

Myxozoan parasites disseminated via oligochaete worms as live food for aquarium fishes: descriptions of aurantiactinomyxon and raabeia actinospore types

Sascha L. Hallett^{1,3}, Stephen D. Atkinson^{1,3}, Christer Erséus^{2,4}, Mansour El-Matbouli^{1,*}

¹Institute of Zoology, Fish Biology and Fish Diseases, University of Munich, Kaulbachstrasse 37, 80539 Munich, Germany

²Department of Invertebrate Zoology, Swedish Museum of Natural History, Box 50007, 104 05 Stockholm, Sweden

³Present address: Center for Fish Disease Research, Department of Microbiology, Oregon State University, Nash Hall 220, Corvallis, Oregon 97331, USA

⁴Present address: Department of Zoology, Göteborg University, Box 463, 405 30 Göteborg, Sweden

ABSTRACT: A total of 7 samples of live freshwater oligochaetes (mixed species), sold as 'tubifex' worms as food for aquarium fishes, were purchased over a 1 yr period from several pet shops in Munich, Germany, and screened for parasitic infections of myxozoans. The water associated with 5 samples contained actinospores at the time of purchase; 6 samples subsequently released spores in the laboratory. In all, 12 types of actinospores (Myxozoa: Myxosporea) from 4 collective groups were released by the oligochaetes. In the current study we provide descriptions of 2 aurantiactinomyxons (*Myxobolus intimus* Zaika, 1965 and type 1 nov.) and 3 raabeias (type 1 and 2 nov., Raabeia type 1 of Oumouna et al., 2003); descriptions of the 5 triactinomyxon and 2 hexactinomyxon types have been published previously. We include both raabeia and echinactinomyxon types in differential diagnoses of our raabeia forms because a clear distinction between these groups no longer exists in the literature. Comparison of 18S rDNA sequence data revealed that 1 of the novel aurantiactinomyxons was *Myxobolus intimus*. The sale of worms hundreds of km away from their point of origin is a means of dissemination of myxozoan parasites.

KEY WORDS: Actinospore · Myxozoa · Oligochaete · Pet shop · Parasite dispersal

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INTRODUCTION

Freshwater 'tubifex' worms (Annelida: Oligochaeta) are a common fish food sold in pet shops in a variety of forms including dried, frozen and live. Despite the name, 'tubifex' worms often comprise mixed oligochaete taxa, including lumbriculids (*Lumbriculus variegatus*), naids (e.g. *Dero digitata*) and tubificids (e.g. *Tubifex tubifex*, *Limnodrilus hoffmeisteri* and *Rhyacodrilus coccineus*) (Beauchamp et al. 2001). Sources of pet shop worms vary, and supplies may be transported across countries or even continents.

Oligochaetes are hosts for a number of parasites (Raftos & Cooper 1990) and have long been known to facilitate introduction of contaminants and disease into

aquaria. Shipments of 'tubifex' worms from eastern Europe to the United States have been found to be infected with myxozoan parasites (Lowers & Bartholomew 2003) which may lead to inadvertent introduction or dissemination of these organisms. Actinospores are the myxosporean life cycle stage known to infect fish, and the ensuing myxospore development can cause disease in wild and farmed fish populations (see Kent et al. 2001).

Curious about dispersal within Europe, we purchased live 'tubifex' worms of eastern European origin from pet shops in Munich, Germany, and screened them for myxozoan infections. The survey revealed infections by 5 triactinomyxons (Hallett et al. 2004, 2005), 2 hexactinomyxons (Hallett et al. 2003), and the 2 aurantiactinomyxons and 3 raabeias described herein.

*Corresponding author.
Email: elmatbouli@zoofisch.vetmed.uni-muenchen.de

MATERIALS AND METHODS

For detailed methodology on isolation of actinospores, identification of oligochaetes and DNA amplification, see Hallett et al. (2005).

Isolation of spores. Live 'tubifex' oligochaetes were purchased from several pet shops in Munich between March 2001 and February 2002. The water in which the oligochaetes had been kept was filtered and examined for actinospores. When spores were observed, the worm sample was sub-divided and re-examined over successive days, and finally oligochaetes were separated into multi-well plates to isolate the host.

Spores were pipetted onto a glass microscope slide under a coverslip and measured with a calibrated eyepiece micrometer. Spores were also pipetted onto glass slides and air-dried before fixing and staining with Giemsa and Diff-Quik. Spores are described herein in accordance with the guidelines of Lom et al. (1997) although we use the more popular term 'germ cell' instead of 'daughter cell'. In lieu of 'type specimen' in species descriptions, we use the term 'reference material' (Hallett et al. 2005).

Identification of host oligochaetes. Oligochaetes were processed following the protocol for marine forms by Erséus (1994) and standard keys to freshwater Oligochaeta were consulted (i.e. Kathman & Brinkhurst 1998, Timm 1999).

Molecular analysis. To collect spores for DNA extraction, host oligochaetes were placed individually in microcentrifuge tubes in a small amount of fresh tap water for 24 to 48 h, after which time the worm was removed and the spore sample either frozen or processed immediately. DNA was extracted using the QIAamp DNA Mini Kit/Tissue protocol (Qiagen) according to the manufacturer's instructions, except that samples were re-suspended in 50 µl distilled water.

Initial amplification of the 18S small subunit ribosomal RNA gene (18S rDNA) was achieved using the primers 18e and 18g (Hillis & Dixon 1991). Combinations of paired sequencing primers were trialled on the 18e/18g template in 20 µl reactions to assess their suitability. A 5' end fragment of ~1000 bp in length was produced using the nested primers MYX1f and ACT1r and sequenced with those primers as well as with ACT1f and ACT1fr (Hallett & Diamant 2001). The 3' end of aurantiactinomyxon was amplified with ACT3f (Hallett & Diamant 2001) and MX3 (Andree et al. 1998) and sequenced with these primers plus ACT2f and ACT2fr (Hallett et al. 2003), whereas raabeia was amplified with ACT1f and MX3 and sequenced with these primers plus ALL1f (Hallett et al. 2002) and ACT2fr. PCR products were purified (QIAquick PCR Purification Kit, Qiagen) and sent to

Sequence Laboratories, Göttingen, Germany, for sequencing in both directions.

The various forward and reverse sequence fragments generated for each actinospore type were aligned manually in BioEdit (Hall 1999) and ambiguous bases clarified using corresponding ABI chromatograms (where a base remained indistinct, an N was designated). Completed sequences were submitted to GenBank and a standard nucleotide-nucleotide BLAST (blastn) search was conducted (www.ncbi.nlm.nih.gov/BLAST/; Altschul et al. 1997).

RESULTS

Over a 1 yr period, 7 samples of 'tubifex' worms from 3 Munich pet shops were screened. Worms were accompanied by a small amount of water, which in 5 cases contained actinospores at the time of purchase (Phylum Myxozoa Grassé, 1970, Class Myxosporea Bütschli, 1881) (Kent et al. 1994). Worms from 6 of the 7 samples (all 3 pet shops) released additional spores while in the laboratory. In total, 12 types of actinospores were identified: descriptions for 5 triactinomyxons and 2 hexactinomyxons have been previously published (Hallett et al. 2003, 2004, 2005); descriptions of the remaining 5 are presented here. 18S rDNA sequences were obtained from the previously undescribed aurantiactinomyxon stage of *Myxobolus intimus* and Raabeia type 1 of Oumouna et al., 2003.

Aurantiactinomyxon stage of *Myxobolus intimus* Zaika, 1965

Description. The spore possesses a spore body and 3 lateral processes (Fig. 1a–e). The spore body in apical view is circular, forms points at the sutures of valve cells, and measures 13.8 µm (13.0 to 14.9 µm) in diameter. In side view it is ellipsoidal with a diameter of 13.6 µm (n = 8). The polar capsules are pyriform, measuring 3.1 × 2.3 µm, are prominent in side view and lie under the sutures. The extended polar filament length is ~21 µm. Capsulogenic cells and their nuclei are prominent. Sporoplasm contains 16 germ cells, arranged in clusters of 4 (Fig. 1f, g). The processes are approximately equal, appearing finger-like to leaf-like in apical view. They are 17.7 µm (15.5 to 22.0 µm) long, 10.7 µm (9.7 to 14.2 µm) wide near the spore body, with the largest span being 43.6 µm (40.2 to 49.9 µm). In side view they curve downwards, are 20.1 µm (18.1 to 22.0 µm) long and 10.4 µm wide. The valve cell nuclei are ~3 µm in diameter.

Host. *Limnodrilus hoffmeisteri* Claparède, 1862.

Site in host. Intestinal epithelium.

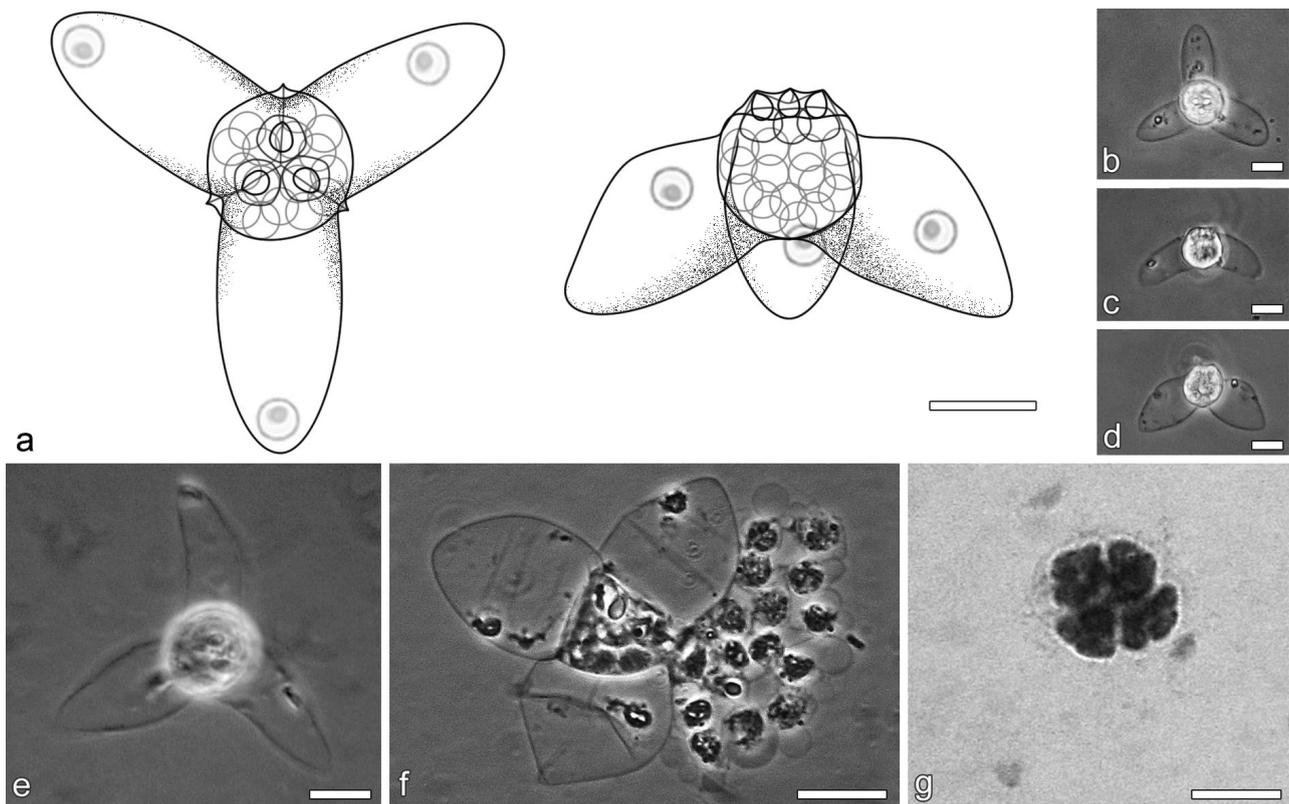


Fig. 1. Aurantiactinomyxon stage of *Myxobolus intimus*. (a) Line drawing of mature spore. Left, apical view; right, side view. (b–e) Fresh, unstained spores viewed at various orientations under a coverslip. (f) Severely compressed spore showing exuded sporoplasm with germ cells. (g) Stained sporoplasm with 4 clusters of 4 germ cells each. Scale bars = 10 µm

Source of material. Pet shop (D), Munich, Germany (purchased 22 June 2001).

Specimens deposited. Diff-Quik and Giemsa slides of air dried spores were deposited in the parasitology collection at Queensland Museum, Brisbane, Australia, accession numbers G464769 and G464770, respectively.

Remarks. We isolated 4 hosts which released this actinospore. The spore dimensions of 3 of these hosts are provided in Table 1. Spores were released for 7 wk but not again in the following 12 mo. Spores were expelled from the worm with faeces, and tended to sink in still water.

Molecular data. 1628 bp of 18S rDNA sequence data were submitted to GenBank (AY495708).

Differential diagnosis. We compared our spore type with 34 aurantiactinomyxon types reported in the literature:

Table 1. Morphometrics (in µm) of aurantiactinomyxon stage of *Myxobolus intimus* from 3 hosts (A–C) and the most similar morphotype, *Aurantiactinomyxon pavinsis* Marquès, 1984 (grande forme)

	Host A	Host B	Host C	<i>A. pavinsis</i>
No. spores measured	8	9	11	
Apical view				
Spore body diameter	13.8 (13.0–14.9)	12.3 (10.4–13.0)	12.9 (11.7–15.5)	12
Polar capsules	3.1 × 2.3	3.0 × 2.7	3.1 × 2.2	
Process length	17.7 (15.5–22.0)	16.7 (14.9–18.1)	17.7 (15.5–20.7)	15–20
Width at base	10.7 (9.7–14.2)	10.1 (9.1–10.4)	10.6 (8.4–13.6)	6–8
Side view				
Spore body diameter	13.6	12.3	13.0	
Process length	20.1 (18.1–22.0)	19.2 (17.5–22.0)	17.5 (16.8–18.1)	
Width at base	10.4	10.6	10.0	
Largest span	43.6 (40.2–49.9)	41.5 (36.3–45.3)	41.4 (35.0–45.3)	
Ratio process:spore body	1:0.78	1:0.74	1:0.73	1:0.6–0.8
Germ cells	16	16	16	16

Marquès 1984 (4 types, one of which is *Chloromyxum truttae*; see Holzer et al. 2004); McGeorge et al. 1997 (1 type); Xiao & Desser 1998b (1 type); El-Mansy et al. 1998a,b (13 types); Székely et al. 2000 (1 type); Negredo & Mulcahy 2001 (1 type); Hallett et al. 2002 (1 type); Oumouna et al. 2003 (1 type); Özer et al. 2002 (3 types); Székely et al. 2003 (1 type). A further 7 types have been documented as part of a myxozoan life cycle: Pote et al. (2000) (*Henneguya ictaluri*); El-Matbouli et al. (1992) (*Hoferellus carassi*); Grossheider & Körting (1992) (*Hoferellus cyprini*); Benajiba & Marquès (1993) (*Myxidium giardi*); Székely et al. (1998) (*Thelohanellus nikolskii*); Lin et al. (1999) (*Henneguya exilis*); and Yokoyama et al. (1997) (*Thelohanellus hovorkai*). Comparison with the actinospore stage of *Hoferellus carassi* (El-Matbouli et al. 1992) or *Myxidium giardi* (Benajiba & Marquès 1993) was not possible since the authors provide only a photograph with magnification but no scale bar, and no dimensions are given in the text. Nor was a comparison with *Aurantiactinomyxon* sp. 1 identified in Yokoyama et al. (1993) possible, for although the authors record several biological characteristics (including seasonality, longevity, circadian rhythm of spores release and chemoreception to fish mucous) they omit phenotypic details of the spores which would permit re-identification.

Initial phenotypic comparison of aurantiactinomyxons indicated closest affinity of our sample to the 'grande form' of *Aurantiactinomyxon pavinsis* Marquès, 1984 (Table 1); however, the host oligochaete species differed, and pairwise alignment in BioEdit of 887 bases of 18S rDNA sequence revealed only 72% similarity.

Genetic data exist for 10 aurantiactinomyxons in GenBank: *Aurantiactinomyxon* sp. (AF378356); *A. mississippiensis* (AF021878); *A. janiszewskai-Henneguya exilis* (AF021881); *Thelohanellus hovorkai* (AJ133419); *Aurantiactinomyxon* type 1 of Negredo & Mulcahy, 2001 (AF483598); *Aurantiactinomyxon* of Hallett et al., 2002 (AF487455); *A. pavinsis* (AJ582006); *A. ictaluri* — *H. ictaluri* (AF0298320); and *Aurantiactinomyxon* types 1 and 3 of Özer et al., 2002 (AJ582004, AJ582005). A BLAST search showed less than 84% similarity between our aurantiactinomyxon and other sequenced aurantiactinomyxons. The search indicated

a 99.9% match with *Myxobolus intimus* (AY325285) with the 2 sequences differing in just 1 nucleotide. A subsequent pairwise alignment of the 2 sequences in BioEdit revealed 3 nucleotide differences over the aligned 1589 bases (99.8% similarity) (Fig. 2).

Aurantiactinomyxon type 1 nov.

Description of reference material. The spore possesses a spore body and 3 lateral processes (Fig. 3a–c). The spore body in apical view is sub-circular with puckering at the sutures, and has a diameter of 12 (11.7 to 13.0) μm ; in side view it is ellipsoidal ($n = 4$). The polar capsules ($2.3 \times 3.0 \mu\text{m}$) are pyriform, are prominent in side view and lie under the sutures. The sporoplasm most likely contains 16 germ cells (Fig. 3d). The processes are approximately equal, in apical view they appear leaf-like and are 26.6 (24.6 to 31.1) μm long, 10.1 (9.1 to 10.4) μm wide at the base near the spore body; they widen in the middle 12.9 (12.3 to 13.6) μm , taper almost to a point at the end with the largest span being 49.9 (44.0 to 54.4) μm ; in side view they curve slightly downwards. The valve cell nuclei are $\sim 2.5 \mu\text{m}$ in diameter.

Host. Not isolated.

Source of material. Pet shop (D), Munich, Germany (purchased 12 August 2001).

Specimens deposited. A Giemsa-stained slide of air dried spores was deposited in the parasitology collection at Queensland Museum, Brisbane, Australia, accession number G464771.

Remarks. Spores tend to sink in still water. The oligochaete sample was sub-divided after observation of waterborne spores; however, no further spore release occurred. The host was not determined and no DNA sample was acquired.

Differential diagnosis. The spore was most similar morphometrically to the *Aurantiactinomyxon* of McGeorge et al., 1997 (Table 2) but the 2 differ somewhat morphologically: the processes of the latter narrow more severely than those of our type, the spore body is spherical rather than triangular/globular and the polar capsules are spherical not pyriform. Thus, our aurantiactinomyxon appears novel.

Auranti	29	tat-ctgtttgattgtcttccccattggataaccgtgggaaatctagagctaatacatgcagtt	91
<i>M. intimus</i>	1	g..c.....c.....	64

Fig. 2. Segment of the 18S rDNA sequence alignment comparing our aurantiactinomyxon (Auranti) (AY495708) and the myxospore of *Myxobolus intimus* (AY325285) showing position of the 3 base differences over 1589 bases. Numbers represent base position from 5' end of submitted sequence

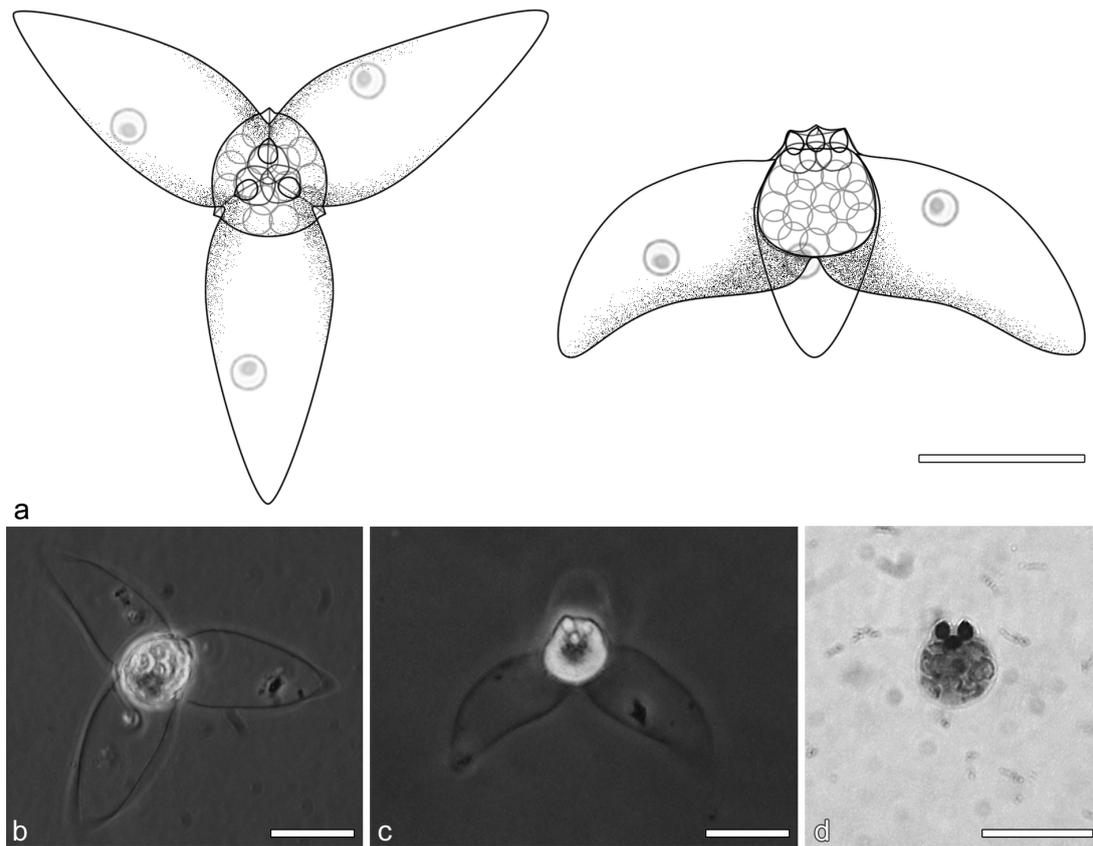


Fig. 3. Aurantiactinomyxon type 1 nov. (a) Line drawing of mature spore. Left, apical view; right, side view. (b,c) Fresh, unstained spores viewed at various orientations under a coverslip. (d) Giemsa-stained spore body highlighting the germ cells and polar capsules. Scale bars = 10 μm

Raabeia type 1 nov.

Description of reference material. The spore possesses a spore body and 3 caudal processes (Fig. 4a,b, Table 3). The spore body is ovate in side view, 27.2 (27.2) μm long and 16.8 (15.5 to 18.1) μm wide ($n = 2$).

Polar capsules are prominent at the spore apex, are squat pyriform, directed 30° outwards from the axis of the spore, are 3.9 (3.9) μm long and 3.2 (3.2) μm wide. The sporoplasm contains at least 12 germ cells (Fig. 4c). The caudal processes are approximately equal, begin two-thirds down the spore body, curve slightly outwards, then taper smoothly to a rounded point. The measurements are: length 213.2 (207.2 to 225.3) μm , width at base 11.2 (10.4 to 13.0) μm .

Host. Not isolated.

Source of material. Pet shop (D), Munich, Germany (purchased 8 July 2001).

Remarks. The oligochaete sample was sub-divided after observation of waterborne spores. However, no further spore release occurred; thus the host could not be segregated, and a DNA sample was not acquired.

Differential diagnosis. We compared our spore with 25 published raabeia descriptions: Janiszewska (1955, 1957)

Table 2. Morphometrics (in μm) of Aurantiactinomyxon type 1 nov. and most similar morphotype

	Aurantiactinomyxon type 1 nov.	Aurantiactinomyxon of McGeorge et al., 1997
No. spores measured	4	20
Spore body/spore cavity	12.0 (11.7–13.0)	13.7 (12–15)
Polar capsules		2.7 (2–3)
Lateral processes		
Length	26.6 (24.6–31.1)	25.6 (19–31)
Greatest width	13.0 (12.3–13.6)	12.0 (10–14)
Width at base	10.1 (9.1–10.4)	
Largest span	49.9 (44.0–54.5)	
Ratio process:spore body	1:0.45	1:0.54
Germ cells	>8 (12?)	
Host		<i>Tubifex</i> sp.

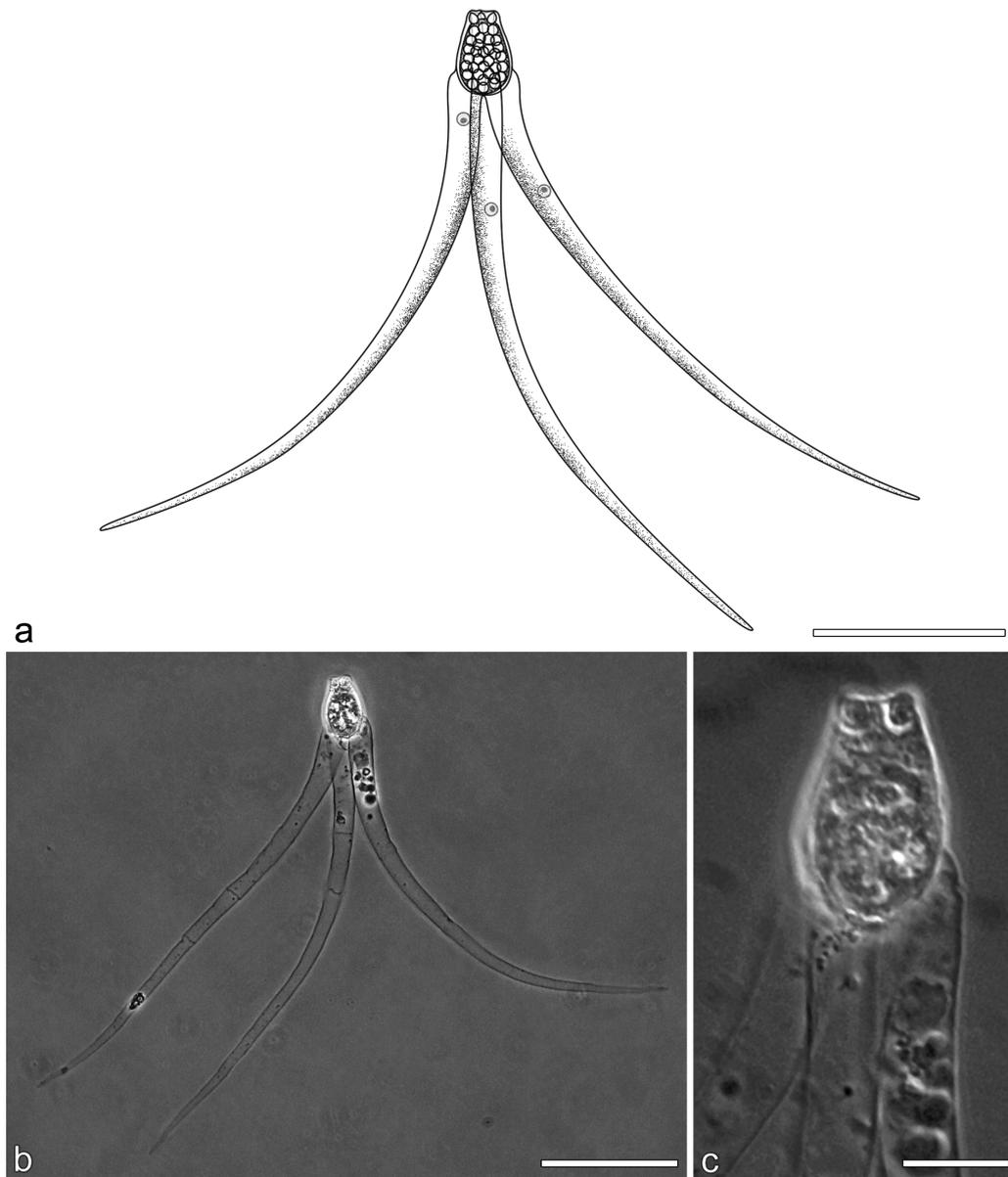


Fig. 4. *Raabeia* type 1 nov. (a) Line drawing of mature spore. Scale bar = 50 μm . (b) Fresh, unstained spore viewed under a coverslip. Scale bar = 50 μm (c) Higher magnification of spore body. Scale bar = 10 μm

(2 types), Janiszewska & Krzton (1973) (1 type), McGeorge et al. (1997) (1 type), Xiao & Desser (1998a) (6 types), El-Mansy et al. (1998a,b) (4 types), Oumouna et al. (2003) (2 types), Koprivnikar & Desser (2002) (1 type), Özer et al. 2002 (6 types, one of which is *Myxidium truttae* [Holzer et al. 2004]), and those whose myxospore stage is known: Yokoyama et al. 1995 (*Myxobolus cultus*) (note that this actinospore was also documented in Yokoyama et al. 1991, 1993) and Molnár et al. 1999 (*Myxobolus dispar*). Spore morphology and morphometrics of our *raabeia* are not consistent with any hitherto described form.

***Raabeia* type 2 nov.**

Description of reference material. The spore possesses a spore body and 3 caudal processes (Fig. 5a,b, Table 3). Spore body, in side view sub-circular, length 22.0 (22.0) μm , width 14.2 (13.0 to 15.5) μm ($n = 2$). The sporoplasm contains 8 germ cells each paired with a polar body (Fig. 5c). The polar capsules at the spore apex are pyriform, directed 30° outwards, 4.2 (2.6 to 5.8) μm long, and 3.6 (3.2 to 3.9) μm wide. The caudal processes are approximately equal, begin two-thirds down the spore body, are relatively straight (but not

Table 3. Morphometrics (in μm) of raabeia spores

	Raabeia type 1 nov.	Raabeia type 2 nov.	Raabeia type 1 of Oumouna et al., 2003 (current study)	Raabeia type 1 of Oumouna et al., 2003 (original description)
No. spores	2	2	7	12
No. germ cells	>12	8 paired with polar body		20–28
Polar capsules				
Length	3.9 (3.9)	4.2 (2.6–5.8)	5.3 (5.1–6.5)	5 \pm 0.4
Width	3.2 (3.2)	3.6 (3.2–3.9)	3.0 (2.6–3.9)	3 \pm 0.2
Spore body				(Spore cavity)
Length	27.2 (27.2)	22.0 (22.0)	40.3 (36.3–42.7)	35 \pm 4
Width	16.8 (15.5–18.1)	14.2 (13–15.5)	10.5 (9.1–11.7)	12 \pm 2
Process				
Length	213.2 (207.2–225.3)	120.7 (114–126.9)	241.8 (220.2–251.6)	245 \pm 13
Width	11.2 (10.4–13.0)	7.7 (6.5–9.1)	9.2 (7.7–9.7)	

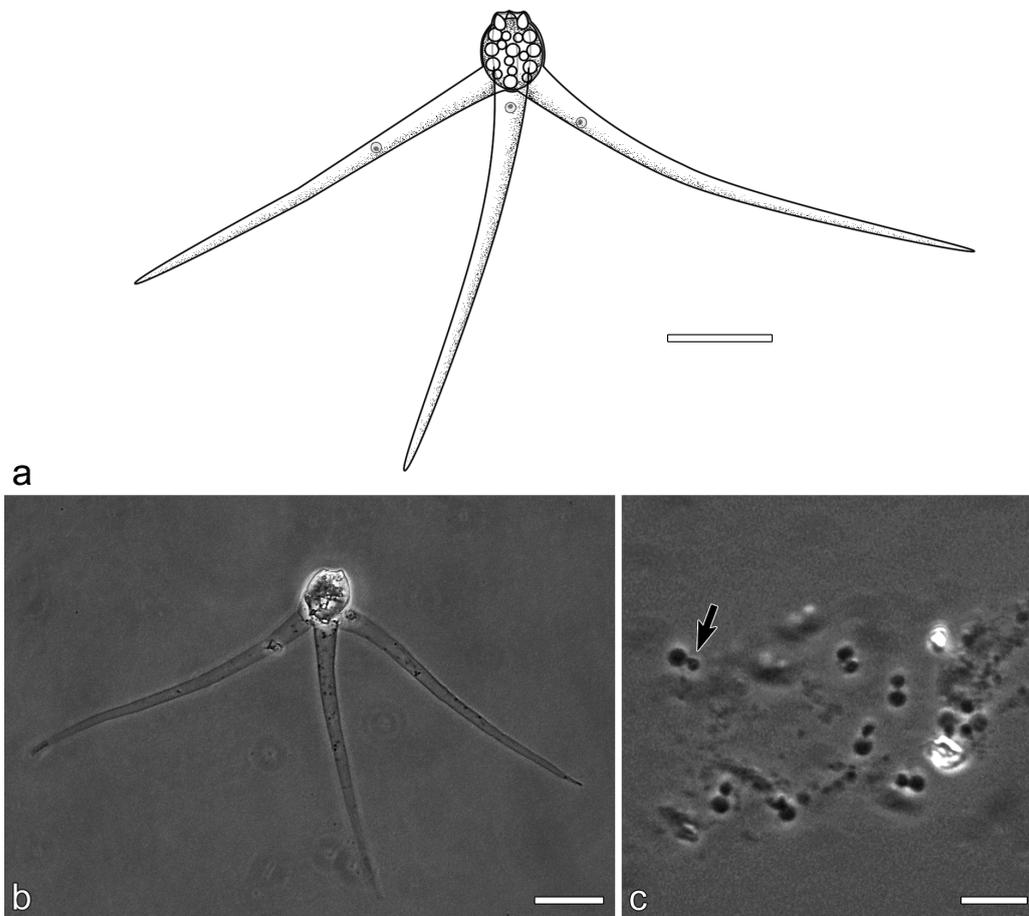


Fig. 5. Raabeia type 2 nov. (a) Line drawing of mature spore. Scale bar = 20 μm . (b) Fresh, unstained spore under a coverslip. Scale bar = 20 μm . (c) Contents of exuded sporoplasm showing the 8 germ cells each paired with a polar body (arrow). Scale bar = 10 μm

rigid or spine-like), taper smoothly to a point, and measure 120.7 (114.0 to 126.9) μm in length and 7.7 (6.5 to 9.1) μm in width.

Host. Not isolated.

Source of material. Pet shop (D), Munich, Germany (purchased 8 July 2001).

Remarks. The oligochaete sample was sub-divided after observation of waterborne spores. However, no further spore release occurred, the host could not be isolated and no DNA sample was acquired.

Differential diagnosis. We compared our spore both with raabeia forms and, given its resemblance to echi-

nactinomyxons, with 13 published echinactinomyxon descriptions: Janiszewska (1957) (1 type); Marquès (1984) (1 type); Xiao & Desser (1998b) (5 types); Negredo & Mulcahy (2001) (2 types); Székely et al. (2002) (1 type); Özer et al. (2002) (1 type), Oumouna et al. (2003) (1 type); and 1 type whose myxospore stage is known (*Sphaerospora truttae*, see Özer & Wootten 2000). Kent et al. (2001) purport that an echinactinomyxon is the alternate stage of *Zschokkella* sp.; however, in the original report it is ambiguous whether this myxospore alternates with an echinactinomyxon, aurantiactinomyxon or a neoactinomyxon, as the life cycle connection between these organisms was speculative (Yokoyama et al. 1991). Irrespective of this, no phenotypic information about the spores was provided.

Spore morphology and morphometrics are not consistent with any of the hitherto described raabeia or echinactinomyxon forms; they most closely identify with Echinactinomyxon 1 of Negredo & Mulcahy, 2001 and Echinactinomyxon type 1 of Özer et al., 2002 with regard to lengths but not to widths or number of germ cells. Furthermore, neither of these types exhibits process curvature proximal to the spore body.

Raabeia type 1 of Oumouna et al., 2003

Description. The spore possesses a spore body and 3 caudal processes (Fig. 6a,b, Table 3). The spore body in side view is elongated oval, pinched in the middle to varying degrees, measures 40.3 (36.3 to 42.7) μm in length, and 10.5 (9.1 to 11.7) μm in width ($n = 7$). The polar capsules are prominent at the apex with distinct nuclei and capsulogenic cells and measure 5.3 (5.1 to 6.5) μm in length, and 3.0 (2.6 to 3.9) μm in width; the extended polar filament length is 39.5 (33.7 to 45.3) μm . The caudal processes are approximately equal, are curved and taper to a sharp point, are 241.8 (220.2 to 251.6) μm long, and 9.2 (7.7 to 9.7) μm wide at the base; the valve cell nuclei are clustered at the base of the spore body.

Host. *Tubifex tubifex* Müller, 1774.

Site in host. Not determined.

Source of material. Pet shop (D), Munich, Germany (purchased 8 August 2001).

Specimens deposited. A Diff-Quik slide of air dried spores was deposited in the parasitology collection at Queensland Museum, Brisbane, Australia, accession number G464772.

Remarks. Waterborne spores of this type were found in a second worm sample (Pet shop D, purchased 22 June 2001) but the second host worm could not be

isolated. Neither squashes of fresh spores nor stained spores exhibited discrete germ cells in the sporoplasm (Fig. 6b inset, c).

Molecular data. 1640 bp of 18S rDNA sequence data were submitted to GenBank (AY495709).

Differential diagnosis. Spore morphology and morphometrics are consistent with Raabeia type 1 of Oumouna et al., 2003 and the spore is identified as such (Table 3). The 18S rDNA sequence data did not match any of the 5 raabeias in GenBank: Raabeia B of Xiao & Desser, 1998a (AF378352); Raabeia stage of *Myxobolus dispar* (AF507972); Raabeia type 1 of Özer et al., 2002 (AJ582008); Raabeia type 3 of Özer et al., 2002 (AJ582009); Raabeia type 4 of Özer et al., 2002 (AJ582010). A BLAST search showed greatest similarity (96%) with *Myxidium truttae* (AF201374).

DISCUSSION

The 'tubifex' samples purchased from pet shops in Munich, Germany, originated from eastern European countries and comprised at least 3 oligochaete species: *Tubifex tubifex*, *Limnodrilus hoffmeisteri* and *L. udekemianus* Claparède, 1862. Myxozoan infections were found in all 3 species, and some worms were releasing spores at the time of purchase. Comparison of these spores with forms in the literature indicated that the majority were novel records.

Taxonomy

Although the Class Actinosporea is suppressed and its members no longer recognised as species in their own right (pending further life cycle information) (Kent et al. 1994), it is nevertheless important that these parasites are accurately and unambiguously documented since at least some infect fish and cause disease. Actinospores are classified into collective groups (formerly genera) which appear distinct based on the original genus descriptions of spore morphology. However, as more actinospores have been encountered, it appears that, rather than fall neatly into morphological groups, they may have a continuum of form leading to some taxonomic ambiguities.

Of the spore types described herein, we found that 2 fitted clearly within the collective group Aurantiactinomyxon and 2 distinctly within Raabeia. However, a fifth spore type which we assigned as Raabeia type 2 resembled members of both Echinactinomyxon and Raabeia collective groups, and we noted from our differential diagnosis that there was no longer a clear distinction between these 2 groups in the literature.

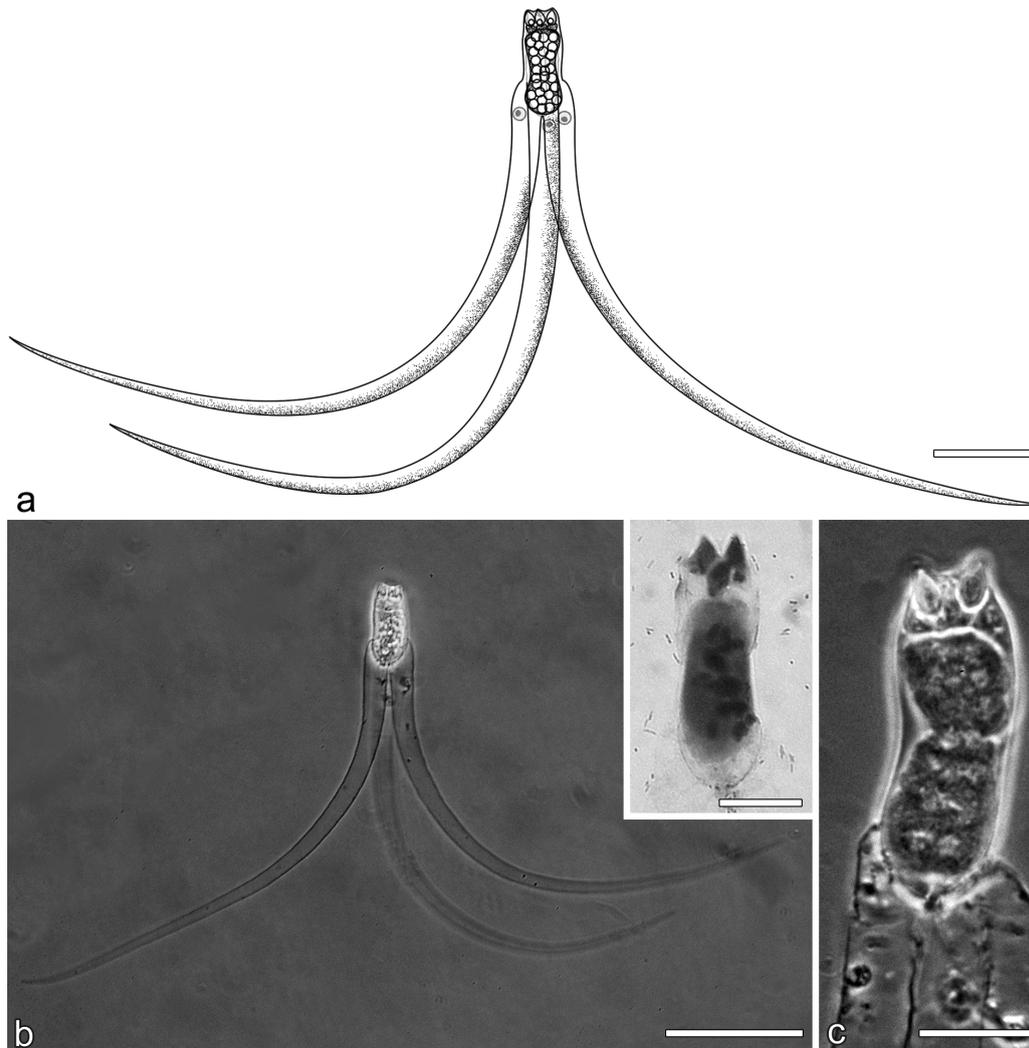


Fig. 6. *Raabeia* type 1 of Oumouna et al., 2003. (a) Line drawing of mature spore. Scale bar = 50 μ m. (b) Fresh unstained spore viewed under a coverslip. Scale bar = 50 μ m. Inset: Giemsa-stained spore body highlighting germ cells and polar capsules. Scale bar = 10 μ m. (c) Higher magnification of the spore body of a second fresh spore showing 'pinching'. Scale bar = 10 μ m

Raabeia spores are defined as: 'episporer' (now an obsolete term; Lom et al. 1997), with 3 long, pointed and curved processes arising from the episporer without a style (Janiszewska 1955). Echinactinomyxon, however, are defined by 3 equal, spiny, straight, rigid and pointed processes (Janiszewska 1957). The difficulty in assigning spores to either taxon arises when spores have shorter, slightly curved processes. We feel that the overriding echinactinomyxon characters are 'spiny, straight, rigid' processes, which our *Raabeia* type 2 did not possess. Its processes, however, were shorter than an architypal *raabeia* form and lacked a distinct deep curve. Our spore type morphologically (but not morphometrically) resembled Echinactinomyxon C of Xiao & Desser, 1998, whose processes are

straight to slightly curved, and other echinactinomyxons (see 'Differential diagnosis' in 'Results').

Janiszewska & Krzton (1973) describe a new actinospore form with uniquely branched processes. Because the processes are also 'arched up' they assign it, appropriately, to *Raabeia*. Since then, several research groups have placed spores in *Raabeia* based on this branching characteristic, even when the spores have straight, rigid processes characteristic of echinactinomyxon forms (e.g. Molnár et al. 1999, Koprivnikar & Desser 2002, Özer et al. 2002); this has further blurred the distinction between the 2 groups. We therefore suggest that differential diagnoses involving either *raabeia* or echinactinomyxon types should include members of the other collective group.

Genetic characterisation and life cycle connections

Aurantiactinomyxon

We identified 2 spore types which we believed to be novel phenotypes. The first type was distinguished genetically from other sequenced aurantiactinomyxons, but without sequence data for our second type we could not be as definitive about its uniqueness. We were acutely aware of our previous case study (Hallett et al. 2002) in which 2 morphologically distinct aurantiactinomyxon spore types, released simultaneously from the one oligochaete, have the same 18S rDNA sequence. Although we advocate augmentation of taxonomic descriptions with molecular sequence data and consider anything else to be less than ideal, we nonetheless believe the types we encountered are worthy of record.

Comparison of 18S rDNA sequences has been used to match or confirm the life cycle stages of 9 myxozoans: *Myxobolus cerebralis* (Andree et al. 1997), *Ceratomyxa shasta* (Bartholomew et al. 1997), *Tetracapsuloides bryosalmonae* (Anderson et al. 1999, Longshaw et al. 1999), *Henneguya exilis* (Lin et al. 1999), *H. ictaluri* (Pote et al. 2000), *Ellipsomyxa gobii* (Koie et al. 2004), *Chloromyxum* sp. (Holzer et al. 2004), *C. truttae* (Holzer et al. 2004) and *Myxidium truttae* (Holzer et al. 2004). A database search revealed a high similarity of the 18S rDNA sequence of 1 of our aurantiactinomyxons to the myxospore stage of *Myxobolus intimus* (AY325285), and a subsequent pairwise alignment of the 2 sequences revealed differences in only 3 base positions: a similarity of 99.8% over 1589 bases, which strongly suggests that our aurantiactinomyxon is *M. intimus*. This similarity is within the range for counterparts in the above studies (98.9 to 100%), and is less than the level of intraspecific divergence (<2%) observed for isolates of several myxozoans from different hosts or geographic locations (see Hallett et al. 2004).

The life cycles of 13 *Myxobolus* species are known and most (85%) involve a triactinomyxon actinospore stage (see Kent et al. 2001, Székely et al. 2002). *M. cultus* and *M. dispar*, involve a raabeia actinospore, whereas the lifecycle of *M. pavlovskii* involves a hexactinomyxon (Kent et al. 2001). Aurantiactinomyxons have been reported as the actinospore stages of a range of myxosporean genera, including members of both Platysporina and Variisporina: *Henneguya* (*H. exilis*, *H. ictaluri*); *Hoferellus* (*H. carassii*, *H. cyprini*); *Thelohanellus* (*T. nikolskii*, *T. hovorkai*); *Myxidium* (*M. giardi*) (Kent et al. 2001); and *Chloromyxum* (*C. truttae*) (Holzer et al. 2004); ours is the first report of a possible myxobolus-aurantiactinomyxon combination.

Recently, the life cycle of *Myxobolus intimus* has been experimentally investigated (Rácz et al. 2004). In conflict with our molecular data, these authors report development of triactinomyxon spores in oligochaetes exposed to *M. intimus* myxospores from gills of roach *Rutilus rutilus*. However, they could not complete the life cycle and infect fish with these triactinomyxons, and also, sequence data for the triactinomyxon and myxobolus spores did not match (Cs. Székely pers. comm.). Furthermore, *Tubifex tubifex* was the host oligochaete of the triactinomyxons, whereas we found *L. hoffmeisteri* produced our aurantiactinomyxons. *Myxobolus intimus* is known from Hungary, one of the countries identified as a source of the pet shop worms we purchased, so it is conceivable that our worms were infected with this parasite. We feel these data, collectively, support our molecular determination that *M. intimus* alternates between a myxobolus myxospore and an aurantiactinomyxon actinospore.

Another life cycle misidentification may have occurred for *Sphaerospora truttae* whose myxospores were linked experimentally with an echinactinomyxon (Özer & Wootten 2000). An echinactinomyxon later described as Echinactinomyxon type 5 by Özer et al. (2002) was identified morphologically as the *Sphaerospora truttae* echinactinomyxon. Subsequently, however, Holzer et al. (2004) found that Özer et al.'s (2002) Echinactinomyxon type 5 and *S. truttae* myxospores share less than 50% of their 18S rDNA sequences, raising questions as to whether this echinactinomyxon was misidentified as the earlier type shown experimentally to be *S. truttae*. Unfortunately, Özer et al. (2002) provide neither morphometrics nor an image of Echinactinomyxon type 5 (and only restate the original measurements given by Özer & Wootten 2000). The data conflict highlights the need to either follow the complete life cycle in the laboratory (i.e. fish-worm-fish or worm-fish-worm) or confirm the putative alternate stages with molecular sequence data, and for subsequent reports of parasites to be supported by both morphological and morphometrical data.

Raabeia

Given the phenotypic similarity of our Raabeia type 2 spore to an echinactinomyxon, we conducted a literature search of both collective groups. Raabeia forms have been identified through laboratory infection experiments as species of *Myxobolus*, *M. cultus* and *M. dispar* (Yokoyama et al. 1995, Molnár et al. 1999), and through DNA sequence comparisons as a species of *Myxidium*—*M. truttae* (Holzer et al. 2004). A BLAST search we conducted indicated that Raabeia type 1 of Oumouna et al., 2003 identified most closely

with *M. truttae* ex *Oncorhynchus kisutch* (AF201374), *M. truttae* ex *Salmo trutta* (AJ582061) and the Raabeia type 3 of Özer et al., 2002 (all > 96%). Our alignment of the above 4 sequences in BioEdit and subsequent pairwise alignment of 1530 bp revealed that Raabeia type 1 shared more bases with *M. truttae* ex *S. trutta* than *M. truttae* ex *O. kisutch* (96% vs. 95%) and *M. truttae* ex *S. trutta* was more similar to the Raabeia type 3 of Özer et al., 2002 (100%; and subsequently both were identified as life cycle counterparts [Holzer et al. 2004]) than to the other *M. truttae* isolate (99.2%; 12 base differences), indicating clear genetic differences between the UK and Canadian myxospore isolates. In the phylogenetic analyses conducted by Kent et al. (2001) and Holzer et al. (2004), Raabeia type B of Xiao & Desser, 1998, also grouped with *Myxidium truttae* (AF201374) and a second *Myxidium* species. Thus, as with aurantiactinomyxon actinospores, raabeia actinospores also belong to myxosporean genera that span 2 bivalvulid suborders, Variisporina and Platysporina. Holzer et al.'s analysis also showed that Raabeia type 1 of Özer et al., 2002 clustered with *Myxobolus portu-calensis*; in our opinion this actinospore more closely resembles an echinactinomyxon than a raabeia.

Life cycle experiments have demonstrated that echinactinomyxons belong to different myxosporean genera from those of raabeia: *Sphaerospora truttae* (Özer & Wootten 2000) and *Zschokkella* sp. (Yokoyama et al. 1993). However, a second *Zschokkella* species, *Z. nova*, is a siedleckiella (Uspenkaya 1995) and a second *Sphaerospora*, *S. renicola*, is a neoactinomyxum (Grossheider & Körting 1993). These are further examples of the inconsistent nature of which myxospore form alternates with which actinospore form.

Origin and dispersal of worms

Only 1 of the 3 Munich pet shop owners was forthcoming with details of the source of their worms: Romania and Hungary. Unlike Romania, myxozoans from Hungary have been relatively well documented. Some 34 types of actinospores are known, 13 of which are aurantiactinomyxons and 4 are raabeias (El-Mansy et al. 1998a,b). None of the types we isolated appear to be any of these. Hosts recorded by El-Mansy et al. (1998) for these types included *Tubifex tubifex*, *Limnodrilus hoffmeisteri* and a species not encountered in our samples, *Branchiura sowerbyi*. A similar number of myxospores are known from Hungarian fishes: El-Mansy et al. (1998a,b) report on 10 species from a local lake and about 28 species from a fish farm, and a further 7 are listed in recent molecular analyses (Molnár et al. 2002, Eszterbauer 2004). Life cycles involving aurantiactinomyxon, echinactinomyxon or raabeia

stages are known for at least 5 of these Myxosporea: *Hoferellus carassi*, *H. cyprini*, *Thelohanellus nikolskii*, *T. hovorkai* (all aurantiactinomyxon) (El-Matbouli et al. 1992, Grossheider & Körting 1992, Yokoyama et al. 1997, Székely et al. 1998) and *Myxobolus dispar* (raabeia) (Molnar et al. 1999); 2 of the fish farm aurantiactinomyxons are the 2 *Thelohanellus* species (Székely et al. 1998).

Growth in the aquarium industry has led to translocation of 100s of species of fishes and invertebrates whose survival rates have improved with expedited transportation (Carlton 1992). Some fish species are now found throughout most of the world, e.g. common carp *Cyprinus carpio*, common goldfish *Carassius auratus*, rainbow trout *Oncorhynchus mykiss*, and brown trout *Salmo trutta* (Ganzhorn et al. 1992), and hence the probability of both myxozoan hosts (vertebrate and invertebrate) being present in a novel location has also increased.

The transfer of parasites with their aquatic fish hosts is well documented: for example *Myxobolus cerebralis*, which is responsible for whirling disease in salmonids and remains a significant problem for farmed and wild populations of trout (Gilbert & Granath 2003), and is a disseminated fish pathogen (Ganzhorn et al. 1992). Nine myxozoans (5 genera), including *M. cerebralis*, are listed in Blanc's (2001) table of major introductions and translocations of pathogens in inland European aquatic ecosystems. Yet little attention has been paid to the degree of parasite dispersal due to commercial movement (or otherwise) of their invertebrate hosts. We have reported the presence of 12 myxozoans in pet shop oligochaetes from eastern Europe sold in Germany (Hallett et al. 2003, 2004, 2005, present study), and Lowers & Bartholomew (2003) report 7 types also from eastern Europe but sold in the United States. These findings demonstrate that both intra- and inter-continental movement of parasites with their invertebrate host facilitates widespread dispersal of these organisms.

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