Microsporidians infecting eel gobies (Gobiidae: Amblyopinae) from Malaysia, with a description of *Microgemma tilanpasiri* n. sp. from the burrowing goby *Trypauchen vagina*

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Abstract

A new species of microsporidia, *Microgemma tilanpasiri* n. sp., is described infecting the burrowing goby, *Trypauchen vagina*, from Malaysia. The microsporidian forms macroscopic xenomas in the host liver which are packed with mature spores. Mature spores are slightly pyriform to oval in shape measuring 3.92 ± 0.21 μm in length and 2.87 ± 0.16 μm in width. No pre-spore stages were observed during electron microscopy studies and mature spores had a single nucleus and 12-13 turns of an isofilar polar filament, arranged in two rows. Sequencing of the ribosomal DNA indicated a strong phylogenetic relationship within the Tetramicridae and to other members of the genus *Microgemma*. The most similar species in terms of genetic distance is *M. carolinus* with a similarity of 99.23% over 1295 bases of the small subunit of ribosomal DNA. However, differences in the number of turns of the polar filament combined with host and geographical differences, support *M. tilanpasiri* as a novel microsporidian species. This represents the first description of *Microgemma* from the Western Pacific and the first from the Gobiidae family of fishes. Related blackfin eel gobies from the same sampling site were found to be uninfected with *M. tilanpasiri*; however one fish was infected with *Glugea* sp. in the visceral mesentery.

We conclude that in spite of the low genetic distances observed in ribosomal DNA sequences between geographically distant xenoma-forming microsporidians from both *Microgemma* and *Glugea*, that they probably represent a number of different species of parasite that may actually be quite host specific.

Introduction

Microsporidians are common intracellular parasites of fish. Some form large xenoparasitic complexes, characterised by an extensive hypertrophic growth of host cells, referred to as xenomas. Currently there are six species described from the xenoma-forming genus *Microgemma*, all of which infect fishes from the Atlantic (Casal et al., 2012). Infections are typically found as macroscopic xenomas in the liver of the host, but may also occur in the skeletal muscle (Canning et al., 2005). Microsporidians from the genus *Glugea* also form xenomas in

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fish. However, it is less clear how many *Glugea* species are involved, and the type species, *G. anomala*, could be responsible for infections in multiple hosts globally (Lovy et al., 2009).

Gobies are one of the most diverse families of vertebrates, and comprise over 1700 species in more than 200 genera of marine, brackish and freshwater fishes (Gill and Mooi, 2012). The subfamily Amblyopinae (eel gobies) contains 12 genera and 23 species (Murdy, 2011). Thirty five species of goby are listed from Malaysia with seven in the Amblyopinae: *Paratrypauchen microcephalus*, *Taenioides anguillaris*, *T. cirratus*, *T. gracilis*, *T. nigrimarginatus*, *Trypauchen vagina* and *T. pelaeos* (Ambak et al., 2010; Murdy, 2006; Randall and Lim, 2000).

Trypauchen vagina, the burrowing goby (locally called *tilan pasir*), is a species of goby native to marine and brackish waters of the Indian Ocean and the Indo-West Pacific. They inhabit burrows in mud or shingle areas, often near river mouths and mangrove forests. *Taenioides nigrimarginatus*, the blackfin eel goby are found in similar habitats to *T. vagina*, typically along the muddy bottoms of coastal rivers and estuaries (Rainboth, 1996).

We describe a new species of microsporidian infecting the liver of *T. vagina* from Malaysia and report a *Glugea* sp. from the visceral mesentery in *T. nigrimarginatus*. We demonstrate that the microsporidian infecting the liver of the burrowing goby, *T. vagina*, belongs in the genus *Microgemma* and propose the name *Microgemma tilanpasiri* n. sp.

Materials and methods

Fish were collected using a fyke net from Bagan Sungai Buloh in the Kuala Selangor mudflats on the west coast of Peninsular Malaysia. All eel gobies sampled were transported to the laboratory on ice and kept frozen till required. Thawed fish were dissected and examined for the presence of microsporidian parasites. Macroscopic cysts from the viscera of all fish were examined with an Olympus BX-41 compound microscope using phase contrast at high magnification. Images were taken using a Leica DMLB digital camera and the length and width of 20 spores were calculated using ImageJ. Infected tissue samples, and a sample of uninfected fish muscle, were fixed in 95% ethanol for DNA analysis and dissected xenomas fixed in 2.5% glutaraldehyde, washed in Sorenson's buffer (0.1M, pH 7.4) before being post-fixed in 1% osmium tetroxide and prepared for TEM as previously described (Freeman and Kristmundsson, 2013). Semi-thin sections of 0.5 μm thickness were cut using a glass knife on a Reichert Ultracut E ultramicrotome and stained with 1% Azur II followed by 1% methylene blue in 1% borax (50:50).

Total DNA was extracted from tissue samples using a GeneMATRIX DNA isolation kit (EURx Poland) following the tissue protocol. The extracted DNA was used to amplify the small subunit (SSU), internal transcribed spacer (ITS) and partial large subunit (LSU) regions of the ribosomal DNA (rDNA) for the microsporidians and the mitochondria cytochrome c oxidase subunit 1 gene (CO1) for the fish. PCR reactions were performed using microsporidian primers as previously described (Freeman et al., 2013) with PCRs being performed according to the original descriptions. In addition, primer MG-1350r 5′tccagctacaggttctcctac designed to bind at the 3′ end of the SSU, being specific for numerous microsporidia, was used with the universal forward primer

870f 5´tgcggcttaatttgactcaac and used to confirm the initial sequence reads. CO1 for the fish host was amplified using the primers Fish-F1 \ R1 described by Ward et al. (2005). Positive PCR products were purified using GeneMATRIX PCR purification kit (EURx Poland), and direct sequencing reactions performed using BigDyeTM Terminator Cycle Sequencing chemistry utilising the same primers. DNA sequencing was performed in both directions on all positive PCR products of the expected sizes, and compared to sequences available in the GenBank databases. CLUSTAL_X was used for the sequence alignments and percentage divergence matrices were constructed using the neighbour-joining method based on the Kimura 2-parameter model (Saitou and Nei, 1987). Phylogenetic analyses were performed using the maximum likelihood methodology in PhyML (Guindon et al., 2010) with the general time-reversible substitution model selected and 1000 bootstrap repeats, and Bayesian inference analysis using MrBayes v. 3.2.2 (Ronquist and Huelsenbeck, 2003). For the BI analysis models of nucleotide substitution were first evaluated for the alignment using MrModeltest v. 2.2 (Nylander et al., 2004).

Results

A total of 5 specimens (167-181 mm TL) of *T. vagina* were caught and identified morphologically as having: total elements in dorsal fin 54-58 (mean 55.2); total elements in anal fin 43-49 (mean 46.8). A total of 15 specimens (149-278 mm SL) of *T. nigrimarginatus* were caught and identified by having: total elements in dorsal fin 50-55 (mean 51.5); total elements in anal fin 43-48 (mean 44.2). CO1 sequences were generated for two fish from each species. Sequences for the two specimens of *T. vagina* differed at 3 nucleotide positions over 698 bp of sequence data but were both >99.9% similar to another CO1 sequence (KJ000236) in the databases for *T. vagina* from the Indian Ocean. The CO1 sequences for the two *T. nigrimarginartus* were both identical over 699 bp of sequence data and were most closely related to the CO1 sequence (HM180729) for *Odontamblyopus rubicundus* (Gobiidae: Amblyopinae) from South Korea at 91% similarity. Fish CO1 sequences generated from this study have been submitted to GenBank under the accession numbers KJ865405-7.

All five *T. vagina* had numerous round to oval-shaped macroscopic cysts randomly distributed throughout the liver tissue, some visible at the surface with others more deeply embedded in the liver. Light microscopy of the cysts revealed large numbers of slightly pyriform to oval-shaped microsporidian spores (Figure 1A.). Twenty spores from 4 fish (n = 80) measured 3.3 - 4.4μm (mean ± S.D. = 3.92 ± 0.21) in length and 2.5 - 3.3 μm (2.87 ± 0.16) wide (Table 1). Xenomas ranged in size to over 1 mm in diameter and could be discrete or in small clusters. In semi-thin histological sections, xenomas packed with spores were found within the liver parenchyma but there was little evidence of a significant host response to infection. Instead the xenoma had a relatively thin xenoma wall, which was probably parasite derived (Figure 1B.). Infected fish showed no other obvious clinical signs, however, no uninfected fish were available for comparisons. Only one specimen of *T. nigrimarginartus* had a microsporidian infection, with a single small xenoma (<1mm in diameter) visible on the visceral mesentery, not embedded in the liver. This xenoma was used for DNA analysis.

On the TEM sections no developing stages were found, just mature spores which had a diffuse

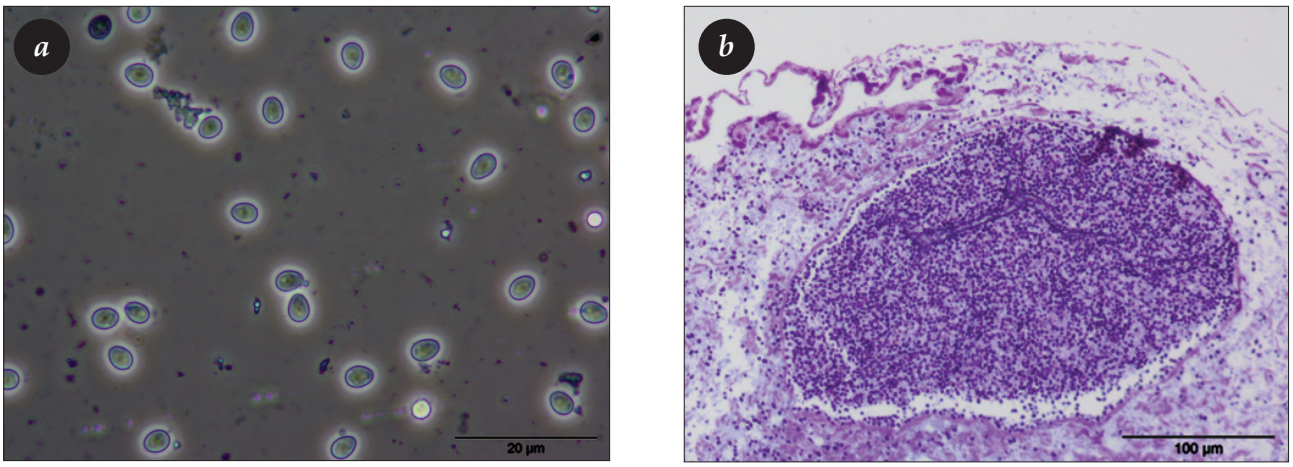

Figure 1. (a) Fresh spores of *Microgemma tilanpasiri* n. sp. (b) Semi-thin section from an infected liver showing a xenoma filled with mature spores. (Material previously frozen).

Table 1. Measurements of *Trypauchen vagina* and fresh microsporidian spores (20 per fish).

Sample	Host	Spore measurements (µm)	
	Total length (cm)	Length	Width
Fish 1	18.1	3.9 ± 0.17	2.8 ± 0.16
Fish 2	17.5	3.9 ± 0.23	2.9 ± 0.13
Fish 3	17.7	3.9 ± 0.19	2.9 ± 0.2
Fish 4	16.7	4.0 ± 0.23	2.9 ± 0.13
	Mean (n=80)	3.9 ± 0.21	2.9 ± 0.16

but single nucleus and 12-13 turns of the polar filament, arranged in two rows (Figure 2.). Developing stages, such as meronts, are sometimes not found in mature xenomas, however, it is possible that prior freezing of this material disrupted these more fragile developmental stages.

A contiguous rDNA sequence of 1854 bp was generated from 4 of the infected *T. vagina* which included the SSU, complete ITS and partial LSU. No interspecific variation was observed between the sequences from individual fish. BLAST searches revealed a 99% identity to members of the genus *Microgemma* over the SSU part of the new contiguous sequence (Table 2); no data currently exists from *Microgemma* spp. for ITS and LSU regions of the gene. A shorter sequence of 493 bp was generated for the single xenoma from *T. nigrimarginatus*, which consisted of the 3′ end of the SSU obtained with primers 870f/MG-1350r. This sequence had very high identities (>99%) to numerous *Glugea* isolates: *G. anomala* (AF056016), *G. hertwigi* (GQ203287), *G. stephani* (AF056015), *G. plecoglossi* (AB623035), *G. atherinae* (U15987) and *Glugea* sp. GS1 (AJ295325). Microsporidian rDNA sequences generated from this study have been submitted to GenBank under the accession numbers KJ865404 (*M. tilanpasiri*) and KJ865408 (*Glugea* sp.).

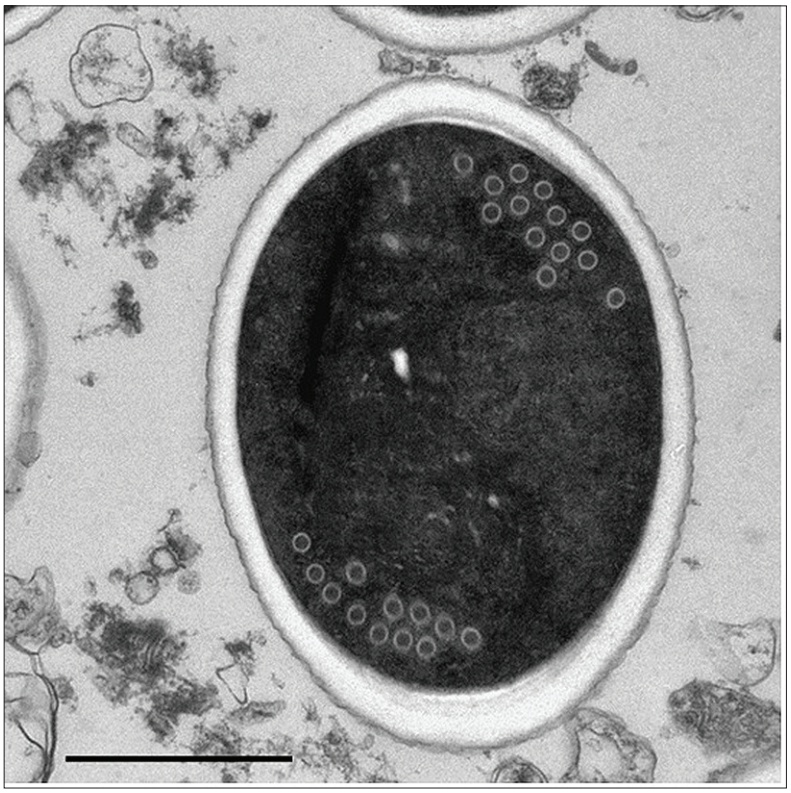

Figure 2. Transmission electron micrograph of a mature spore of *Microgemma tilanpasiri* n. sp. showing the polar filament with 12-13 turns arranged in two rows, bar = 1 µm. (Material previously frozen).

Table 2: Percentage identities of SSU rDNA sequences, above diagonal, and number of bases compared, below diagonal, for available members of the Tetramicridae

	(1)	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**
(1) ***Microgemma tilanpasiri***	-	99.23	98.51	93.19	98.80	94.35
(2) ***Microgemma carolinus***	1295	-	98.84	93.28	99.03	94.61
(3) ***Microgemma vivaresi***	1343	1292	-	93.25	99.28	94.33
(4) ***Microgemma caulleryi***	1233	1220	1230	-	95.31	96.69
(5) ***Microgemma tincae***	835	822	833	832	-	95.55
(6) ***Tetramicra brevifilum***	1238	1225	1235	1239	832	-

Molecular phylogenetic analyses consistently place *Microgemma tilanpasiri* n. sp. with other members of the genus *Microgemma* within the family Tetramicridae, irrespective of methodology used, being most closely related to *M. carolinus* from the Florida pompano, *Trachinotus carolinus*, from Brazil (Figure 3.). Percentage divergence matrices also showed *M. carolinus* to be the most similar, in terms of genetic distance, with a percentage similarity of 99.23% over 1295 bases of the small subunit rDNA (Table 2).

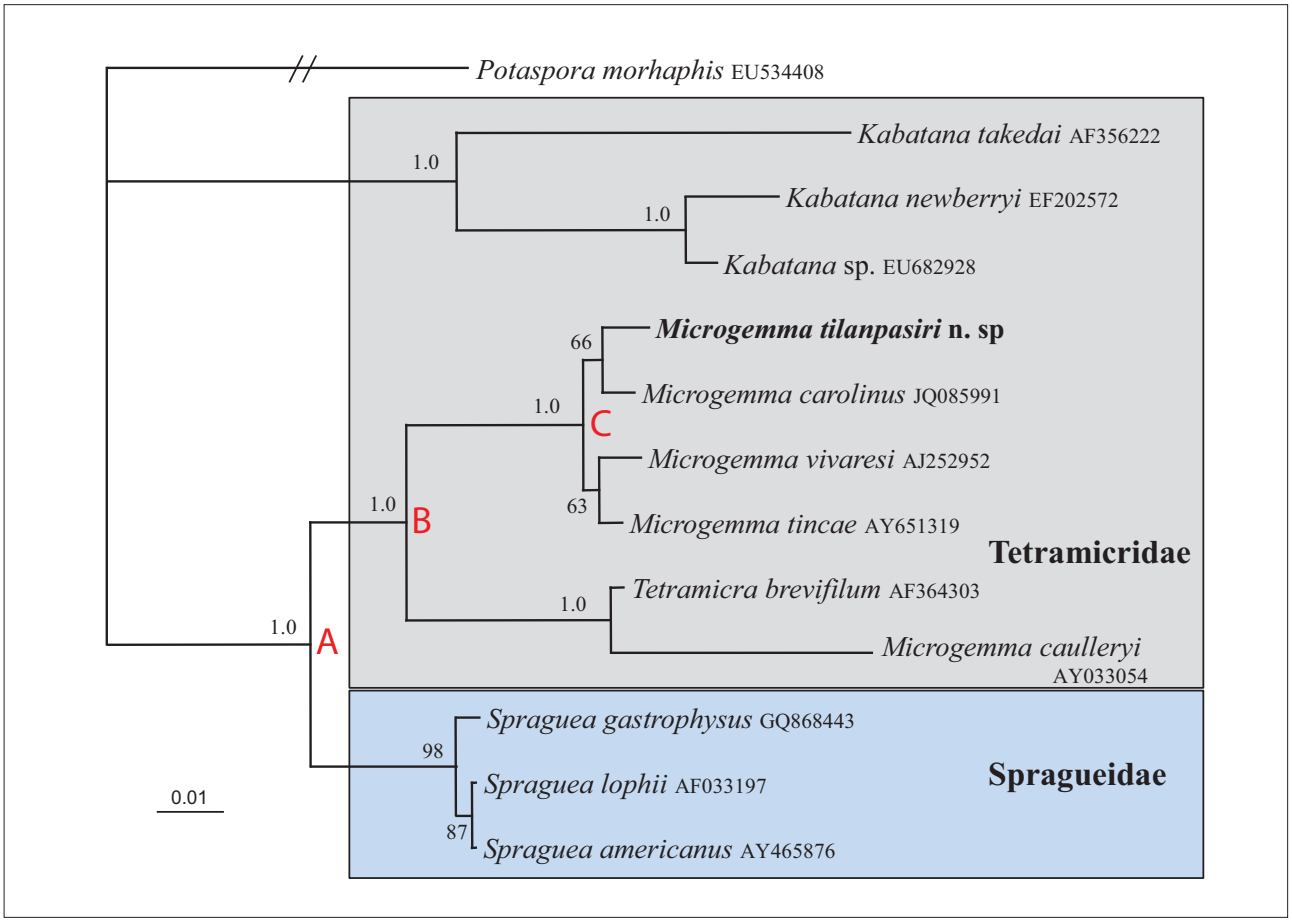

Figure 3. Bayesian inference phylogenetic tree showing the strong relationship between the Tetramicridae and Spragueidae from node A. *Microgemma tilanpasiri* n. sp. is fully supported as a member of the Tetramicridae from node B, and is robustly placed from node C, with three congeners.

Taxonomic summary

Phylum: Microsporidia Balbiani, 1882

Class: Marinosporidia Vossbrinck and Debrunner-Vossbrinck, 2005

Family: Tetramicridae Matthews and Matthews, 1980

Genus: *Microgemma* Ralphs and Matthews, 1986

Species: *Microgemma tilanpasiri* n. sp.

Type host: *Trypauchen vagina* (Bloch and Schneider, 1801), (Gobiidae: Amblyopinae).

Type locality: Bagan Sungai Buloh, Kuala Selangor, Peninsular Malaysia.

Site of infection: liver

Etymology: specific name refers to the Malay name for the fish host *tilan-pasir* (sand eel).

Type material: semi-thin sections have been lodged in the collection at the Institute for Experimental Pathology, at Keldur, University of Iceland, under the accession numbers 2013:Mi-Mt-1-3.

Discussion

This is the first description of *Microgemma* infecting fish in the Gobidae, and the first report of *Microgemma* from the Western Indo-Pacific. *M. ovoidea* has been reported from the Eastern Pacific in Peru, but from gadoid fish where mature spores only have 7-9 coils of the polar filament (Amigó et al., 1996). *M. carolinus* is >99% similar in the SSU rDNA, but is found

infecting the Florida pompano from Brazil and only has 8-9 turns of the polar filament (Casal et al., 2012).

In this study we found that the prevalence of infection in the burrowing goby, *T. vagina*, was 100% (5/5 fish). However, the blackfin eel goby, *T. nigrimarginatus*, from the same sub-family (Amblyopinae), and caught at the same location during the same sampling trip, were not infected with *Microgemma* (0/15 fish). These findings suggest that *M. tilanpasiri* n. sp. is host specific and only infects certain very closely related fish (*Trypauchen* spp.) or possibly only *T. vagina*. To date, all but one species of *Microgemma*, *M. ovoidea*, have been reported from a single species of fish (Casal et al., 2012). *M. ovoidea* has been reported from seven species of fish ranging in location from the Mediterranean Sea, the Atlantic coast of France, the Patagonian coast of Argentina to the Pacific Ocean of Peru (Canning & Lom, 1986; Amigó et al., 1996). However, no DNA data exist and ultrastructural studies are limited for these descriptions, therefore, it is not known if they truly represent a single species. Similar complications have also arisen for some members of the genus *Glugea* (Lovy et al., 2009), and existing SSU rDNA sequence data fail to provide sufficient resolution in phylogenetic studies, so it remains unknown whether *G. anomola*, the type species, infects multiple fish species or not. The *Glugea* sp. found infecting *T. nigrimarginatus* in this study adds another host to further complicate this situation, as the SSU rDNA sequence obtained was almost identical to numerous sequences in the databases.

Transmission of xenoma-forming microsporidians is known to take place directly between fish or involve paratenic hosts, such as small crustaceans that are consumed by the fish (Lom and Dyková, 2005). *T. vagina* are omnivorous fish, mostly preying on small crustaceans that stray close to their burrows (Murdy, 2006), so it is possible that either unaided direct transmission or the consumption of crustaceans containing viable spores is the route of transmission in *M. tilanpasiri*. The blackfin eel goby, *T. nigrimarginatus*, lives in the same habitat and is also known to feed on small crustaceans and other invertebrates (Rainboth, 1996), therefore, it would almost certainly be exposed to the same methods of infection experienced by *T. vagina*, suggesting that it is not readily infected by *M. tilanpasiri*.

Although *M. tilanpasiri* has a high similarity to other species of *Microgemma* with respect to SSU rDNA sequences, it is the first species described from the Western Pacific, and data from the current study suggests that it is probably host specific. We have provided ITS and partial LSU regions of the rDNA that may prove useful for future comparisons to be made within the Tetramicridae and recommend that future descriptions provide these additional regions of the gene, that may allow better phylogenetic relationships to be inferred.

It is therefore possible that many geographically distant xenoma-forming microsporidians from *Glugea* and *Microgemma* represent a number of different species of parasite that may actually be quite host specific. However, more research effort, including infection trials, will be required to unambiguously demonstrate this.

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