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ARTICLE

First Record of the Water Mold *Achlya bisexualis* (Saprolegniaceae) Isolated from Ornamental Fish in South Africa

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Abstract

The order Saprolegniales (Class Oomycota) is a group of fungus-like eukaryotic microorganisms that have been associated with infections in fish and fish eggs. Infections with microorganisms from this order are clearly evident because they have a cotton wool-like appearance. The aim of this study was to characterize and identify an oomycete that was isolated from the eye of an Orange Blotched Peacock Cichlid *Aulonacara* sp. A sample of cotton wool-like mycelia was isolated and single-spore isolations were conducted. Molecular characterization and phylogenetic analysis of the ITS1-5.8-ITS2 rDNA region for all isolates were used for species identification. Following molecular identification, one isolate was used to culture and characterize the reproductive structures. Physiological characterization entailed incubating the isolate on potato dextrose agar (PDA) at five different temperatures, ranging from 5°C to 25°C, to monitor growth rates. A multiple sequence alignment showed 100% similarity between all of the single-spore isolates and alignment with other *Achlya bisexualis* strains. Long, coarse hyphae with zoosporangia and gemmae typical of the order Saprolegniales were observed with an optimal growth rate at 25°C. The oomycete that was isolated from an Orange Blotched Peacock Cichlid was identified as *A. bisexualis*, the first record of this species in South Africa.

The oomycetes (Phylum Oomycota) are a group of fungus-like eukaryotic microorganisms that are globally distributed and often referred to as water molds (Webster and Weber 2007). These are among the most significant disease-causing organisms in both terrestrial agriculture and aquaculture alike and continually present a realized threat to global food security (Derevnina et al. 2016). In contrast with their terrestrial counterparts, aquatic oomycetes remain understudied. This applies especially to those that are pathogenic to animals in the order Saprolegniales such as *Aphanomyces, Saprolegnia* and *Achlya* (Derevnina et al. 2016). Representatives of this order are characterized by coenocytic mycelia, motile zoospores, and their ability to reproduce both sexually and asexually (Webster and Weber 2007).

Water molds of the order Saprolegniales are mostly saprophytic. *Achlya* species have been reported as either obligate or facultative pathogens infecting both wild and cultured fish and their eggs (Lawhavinit et al. 2002;

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Chukanhom and Hatai 2004; Czeczuga et al. 2005; Prasad and Rajanaika 2010; Hunjavanit et al. 2012; Mastan et al. 2012; Dubey et al. 2018). Infections with *Achlya* species are known as occasional etiological agents in aquaculture, particularly in tropical countries (Lilley and Roberts 1997; Lau et al. 2018). Due to the common occurrence of *Achlya* species in natural water systems and their wide range of susceptible hosts under conditions that favor facultative infections (Dubey et al. 2018; Lone et al. 2018), it is not surprising that *Achlya* infections have been widely reported as secondary infections on physical injuries or in combination with other, primary pathogenic agents (Lawhavinit et al. 2002). Records of secondary infections include those from aquaculture facilities in Thailand where *Achlya klebsiana* has been isolated from farmed, adult Common Carp *Cyprinus carpio* (Chukanhom and Hatai 2004), *A. bisexualis* from Nile Tilapia *Oreochromis niloticus* eggs (Panchai et al. 2007), and *A. ambisexualis* from African Catfish *Clarias gariepinus* eggs (Hunjavanit et al. 2012). Another species, *A. inflata*, has been isolated from Brown Trout *Salmo trutta* in Poland (Czeczuga et al. 2005). More recently, *A. oblongata* has been reported as a pathogen of farmed Asian Seabass fry *Lates calcarifer* in Malaysia (Lau et al. 2018).

The genus *Achlya* typically shares phenotypical characteristics with other members of the order Saprolegniales, including similar environmental requirements for growth and reproduction, morphology, and cotton wool-like mycelia often covering dermal erosions or skin lesions (Chukanhom and Hatai 2004; Refai et al. 2016; Dubey et al. 2018; Lau et al. 2018). This physical similarity with other genera in Saprolegniales often results in the incorrect or inaccurate diagnosis as a *Saprolegnia* species or *Saprolegnia*-like infection. Furthermore, the mode of discharge of the zoosporangia from the sporangium often forms the basis of discrimination between genera in practice, despite the lack of specificity of diagnostic characteristics at the genus or species level (Daugherty et al. 1998; Leclerc et al. 2000).

The aim of this study was to characterize and identify an oomycete infection that was isolated from the eye of an Orange Blotched Peacock Cichlid *Aulonacara* sp. (Cichlidae) in South Africa.

METHODS

Fungus-Like Isolation

During a disease inspection at an ornamental fish farm in the Western Cape of South Africa, an individual Orange Blotched Peacock Cichlid presented with a superficial fungus-like infection on its left eye. A small sample of cotton wool-like mycelia was removed from the eye and inoculated onto modified phycomycetes isolation agar (12.00-g agar, 1.00-g glucose, 1.00-g gelatin hydrolysate, 0.01-g liver extract, 0.10-g yeast extract, 1.00-L water) supplemented with streptomycin sulphate and penicillin G sodium salt (0.05% w/v) to hinder bacterial growth (Fuller et al. 1964). The plates were incubated at 20°C and monitored daily for fungus-like growth. The infected fish was removed from the system.

Single-Spore Isolation

A modification of the method described by Sandoval-Sierra and Diéguez-Uribeondo (2015) was used for single-spore isolations. Isolates were left to grow on potato dextrose agar (PDA) for 3 d. Agar plugs were taken from the edge of the growing colonies with a cork borer (5.5 mm in diameter). Each agar plug was aseptically transferred to a separate, sterile 90-mm petri dish containing 20 mL autoclaved Milli-Q water (MilliporeSigma) and approximately 0.1 g of sterile hemp seeds, after which they were incubated overnight at room temperature to grow and sporulate. After 24 h, 80–100 µL of the zoospore suspension was spread onto four plates (two PDA and two 1.5% water agar plates) and incubated overnight at room temperature. The following day, each plate was examined for germinated spores using a Nikon SMZ1500 stereo microscope. From each plate, four single germinated spores were transferred onto new plates containing glucose peptone agar (GPA) with a sterile needle. After 3 d of incubation at room temperature, all colonies were examined and actively growing colonies were transferred individually onto PDA plates. Plates were kept for an additional 2 d in case slower-growing, single-spore colonies started to develop. Isolates were maintained on PDA and were regularly subcultured as required, approximately every 7 d.

Genomic DNA Extraction and Sequencing

Total genomic DNA was extracted from all single-spore colonies following incubation for 7 d on PDA (Greeff et al. 2012). DNA quality and concentrations were confirmed using a Jenway Genova NanoDrop spectrophotometer. Samples were stored at −20°C until needed.

A universal fungal primer set (ITS1 and ITS4) was used to amplify the ITS1-5.8S-ITS2 rDNA region for all isolates (White et al. 1990). The 25-µL reaction mixtures consisted of 12.5 µL Taq DNA polymerase 2× Master mix (Amplicon), 0.5 µL (0.25 µM) of each primer, 9.5-µL PCR grade water, and 2-µL DNA template. Amplification was conducted using a Labnet Thermal Cycler and entailed an initial denaturation for 5 min at 94.0°C, followed by 35 cycles of 45 s at 94.0°C, 45 s at 51.1°C, and 1 min at 72.0°C with a final extension of 7 min at 72.0°C. The amplified PCR products were analyzed by agarose gel (1%) electrophoresis to verify the fragment size. The PCR products were sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI 3730xl Genetic Analyzer (Applied Biosystems) according

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to the sequencer manufacturer's instructions. The nucleotide sequences were deposited in the GenBank database (available at www.ncbi.nlm.nih.gov/genbank) under the accession numbers MN317321 through MN317329. Each sequence was edited and assembled using MEGA X software (MEGA, Pennsylvania State University, State College). Homology searches were carried out using the BLASTn algorithm (Altschul et al. 1990) provided by the National Centre for Biotechnology Information (available at https://blast.ncbi.nlm.nih.gov/Blast/).

Molecular Phylogeny

Additional *Achlya* species sequences were downloaded from Genbank (Table 1) and used in subsequent phylogenetic analyses. Alignments were done with MAFFT software (Berkley Software Distribution, University of California; version 7.221) using the L-INS-I option. The MEGA X software package was used to construct a maximum likelihood phylogenetic tree using a bootstrap analysis of 1,000 replicates, with p-distance nucleotide substitution using *Saprolegnia* spp. (*S. parasitica* [HQ644002] and *S. ferax* [HQ643987]) as an outgroup.

Morphological Characteristics

For morphological observations, agar plugs from a single isolate (ML18040) were aseptically transferred to a sterile 90-mm petri dish containing 20 mL autoclaved Milli-Q water and approximately 0.1 g sterile hemp seeds and incubated at 25°C. Cultures were monitored daily for key developmental structures using a NIKON SMZ1500 stereo microscope. When these structures were observed, temporary wet mounts were prepared for observation and documentation using a Nikon Eclipse Ni-E compound microscope at 100× magnification. Structures were photographed using a Nikon DS-Fi2 microscope-attached camera, and measurements were made using NIS-Elements BR software (Nikon, Tokyo; version 4.00). Thirty measurements of each of the following characteristics were taken: zoosporangium length, zoosporangium breadth, diameter of the spores, and diameter of the gemmae. Zoosporangium breadth was measured perpendicularly to the hyphal wall at one-third of the length of the zoosporangium from the distal end. The diameter of the spores and gemmae was calculated as the spherical diameter from a measurement of their surface area. Along with the above measurements, the discharge pattern of zoospores was also documented. All measurements were taken in μm and are presented as the mean ± SD (range).

Physiological Characteristics

Effect of temperature on growth rate.— Isolate ML18040 was inoculated onto GPA plates (Lilley et al. 1998) and incubated at room temperature for 5 d to encourage growth of large colonies. Following incubation, agar plugs were taken from the distal edges of the growing colonies with a cork borer (5.5 mm in diameter). Agar plugs were aseptically transferred to the center of fresh GPA plates. Each plate was incubated at one of five different temperatures (5, 10, 15, 20, and 25°C) with a total of four replicate plates per temperature. At temperatures where little to no growth was observed after 7 d, plates were further incubated at 25°C to assess the viability of the respective cultures. The growth rate of isolate ML18040 at each temperature was recorded every day for a maximum of 7 d by photographing the plates with a DSLR Canon EOS 600 d camera. For image analysis, a ruler was included on each photograph to serve as a reference for calibrating the digital images. Image J 1.52a software (Image J, National Institutes of Health and the University of Wisconsin, Madison) was used to measure the surface area of each colony, and the data was expressed as the mean spherical diameter (mm). The size of the agar plug (5.5 mm) was used as the starting point and was subtracted from all subsequent measurements. SigmaPlot software for Windows 12.0 (Systat Software, San Jose, California) was used for all data analyses.

Effect of temperature on reproductive structures.— To determine the effect of temperature on the development of reproductive structures of isolate ML18040, agar plugs that were taken from the growing edge of the colonies were aseptically transferred to sterile 90-mm petri dishes, each containing 20 mL autoclaved Milli-Q water and approximately 0.1 g sterile hemp seeds. Each petri dish was incubated at one of five different temperatures (5, 10, 15, 20, and 25°C) with a total of four replicate petri dishes per temperature.

RESULTS

Gross Observation and Isolation

Cotton wool-like mycelia were observed covering only the left eye of an individual Orange Blotched Peacock Cichlid (Figure 1). Once the superficial mycelial crust was removed, the underlying cornea of the affected eye appeared unharmed and functional with no obvious lesions or abrasions, suggesting that the fungus-like growth did not penetrate into the underlying tissue. However, no microscopy or additional staining techniques were used to further assess damage to the cornea. Following the single-spore isolations from the mycelia that were isolated from the eye, nine single-spore isolates were obtained and subsequently classified as belonging to the order Saprolegniales based on the presence of stiff, coarse hyphae with terminal sporangia.

Molecular Phylogeny

To identify the nine single-spore isolates that were cultured from the infected fish to the species level, the ITS1-5.8S-ITS2 gene region sequences were analyzed. Multiple sequence alignment of these nucleotide sequences confirmed 100% similarity between all single-spore isolates.

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TABLE 1. Internal transcribed spacer (ITS) sequences used in this study for the molecular phylogenetic analysis.

Taxon	Host species or substrate	Locality	GenBank accession number	Reference
A. ambisexualis	Lake Calumet	Chicago, USA	HQ643083	Robideau et al. (2011)
A. ambisexualis	Water	France	HQ643082	Robideau et al. (2011)
A. ambisexualis	Water	France	AF218147	Leclerc et al. (2000)
A. ambisexualis	Not specified	USA	FJ545256	Panchai et al. (2014)
A. americana	River water	New York	HQ643084	Robideau et al. (2011)
A. aquatica	Pool water	Uttar Pradesh	HQ643085	Robideau et al. (2011)
A. bisexualis	Estuary water	Florida, USA	DQ403201	Sosa et al. (2007)
A. bisexualis	Not specified	Sweden	HQ643088	Robideau et al. (2011)
A. bisexualis	River water	Illinois	HQ643086	Robideau et al. (2011)
A. bisexualis	Not specified	Iran	KF225573	Panchai et al. (2014)
A. bisexualis	Pond water	France	AF218153	Leclerc et al. (2000)
A. bisexualis	Pond water	France	AF218151	Leclerc et al. (2000)
A. bisexualis	Pond water	North Carolina	HQ643087	Robideau et al. (2011)
A. bisexualis	Not specified	Not specified	EU441154	Unpublished
A. bisexualis	*Poecilia reticulata*	Thailand	AY647189	Phadee et al. (2004)
A. bisexualis	Not specified	Not specified	KP663631	Lau et al. (2018)
A. bisexualis	Not specified	Not specified	KP663631	Lau et al. (2018)
A. bisexualis	*Aulonocara* sp.	South Africa	MN317321	ML18040 (present study)
A. bisexualis	*Aulonocara* sp.	South Africa	MN317322	ML18041 (present study)
A. bisexualis	*Aulonocara* sp.	South Africa	MN317323	ML18042 (present study)
A. bisexualis	*Aulonocara* sp.	South Africa	MN317324	ML18043 (present study)
A. bisexualis	*Aulonocara* sp.	South Africa	MN317325	ML18044 (present study)
A. bisexualis	*Aulonocara* sp.	South Africa	MN317326	ML18045 (present study)
A. bisexualis	*Aulonocara* sp.	South Africa	MN317327	ML18046 (present study)
A. bisexualis	*Aulonocara* sp.	South Africa	MN317328	ML18047 (present study)
A. bisexualis	*Aulonocara* sp.	South Africa	MN317329	ML18048 (present study)
A. caroliniana	Water	Spain	JX418018	Mélida et al. (2013)
A. caroliniana	Not specified	Nigeria	HQ643089	Robideau et al. (2011)
A. dubia	Not specified	Nigeria	HQ643093	Robideau et al. (2011)
A. dubia	Not specified	France	AF218155	Leclerc et al. (2000)
A. flagellata	Not specified	Not specified	HQ643097	Robideau et al. (2011)
A. flagellata	Not specified	Not specified	HQ643096	Robideau et al. (2011)
A. flagellata	Not specified	Not specified	HQ643095	Robideau et al. (2011)
A. glomerata	Not specified	Not specified	HQ643098	Robideau et al. (2011)
A. oblongata	Not specified	India	DQ324365	Lau et al. (2018)
A. oblongata	Asian Seabass	Subah, Malaysia	LC149928	Lau et al. (2018)
A. oblongata	Tilapia	Northeast Thailand	KJ511773	Panchai et al. (2014)
A. oblongata	Tilapia	Northeast Thailand	KJ511777	Panchai et al. (2014)
A. klebsiana	Not specified	Not specified	AF218156	Leclerc et al. (2000)
A. klebsiana	Not specified	Not specified	KP006460	de Jesus et al. (2015)
A. prolifera	Shortnose Gar	Japan	AY647196	Phadee et al. (2004)
A. prolifera	Not specified	France	EU849169	Panchai et al. (2014)
S. parasitica	Northern Pike	Netherlands	HQ644002	Robideau et al. (2011)
S. ferax	Not specified	France	HQ643987	Robideau et al. (2011)

The edited and assembled nucleotide sequences of the internal transcribed spacer (ITS) gene region of all isolates used in the alignment were 679 bp long. A BLAST search in the GenBank database revealed that the ITS gene sequences of all of the isolates showed high similarity to other *Achlya* species. The strains from the GenBank database with the highest similarity to our isolates were *A. bisexualis* (namely, EU441154.1), which was 100% identical with 100% coverage. Published strains with the highest similarity included KF225573 (Panchai et al. 2014) and

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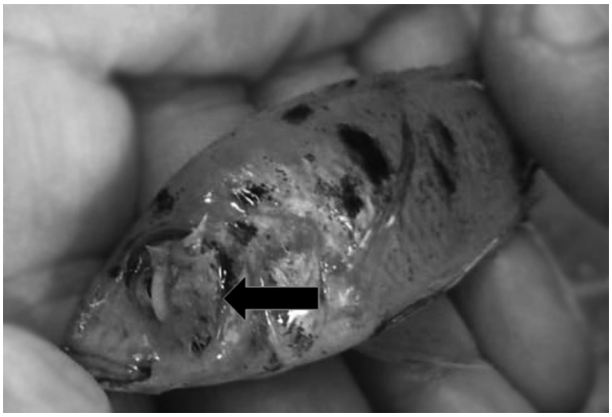

FIGURE 1. A photograph showing the cotton, wool-like mycelia covering the eye of an Orange Blotched Peacock Cichlid (black arrow).

HQ643087 (Robideau et al. 2011), which had 100% and 98% identity, respectively, with 99% coverage relative to all of the isolates. These strains, along with other closely-related published strains, were included in the phylogenetic analyses (Table 1; Figure 2).

The resulting maximum likelihood phylogenetic tree grouped the *Achlya* strains into three major clades. The first major clade consisted of two monophyletic groups. The first monophyletic group comprised only *A. prolifera* strains, while the second comprised *A. dubia*, two strains of *A. klebsiana* and one strain of *A. bisexualis* (AY647189). The second major clade consisted of two subclades. The first subclade comprised two groups, with the first group consisting of *A. oblongata* and the second consisting of 8 of the 11 *A. bisexualis* strains used in this analysis, as well as all of the isolates from this study. The third major clade was made up of five dominant groups, with the first consisting mostly of *A. ambisexualis* strains. The second comprised mostly *A. flagellata* strains, the third comprised mostly *A. caroliniana* strains, and the fourth comprised three *Achlya* species. The latter included *A. americana* (HQ643084), *A. aquatica* (HQ643 085), and *A. glomerata* (HQ643098).

Phylogenetic analysis confirmed similarity between isolates from this study and *A. bisexualis* by forming a monophyletic group with high bootstrap support. The support applied to eight of the *A. bisexualis* strains (HQ643087, AF218151, KP663631, KP663630, KF225573, AF218153, EU441154, DQ403201) included in the phylogenetic analysis, forming a sister group to *A. oblongata* (Figure 2).

Based on the results of the multiple sequence alignment and phylogenetic analysis, only one isolate (ML18040) was selected for subsequent morphological examination.

Morphological Characteristics

When isolate ML18040 was cultured in sterile water containing sterile hemp seeds at 25°C, its morphological characteristics included long, coarse hyphae (a typical observation) with terminal clavate or filiform zoosporangia. The zoosporangia exhibited a length of 258.11 ± 65.50 µm (range: 164.00–425.00 µm) and width of 17.61 ± 5.50 µm (range: 11.00–30.00 µm) and were renewed through both internal and lateral proliferation. Zoospore discharge took place in an achlyoid manner with the emergent zoospore cluster persisting at the apical pore (Figure 3). The average spherical diameter of the spores was 11.61 ± 0.10 µm (range: 11.00–12.00 µm). No oogonia were observed after 21 d of incubation at 25°C. Single spherical gemmae and spherical, pyriform, or obovate-shaped moniliform gemmae were observed at the terminal protrusion of the hypha (Figure 4). The average spherical diameter of the spherical gemmae measured 63.59 ± 14.90 µm (range: 36.00–89.00 µm), and the average spherical diameter of pyriform gemmae had an average of 69.53 ± 21.30 µm (range: 33.00–112.00 µm). Morphological similarities were observed in many characteristics of isolate ML18040, such as zoosporangium morphology, zoospore discharge, zoospore shape and size, and gemmae shape, as compared to the original species description of *A. bisexualis* (Coker 1927) and other published reports of this species (Barksdale 1962; Lawhavinit et al. 2002) (Table 2).

Physiological Characteristics

Effect of temperature on growth rate.— Isolate ML18040 grew at a wide range of temperatures (5–25°C), but the maximum growth rate was reached at 25°C (Figure 5). From days one to five at this temperature, the average growth rate of the isolate's spherical diameter was 19.00 ± 0.68 mm/d (range: 16.00–21.40 mm/d). All of the cultures that were incubated at 25°C reached the maximum allowable growth size (i.e., the total growth was a spherical diameter of 84.50 mm in a 90-mm petri dish) after 5 d. Plates that were incubated at 5°C only started to show visible growth after 3 d and reached a total diameter of 7.30 ± 0.22 mm (range: 6.90–7.40 mm) after 7 d.

Effect of temperature on reproductive structures.— Isolate ML18040 exhibited growth in sterile water containing hemp seeds at all the temperatures tested (5–25°C). The growth rate in sterile water containing hemp seeds was not measured but our observations appeared to conform to those made for the growth on solid media. The growth rate at 25°C was faster than at 5°C; however, the presence of reproductive structures remained the same at all temperatures. Zoosporangia and gemmae were clearly visible after 24 h.

DISCUSSION

Opportunistic *Achlya* spp. are known for causing secondary infections in both farmed and wild freshwater fish

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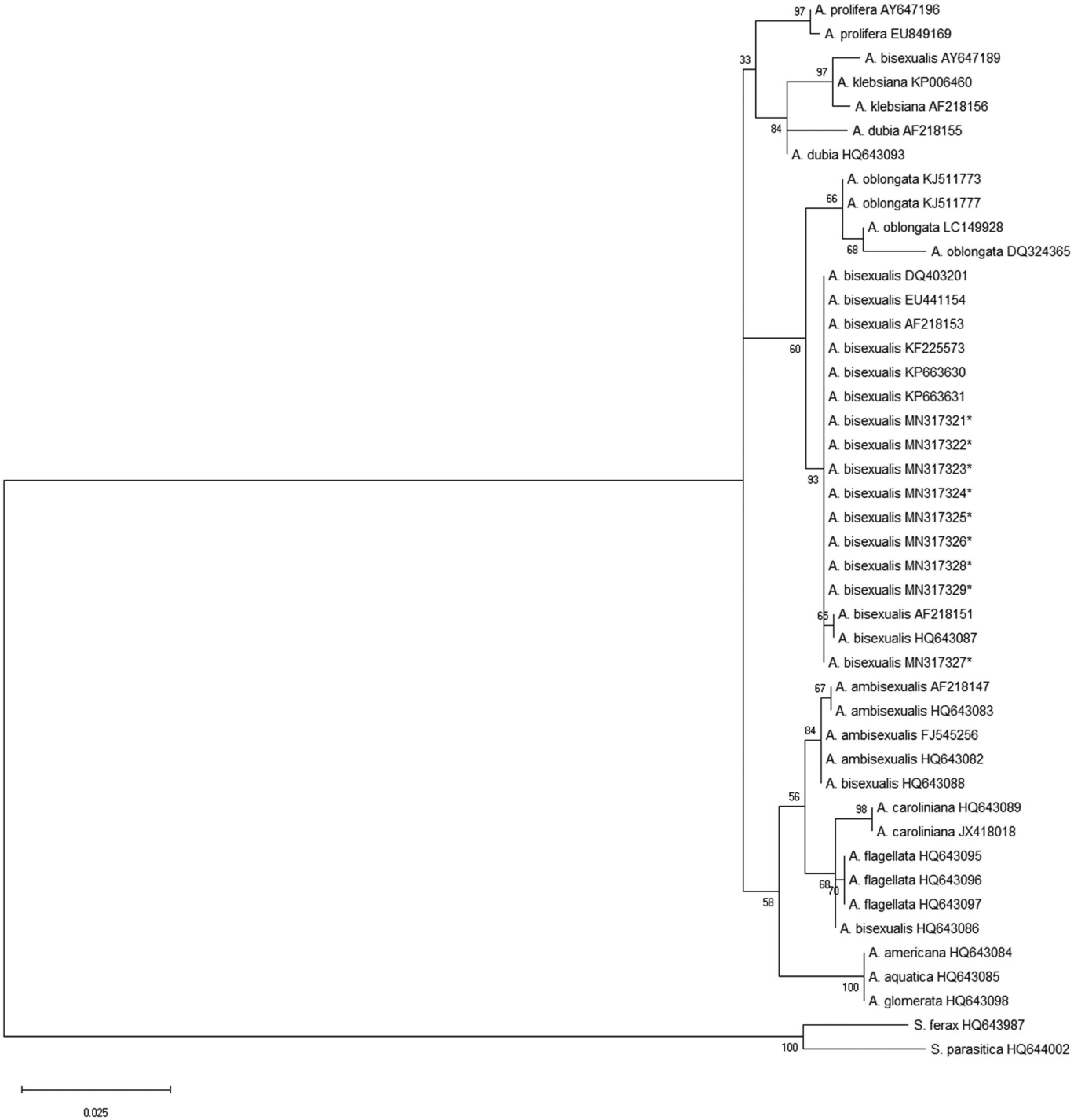

FIGURE 2. The maximum likelihood phylogenetic tree based on the ITS (rRNA) gene sequences of 9 *A. bisexualis* isolates from the present study, 11 other *Achlya* spp., and an outgroup with 2 *Saprolegnia* spp. The reliability of the inferred phylogenetic tree was assessed using the bootstrap test with 1,000 replicates. The numbers on the branches show the bootstrap values. The asterisk indicates isolates that were isolated during the present study.

species and their eggs (Chukanhom and Hatai 2004; Panchai et al. 2007; Hunjavanit et al. 2012). *Achlya* spp. have been confirmed as pathogenic agents causing mycoses in aquaculture, specifically in tropical environments where infection has been reported to affect economically important cultured fish species (Lawhavinit et al. 2002; Webster and Weber 2007; Lau et al. 2018). Infections with *Achlya* spp. are often more superficial, with a clearly visible,

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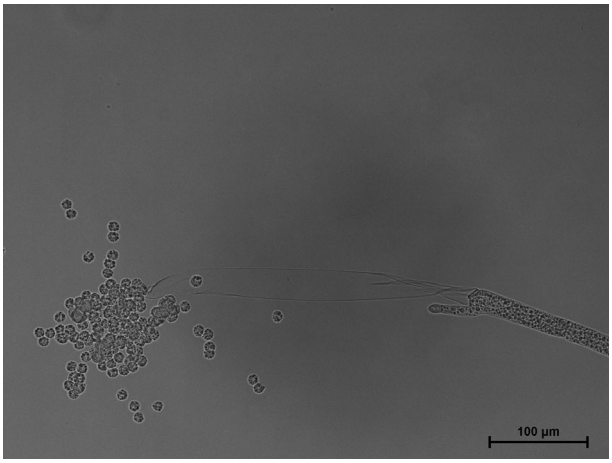

FIGURE 3. A micrograph showing the zoospore discharge pattern with zoospores encysted at the tip of the zoosporangium.

cotton, wool-like appearance of mycelia covering dermal erosions or skin lesions (Chukanhom and Hatai 2004; Andrew et al. 2008; Refai et al. 2016; Lau et al. 2018). The infectious agent from this study showed similar clinical signs, with superficial, cotton, wool-like mycelia covering the eye of an Orange Blotched Peacock Cichlid. Preliminary characterization classified the isolate within the order Saprolegniales due to the clearly visible terminal sporangia and zoospore discharge pattern. This was verified by observing zoospores being discharged through an apical pore from zoosporangia and accumulating at the tip of the sporangia. *Aphanomyces* spp. and *Achlya* spp. share this key mode of zoospore discharge. However, for *Saprolegnia* spp., zoospores are released in a saprolegnoid manner (i.e., when zoospores move quickly as a column through an open papilla at the tip of the sporangium and escape within seconds) (Leclerc et al. 2000; Webster and Weber 2007). Although the zoospore release type can be useful during presumptive diagnoses, it lacks specificity for identification to species level.

The maximum likelihood phylogenetic tree of the ITS region is presented in Figure 2. The ITS gene provides acceptable taxonomic resolution amongst closely-related oomycetes and was therefore amplified in this study (Robideau et al. 2011). The oomycete isolates from this study formed a monophyletic group with 8 of the 11 *A. bisexualis* strains (HQ643087, AF218151, KP663631, KP663630, KF225573, AF218153, EU441154, DQ403201) included in the phylogenetic analysis, with an average of 3.36×10^{-4} base substitutions per site between the sequences within this group (Figure 2), confirming the likelihood that our isolates are *A. bisexualis*. Three *A.*

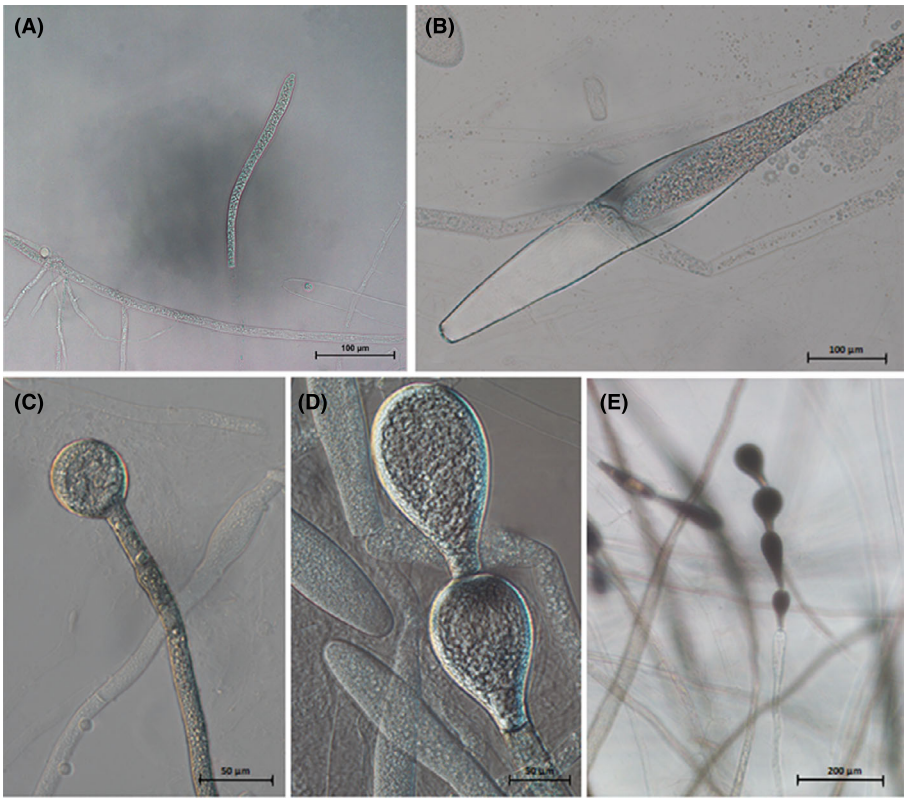

FIGURE 4. Micrographs showing **(A)** zoosporangium, **(B)** internal renewal of the zoosporangium, **(C)** spherical gemmae, **(D)** pyriform gemmae, and **(E)** gemmae of isolate *A. bisexualis* isolate ML18040 in moniliform,. [Color figure can be viewed at afsjournals.org]

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TABLE 2. A comparison of morphological characteristics of isolate ML18040 and *A. bisexualis*. The measurements for ML18040 are expressed as the mean ± SD (range), while those presented in the other studies are expressed as the range.

Morphological characteristic	ML18040 (present study)	*A. bisexualis* (Coker 1927)	*A. bisexualis* (Barksdale 1962)	*A. bisexualis* (Lawhavinit et al. 2002)
Zoosporangia				
Number	Abundant	n/a	n/a	Abundant
Shape	Clavate, filiform	Filiform	Fusiform, filiform	Fusiform
Width (μm)	17.61 ± 5.5 (11–30)	30–60	23–25	22–43
Length (μm)	258.11 ± 65.5 (164–425)	300–950	240–420	100–310
Zoospore discharge	Achlyoid	Achlyoid	Achlyoid	Achlyoid
Zoospores				
Shape	Spherical	Spherical	Spherical	n/a
Spherical diameter (μm)	11.61 ± 0.1 (11–12)	9.6–10.8	9.3–11.7	n/a
Gemmae				
Shape	Spherical, pyriform, obovate	Pyriform, flask-shape, nearly spherical, elongated	Spherical, pyriform, obovate, cylindrical, fusiform, filiform	n/a

bisexualis strains (AY647189, HQ643088, and HQ643086) were clumped within the *A. klebsiana, A. ambisexualis*, and *A. flagellate* groups, respectively. The absence of the *A. bisexualis* type strain sequence in the current available literature and online databases leads to uncertainty regarding the true *A. bisexualis* representative strain. Following the criteria recommended by Nilsson et al. (2012) in validating the reliability of sequences, it was concluded that the sequences in the database for the three *A. bisexualis* strains may have previously been given incorrect taxonomic annotations (Nilsson et al. 2006). This finding was based on the lack of available taxonomic and morphological information of strains and the strong representation of *A. dubia, A. ambisexualis,* and *A. flagellate* strains in the respective groups (Nilsson et al. 2012).

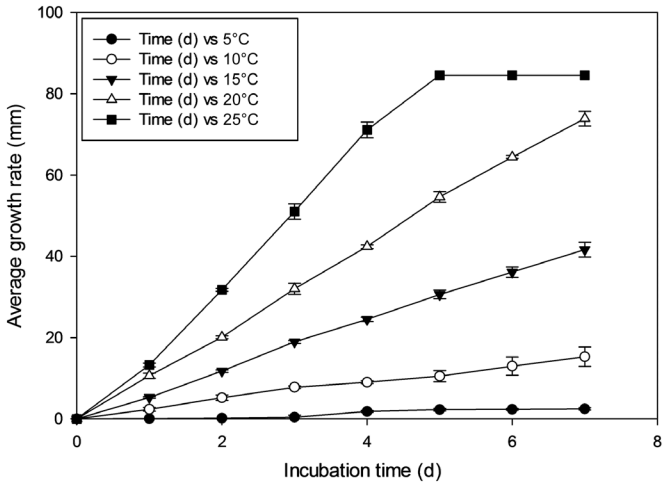

FIGURE 5. A graph showing the effect of temperature on vegetative growth (as the mean spherical diameter of the colony) of *A. bisexualis* over 7 d at various temperatures.

Morphological characteristics of our isolates conform to the original species description of Coker (1927). These characteristics also support the molecular findings, further confirming the likely identity of the isolate from this study as *A. bisexualis*. Spherical and pyriform gemmae were observed in isolate ML18040 at all five temperatures tested. Gemmae are produced during asexual reproduction and are regarded as important morphological characteristics. Neither oogonia nor oospores were observed in isolate ML18040 after 21 d of incubation at 25°C. Barksdale (1962) reported that *A. bisexualis* is the only *Achlya* species to produce spherical gemmae. Table 2 provides a summarized comparison of the morphological characteristics of isolate ML18040 from this study and the original species description of *A. bisexualis.*

Most *Achlya* spp. are able to grow across a broad range of temperatures (Lawhavinit et al. 2002; Chukanhom and Hatai 2004; Panchai et al. 2007; Hunjavanit et al. 2012). Isolate ML18040, identified in this study as *A. bisexualis,* was able to grow between 5°C and 25°C, with maximum vegetative growth between 20°C and 25°C and minimum growth at 5°C (Figure 5). The growth at 5°C (albeit minimal) that we observed is in contrast with Panchai et al. (2007), who reported no growth at 5°C after 4 d. Table 3 summarizes a comparison of the growth rates of isolate ML18040 and other closely-related species in the order Saprolegniales at different temperatures. Plates incubated at 25°C developed the typical, white, cotton-like colonies that reach full plate growth after 7 d. Plates that were incubated at 5°C not only survived, but

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TABLE 3. A comparison of radial colony growth rates after 4 d (measured as the diameter in mm) of isolate ML18040 (from the present study) with other closely-related species in the family Saprolegniaceae at different temperatures (°C) based on the literature.

Species	Temperature (°C)					Reference
	5	10	15	20	25	
Achlya bisexualis	7.30 ± 0.22 (6.90–7.40)	14.48 ± 0.35 (14.01–14.75)	29.98 ± 0.52 (29.47–30.48)	47.87 ± 0.46 (47.30–48.40)	76.61 ± 1.94 (75.54–79.53)	Present study
Achlya bisexualis	0	~2–10	~10–30	~30–36	~30–41	Panchai et al. (2007)
Achlya ambisexualis	n/a	0.0–2.4	19.0–39.8	64.4–65.8	65.8–85	Hunjavanit et al. (2012)
Achlya klebsiana	1.0	5.0	36.6	59.0	73.6	Chukanhom and Hatai (2004)
Saprlegnia diclina	n/a	21.6–38.4	61–85	85.0	85.0	Hunjavanit et al. (2012)
Saprolegnia diclina	16.0	39.6	75.0	75.0	75.0	Chukanhom and Hatai (2004)

demonstrated satisfactory growth after being re-incubated at 25°C. Lower temperatures clearly slow growth, and growth rate increases as temperatures increase. All of these physiological characteristics are corroborated by species descriptions from previous studies (Chukanhom and Hatai 2004; Panchai et al. 2007; Lau et al. 2018). Overall, *A. bisexualis* is well-adapted to staying viable across a wide range of temperatures and will likely flourish in tropical waters.

The oomycete that was isolated from the eye of the individual Orange Blotched Peacock Cichlid was identified as *A. bisexualis* and is the first report of the genus *Achlya* from South Africa. Little is currently known about the taxonomy of not only the genus *Achlya*, but of the entire Saprolegniales order as well; this makes it difficult to perform further studies to understand its pathology (Sandoval-Sierra and Diéguez-Uribeondo 2015). *Achlya bisexualis* shares key morphological characteristics with closely-related species in the order Saprolegniales; therefore, it is suggested that future diagnostics make use of a combination of morphological and molecular analyses as well as agent isolation and culture, instead of relying only on clinical signs and presumptive diagnostics. The information that will be gained from these molecular analyses and culture will not only assist in more accurate diagnostics, but may also contribute to the description, identification, and classification of these species.

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