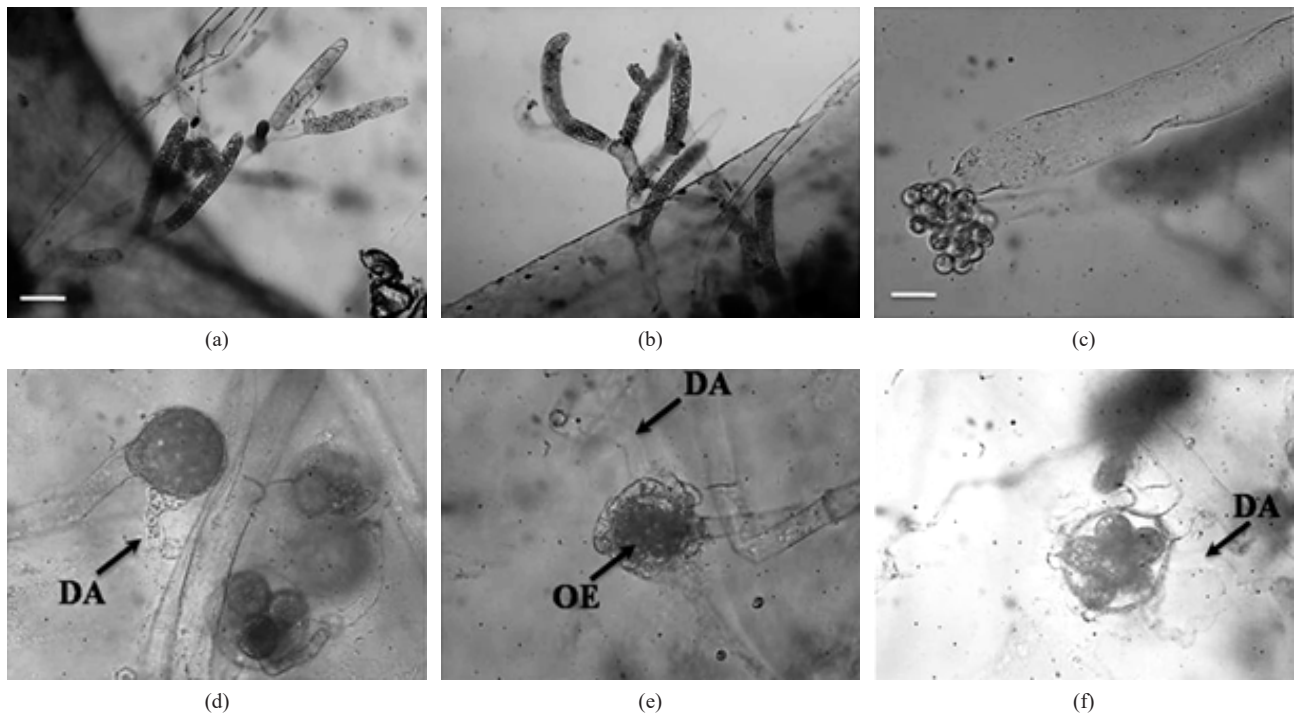


Fig. 1. *Achlya ambisexualis*: (a) Young vegetative thallus, (b) Aseptate and hyaline hyphae emerging from the snake skin with primary and secondary sporangia renewed sympodially, (c) Achlyoid discharge and behaviour with spore cluster persisting at exit orifice immediately upon emergence, (d) Diclinous antheridial branch on smooth-walled maturing oogonium, (e) Eccentric oospores with antheridial branches having exclusively diclinous origin wrapping or clasping around the wall pitting oogonia, (f) Eccentric oospores with antheridial cells and branches attached in a digitate fashion or laterally/apically encircling the oogonia. Bars = 60 μm for a and b; 20 μm for c-f. OE: Oospores eccentric, DA: Diclinous antheridium

usually 3-14 per oogonium, spherical, eccentric and generally filling it; germination not observed. Oospores 18-26 μm in dia. Antheridial branches always present, long and arising from one hypha ("male") or exclusively diclinous origin; branched, irregular, long, diffused and slender often wrapping or clasping around the oogonia, laterally appressed, persisting (Fig. 1e). Antheridial cells compound; tubular or clavate, branched or unbranched; persisting; attached in a digitate fashion or laterally/apically encircling the oogonia; fertilization tubes not observed (Fig. 1f). Based on the above characteristics, the strain was identified as *A. ambisexualis*.

Material examined: Fungal strains isolated using snake skin as a preferred substrate, from water and soil of Chandraprabha dam, Chandauli District, Uttar Pradesh, India during February 2015 - January 2016 (Acc. No. 178).

Distribution: USA, Taiwan, Thailand, France, Iraq, China, South America, Africa, British Isles, India, Philippines, USSR, Mexico, Poland.

Remarks: The species is morphologically similar to its close relative *Achlya bisexualis* in general configuration of reproductive structures, although the oospheres nearly always mature into oospores that usually fill the oogonial cavity of the former and that of the latter the maturation is decidedly uncommon and seldom fully occupy the cavity. Similar to most species of *Achlya*, *A. ambisexualis* has been reported as a fish pathogen but many reports demonstrated that most strains were acting as saprotrophs. It grows commonly on organic debris in the soil as well as water bodies and has been isolated often from paddy soil. *A. ambisexualis* thus likely perform an active role in decomposition of cellulosic and keratinous substrates within the ecosystem. It is mostly recovered from soil samples in comparatively low temperature months.

During the present study, we investigated the naturally occurring *Achlya* sp. identified as *A. ambisexualis* based on morphological and reproductive characteristics. Although the pathogenicity of the isolate is still under investigation, there are numerous reports suggesting *A. ambisexualis* as etiologic agents of fish mycoses and may primarily cause a clinically identical saprolegniasis as *Saprolegnia* spp. Hunjavanit *et al.* (2012) isolated virulent *A. ambisexualis* from eggs of African catfish (*C. gariepinus*) while Panchai *et al.* (2005) and Hanjavanit *et al.* (2013) isolated it from Tilapia eggs (*O. niloticus*). Panchai *et al.* (2007) succeeded in isolating *A. ambisexualis* from the eggs and fry of tilapia (*O. niloticus*). These investigations and keratinophilic nature of the species strongly suggest that *A. ambisexualis* like other *Achlya* spp. are the major cause of saprolegniasis in aquaculture. During the course of study, maximum

occurrence of the species was recorded during winter spring months followed by summer and rainy season respectively. These findings are clearly in agreement with the reports of Dayal and Tandon (1962), Srivastava (1967), Manoharachary (1981) and Chauhan and Qureshi (2012) that high temperatures and rainy season are unfavourable for most of the aquatic fungi.

In India, association of *Achlya* spp. with diseased fishes have been reported by many workers (Bhargava *et al.*, 1971; Srivastava and Srivastava, 1976; Srivastava and Srivastava, 1977a, b; Hasija and Batra, 1978; Srivastava, 1980; Sati and Khulbe, 1981; Khulbe and Sati, 1981; Srivastava *et al.*, 1984; Sati, 1985; Khulbe *et al.*, 1994; Devi and Rajanaika, 2010; Chauhan, 2012; Chauhan *et al.*, 2012; Chauhan and Qureshi, 2012; Chauhan and Bankhede, 2013; Chauhan *et al.*, 2013; Chauhan, 2014; Mastan *et al.*, 2015). These investigations clearly indicate that *Achlya* is one of the most prevalent and frequent genera recorded in water bodies all over India. Only a few studies on *Achlya* were focused on the morphology and taxonomy. Identification of *Achlya* spp. is complex, difficult and sometimes confusing. However, several typical morphological characteristics involving asexual and sexual reproductive structures serve for classical *Achlya* identification. Many *Achlya* spp. are endemic to all freshwater habitats around the world and they cause saprolegniasis in fish. Thus studies on genus *Achlya* are of major environmental and economic importance due to their negative impact on aquaculture and aquatic ecosystems by parasitising fishes, amphibians and crustaceans (Srivastava and Srivastava, 1977a, b; El-Sharouny and Badran, 1995; Kitancharoen *et al.*, 1995; Kitancharoen *et al.*, 1997; Nejadstari, 2000; Steciow, 2001; Czczuga *et al.*, 2002; Mazurkiewicz-Zapałowicz *et al.*, 2008; Hunjavanit *et al.*, 2012). Taxonomy and phylogenetic relationships remain unresolved and suffer from many inconsistencies, which are the major obstacles to the widespread application of molecular barcoding to identify pathogenic strains with quarantine implications.

Studies in the field of zoosporic fungi is continuously going down as compared to higher fungi as far as the Indian scenario is concerned (Dubey *et al.*, 2016). The present study attempted to bridge this gap. To assess the diversity and distribution pattern of this group, extensive surveys are required. The taxonomy of the water moulds is known to be difficult and the study of oomycetes in general has not received much attention in India. The encouraging results of the present study, emphasises that more efforts and surveys are needed for better understanding the members of the *Achlya*. Therefore, isolation of this taxon from multiple disparate

geographic locations and habitats will further provide a better understanding of microhabitat, distribution, the frequency of occurrence or relative abundance as well as genetic divergence of the members from an ancient lineage.

The present study reported on occurrence of *A. ambisexualis*, for the second time in India, which is also the first record of the species on keratin bait. The results from the present study may contribute in knowledge of the occurrence and distribution of *A. ambisexualis* in India.

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