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Morphological re-description and phylogenetic relationship of five myxosporean species of the family Myxobolidae infecting Nile tilapia

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ABSTRACT: Freshwater fish have a major economic and nutritional importance worldwide. Myxosporeans are highly dangerous parasites that infect different fish species, causing severe damage to a large number of economically important species, especially in aquaculture. We conducted a survey of myxosporean parasites infecting Nile tilapia *Oreochromis niloticus* (Perciformes: Cichlidae) collected from different localities along the River Nile passing through Giza province, Egypt. Out of 100 fish specimens collected, 45 were found to be naturally infected with these parasites in the region of the trunk kidney. Light microscopic examination revealed the presence of 5 distinct myxosporean species belonging to 2 different genera, viz. *Myxobolus* and *Triangula*, belonging to the family Myxobolidae; all 5 species have been previously described. Morphological characteristics, host specificity and geographical distribution, tissue tropism, and molecular analysis of the partial sequence of small subunit ribosomal DNA gene revealed that the recovered myxosporean species described herein were genetically distinct from other myxozoan species but had 95% sequence similarity to *M. cerebralis*. Also, phylogenetic analysis placed the present myxosporean species in the freshwater *Myxobolus* clade, which is a sister group of freshwater *Myxobolus*/*Henneguya* species.

KEY WORDS: Bivalvulida · Myxosporea · Light microscopic study · Molecular phylogeny · *Oreochromis niloticus*

INTRODUCTION

Myxosporidiasis is a disease caused by myxosporean parasites (Szczepaniak et al. 2011). Although these parasites are mostly extracellular, some species present intracellular stages which may be localized in the different organs and tissues of fish (Zhang et al. 2010) and rarely amphibians or reptiles (Moser & Kent 1994). The Phylum Myxozoa contains about 52 genera (Milanin et al. 2010) with up to 2300 described species (Kaur 2014). In Africa, approximately 135 myxosporean species are currently known to infect freshwater, brackish, and marine fish (Fomena et al. 2006). Fomena & Bouix (1997) counted 100 species in African freshwater fish belonging to different myxosporean genera, including *Myxobolus* Bütschli, 1882, *Myxobilatus* Davis, 1944, *Henneguya* Thélohan, 1892, *Sphaerospora* Thélohan, 1892, *Chloromyxum* Mingazzini, 1890, *Thelohanellus* Kudo 1933, *Myxidium* Bütschli, 1882, *Unicauda* Davis, 1944, and *Parahenneguya* Sakiti, 1997. The genus *Myxobolus* is the most speciose myxozoan, contain-

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ing approximately 850 species reported worldwide. Some of these species were described based only upon simple morphological features of the spores (Eiras et al. 2014).

In Egypt, parasitological research on Myxosporea infecting Nile fish is fragmentary. The first such study was conducted by El-Naffar (1970), followed by Fahmy et al. (1971), who described *Myxobolus niloticus* from Nile carp *Labeo niloticus* from the River Nile in Assiut Governorate. Fahmy et al. (1975) described 3 *Myxobolus* species, 1 species of *Myxidium*, and 1 species of *Cocomyxa* from Nile carp, as well as 3 *Myxobolus* species from Nile tilapia *Oreochromis niloticus* and 1 species of *Myxosoma* from tiger fish *Hydrocyon forskalii* from the River Nile in Assiut. Abdel-Ghaffar et al. (1995) described 5 *Myxobolus* species from Nile tilapia, blue tilapia *O. aureus,* binny *Barbus bynni*, and Nile carp; *Myxidium schalli* from wahrindi *Synodontis schall*; and *Henneguya branchialis* from African catfish *Clarias lazera*. Ali et al. (2002) described *Myxobolus naffari* infecting gills of Nile carp and mouth of binny, *M. caudatus* infecting the tail fin of binny*, M. fahmii* infecting gills of binny, *M. imami* infecting kidney of Nile carp, *M. intestinalis* infecting intestine of binny, and *M. perforata* infecting the operculum of tiger fish. Ali et al. (2003) described *M. stomum* infecting the oral cavity and lips of the black-spotted grunt *Plectorhynicus gaterinus*. Abdel-Ghaffar et al. (2008) described *Zschokkella nilei*, *Ortholinea africanus*, *Triangula egyptica*, *M. fomenai*, and *M. branchiophilus* from Nile tilapia in Bahr Shebin, Nile Tributary, River Nile. Abdel-Ghaffar et al. (2013) studied *Thelohanellus niloticus* infecting gills of Nile carp from the River Nile. Abdel-Ghaffar et al. (2015) described *M. niloticus* from the pectoral, dorsal, and tail fins of Nile carp, and *M. naffari* and *M. imami* from the kidney of binny and Nile carp. *M. caudatus* was found in the tail fin of binny, *M. fomenai* in the kidney and intestinal tissue of Nile tilapia, and *H. suprabranchiae* and *H. branchialis* in gills and suprabranchial organ of *Clarias gariepinus* from the River Nile, Giza Governorate. Abdel-Baki et al. (2015a,b) described *M. brachysporus* and *M. israelensis* from Nile tilapia.

Molecular analyses have become an important tool in the study of myxosporean parasites to complete the identification procedure and in combination with traditional methods such as spore morphology, exact location of sporulation, and tissue and host specificity to be widely applied to the taxonomy of these parasites (Bartošova et al. 2009, Evans et al. 2010, Iwanowicz et al. 2013) and to determine the phylogenetic position of myxosporeans inside metazoans (Evans et al. 2010, Mallatt et al. 2012). In addition, this has led to correct identification and differentiation of morphologically indistinguishable myxobolid species belonging to both genera of *Myxobolus* and *Henneguya* (Urawa et al. 2011, Liu et al. 2013).

Here we aimed to provide small subunit (SSU) rDNA sequence data for the recovered myxosporean parasites coupled with morphological re-descriptions and comparisons to other morphologically related myxosporean species.

MATERIALS AND METHODS

Fish sample collection and parasitological examination

In total, 100 freshwater specimens of Nile tilapia *Oreochromis niloticus* were randomly collected from different localities along the River Nile passing through Giza province (about 20 km) during summer (n = 50) and winter (n = 50) from June 2015 to July 2016. All fish were kept alive in glass aquaria, supplied with chlorine-free tap water with continuous aeration and filtration, and then transported immediately to the Laboratory of Parasitology Research in the Zoology Department, Faculty of Science, Cairo University, Egypt. Identification of fish specimens was carried out according to Eccles (1992). Collected fish samples were examined for the presence/absence of mature stages of myxospores. Gross external examination was carried out for the detection of any macroscopically visible lesions or cysts throughout the whole body length. Gills and internal organs were removed, washed, and microscopically examined for histozoic and coelozoic myxosporean parasites according to normal routine examination. For light microscopic examination, myxosporean cysts or plasmodia were collected and ruptured to release their spores. Fresh spores were examined using a Zeiss Axiovert 135 microscope and measured with an elaborated ocular micrometer. Measurements were based on 30 fresh mature spores, and data are presented as a range followed by the arithmetic mean and standard deviation in parentheses. Minimum and maximum values of spore measurements are provided in µm. Classification, measurement, and description of spores were carried out according to the guidelines of Lom & Arthur (1989). Line drawings of spores were made based on fresh wet mounts with the help of camera lucida (Weesner 1965). Parasite prevalence (total number of infected fish/total num-

ber of fish hosts examined × 100) of *O. niloticus* was calculated according to Bush et al. (1997).

Molecular methods

DNA extraction

Ethanol-preserved myxosporean samples were centrifuged at 5000 × *g* (10 min) to pellet myxospores. The supernatant was discarded, the spore pellet was dissolved in 500 µl lysis STE buffer (10 mM Tris–HCl [pH 8], 100 mM NaCl, 10 mM ethylenediaminetetraacetic acid [EDTA], 0.2% sodium dodecyl sulfate, and 0.4 mg Proteinase K) and incubated at 55°C for 3 to 4 h. Genomic DNA was extracted using a QIAamp DNA MiniKit (Qiagen) according to the manufacturer's instructions. The SSU rDNA gene cluster was targeted for PCR amplification.

PCR amplification

SSU rDNA genes were amplified with the universal primer pair 18e–18g′ (Table 1), in a 25 µl reaction mixture comprising 1 µl of extracted genomic DNA, 5 µl of 1 mM deoxyribonucleotide triphosphates (dNTPs, MBI Fermentas), 0.25 µl of each primer (50 pmol μl^{-1}), 2.5 µl of 10× *Taq* polymerase buffer (MBI Fermentas), 2 µl of 25 mM $MgCl_2$, 1 µl *Taq* DNA polymerase (2 U) (MBI Fermentas), and 13 µl of distilled water. The PCR cycle consisted of an initial denaturation step of 94°C for 4 min, followed by 35 cycles of 94°C for 50 s, 56°C for 50 s, 72°C for 80 s, and was finished with terminal extension at 72°C for 7 min, and then rested at 4°C. This was followed by a second round of nested PCR with the primer-pair specific for the family Myxobolidae, MX5–MX3. The total volume of the nested PCR reactions was 50 µl, which contained 1 µl of amplified DNA, 10 µl of 1 mM dNTP, 0.5 µl of each primer, 5 µl of 10× *Taq* buffer, 2.5 µl of 25 mM $MgCl_2$, 2 µl of *Taq* polymerase (2 U), and 28.5 µl of diethylpyrocarbonate-treated water. Amplification conditions in the second round were 94°C for 50 s, 56°C for 50 s, 72°C for 60 s for 30 cycles, and then terminated with an extension period at 72°C for 10 min, then rested at 4°C. The PCR products were electrophoresed in 1.0% agarose gel in Tris–acetate–EDTA (TAE)-buffered gel stained with 1% ethidium bromide, and bands of predicted sizes were purified using standard techniques (QIA-quick PCR purification KIT, Qiagen).

Sequencing

Three primer pairs were used for sequencing: MX5–MX3 described above, MC5–MC3, and MB3–MB5, based on the 18S rDNA sequences of *Myxobolus* species available in GenBank. Purified PCR products were sequenced in both directions using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems) with an ABI PRISM 310 Automatic DNA Sequencer (Applied Biosystems).

Phylogenetic analysis

The various forward and reverse sequence segments were aligned using CLUSTAL-X v1.83 (Thompson et al. 1997). The alignment was corrected manually using the alignment editor of the software MEGA 4.0 (Tamura et al. 2007). DNA sequence similarities were calculated with the Sequence Identity Matrix of the software BioEdit 4.8.9 (Hall 1999). Phylogenetic calculations were performed with PAUP 4.0b10 (Swofford 2000). The data were analyzed with maximum parsimony (close neighbor-interchange level 3, random addition trees 100). Also, neighbor-joining was calculated by MEGA 4.0 using

Table 1. Primers used in PCR and sequencing of the recovered myxosporean parasites

Primer	Sequence	GC content (%)	Melting temperature (°C)	Source
18e	5′-CTG GTT GAT TCT GCC AGT-3′	50	48	Hillis & Dixon (1991)
18g′	5′-CGG TAC TAG CGA CGG GCG GTG TG-3′	70	64	Hillis & Dixon (1991)
MX5	5′-CTG CGG ACG GCT CAG TAA ATC AGT-3	54	59	Andree et al. (1999)
MX3	5′-CCA GGA CAT CTT AGG GCA TCA CAG A-3′	52	59	Andree et al. (1999)
MC5	5′-CCT GAG AAA CGG CTA CCA CAT CCA-3′	54	59	Molnar et al. (2002)
MC3	5′-GAT TAG CCT GAC AGA TCA CTC CAC GA-3′	50	60	Molnar et al. (2002)
MB3	5′-GAT GAT TAA CAG GAG CGG TTG G-3′	50	55	Eszterbauer (2004)
MB5	5′-ACC GCT CCT GTT AAT CAT CAC C-3′	50	55	Eszterbauer (2004)

the Tamura-Nei model and pairwise deletion for gaps. Clade support was assessed with bootstrapping (100 replicates for maximum likelihood and maximum parsimony and 1000 replicates for neighbor-joining) to assess the relative robustness of the branches of the tree.

RESULTS

Macro- and microscopic examination revealed that 45 of the 100 collected Nile tilapia *Oreochromis niloticus* were naturally infected with 5 histozoic myxospores which were only detected in the region of the trunk kidney. No mixed infections were found. Seasonal prevalence of infection was recorded to be 28.0% during winter and 17.0% during summer (Table 2). All recovered mature myxosporean parasites belonged to 2 different genera, namely *Myxobolus* and *Triangula* (family Myxobolidae): *M. agolus*, *M. fomenai*, *M. brachysporus*, *M. tilapiae*, and *T. egyptica*.

Myxobolus agolus Landsberg (1985) (Fig. 1a,b)

Plasmodia: Plasmodia not found.

Spore description: The spores were oval to slightly pyriform, with a rounded and slightly wider posterior end, and measured 9.1–11.9 (9.8 ± 0.3) µm in length and 8.5–9.1 (8.7 ± 0.1) µm in width. Two elongated and pyriform polar capsules were hanging at the anterior end of the spore and extended about two-thirds of the spore length and measured 4.8–6.7 (6.3 ± 0.2) µm in length and 3.1–3.8 (3.5 ± 0.1) µm in width. The polar filament turned in 8 to 9 coils along the inner wall of the capsule. The sporoplasm occupied the remaining third of the spore cavity. A large and rounded iodinophilous vacuole was located in the sporoplasm.

Remarks: Nile tilapia were naturally infected with mature spores of *M. agolus* inhabiting the trunk kidney, with a prevalence of infection reaching 8.0% throughout the whole year. This prevalence is lower than that reported by Abdel-Baki et al. (2015b), who found that the infection rate of *O. niloticus* with *M. tilapiae* and *M. agolus* was 61% for both species combined. This myxosporean parasite resembles many other related species such as *M. microcystus* Price and Mellen, 1980 from the gills of largemouth bass *Micropterus salmoides* in the USA; *M. agolus* Landsberg, 1985 from the blue tilapia *O. aureus* and Nile tilapia *O. niloticus* in Israel; *M. cotti* El-Matbouli and Hoffmann, 1987 from the brain and spinal cord of the European bullhead *Cottus gobio* in Germany; *M. zillii* Sakiti et al. 1991 from gills of the redbelly tilapia *Tilapia zillii* in Benin; and *M. cognati* Cone et al. 1996 from the skin of the slimy sculpin *C. cognatus* in Canada (Table 3). *M. microcystus* differs from the present species in having spores with a different number of polar filament turns (6–7 vs. 8–9). *M. cotti* also has a different number of polar filament turns (7–8 vs. 8–9) and also differs from *M. agolus* described here in having spores with a pointed anterior end. *M. cognati* can also be distinguished by its larger spores and the sutural folds present at their posterior margin. The only 2 species which closely resemble the species described here are *M. zillii* and *M. agolus*. Of these, however, *M. zillii* exhibits relatively smaller polar capsules confined in an intercapsular process. All these species show different host and sites of infection from the present form. Consequently, *M. agolus* resembles the present species in shape and size.

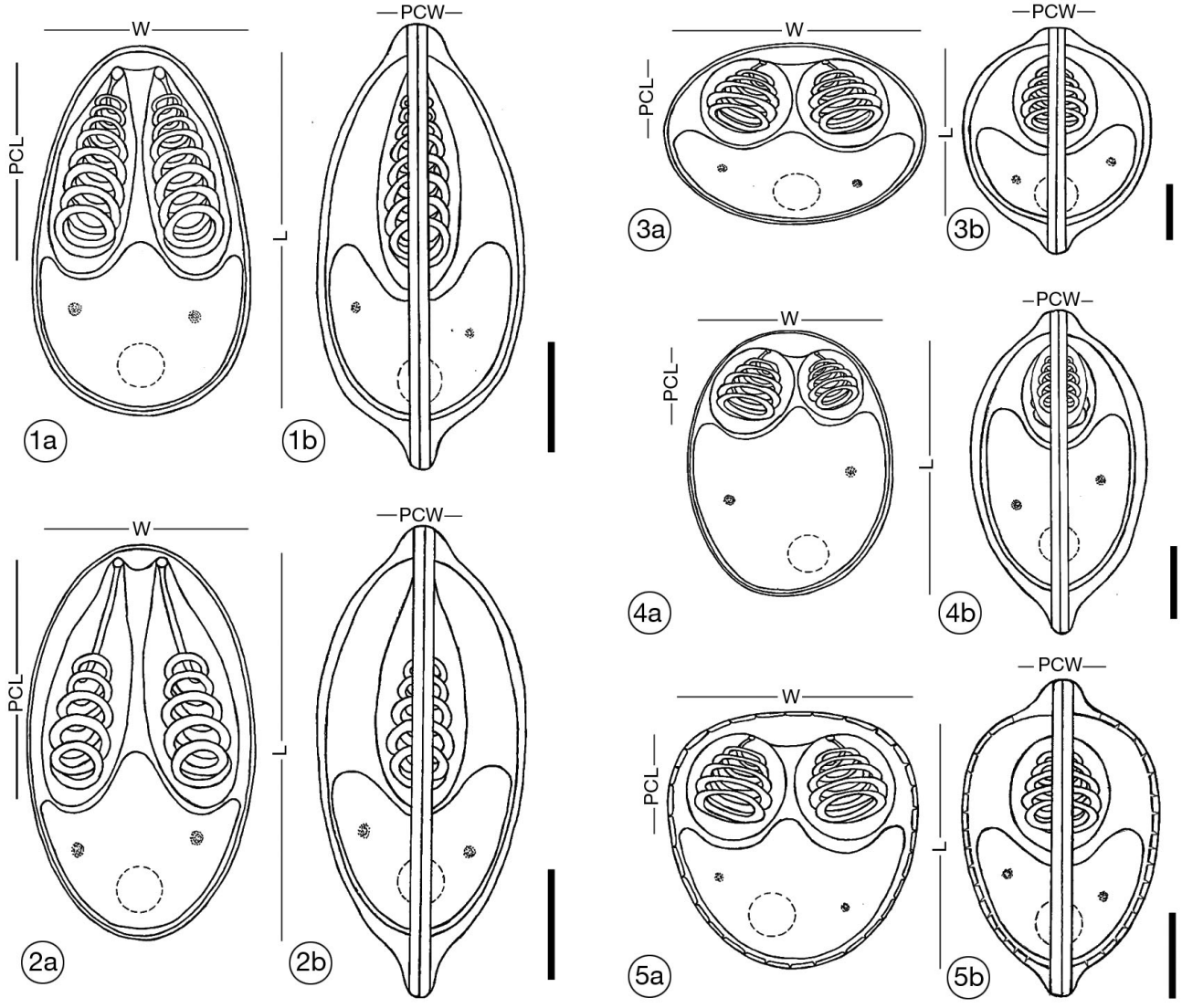
Myxobolus fomenai Abdel-Ghaffar et al. (2008) (Fig. 2a,b)

Plasmodia: Plasmodia were observed to be whitish in color, rounded to oval, and measured 0.23–0.41 (0.35 ± 0.01) mm in diameter.

Spores description: Spores were pyriform with a pointed anterior end and rounded posterior end in valvular view and fusiform in sutural view. A small intercapsular process was observed at the apex of the spore. Spores measured 14.1–15.4 (14.9 ± 0.2) µm in length and 7.8–9.2 (8.8 ± 0.2) µm in width. Two large

Table 2. Prevalence of infection for the recovered mature stages of myxosporean parasites found in the trunk kidney of infected Nile tilapia *Oreochromis niloticus* (Cichlidae) collected at different localities along the River Nile, Giza Province, Egypt. Voucher specimens were deposited in the Zoology Department, Faculty of Science, Cairo University, Egypt. Values are percentages (number of infected fish/number of sampled fish)

Season	*Myxobolus agolus*	*Myxobolus fomenai*	*Myxobolus brachysporus*	*Myxobolus tilapiae*	*Triangula egyptica*
Total	8.0 (8/100)	14.0 (14/100)	12.0 (12/100)	6.0 (6/100)	5.0 (5/100)
Winter	12.0 (6/50)	22.0 (11/50)	14.0 (7/50)	8.0 (4/50)	10.0 (5/50)
Summer	4.0 (2/50)	6.0 (3/50)	10.0 (5/50)	4.0 (2/50)	0.0 (0/50)



Figs. 1 to 5. Line drawings made with camera lucida of the spores of 5 myxosporean parasites recovered in the present study. Fig. 1. *Myxobolus agolus*. Fig. 2. *M. fomenai*. Fig. 3. *M. brachysporus*. Fig. 4. *M. tilapiae*. Fig. 5. *Triangula egyptica*. In all figures, (a) shows the frontal view, (b) shows the lateral view. Scale bars = 5 µm. PCL: polar capsule length; PCW: polar capsule width; L: length; W: width

and elongated polar capsules located at the anterior end of the spores reached almost three-quarters of the whole spore length and measured 9.0–9.8 (9.4 ± 0.1) µm in length and 2.4–3.4 (2.9 ± 0.1) µm in width. The polar filament coiled spirally in 5 to 6 loops arranged along the inner wall of the polar capsule in an oblique pattern. The filament occupied the hind two-thirds of the whole capsule and displayed a convergent anterior end. The sporoplasm was binucleated, finely granulated, and restricted only to a small portion in the spore cavity. A large and rounded iodinophilous vacuole was located in the sporoplasm.

Remarks: Nile tilapia were found to be naturally infected with mature spores of *M. fomenai* inhabiting the trunk kidney, with a prevalence of infection of 14.0%. These results are lower than data obtained by Abdel-Ghaffar et al. (2015), who reported that the infection rate was 49.46%. Spore measurements of the present species were different from the majority of the previously described species (Table 4). Our spore observations agreed with those recorded by Abdel-Ghaffar et al. (2008, 2015) from muscles, kidney, and intestine of *O. niloticus*. The current spores were smaller than in other described species such as *M. heterosporus* Baker (1963) from *O. niloticus*, *M. moruliensis* Sarkar et al. (1985) from bullseye snakehead *Channa marulius*, and *M. amieti* Fomena et al. (1985) from dwarf climbing perch *Ctenopoma na-*

Table 3. Comparative measurements (in µm) of *Myxobolus agolus* described in this study and other species described previously. Measurements are the range, with mean (±SD, where provided) in parentheses. (–) no information provided

Species	Host	Site of infection	Locality	Spore		Polar capsule		Reference
				Length	Width	Length	Width	
Myxobolus cotti	*Cottus gobio*	Central nervous system	Alpine lake (Königssee, Germany)	8.9–15.11 (12.4)	8.01–12.42 (9.62)	4.34–9.01 (6.44)	2.07–3.76 (2.89)	El-Matbouli & Hoffmann (1987)
M. zillii	*Tilapiae zillii*	–	–	8–11 (9.8)	6–8 (7.5)	4–6 (5.1)	2–3 (2.5)	Sakiti et al. (1991)
M. cognati	*C. cognatus*	Opercular integument	Lake Michigan, USA	12–14 (13.3)	9.5–10.5 (10)	5.5–7.5 (6.6)	3	Cone et al. (1996)
M. agolus	*Oreochromis niloticus*	Kidney	Egypt	9.9–11.6 (10.9±0.6)	8.2–8.7 (8.5±0.1)	5.4–7.1 (6.3±0.5)	2.8–3.5 (3.1±0.3)	Abdel-Baki et al. (2015b)
M. agolus	*O. niloticus*	Trunk kidney	Egypt	9.1–11.9 (9.8±0.3)	8.7–9.1 (8.5±0.1)	4.8–6.7 (6.3±0.2)	3.1–3.8 (3.1±0.1)	This study

num, and they presented different tissue tropisms. In addition, the present species could be distinguished from *M. koi* Kudo (1919) recorded from the gills of common carp *Cyprinus carpio* in Japan, which have polar capsules with heavily coiled filaments that fill the entire capsule, versus the filaments in the present species that fill only the hind two-thirds of the capsules. The morphological and morphometric data recorded here closely resemble data for the same species recorded by Abdel-Ghaffar et al. (2008, 2015).

Myxobolus brachysporus Abdel-Baki et al. (2015a) (Fig. 3a,b)

Plasmodia: Plasmodia not found.

Spore description: The spores were ellipsoidal in shape, characterized by their width (11.9–13.6 [12.51 ±0.2] µm), which greatly exceeded their length (7.2–8.9 [8.2±0.2] µm). The polar capsules were subcircular, mostly equal in size, and measured 4.6–5.8 (4.8±0.1) µm in length and 3.4–4.9 (4.2±0.1) µm in width. The polar filament coiled in 5–6 turns perpendicular or slightly oblique to the longitudinal axis of the capsule. The sporoplasm filled up the rest of the spore cavity. A large and rounded iodinophilous vacuole was located in the sporoplasm.

Remarks: Nile tilapia were naturally infected with mature spores of *M. brachysporus* inhabiting the trunk kidney, with 12.0% prevalence of infection. These results are lower than data obtained by Abdel-Baki et al. (2015a), who reported a 51.9% infection rate of *O. niloticus* with *M. brachysporus*. The morphological and morphometric data of the present spores were in agreement with the same species recorded previously by Baker (1963) from Singida tilapia *O. esculentus* and by Abdel-Baki et al. (2015a) from *O. niloticus*, in addition to having the same host specificity and tissue localization as shown in Table 5. While the specimens examined here closely resembled the *M. brachysporus* reported by Abdel-Baki et al. (2015a), they differed from the *M. brachysporus* reported by Baker (1963) in having larger spore measurements and different host species. When *M. artus* Akhmerov (1960) described from common carp *Cyprinus carpio* and *M. jahricei* Landsberg & Lom (1991) described from small mouth buffalo *Ictiobus bubalus* are compared to other related species, some resemblance in the general morphology can be found. However, the polar capsule size and angle of orientation of these 2 species differs from the present species.

Myxobolus tilapiae Abolarin (1974) (Fig. 4a,b)

Plasmodia: Plasmodia not found.

Spore description: The spores were relatively large, ellipsoidal to oval, with rounded anterior and posterior ends. The posterior end appeared slightly wider than the anterior end. The spore measurements were 14.9–15.8 (15.3±0.2) µm in length and 9.5–10.8 (10.3±0.1) µm in width. The 2 polar capsules were slightly unequal, oval, and located at the anterior end of the spore. They occupied about one-quarter of the entire spore length and measured 4.7–6.4 (5.6±0.1) µm in length and 3.1–4.2 (3.5±0.1) µm in width. The polar filaments were closely coiled within the polar capsules in 5 to 6 turns and were situated obliquely to the longitudinal axis of the polar capsule. The remaining space of the spore behind the polar capsules was filled by binucleated sporoplasm with a rounded iodinophilous vacuole.

Table 4. Comparative measurements (in µm) of *Myxobolus fomenai* described in this study and other species described previously. Measurements are the range, with mean (±SD, where provided) in parentheses. (–) no information provided

Species	Host	Site of infection	Locality	Spore		Polar capsule		Reference
				Length	Width	Length	Width	
Myxobolus heterosporus	*Oreochromis niloticus*	Gills	Cameroon	13–16 (14.65)	7–9.2 (7.97)	8–10.5 (9.47)	2–3.5 (2.8)	Baker (1963)
M. moruliensis	*Channa marulius*	–	India	12–17.5 (15.94)	2.5–5 (4.23)	7.5–13 (10.05)	1–2 (1.55)	Sarkar et al. (1985)
M. amieti	*Ctenopoma nanum*	Gills and eye	Cameroon	11.3–15.8 (14.6)	5.4–8.7 (7.4)	6–10 (8.49)	1.4–2.5 (1.96)	Fomena et al. (1985)
M. fomenai	*O. niloticus*	Muscles	Egypt	13.09–16.5 (15.02)	6.16–9 (7.91)	8.47–0.13 (9.26)	2.31–3.5 (2.77)	Abdel-Ghaffar et al. (2008)
M. fomenai	*O. niloticus*	Intestine	Egypt	14.0–15.2 (14.4±0.3)	8.7–9.5 (9.0±0.3)	9.0–9.4 (9.2±0.12)	2.5–3.5 (2.8±0.2)	Abdel-Ghaffar et al. (2015)
M. fomenai	*O. niloticus*	Trunk kidney	Egypt	14.1–15.4 (14.9±0.2)	7.8–9.2 (8.8±0.2)	9.4–9.8 (9.5±0.1)	2.4–3.4 (2.9±0.1)	This study

Table 5. Comparative measurements (in µm) of *Myxobolus brachysporus* described in this study and other species described previously. Measurements are the range, with mean (±SD, where provided) in parentheses. (–) no information provided

Species	Host	Site of infection	Locality	Spore		Polar capsule		Reference
				Length	Width	Length	Width	
Myxobolus artus	*Cyprinus carpio*	–	–	6.5–8.5	9–12	4–6	2.3–5	Akhmerov (1960)
M. brachysporus	*Oreochromis esculentus*	Kidney	Egypt	7–7.5 (7.3)	12–13.5 (12.5)	2.5–3.8 (3.1)	2.3–2.5 (2.3)	Baker (1963)
M. jahnricei	*Ictiobus bubalus*	–	–	12.4	15.5	7	4.4	Landsberg & Lom (1991)
M. brachysporus	*O. niloticus*	Kidney	Egypt	7.8–9.2 (8.6±0.4)	12.1–14.2 (13.7±0.6)	4.2–5.14 (4.7±0.3)	3.2–4.2 (3.6±0.3)	Abdel-Baki et al. (2015a)
M. brachysporus	*O. niloticus*	Trunk kidney	Egypt	7.2–8.9 (8.2±0.2)	11.9–13.6 (12.5±0.2)	4.6–5.8 (4.8±0.1)	3.4–4.9 (4.2±0.1)	This study

Remarks: Nile tilapia were naturally infected with mature spores of *M. tilapiae* inhabiting the trunk kidney, with 6.0% prevalence of infection, which was lower than the rate obtained by Abdel-Baki et al. (2015b), who reported that the active plasmodial spores of *M. tilapiae* and *M. agolus* in *O. niloticus* was 61%. The shape and measurements of the present species resembled those of many other previously described related species, including *M. heterosporus* Baker, 1963 described from *O. niloticus*; *M. tilapiae* Abolarin, 1974 from *O. niloticus*; *M. polycentropsis* Fomena et al., 1985 from the African leaf fish *Polycentropsis abbreviata*; *M. tilapiae* Reed et al., 2002 from the redbreast tilapia *Tilapia rendalli* in the Okavango River, Botswana; and *M. tilapiae* Abdel-Baki et al., 2015 from *O. niloticus* in Egypt (Table 6). The spores of the present species have spherical polar capsules, while those of *M. heterosporus* have pyriform polar capsules. *M. polycentropsis* differs in having thinner spores with smaller pyriform polar capsules. The shape and measurements of the present species were very similar to those of *M. tilapiae* originally described by Abolarin (1974) from freshwater tilapia in Nigeria, and by Reed et al. (2002) and Abdel-Baki et al. (2015b) from Egypt.

Triangula egyptica Abdel-Ghaffar et al. (2008) (Fig. 5a,b)

Plasmodia: Plasmodia were microscopic, rounded, polysporous, and measured 50–65 (61.47 ± 2.5) µm in diameter.

Spore description: The spores were triangular, with a blunt anterior end and tapering into a rounded posterior end. The 2 shell valves were symmetrical and characterized by relatively thickened sutural edges and a large prominent intercapsular process.

Distinct sutural markings (n = 9–15) were arranged all around the circumference of the spore, being more widely spaced posteriorly than at the anterior end. The spores measured 12.32–13.92 (13.12 ± 0.2) µm in length and 10.21–11.99 (11.56 ± 0.2) µm in width. The sub-spherical polar capsules occupied the anterior half of the spore body and measured 4.32–5.34 (4.75 ± 0.1) µm in length and 3.95–4.87 (4.21 ± 0.1) µm in width. The polar filament coiled into 5 to 6 turns, coiling tightly along the inner wall of the capsule and arranged perpendicular to the longitudinal axis of the polar capsule. The large and rounded iodinophilous vacuole was located in binucleated sporoplasm.

Remarks: Nile tilapia were naturally infected with mature spores of *T. egyptica* inhabiting the trunk kidney, with a prevalence of infection of 5.0%. These results were slightly lower than data obtained by Abdel-Ghaffar et al. (2008), who reported that the infection rate of *O. niloticus* with *T. egyptica* was 9.21%. Only 5 species of *Triangula* have been described to date: *T. percotti* Akhmerov, 1960 from fins and gills of the big-mouthed sleeper *Gobiomorus dormitor* in eastern Asia; *T. yangkiangensis* Chen and Hsieh, 1984 from the skin and gills of the goby *Rhinogobius giurinus* in China; *T. percae* Langdon, 1987 from the brain of the European perch *Perca fluviatilis* in Australia; *T. illinoisensis* Lom and Cone, 1996 from the gills of the bigmouth buffalo *Ictiobus bubalus* in the USA; and *T. egyptica* Abdel-Ghaffar et al., 2008. *T. percotti* and *T. yangkiangensis* can be differentiated by the recognizable small size of their spores in comparison with those of the present study. Spores of *T. percae* differ from those of the present species in the sharp triangular shape compared with the rounded triangular spores of the spores described here. *T. illinoisensis* has a recognizable asymmetrical oval and vacuolated posterior end of the spores, while the present species had rounded triangular spores with rounded posterior ends. All of these morphological features of the previously described species differed from those of the present species. Moreover, the site of infection, host, and locality of the previously discussed species differed markedly in contrast with those described here (Table 7). Taking these differences into consideration, it appeared that the present species does not satisfy the characters of any species except that recorded by Abdel Ghaffar et al. (2008) with which it shares very close morphological and morphometric data.

Phylogenetic analysis

Molecular analyses based on partial 18 SSU rDNA gene sequences were performed to investigate the taxonomy and classification of the recovered myxosporean parasites. The amplified and sequenced SSU rDNA gene regions and GC content for these parasites were 1400 (40.85%), 1377 (40.59%), 1339 (40.55%), 1348 (40.50%), and 1395 (40.86%) bp in length and were deposited in GenBank under accession numbers KX632948, KX632947, KX632949, KX632950, and KX632951 for *M. agolus*, *M. fomenai*, *M. brachysporus*, *M. tilapiae* and *T. egyptica*, respectively. The obtained gene sequences were compared with each other and with other sequences from dif-

Table 6. Comparative measurements (in µm) of *Myxobolus tilapiae* described in this study and other species described previously. Measurements are the range, with mean (±SD where provided) in parentheses. (–) no information provided

Species	Host	Site of infection	Locality	Spore		Polar capsule		Reference
				Length	Width	Length	Width	
Myxobolus heterosporus	*Oreochromis niloticus*	–	Africa	8.5–17.0 (12.5)	6.5–11 (8.3)	2.0–5.5 (4.1)	1.5–3.5 (2.3)	Baker (1963)
M. polycentropsis	*Polycentropsis abbreviata*	–	–	11.8–14.4 (13.2)	5.6–10.0 (7.0)	3.5–6.4 (4.0)	1.5–2.3 (1.7)	Fomena et al. (1985)
M. tilapiae	*Tilapia zillii*	–	Nigeria	14.0–15.5) (15 ± 0.4	12.0–12.6 (12.3±0.3)	3.8–5.0 (4.6±0.6)	3.0–4.0 (3.5±0.4)	Abolarin (1974)
M. tilapiae	*T. rendalli*	Buccal cavity	Botswana	14.0–15.5 (15.0±0.39)	12.0–12.6 (12.3±0.27)	3.8–5.0 (4.6±0.55)	3.0–4.0 (3.5±0.44)	Reed et al. (2002)
M. tilapiae	*O. niloticus*	Kidney	Egypt	11.4–14.2 (13.1±0.7)	8.5–12.8 (11.1±0.9)	3.5–5.7 (5.1±0.6)	3.0 4.9 (4.1±0.4)	Abdel-Baki et al. (2015b)
M. tilapiae	*O. niloticus*	Trunk kidney	Egypt	14.9–15.8 (15.3±0.2)	9.5–10.8 (10.3±0.1)	4.7–6.4 (5.6±0.1)	3.1–4.2 (3.5±0.1)	This study

Table 7. Comparative measurements (in µm) of *Triangula egyptica* described in this study and other species described previously. Measurements are the range, with mean (±SD where provided) in parentheses. (–) no information provided

Species	Host	Site of infection	Locality	Spore		Polar capsule		Reference
				Length	Width	Length	Width	
Triangula percotti	*Gobiomorus dormitor*	Fins and gills	East Asia	8	8	3	3	Akhmerov (1960)
T. yangkiangensis	*Rhinogobius giurinus*	Skin and gills	China	9.6	9.1	3	2.8	Chen & Hsieh (1984)
T. percae	*Perca fluviatilis*	Brain	Australia	10.6	10.4	3.8	3	Langdon (1987)
T. illinoisensis	*Ictiobus bubalus*	Gills	USA	–	–	4.4–5.2 (4.9)	3.9–4.5 (4.2)	Lom & Cone (1996)
T. egyptica	*Oreochromis niloticus*	Kidney	Egypt	11.55–14.63 (12.85)	9.24–12.32 (11.22)	3.85–5.39 (4.69)	3.08–4.62 (3.91)	Abdel-Ghaffar et al. (2008)
T. egyptica	*O. niloticus*	Trunk kidney	Egypt	12.32–13.92 (13.12±0.2)	10.21–11.99 (11.56±0.2)	4.32–5.34 (4.75±0.1)	3.95–4.87 (4.21±0.1)	This study

ferent geographic regions available in GenBank. BLAST searches demonstrated that these sequences exhibit a close relationship with those of other myxosporeans that have been previously sequenced, but they were not identical to any of them.

Two clades were clustered during the construction of the phylogenetic tree using maximum likelihood and maximum parsimony of the 5 parasite species described herein (Fig. 6). The major clade represented the species of the phylum Myxozoa and was divided into 2 branches: the marine branch was represented by the order Multivalvulida (*Kudoa* species), while the freshwater branch was represented by the order Bivalvulida (*Myxobolus, Henneguya*, *Myxidium*, *Sphaeromyxa*, and *Zschokkella* spp.). We recorded a single exception for the presence of a freshwater species, viz. *Ceratomyxa shasta*, that infects salmonid fish but clustered with the marine myxozoan clade, in addition to a great similarity to the narcomedusae *Polypodium hydriforme*. Calculation of the percentage of identity between these novel sequences and a range of sequences for other mxyosporeans from other hosts demonstrated a high degree of similarity, up to 95%, with intra-specific differences that varied from 0.5 to 3.9% with all *Myxobolus* species available in GenBank. The results revealed that the recovered myxosporean parasites described herein are deeply embedded within the genus *Myxobolus*, which belonged to the clade containing *M. cerebralis* with a high bootstrap value and a paraphyletic origin. The minor clade included species belonging to the phylum Cnidaria and was represented by the classes Hydrozoa, Cubozoa, and Scyphozoa. This places the Myxozoa as a sister taxon to Cnidaria.

DISCUSSION

Tilapia are among the most productive and internationally traded food fish in the world (Yakubu et al. 2014). The production of farmed tilapia is among the fastest expanding food sectors globally (Soto-Zarazúa et al. 2010). Nile tilapia *Oreochromis niloticus* is the most cultured freshwater species among the farmed tilapia and contributes about 71% of the world total tilapia production (Soto-Zarazúa et al. 2010). In this study, a total of 45 out of 100 examined fish specimens were found to be infected with spores of myxosporean parasites. Infections by these parasites may occur to a lower degree in fish organs as a result of tissue tropism or as a consequence of systemic infection. Thus, infections have been reported in various organs such as gills, fins, intestine, heart, kidney, brain, and ovaries (Lewisch et al. 2015, Abdel-Ghaffar et al. 2016). Some parasite species may cause economic losses through induction of post-mortem myoliquefaction of fish muscles in addition to the production of hardly visible cysts in fish musculature (Schmidt-Posthaus et al. 2012).

The rate of parasitism was higher during winter than summer due to seasonal changes representing a combination of many factors influencing the success of parasites to penetrate different hosts. Zhang et al. (2010) observed the seasonal occurrence of plasmodia-forming myxosporeans, including *Kudoa ciliatae* infecting trumpeter whiting *Sillago maculata*; *Thelohanellus wuhanensis* and *Myxobolus gibelioi* infecting goldfish *Carassius auratus*; and *M. intimus* infecting common roach *Rutilus rutilus*, which showed the highest prevalence and abundance in winter and the lowest in spring and summer

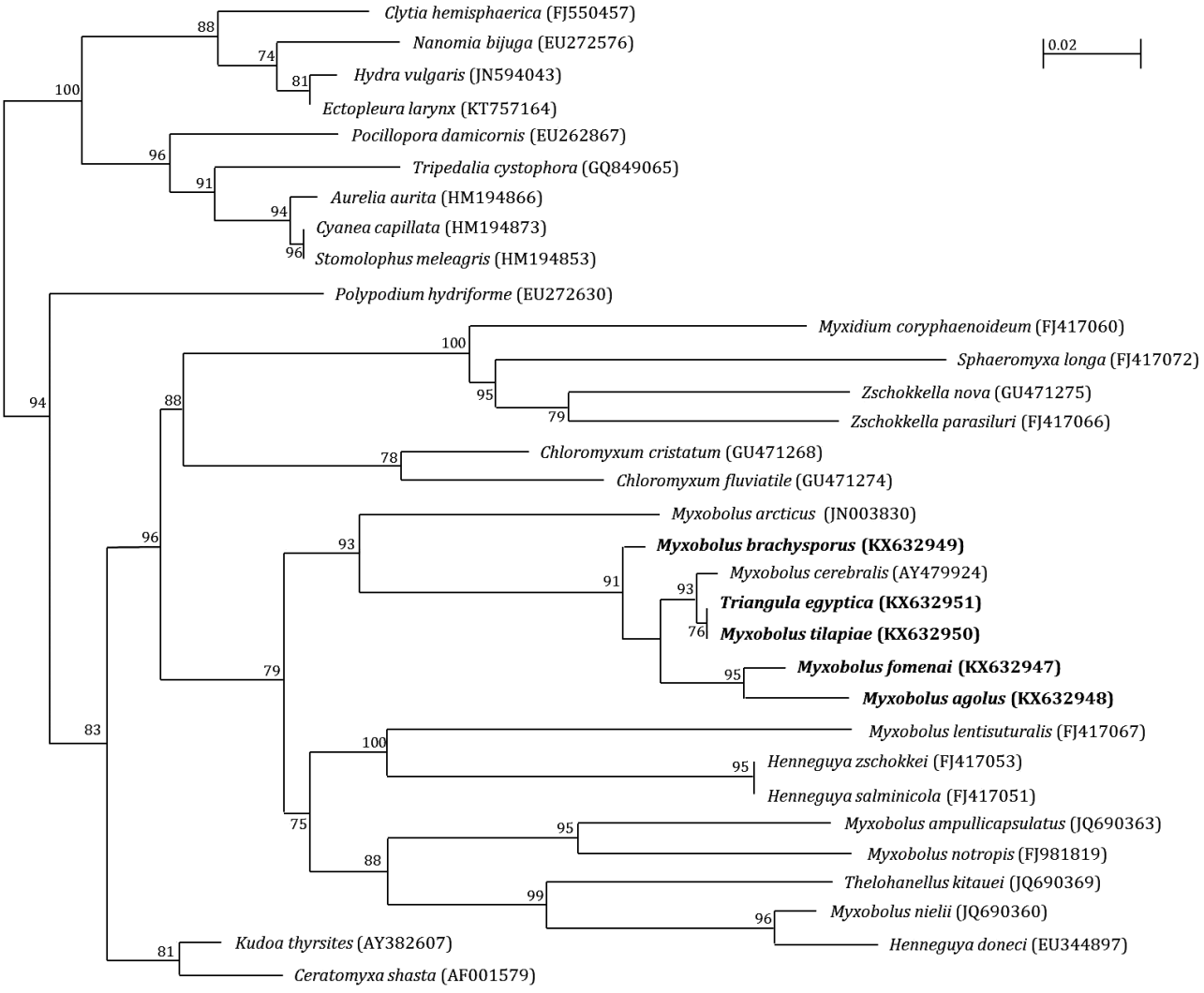


Fig. 6. Phylogenetic tree generated by maximum parsimony analyses of the 18S rDNA sequences. Numbers at nodes indicate bootstrap confidence values (100 replications). GenBank accession numbers are given in parentheses. Myxosporeans examined in the present study are highlighted in **bold**

seasons. They added that rising water temperature in spring promotes cyst rupture and loss. This fluctuation in the infection rate and seasonal changes were explained by Cone (1994), who mentioned that the myxosporean *Henneguya doori* had an annual developmental cycle on the gills of the adult yellow perch *Perca flavescens*, and this cycle involved a period of summer invasion, fall development of plasmodia and initiation of sporogenesis, winter completion of sporogenesis, and spring release of spores.

Our study included 5 myxosporean parasites. Identification of the different myxosporean species, especially of *Myxobolus* species based only on morphological and morphometric criteria, is rather difficult due to their great similarity. During the past 2 decades, use of genetic sequence information has clearly offered a powerful diagnostic tool that is capable of clarifying the identity and relationships between morphologically close myxosporean species (Halanych et al. 1995). Iwanowicz et al. (2008) and Bahri et al. (2010) stated that phylogenetic analyses based on SSU rDNA suggest that spore morphology is of minor importance in phylogenetic relationships due to the frequent change in spore shape during myxosporean evolution.

The phylogenetic tree in our study, constructed using maximum likelihood and maximum parsimony, confirmed that myxozoans are not protists and showed a great similarity to narcomedusae *Polypodium hydriforme*. This postulation was already stated by Stolc (1899), Weill (1938), Dogeil (1965), and Grasse & Lavette (1978), who claimed that myxozoans

are not protists and asserted that their spores are multicellular and should be included within Metazoa. Siddall et al. (1995) showed that myxozoans nested within the Cnidaria as a sister group of the narcomedusan fish parasite *P. hydriforme*. Ultrastructural comparisons, particularly comparing polar capsules, nematocysts, and nucleotide data, showed close similarity between Myxozoa and Cnidaria (Dyková & Lom 1997, Siddall & Whiting 1999). Our phylogenetic tree indicated that marine and freshwater taxa belonging to the myxozoan clade are separated into 2 major branches. These data coincide with those of Kent et al. (2000), who stated that the marine branch is represented by the order Multivalvulida (*Kudoa* species), while the freshwater branch is represented by the order Bivalvulida (*Myxobolus, Henneguya*, *Myxidium*, *Sphaeromyxa*, and *Zschokkella* spp.). We recorded a single exception to this based on the presence of the freshwater species *Ceratomyxa shasta* that infects salmonid fish and clustered with the marine myxozoan clade. In addition, *Myxobolus* species appeared to be a paraphyletic group, in agreement with Andree et al. (1999) and Smothers et al. (1994), who reported that 18S rDNA sequence data did not support the phylogenetic separation of the 2 major genera *Henneguya* and *Myxobolus*.

Species of the genus *Myxobolus* are mostly histozoic, infecting various tissues. Shulman (1966) suggested that histozoic species evolved from coelozoic myxosporeans. The overall results of our phylogenetic analysis based on SSU rDNA support this hypothesis. The recovered myxospores corresponded to histozoic species infecting trunk kidney and clustered with other histozoic *M. cerebralis* within the *Myxobolus*/*Henneguya* clade. These data coincided with Andree et al. (1999), who suggested that members of the genus *Myxobolus* tend to group by tissue locations. In contrast, Salim & Desser (2000) used partial 18S rDNA sequences of 7 different *Myxobolus* species from cyprinid fish and found that they group by spore morphology. The recovered parasite species exhibited up to 95% similarity with other *Myxobolus* species, especially *M. cerebralis*. These results coincided with data obtained by Andree et al. (1997), who stated that *M. cerebralis* isolated from different hosts and geographic areas had 99.2–99.3% similarity. Our results clarified that the presence of intraspecific differences varied from 0.5 to 3.9% with other *Myxobolus* species. In taxonomic analyses of Myxozoa, Andree et al. (1999) observed a variation of only 0.8% among isolates of *M. cerebralis* from different hosts and geographic locations. Easy et al. (2005) stated that even though *M. intramusculi* Easy et al. (2005) and *M. procerus* Kudo (1934) were both found in the muscles of trout perch *Percopsis omiscomaycus* and presented differences in only 2% of their 18 SSU rDNA sequences, they were considered 2 separate species, because *M. intramusculi* was found in intramuscular tissue, whereas *M. procerus* was found infecting the adjacent connective tissue. Molnár et al. (2006) observed a variation of 3.6% among samples of *M. dujardini* Thélohan, 1892, and Ferguson et al. (2008) showed only 0.5% variation between *M. fryeri* Ferguson et al. (2008) and *M. insidiosus* Wyatt & Pratt (1963) with different infection sites and spores with significantly different lengths; they were therefore considered 2 separate species.

Phylogenetic analysis in our study clearly showed that the recovered myxosporean species belonged to the clade containing *M. cerebralis* with high bootstrap support, suggesting that spore morphology shows congruence with molecular phylogeny to some extent. Similar relationships have been reported in other groups of *Myxobolus* species (Salim & Desser 2000, Eszterbauer 2004, Zhao et al. 2008). Thus, the high degree of molecular similarity (up to 95%) and morphological data with intra-specific differences in the samples of *M. fomenai*, *M. agolus*, *M. brachysporus*, *M. tilapiae* and *T. egyptica* led us to consider these samples as 5 different species with unique gene sequences.

CONCLUSION

We concluded that the combination of morphological features, host specificity, tissue affinity, and molecular analyses supply sufficient evidence for the detection and interpretation of myxozoan phylogeny. In addition, our study supports the phylogenetic hypothesis for the placement of myxozoans within the Cnidaria, as a sister taxon to *Polypodium hydriforme*. However, further investigation should focus on analyses of other appropriate genes to help in clarifying the phylogenetic relationships of myxosporeans.

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