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First record of a *Kabatana* sp. microsporidium infecting fish in the Atlantic Ocean

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ABSTRACT: Two-spotted goby *Gobiusculus flavescens* from the Swedish Gullmarsfjord regularly present subcutaneous creamy-white patches in the body musculature, associated with *Kabatana* sp. infection. Analysis of the *16S rRNA* gene of the microsporidium showed 98.54% homology with *Kabatana newberryi* infecting a marine goby from California, indicating that the Swedish microsporidium is either a different strain of *K. newberryi* or a closely related species. This represents the first record of a *Kabatana* species in the Atlantic Ocean. The genetic similarity of the 2 microsporidia was paralleled by close infection phenotypes. Infected muscle fibres were swollen compared to adjacent non-infected fibres, and mature spore masses were found throughout the skeletal musculature. No xenoma formation was detected. Since *G. flavescens* is an established model species in behavioural ecology, the host–parasite system is ideally suited for testing how microsporidian infections affect host behaviour and fitness.

KEY WORDS: Microsporidia · *Kabatana* · Two-spotted goby · *Gobiusculus flavescens* · Parasites · Tidewater goby · *Eucyclogobius newberryi*

INTRODUCTION

Microsporidia are intracellular parasites that commonly infect teleost fishes (Canning & Lom 1986, Dyková 2006). Species of the microsporidian genus *Kabatana* Lom, Dyková & Tonguthai, 2000 infect the skeletal musculature of marine and freshwater fish. Until recently, all of the few known *Kabatana* species had been reported from eastern Asia (Lom 2002). *Kabatana arthuri* (Lom, Dyková & Shaharom, 1990) is recorded from the trunk musculature of the freshwater catfish *Pangasius sutchi* in Thailand (Dyková & Lom 2000), *K. seriolae* (Egusa, 1982) from the trunk muscles of yellowtail *Seriola quinqueradiata* in Japan (Sano et al. 1998), and *K. takedai* (Awakura, 1974) from the heart, trunk and other muscles of freshwater salmonids in Japan and far eastern Russia (Lom et al. 2001). Recently, however, a new species of *Kabatana*, *K. newberryi*, has been described from the tidewater goby *Eucyclogobius newberryi* (Girard, 1856) in coastal lagoons in Northern California (McGourty et al. 2007).

Two-spotted goby *Gobiusculus flavescens* caught in the Swedish Gullmarsfjord regularly present externally conspicuous creamy-white patches in the body musculature (Pélabon et al. 2005) that resemble closely those associated with *K. newberryi* infections of the tidewater goby (McGourty et al. 2007). The present study describes a molecular phylogenetic analysis of the microsporidian parasite infecting *G. flavescens* and presents macro- and microscopic investigations of the infection.

MATERIALS AND METHODS

Fish collection. Samples of *Gobiusculus flavescens* were hand-netted by snorkelers in early summer 2005 from the Gullmarsfjord, adjacent to the Sven Lovén

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Centre for Marine Sciences at Fiskebäckskil, on the west coast of Sweden (58° 15′ N, 11° 27′ E).

DNA isolation, PCR, DNA sequencing and phylogenetic analysis. Freshly dissected infected trunk muscle of *Gobiusculus flavescens* was stored in RNAlater (Qiagen) for processing and sequencing. Genomic DNA was extracted from 1 sample using a Qiagen DNeasy DNA purification kit, following manufacturer's instructions. A 1352 bp fragment of the microsporidian *16S rRNA* gene was amplified from the resulting mixture of host and parasite genomic DNA using the primer combination V1:1492R (Table 1 gives primer sequences). All PCR amplifications were performed using recombinant *Taq* polymerase (Invitrogen) with an annealing temperature of 50°C and an extension time of 60 s.

An initial BLASTn sequence similarity search of GenBank indicated that the *16S rRNA* sequence was highly similar to that of both *Kabatana takedai* (Lom et al. 2001) and *K. newberryi* (McGourty et al. 2007). The sequence was therefore aligned with published *16S rRNA* sequences from *Kabatana* species, from the closely related genera *Spraguea*, *Tetramicra* and *Microgemma*, and from representative members of other genera within the main fish-parasitic clade, class Marinosporidia (Vossbrinck & Debrunner-Vossbrinck 2005, but see Larsson 2005 for a criticism of higher taxonomic groupings). GenBank accession numbers for all species are given in Table 2. Representative members of other microsporidian clades were selected as outgroups. Sequences were aligned using Clustal W (Thompson et al. 1994).

Comparison of evolutionary models using hierarchical likelihood ratio tests, implemented in ModelTest (Posada & Crandall 1998), indicated that the Hasegawa-Kishino-Yano (HKY) model with gamma-distributed rate variation across sites and a proportion of invariable sites (HKY+G+I) was most suitable for phylogenetic analysis of the *16S rRNA* alignment. Phylogenetic analysis was performed using maximum likelihood implemented in PAUP* 4b10 (Swofford 2002) and Bayesian inference implemented in MrBayes (Huelsenbeck & Ronquist 2001). For each alignment, a maximum likelihood consensus tree was generated by conducting a heuristic search and bootstrapping with 100 replicates.

A 2730 bp fragment of the microsporidian RNA polymerase II largest subunit gene (*Rpb1*) was amplified in 3 sections using the primer pairs AF3:KR1, *Rpb1*-F1:*Rpb1*-R1 and KF1:GR1 (Table 1). PCR amplifications were performed using Invitrogen's recombinant *Taq* polymerase with an annealing temperature of 50°C and an extension time of 60 s. Because microsporidia undergo extremely rapid rates of nucleotide substitution at synonymous sites, resulting in saturation at third-codon positions of protein-coding genes, nucleotide sequences for *Rpb1* were translated into amino acid sequences prior to alignment with Clustal W (Thompson et al. 1994). Although published *Rpb1* sequences corresponding to the amplified fragment were not available for *Kabatana*, *Tetramicra* or *Microgemma*, sequences were available for other Marinosporidia. These were included in the alignment, together with representatives of other microsporidian groups. Because the Microsporidia have been suggested as a sister clade to the chytrid fungi (Liu et al. 2006), *Neocallimastix frontalis* was used as an outgroup, together with the yeast *Schizosaccharomyces pombi*. GenBank accession numbers for species included in the *Rpb1* alignment are provided in Table 2. Phylogenetic analysis of the *Rpb1* alignment used the same techniques as described for *16S rRNA*, except that 1000 replicates were used in bootstrapping.

Microscopic examination and histology. Visibly infected trunk muscle was selected for microscopic analysis. Skin was removed to reveal the underlying infected myomeres, and associated muscle fibres were teased apart in standard physiological saline, under a coverslip, to release the parasites. Wet-mount preparations were examined by brightfield with a Nikon 80i photomicroscope using a 100× (oil immersion) objective. Images were captured with a Nikon DS-5M digital camera, and spore and other measurements made using a Nikon NIS 2.10 image analysis system, calibrated to a stage micrometer.

Excised, infected myomeres were also prepared for histology following standard fixation in 4% formol-saline and processing into paraffin wax blocks (Bancroft & Gamble 2002). Three whole, infected gobies were cut in half

Table 1. Primers used for PCR and sequencing of *Kabatana* sp. *16S rRNA*, and *Rpb1* genes. The following primer sequences have been published previously: V1 and 1492R (Weiss et al. 1994), *Rpb1*-F1 and *Rpb1*-R1 (Hirt et al. 1999), and AF3 and GR1 (Cheney et al. 2001). Position refers to the position of the first nucleotide of the primer on the relevant DNA fragment: *16S rRNA* (GenBank accession no. EU682928) or *Rpb1* (GenBank accession no. EU682929)

Gene	Primer	Sequence 5′–3′	Position
16S rRNA	V1	CAC CAG GTT GAT TCT GCC TGA C	1
16S rRNA	1492R	GGT TAC CTT GTT ACG ACT T	1348
Rpb1	AF3	GGW CAT TTC GGW CAC ATI GA	1
Rpb1	*Rpb1*-F1	CGC ACT TYG AYG GNG AYG ARA TGA	1098
Rpb1	KR1	GTA AGT CTT CGG ACG CCT AT	1252
Rpb1	KF1	GAA TGA TCA CGG TTG TGA GAT GTA G	1632
Rpb1	*Rpb1*-R1	CCC GCK NCC NCC CAT NGC RTG RAA	2208
Rpb1	GR1	TGR AAM GTR TTI AGI GTC ATY TG	2732

Table 2. DNA sequences used in alignments of *16S rRNA* and *Rpb1* genes. Non-microsporidian outgroups are given in **bold**

Species	Host	GenBank accession no.	
		16S rRNA	*Rpb1*
Antonospora locustae	*Locusta migratoria*	AY376351	AF061288
Cystosporogenes operophterae	*Operophtera brumata*		AJ278949
Encephalitozoon cuniculi	Mammals	AJ005581	NM_001040904
Enterocytozoon bienusi	Mammals	AY331010	XM_001827870
Glugea anomala	*Gasterosteus aculeatus*	AF044391	AJ278952
Heterosporis anguillarum	*Anguilla japonica*	AF387331	
Kabatana sp.	*Gobiusculus flavescens*	EU682928	EU682929
Kabatana newberryi	*Eucyclogobius newberryi*	EF202572	
Kabatana takedai	*Oncorhyncus masu*	AF356222	
Loma acerinae	*Gymnocephalus cernuus*	AJ252951	AJ278951
Microgemma sp.	*Taurulus bubalis*	AJ252952	
Microgemma caulleryi	*Hyperoplus lanceolatus*	AY033054	
Microsporidium sp.	*Pagrus major*	AJ295323	
Microsporidium prosopium	*Prosopium williamsoni*	AF151529	
Microsporidium seriolae	*Seriola quinqueradiata*	AJ295322	
Nosema apis	*Apis mellifera*		DQ996230
Nosema bombycis	*Bombyx mori*	AY259631	DQ996231
Nosema empoascae	*Empoasca fabae*		DQ996232
Nosema granulosis	*Gammarus duebeni*		DQ996233
Nosema trichoplusiae	*Trichoplusia ni*		DQ996234
Nosema tyriae	*Tyria jacobaeae*		AJ278948
Pleistophora sp.	*Litopenaeus setiferus*		CAC33863
Pleistophora hippoglossoideos	*Hippoglossoides platessoides*		AJ278950
Pleistophora typicalis	*Myoxocephalus scorpious*	AJ252956	AJ278946
Spraguea lophii	*Lophius piscatorius*	(a) AF056013, (b) AF033197	AJ278954
Tetramicra brevifilum	*Scophthalmus maximus*	AF364303	
Trachipleistophora hominis	*Homo sapiens*	AJ002605	AJ278945
Vairimorpha cheracis	*Cherax destructor*		DQ996235
Vairimorpha necatrix	*Pseudaletia unipuncta*	DQ996241	DQ996236
Vavraia culicis	*Aedes albopictus*	AJ252961	AJ278956
Neocallimastix frontalis			**EF014394**
Schizosaccharomyces pombe			**NM_001021568**

transversely across the trunk and fixed in formol-saline following decalcification in 2.5% (w/v) ethylene diamine tetraacetic acid (EDTA, BDH Chemicals) in 30% ethanol for 18 to 24 h prior to wax embedding (see Charmantier et al. 1987). Sections, 5 to 6 µm thick, were cut from blocks containing myomeres or whole fish, processed and stained with Giemsa, haematoxylin and eosin (H&E), Masson's trichrome, or verde luz, orange G and acid fuchsin (VOF, Type III-G.S) (Bancroft & Gamble 2002, Sarasquete & Gutiérrez 2005). Stained sections were examined by brightfield, and images were captured digitally and measured by image analysis.

RESULTS

Phylogenetic analysis

The DNA sequence of the *16S rRNA* gene of the microsporidium infecting *Gobiusculus flavescens* showed 98.54% homology with that of *Kabatana newberryi* (McGourty et al. 2007). The *16S rRNA* sequence also shared 93.89% homology with *K. takedai* (Lom et al. 2001) and 91.84% homology with an undescribed isolate from bottlenose dolphin *Tursiops truncatus* faeces (Fayer et al. 2008). Phylogenetic analysis of the *16S rRNA* sequence (Fig. 1a) placed the microsporidium infecting *G. flavescens* within a clade containing 2 *Kabatana* species (Lom et al. 2001) and 2 species infecting muscle of yellowtail *Seriola quinqueradiata* and red sea bream *Pagrus major* in Japan, assigned to the collective genus *Microsporidium* (Bell et al. 2001). This clade nested within a well-supported clade within the class Marinospora, also containing fish parasites of the genera *Tetramicra*, *Microgemma* and *Spraguea*.

The consensus phylogenetic tree for *Rpb1* amino acid sequences (Fig. 1b) was poorly resolved at basal nodes, due to the sparse distribution of species, yet still congruent with the *16S rRNA* tree (Fig. 1a). Within the *16S rRNA* clade containing *Kabatana*, published *Rpb1* sequence data was only available for *Spraguea lophii*, a parasite of *Lophius* spp. anglerfishes (Loubes et al. 1979). The *Kabatana* sp. *Rpb1* sequence formed a well-supported clade with *S. lophii*.

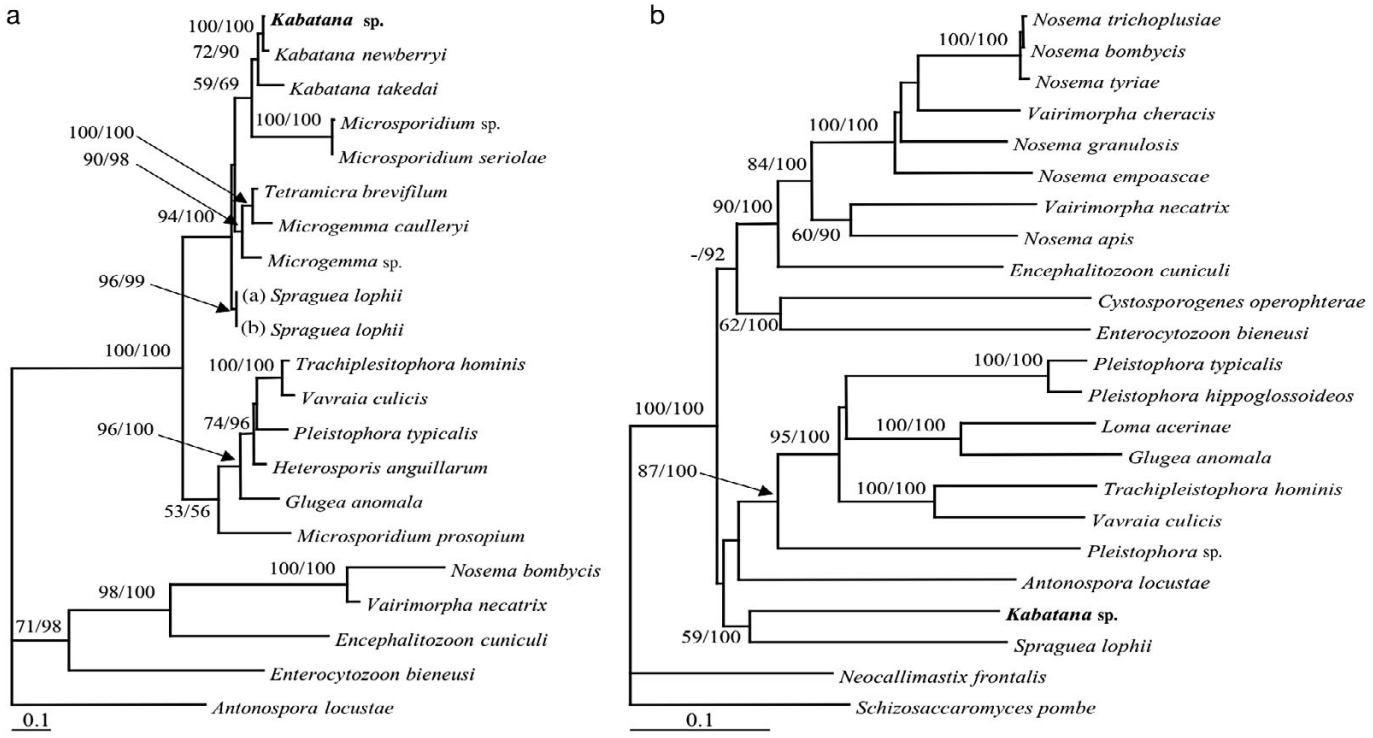

Fig. 1. Consensus phylogenetic trees for the (a) nucleotide sequence of the small subunit ribosomal RNA (*16S rRNA*) gene and (b) amino acid sequence of the *Rpb1* gene of *Kabatana* sp. from the two-spotted goby *Gobiusculus flavescens* (in bold). Maximum likelihood and Bayesian inference criteria produced identical consensus tree topologies. Support for each internal node is given as a maximum likelihood (a) or parsimony (b) bootstrap value followed by a Bayesian posterior probability of partition. Bootstrap values of <50 are not generally considered to indicate significant support, and are shown by '–'. Branch lengths are drawn proportionally to the number of changes assigned to each branch. Scale bar = 0.1 substitutions per site

Gross and microscopic aspects of infection

Foci of parasite infection were externally visible as circular, oval or fan-shaped creamy-white patches under the skin, 0.7 to 3.2 mm at their broadest (Fig. 2a–c). These foci, which raised the skin slightly, were located below the eye, immediately posterior to the operculum and generally on the dorsal and ventral surfaces and the caudal peduncle. On removal of the skin, the infection was visualized as white streaks in the musculature (Fig. 2b). In females, infections were also observed in the muscle underlying the stretched, pigmented skin surrounding the developing egg mass (Fig. 2c). Infected fibres were fragile, often liquefying when disturbed, even in freshly euthanized specimens.

Analysis of stained sections revealed high levels of infection in skeletal muscle, particularly of the flank and tail (Fig. 2d). In sections of excised infected muscle, ~82% of fibres were occupied by spores. The cross-sectional diameter of infected muscle fibres (67.1 ± 14.3 µm, range = 47.6 to 107.3 µm, n = 20) was more than twice that of uninfected fibres (26.4 ± 6.37 µm, range = 17.6 to 39.3, n = 20) (Fig. 2g,h).

Macroscopically visible infections were limited to striated muscle, with other tissues being apparently free from infection. However, Masson's trichrome and VOF (Type III-G.S) stains identified spores at sites not visualized macroscopically, such as muscle blocks within the mandible, blocks adjacent to the orbits, brain and spinal cord, and those surrounding the gill chambers (Fig. 2e). Spores escaped muscle blocks in some areas to form raised accumulations beneath the epidermis (Fig. 2e).

Infected muscle fibres contained 1 or 2 large, circular, oval or elongate cyst-like masses measuring 42 to 106 µm across, each containing many hundreds of spores. Spore masses occasionally (<0.5%) occupied almost the entire cross-sectional area of infected fibres, except for a thin rim of host tissue. Masses were only rarely (<0.1%) surrounded by a narrow, limiting layer (Fig. 2i), but the nature of this surface layer was unclear. Most masses contained mature spores, but some had islets of immature spores up to ~14 µm across, probably indicating regions of spore production. Pre-spore stages were not detected, despite extensive sampling effort and the use of Giemsa's stain, which might have located such stages (Canning & Nicholas 1980). The structure of myofibrils adjacent to spore masses, or their limiting layer (when present), was seemingly unchanged (Fig. 2f,i); furthermore,

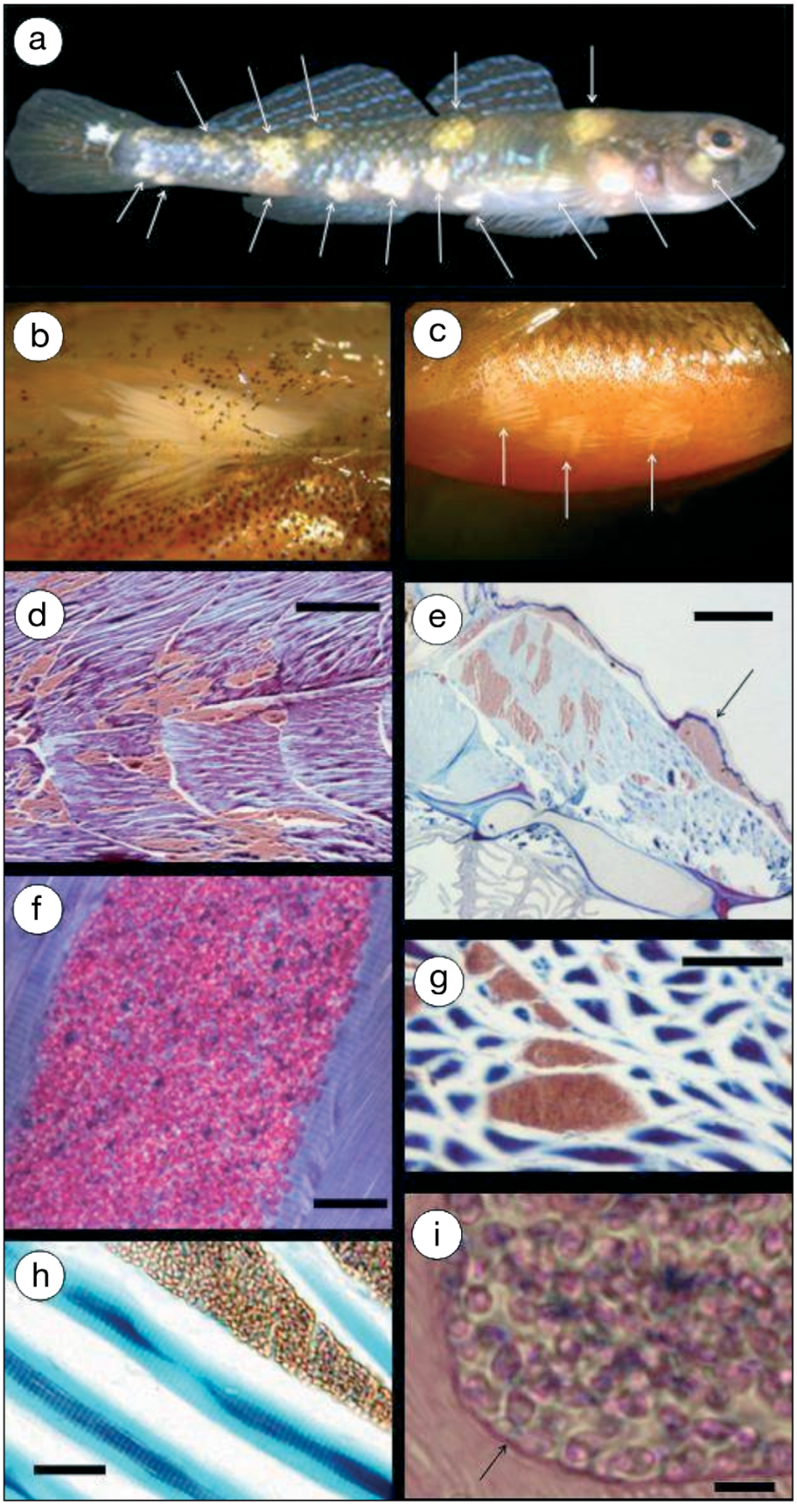

Fig. 2. *Kabatana* sp. infecting *Gobiusculus flavescens*. (a) Male two-spotted goby infected with the *Kabatana* sp. microsporidium. Individual infected muscle patches (arrows). (b) Enlarged image of infected muscle tissue in a specimen from which skin has been lifted. (c) Multiple infected muscle patches (arrows) beneath pigmented skin surrounding the belly of an infected, gravid female. (d) Longitudinal section of infected caudal musculature, with VOF (Type III-G.S) staining the microsporidium orange. (e) VOF-stained longitudinal section of infection in the gill region, within muscle blocks and below the raised cuticle/epidermis (arrow). (f) Longitudinal section through infected flank muscle, with Masson's trichrome staining indicating the spore mass (red) and surrounding, intact myofibrils (blue). (g) VOF-stained cross and oblique sections of swollen infected (orange) and non-infected fibres (blue) of the mandible musculature. (h) VOF-stained longitudinal section of non-infected and enlarged infected muscle fibres in the mandible. (i) H&E-stained longitudinal section through excised infected flank muscle, showing the periphery of the spore mass with a limiting membrane (arrow) and apparently normal myofibrils adjacent to spore mass. Scale bars: (d, e) = 0.5 mm; (f, h) = 20 µm; (g) = 100 µm; (i) = 5 µm

there was no evidence of a host response around spore masses and xenomas were not detected. Secondary bacterial infections were sometimes associated with spore-filled areas of the abdominal wall.

Spore morphology and developmental aspects of infection

Spores released from fresh (unfixed) excised muscle bundles occurred singly or in groups of several hundred and were largely ovoid or slightly curved, with a large posterior vacuole relative to their size (Fig. 3). Spores were released only rarely within a limiting membrane, and thus the existence of a sporophorous vesicle appeared unlikely. Fresh spores (n = 100) were 3.7 ± 0.4 µm (range = 3.1 to 4.8 µm) long by 2.3 ± 0.3 µm (range = 2.1 to 2.3 µm) wide and appeared monomorphic.

DISCUSSION

Analysis of sequence data provided strong genetic evidence that the microsporidium infecting *Gobiusculus flavescens* in the Swedish Gullmarsfjord belonged to the genus *Kabatana*. *16S rRNA* data placed the microsporidium within a clade containing other *Kabatana* species, including the newly described *K. newberryi* from the tidewater goby in Northern California, and 2 *Microsporidium* spp. that have been suggested, on the basis of morphology, to belong to the genus *Kabatana* (Lom et al. 1999, 2000). *Rbp1* data supported the hypothesis that the *Kabatana* sp. microsporidium and *Spraguea* belonged to a separate group within the class Marinospora that may also contain the genera *Tetramicra* and *Microgemma*. *16S rRNA* sequences of the observed microsporidium and *K. newberryi* diverged by only 1.46% over 1352 bp, substantially less than closely related species of *Nosema* (which diverge by 2 to 11%) and *Dictyocoela* (4 to 11%) (Baker et al. 1995, Terry et al. 2004), indicating that the Swedish and Californian microsporidia are either closely related species or strains of a single species.

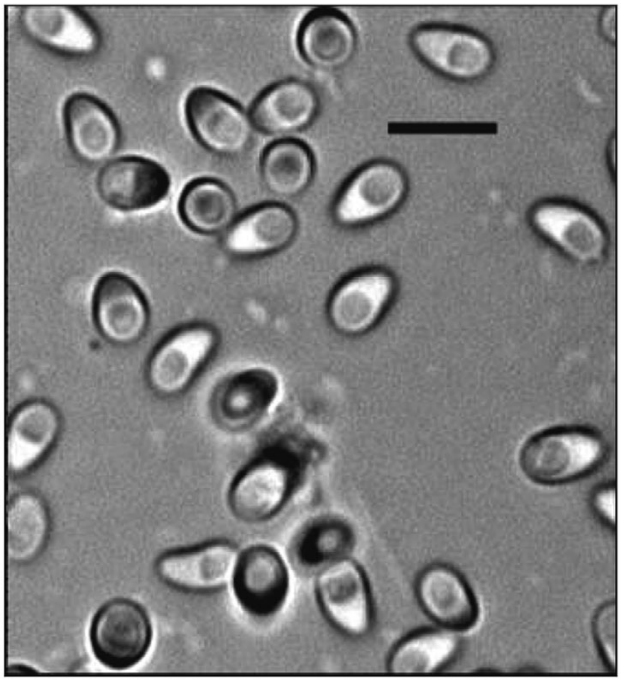

Fig. 3. *Kabatana* sp. Fresh spores of the *Kabatana* sp. released from liquefied muscle fibres of infected *Gobiusculus flavescens*, showing the ovoid or curved shape of the spores and their prominent posterior vacuole. Scale bar = 5 µm

Macro- and microscopic aspects of infection supported the genetic analysis, and there were a number of striking similarities between *Gobiusculus flavescens* and tidewater goby infections (McGourty et al. 2007). Both caused macroscopic lesions that were similar in size and distribution, and neither showed evidence of spores contained within macrophages or of xenoma formation (though spore masses in *G. flavescens* were occasionally membrane-bound). Previously described muscle-dwelling microsporidia from European marine fishes are largely *Glugea* spp. and *Pleistophora* spp., typically associated with xenoma formation or having macro- and microspore populations (see Lom 2002). However, the absence of these characteristics in the present study also supported the genetic evidence that the microsporidium infecting *G. flavescens* belonged to a separate clade from these other European species.

Despite the similarities between the *Kabatana* infections from two-spotted and tidewater gobies, some aspects of infection in the 2 host species appeared to differ. Liquefaction of muscle tissue was not recorded in infected specimens of the tidewater goby, but was a common and obvious feature of infected *Gobiusculus flavescens* that has also been reported in other microsporidian infections (Thélohan 1895, Dyková & Lom 2000). Also, freshly released (unfixed) spores of the microsporidium infecting *G. flavescens* were larger (mean size = 3.7 × 2.3 µm, range = 3.1 to 4.8 × 2.1 to 2.3 µm) than those reported by McGourty et al. (2007) for *K. newberryi* (2.8 × 1.9 µm). However, measurements of the latter appear to have been calculated from electron micrographs, which could explain spore size differences. Furthermore, the size range of spores of the microsporidium infecting *G. flavescens* overlapped substantially with those reported (measured fresh, unfixed) for *K. takedai* (mean 3.4 × 2.0 µm) (Lom et al. 2001), *K. seriolae* (3.3 × 2.2 µm) (Lom et al. 1999) and *K. arthuri* (3.1 × 2.1 µm) (Lom & Dyková 1992, Lom et al. 1999), suggesting that spore sizes of the *Kabatana*

sp. infecting *G. flavescens* are more typical of the genus than those reported for *K. newberryi*.

The origin of the *Kabatana* sp. infection in the *Gobiusculus flavescens* population off the Swedish west coast is unknown. Apart from *K. newberryi* and the microsporidium described here, all previously described *Kabatana* spp. have occurred in eastern Asia (Lom 2002). One possibility is that the parasite is a recent transcontinental colonist, perhaps introduced through anthropogenic activity (Stewart 1991, Wallentinus & Jansson 1999, Hayward et al. 2001). However, because microsporidian infections are probably under-reported in non-commercial species, *Kabatana* spp. may be prevalent or widespread in other fishes of the Northern Pacific and/or Atlantic. Findings presented here, and those of McGourty et al. (2007), suggest a more global distribution for the genus *Kabatana* than previously recognised, and further work is required to assess the extent of such infections. Microsporidian infections can have significant implications for host physiology, ecology and survival (Nepszy et al. 1978, Dezfuli et al. 2004, Khan 2004, Ward et al. 2005), and impact negatively on fisheries and aquaculture (Slifko et al. 2000). The emergence of *G. flavescens* as a model species in behavioural and evolutionary ecology (Skolbekken & Utne-Palm 2001, Forsgren et al. 2004, Pélabon et al. 2005) means the host–parasite system may be ideally suited for examining the fitness consequences of microsporidian infections in fish.

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