Hepatic Microsporidiosis of Mudskipper, *Boleophthalmus Dussumieri* Valenciennes, 1837 (Perciformes: Gobiidae), Due to *Microgemma* Sp.

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Abstract

The present study reports a case of hepatic microsporidiosis caused by *Microgemma* sp in brackishwater fish, *Boleophthalmus dussumieri* (Valenciennes, 1837) (n = 60), from north-west coast of India. An eight-month study from September 2017 to April 2018 revealed a prevalence of 11.6% for this parasite. The microsporidian showed tissue-specific infection and did not reveal any gross pathology in infected fish. Large whitish cysts containing microspores of size 0.3–0.5 mm were observed in the liver of fish. The range of pyriform microsporidian spore size varied from 2.9–3.77 X 1.85–2.67 µm. Histological observations of infected liver revealed large xenoma of the microsporidian filled with spores and encircled by a cyst wall-like layer. Scanning electron microscopy of the spores showed a distinct groove on the anterior end of the spore for polar tube extrusion. Polymerase chain reaction (PCR) amplification of the DNA extracted from the microsporidian spores using primers targeting small ribosomal subunit DNA (SSU rDNA) yielded ~ 1340 bp amplicon and the genetic distance analysis showed a 0.2% variation with the reported *M. tilanpasiri*. Accordingly, in the phylogenetic tree, the present species of *Microgemma* clustered with *M. tilanpasiri.* Even though, the morphomeristic characters of the present *Microgemma* sp. was marginally different from the reported *M. tilanpsasiri;* the SSU rDNA showed considerably higher similarity with *M. tilanpasiri.* Thus, we report the species of *Microgemma* as *Microgemma* aff. *tilanpasiri* from a new host. This is the first report of a microsporidian from *B. dussumieri* and the first record of the genus *Microgemma* from India.

Introduction

Microsporidia are a diverse group of obligate intracellular, spore-forming parasites that infect a wide-range of hosts, including insects, fishes and humans (Dean et al. 2016; Mansour et al. 2020). Among these, fish is the most common vertebrate host for microsporidia and the infection could cause significant losses to fisheries (Dyková 2006; Abdel-Ghaffar et al. 2011). Some of the microsporidians induce hypertrophic growth of host cells, a well-organized xenoparasitic complex (XC) referred to as xenoma (Lom and Dyková 2005). Currently, seven species of *Microgemma*, namely *M. carolinus, M. hepaticus, M. ovoidea, M. tincae, M. vivaresi, M. caulleryi*, and *M. tilanpasiri* have been reported from various fish hosts (Freeman et al. 2015). However, the pathogenic potential of many microsporidians has not been studied as the hosts of these species have relatively low economic value and hence received little attention (Gómez et al. 2014). One such group of fish is the Mudskippers (Gobiidae); these diverse species of amphibious teleosts inhabit swamps, estuaries, mudflats, intertidal habitats and mangrove ecosystems. *Boleophthalmus dussumieri* Valenciénnes, 1837 is one of the most abundant species of mudskippers distributed along the north-west coast of India (Murdy 1989). The mudskippers play an important role in benthic ecology and have been recognized as potential bio-indicators for environmental monitoring. Further, as more species diversification is expected in aquaculture and new potential species such as mudskippers can be brought into culture, diseases caused by parasites such as microsporidians can emerge as a potential threat.

In this background, an investigation was carried out to study the prevalence of parasitic infection in *B. dussumieri* and we observed a microsporidian infection in the liver of the fish. The present study provides information on spore morphology, morphometrics as well as gross and histological evidence of *Microgemma* sp. infection in *B. dussumieri*. The scanning electron microscopic features of spores along with molecular sequence information and phylogenetic relation of the species are also provided. As far as is known, this forms the first report of a microsporidian infecting mudskippers.

Materials And Methods

Sampling

Live *B. dussumieri* (*n*=60) were collected from a brackishwater area located around Pancham Aquaculture Farms, (19°31’32.92”N and 72°47’57.83”E), Saphale, Palghar district, Maharashtra, India. The fish (mean length= 11.8 ± 3.17 cm; range= 7.3-17.2 cm) were transported live to the Aquatic Animal Health Laboratory, ICAR-Central Institute of Fisheries Education (CIFE), Mumbai, Maharashtra, India for parasitic examination.

Parasitic examination and identification of microsporidian

Fish were killed by pithing without any tissue damage, after immobilizing them on ice for adequate time. This method was as per the accepted guidelines (https://fisheries.org/docs/policy_useoffishes.pdf.). Initially, gross observations were carried out under a stereomicroscope to find out the presence of any ectoparasites, external lesions, discoloration, haemorrhage or cysts. Subsequently, all the external and internal organs of the fish were examined for the presence of parasites. Microsporidian cysts found in the liver were carefully removed, placed on a clean glass slide in physiological saline, ruptured with fine needles, mounted with a clean cover glass, and observed under a phase-contrast microscope. The spores were treated with 1-2% KOH to observe the polar tube extrusion. Smears of the infected tissues were air-dried, fixed in methanol, and stained with Giemsa stain. Photomicrographs of fresh and stained materials were taken using a research microscope (Nikon eclipse 80i, Japan) with image capture software (NIS elements BR, Nikon, Japan).

Histology

The infected tissues were fixed in neutral buffered formalin (NBF) for 24-72 h and washed thoroughly to remove the fixative. The tissues were dehydrated in an ascending series of alcohol followed by acetone and cleared in xylene. Paraffin infiltration and embedding of processed tissues were carried out using a histoembedder (LEICA EG 1140C, Germany). Tissue sections of 3-5 µm thickness were made using a rotary microtome (LEICA RM2125RT, Germany) and stained with Harris haematoxylin and eosin. The sections were dehydrated through different grades of alcohol and acetone. Xylene was used for clearing and sections were mounted in DPX (Sigma-Aldrich, USA).

Scanning electron microscopy

For scanning electron microscopy, microsporidian cysts were fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer. The cysts were fixed in 1% osmium tetroxide after washing in cacodylate buffer. Subsequently, after dehydration through graded acetone series, the cysts were transferred to isoamyl acetate and then critical point dried using a Hitachi HCP-2 Critical Point dryer (Hitachi, Japan). The dried cyst was cut open and then mounted on the SEM stub, using an adhesive carbon tape, so as to expose the inner surface of the cyst. Further, it was sputter-coated with gold using Quorum SC76220 mini sputter coater (Quorum Technologies, UK). The processed cyst with microspores was observed and photomicrographs were taken using a TESCAN VEGA 3 scanning electron microscope (TESCAN, Brno, Czech Republic).

Molecular analysis

The total genomic DNA was extracted from microsporidian-infected liver tissue of fish using DNAzol (Invitrogen®) kit following the manufacturer's instructions. The small subunit ribosomal DNA (SSU rDNA) of microsporidian spores was amplified using reported primers MicroSSUF: 5'-GGTTGATTCTGCCTGACGT-3' and MicroSSUR: 5'-GACGGGCGGTGTGTACAAAG-3' (Baker et al. 1994; Pomport-Castillon et al. 1997). The PCR reaction was carried out in a 25 µL reaction volume with 100 ng of template DNA, 10 mM of dNTP, 10 pmol of each primer, 5 U of Taq DNA polymerase and 1× Taq buffer with 1.5 mM $MgCl_2$, (Invitrogen®, USA). The thermal conditions for PCR were as follows: 95°C for 5 min, 30 cycles of 94°C for 1 min, 62°C for 1.5 min, 72°C for 2 min; followed by 72°C for 10 min final extension. PCR products were visualized on 2% agarose-TAE gel containing 0.5 µg mL^{-1} ethidium bromide. The desired PCR amplicon was extracted from the gel using a gel extraction kit and cloned into PTZ57R/T vector using T4 DNA ligase (Thermo Scientific®, India). The recombinant plasmid was transferred into *Escherichia coli* (DH5α) and the positive clones were selected by blue-white colony selection. The plasmid was isolated using GeneJET® plasmid extraction kit (Thermo Scientific®, India) and the insert was reconfirmed by PCR amplification with microsporidian-specific primers. The confirmed plasmid DNA was further sequenced in both directions using the same microsporidian-specific primers by a commercial company (Xcelris Labs, Ahmedabad, India). The quality of each sequence was verified by the Phred score (q value) of each nucleotide using Finchtv software. The sequences were subjected to BLAST (Basic Local Alignment Search Tool) analysis with NCBI 'nr' database and the sequences with more than 85% similarity were downloaded to estimate the genetic divergence values. Kimura 2 parameter model implemented in MEGAX (Kumar et al. 2018) was used to estimate the genetic distance values. JModeltest was used to assess the best evolutionary model (Posada 2008). Based on the Akaike Information Criterion (AIC), the Transitional model with rate variation among sites (TIM3+G) was selected as the best model to reconstruct the phylogenetic tree using Maximum likelihood and Bayesian Inference. Maximum likelihood and Parsimony methods were used to reconstruct the phylogenetic trees using PAUP software (Swofford 2003). Bayesian inference was also implied to deduce the phylogenetic tree using MrBayes (Ronquist and Huelsenbeck 2003).

Results

Gross examination of fish

A total of 60 *B. dussumieri* were collected from brackishwater system located around Pancham Aquaculture Farms, Maharashtra. Gross examination of the fish did not show any abnormalities or lesions.

Microgemma sp.

A microsporidian infection was observed in the liver with a prevalence of 11.6% (7 out of 60 fishes). Gross examination of the liver revealed the presence of numerous whitish, round to oval macroscopic cysts (xenoma) 0.3-0.5 mm in diameter. The cysts were either present on the surface or deeply embedded in the liver tissue (Fig. 1 a). Cysts, when ruptured, released numerous microsporidian spores (Fig. 1 b). Fresh spores were pyriform, in the size range of 2.9-3.87 (3.25) X 1.85-2.67 (2.08) µm (Fig. 1 c). The posterior vacuole was seen occupying the posterior third of the spore (Fig. 1 d). A partially extruded polar tube was also observed (Fig. 1 e). Spores stained with Giemsa's showed the distinct pyriform shape with posterior vacuole (Fig. 1 f).

Histopathology and ultrastructural observations

Infected liver tissues were subjected to histological observation. The parasite showed strict tissue-specificity, as cysts were noticed only in the liver of *B. dussumieri*. Infected liver tissues were subjected to histological observation. Large xenomas surrounded by distinct layer/wall were observed in the histological section (Fig. 2 a). Multiple xenomas were frequently observed. Granuloma formation was not observed in any of the tissue sections examined. A large number of spores were noted inside the xenoma (Fig. 2 b). Ruptured microsporidian cyst under a scanning electron microscope revealed numerous spores attached to the cyst wall (Fig. 3 a-d). Mature microsporidian spores showed a distinct groove at the anterior end of the spore for polar tube extrusion (Fig. 3 e). Many spores also revealed a prominent ridge/fold-like structure on one side of the spore wall (Fig. 3 f).

Molecular and Phylogenetic analysis

Polymerase chain reaction (PCR) amplification of the DNA extracted from the microsporidian spores using primers targeting small ribosomal subunit DNA (SSU rDNA) yielded ~1340 bp amplicon (Fig. 4). The PCR-amplified products were sequenced and almost complete SSU rDNA (1269 bp) was sequenced from *Microgemma* sp. and submitted to GenBank (accession number of MN733420). The sequence similarity analysis using Basic Local Alignment Search Tool (BLAST) with NCBI GenBank database showed ~99.8% sequence similarity with *M. tilanpasiri* (KJ865404) reported from *Trypauchen vagina*. A total of 33 sequences with more than 85% sequence similarity with the present species were downloaded to reconstruct the phylogenetic tree. Alignment and subsequent trimming resulted in a uniform length of 1219 bp. The number of conserved and variable nucleotides is 752 and 467, respectively. Among the variable nucleotides, 300 nucleotides were parsimony informative. The present species showed a genetic

distance value (Kimura 2 parameter model) of 0.2% (nucleotide difference of 2) with *M. tilanpasiri* (Table 1).

The tree topologies reconstructed by different methods were similar and in the consensus phylogenetic tree, the species of *Microgemma* clustered with *M. tilanpasiri* as a single clade with significant bootstrap value (Fig. 5). Further, this group emerged as a sister clade to *M. carolinus* with moderate bootstrap value. Few species of *Sprageua* sp. (GenBank accession number AB623034 & JQ820238) clustered within the Tetramicridae family. Family Spragueidae formed a sister group to *Tetramicridae*.

Discussion

This is the first report of the microsporidium, *Microgemma* sp. from *Boleophthalmus dussumieri* and the first record of the genus *Microgemma* from India. The present *Microgemma* sp. showed close similarity with the diagnostic features described for the genus *Microgemma* (Ralphs and Matthews 1986). The resembling features are: pyriform spore shape; posterior vacuole occupying the posterior third of the spore; spore dimensions (4.2 X 2.4 µm of the genus *Microgemma* and 2.9–3.87 X 1.85–2.67 µm of the present microsporidium); sporogonial development seen within a whitish spherical xenoma (host-parasite complex) in the liver; parasitic in marine fishes.

To date, this genus contains seven species reported from different parts of the world. All the species are known to infect liver except *M. vivaresi* which has been reported to infect both liver and skeletal muscles (Canning et al. 2005). A comparative account of all the *Microgemma* species reported along with the present species is given in Table 1.

The morphometric values of the present species were found to be closer to *M. vivaresi* and *M. tilanpasiri*. However, the species has been recorded from a different host and different geographical location. There were no gross signs of the microsporidian infection in infected fish in the present study. This is in accordance with the previous observations made in *M. tincae* (Mansour et al. 2005) and *M. tilanpasiri* (Freeman et al. 2015). Histological observations revealed large, multiple xenomas surrounded by a distinct layer. Previous studies on *M. tilanpasiri* infection in *T. vagina* have also reported similar observations as in the present study (Freeman et al. 2015). Granuloma formation and extensive necrosis in host fishes have been reported in response to *M. caulleryi* (Leiro et al. 1999) and *M. tilanpasiri* (Freeman et al. 2015) infections. However, such pathological changes were not observed in the present study. This could be attributed to the fact that a symbiotic co-existence might have developed between the host cell and the microsporidian parasite leading to the formation of the xenoparasitic complex as observed by Lom and Dyková (2005).

Under the scanning electron microscope, numerous spores were seen attached to the cyst wall and mature spores were observed to have a distinct groove on the anterior end of the spore for polar tube extrusion and a ridge/fold-like structure on the spore wall. Though *M. caulleryi* spores were studied using SEM, there was no clear description of the surface morphology of the spores (Leiro et al. 1999). As far as is known, the present study forms the first detailed SEM description of a *Microgemma* sp.

Molecular data, particularly small subunit ribosomal DNA, have been used to study the microsporidian phylogeny (Baker et al. 1995; Cheney et al. 2000; Kent et al. 1999; Moser et al. 1998; Nilsen 2000; Bell et al. 2001). Lom and Nilsen (2003) stated that the level of genetic variation between closely related species of microsporidians varies as per the host group. Several reports showed a lack of sufficient genetic variation among closely related species of microsporidia that infect fishes (Nilsen et al. 1998; Cheney et al. 2000;Casal et al. 2012; Freeman et al. 2015). The sequence of the present species of *Microgemma* (1269 bp) showed high genetic similarity (~ 98.9–99.8%) and less genetic divergence value with *M. tilanpasiri* (0.2%), *M. carolinus* (0.7%), and *M. vivaresi* (1.1%). Several previous studies have also reported low divergence values between *M. carolinus* and *M. tilanpasiri* (0.7%), *M. tincae* and *M. vivaresi* (0.7%), and *M. caulleryi* and *Tetramicra brevifilum* (0.3%) (Freeman et al. 2015; Casal et al. 2012). This could be due to the recent evolution of the species and subsequently less divergence time from their most recent common ancestor. However, accurate species delimitation relies on the occurrence of high genetic distance value (minimum 2%) between species.

In the phylogenetic tree, the present species clustered with *M. tilanpasiri* with significant bootstrap. This clade corresponds to group IV of the classification reported by Lom and Nilsen (2003). Although the present species of *Microgemma* displayed unique morphological and morphometric features in the new host, molecular sequence data showed a high affinity to *M. tilanpasiri*. Hence, the present species can be considered as *Microgemma* aff. *tilanpasiri*, a species with close affinity to *M. tilanpasiri.* However, more molecular markers (large subunit ribosomal DNA and ITS) are required for further resolution of these recently evolved microsporidian species. In conclusion, based on the light and scanning electron microscopic studies together with histopathology, molecular sequencing and phylogenetic analysis, the present study identifies and describes a new record of *Microgemma* aff. *tilanpasiri* infecting the hepatic tissue of the brackishwater fish, *Boleophthalmus dussumieri* Valenciénnes, 1837, from India.

Declarations

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Author's contribution: The study was designed and guided by Rajendran K.V. and Sanil N.K. Vandana V.R. carried out the study in detail. The manuscript was written by all the authors.

Compliance with ethical standards: All applicable institutional, national and international guidelines for the

care and use of animals were followed in the present study.

Conflict of interest: The authors declare that there is no conflict of interest or competing interests.

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Tables

TABLE 1 Comparison of the present species of *Microgemma* with previously reported species

Species	Host	The site of infection locus	Spore dimension (µm)	Country/ Region	Reference
Microgemma hepaticus	*Chelon labrosus*	Liver	4.2 X 2.4	United Kingdom	Ralphs and Matthews, 1986
M. ovoidea	*Motella tricirrata, Cepola rubescens, C. macrophthalma, Merluccius hubbsi, M. barbatus, M. gayi, M. hubbsi*	Liver	3.8 x 1.97	Mediterranean Sea, Atlantic coast (France), Peru and Patagonia (Argentina)	Canning and Lom, 1986; Amigó et al., 1996
M. caulleryi	*Hyperoplus lanceolatus*	Liver	2.6 X 1.2	Atlantic coast (France, and Spain)	Leiro et al., 1999
M. tincae	*Symphodus tinca*	Liver	3.6 × 1.2	Tunisian coast	Mansour et al., 2005
M. vivaresi	*Taurulus bubalis*	Liver and Skeletal Muscle	3.6 X 2.1	United Kingdom	Canning et al., 2005
M. carolinus	*Trachinotus carolinus*	Liver	3.8 X 2.4	Brazil	Casal et al., 2012
M. tilanpasiri	*Trypauchen vagina*	Liver	3.92 X 2.87	Malaysia	Freeman et al., 2015
M. aff. *tilanpasiri*	*Boleophthalmus dussumieri*	Liver	2.9-3.77 X1.85-2.67 (mean,3.25 X 2.08)	India	Present study

Figures

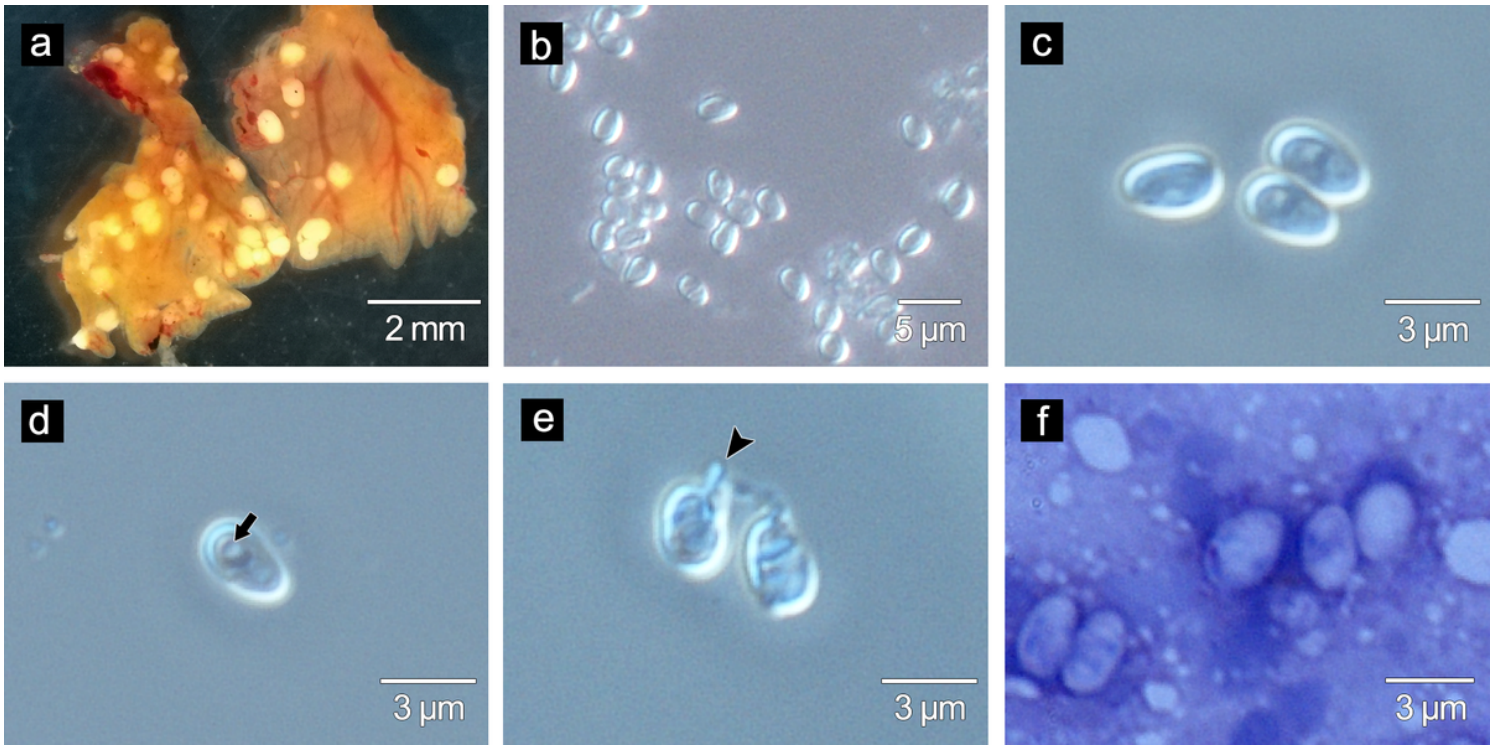

Figure 1

a) Whitish microsporidian cysts found in the liver tissue of the mudskipper, B. dussumieri observed under a stereomicroscope; b) fresh preparation of spores released from the cysts; c) enlarged view of spores; d) spore showing posterior vacuole (arrow); e) spores showing extruded polar tube (arrowhead); f) spores stained with Giemsa's stain

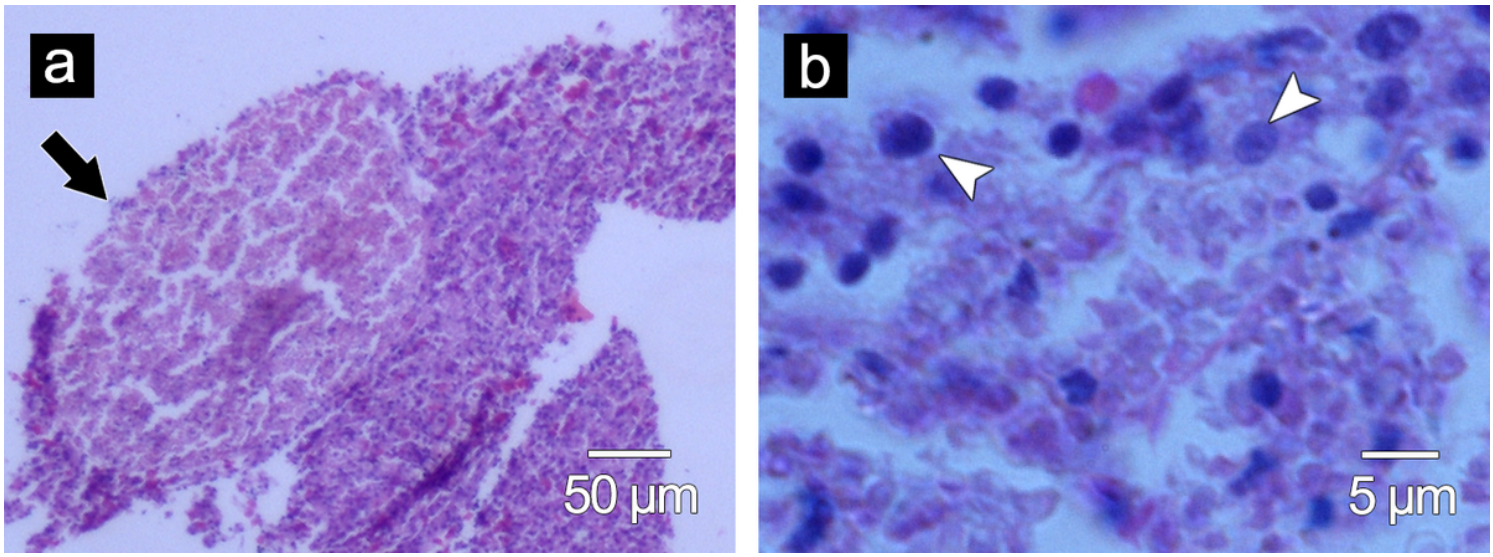

Figure 2

Histological section of infected liver a) xenoma (arrow) observed in the hepatic tissue; b) enlarged view of the xenoma showing spores (arrowhead) (H&E).

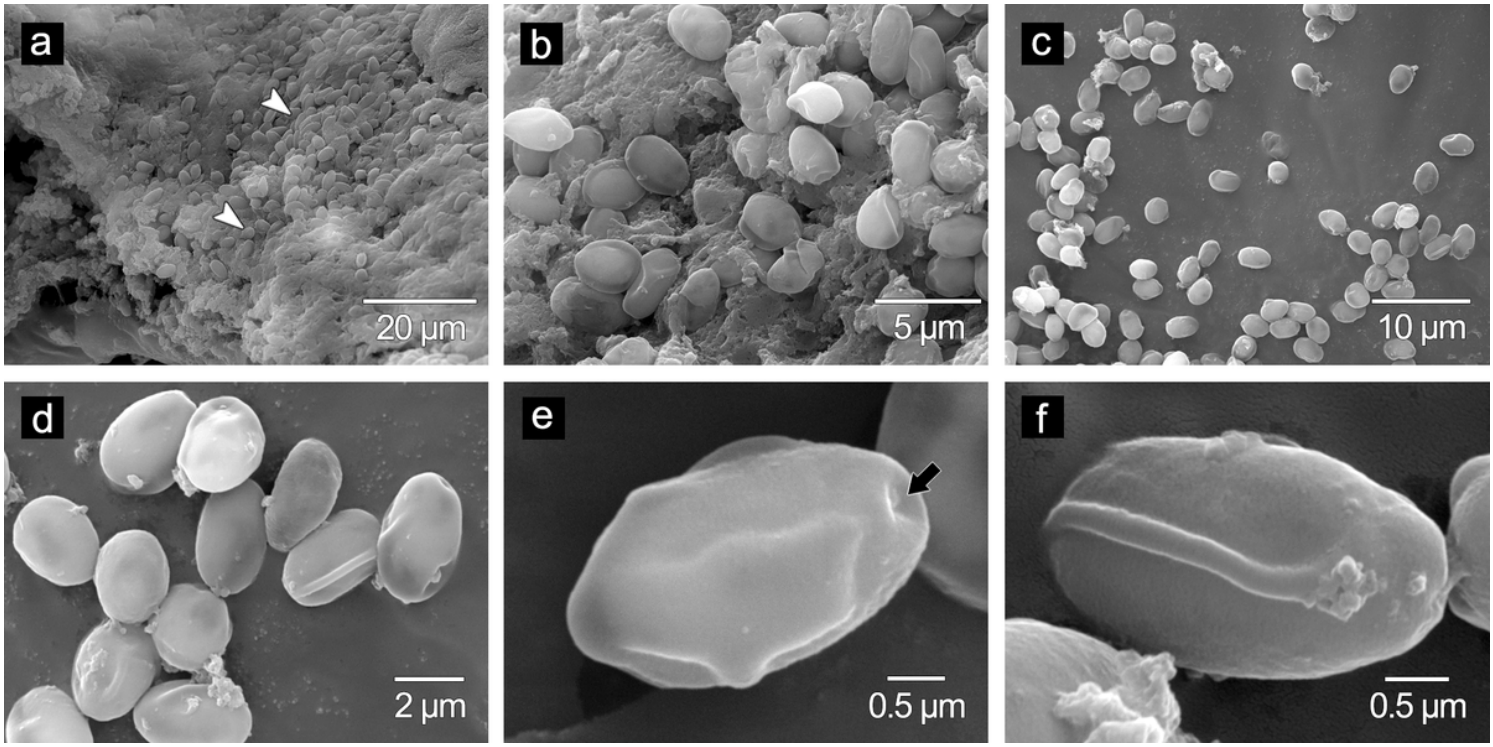

Figure 3

Scanning electron microscopy of spores. a) spores found in the ruptured cyst (arrowhead); b) enlarged view of spores attached to the cyst wall; c) free spores; d) enlarged view of spores. e) enlarged view depicting the polar tube extrusion pore (arrow), f) enlarged view showing ridge/fold-like structure on the spore wall

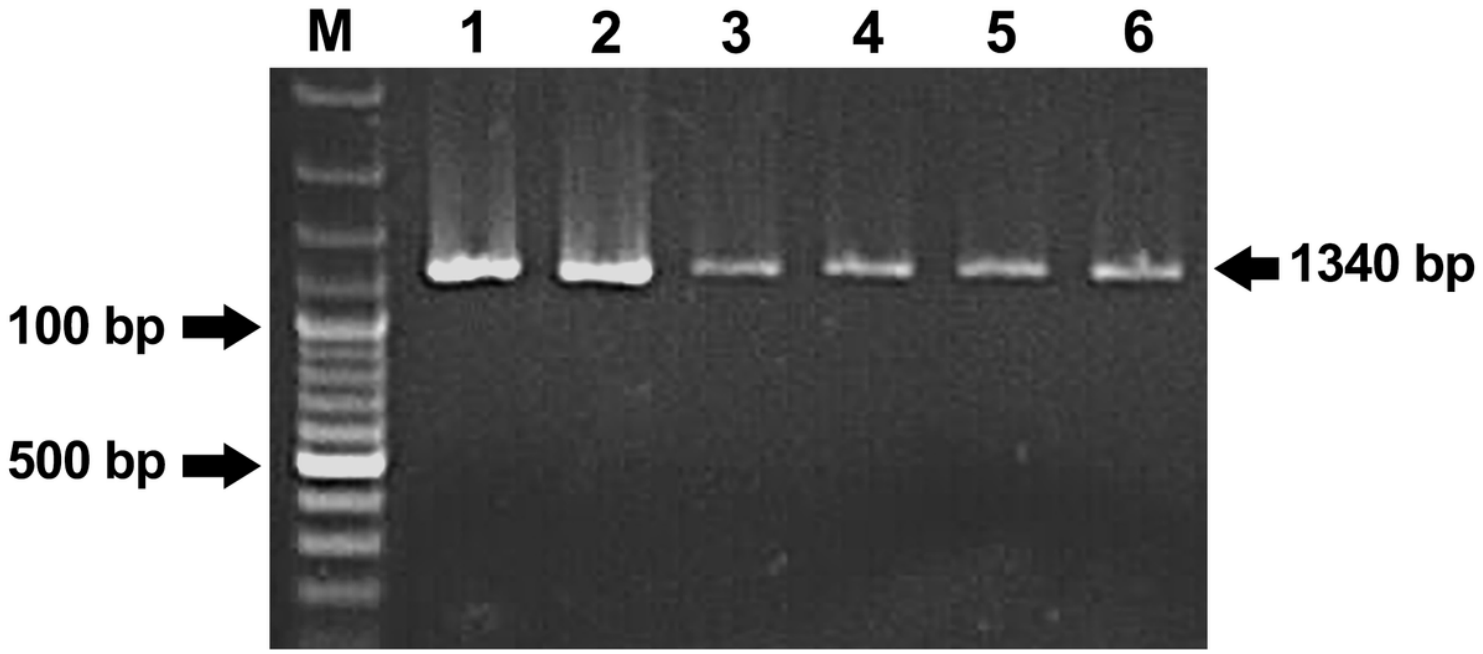

Figure 4

Agarose gel electrophoresis of the PCR product. PCR yielded approximately 1340 bp product. Lane M. 100 bp plus molecular weight marker (Fermentas). Lane 1-6 DNA from infected liver.

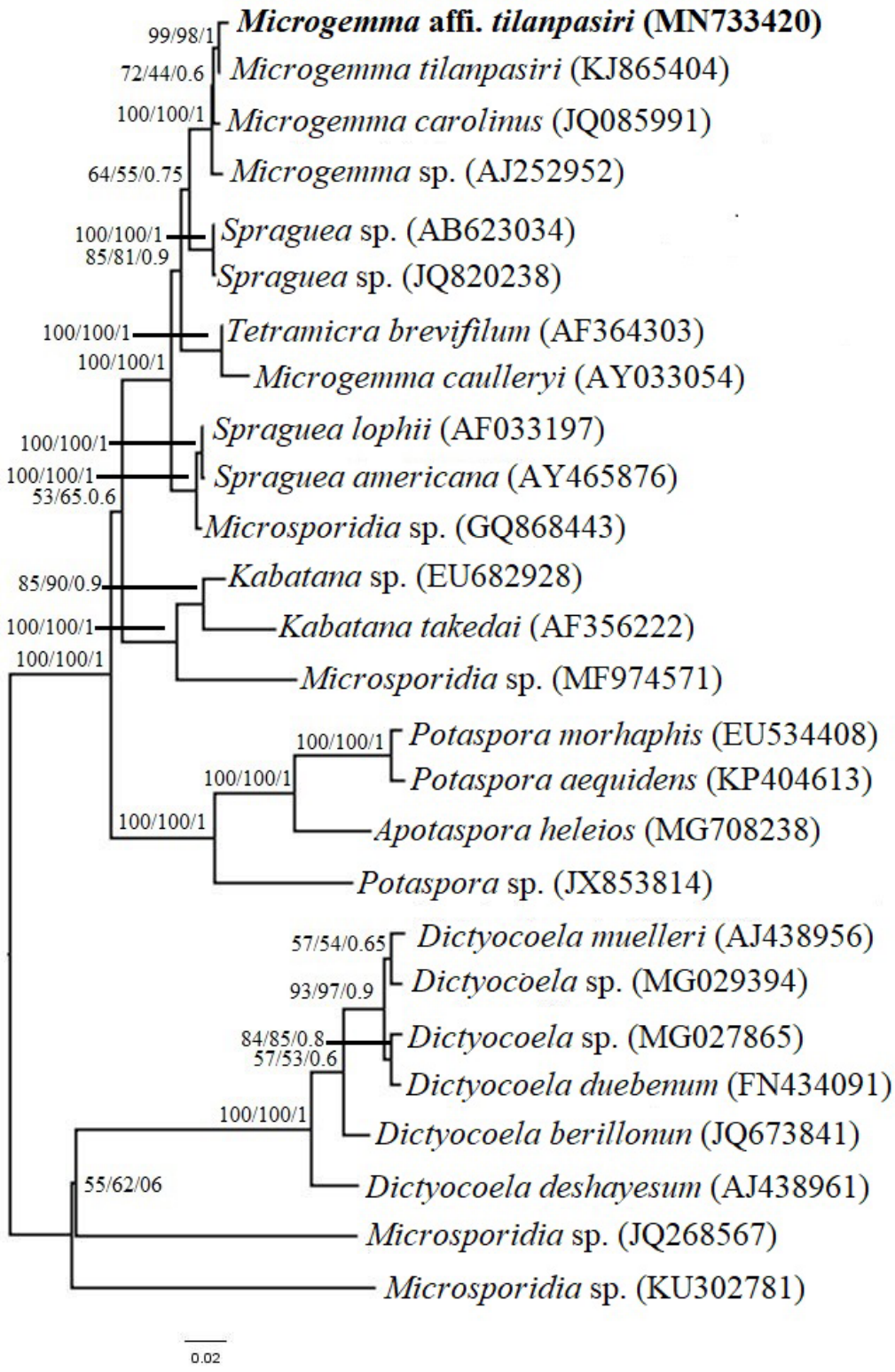

Figure 5

Neighbour-Joining tree of selected microsporidians. Labels on the nodes represent bootstrap values. Branch length shows the divergence between the species.