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Hyperparasitism has wide-ranging implications for studies on the invertebrate phase of myxosporean (Myxozoa) life cycles

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ABSTRACT

All of the actinospore releasing oligochaetes collected in an environmental sample were found to be infected with the microsporidian *Neoflabelliforma aurantiae* n. gen. n. sp. Ultrastructural and phylogenetic studies on this microsporidian indicated similarities with *Flabelliforma magnivora* but not with the type species *Flabelliforma montana*, necessitating the formation of a new genus *Neoflabelliforma* and reassignment of *F. magnivora* as *Neoflabelliforma magnivora* n. comb. The development of *N. aurantiae* is described both parasitising the oligochaete worm and hyperparasitising the concurrent myxosporean infection. The effect of *N. aurantiae* on the myxosporeans was deleterious and progressive, eventually stopping all actinospore formation. Its discovery has the potential to impact on areas examining the phase of myxosporean life cycles in the invertebrate host, from transmission studies and epidemiology to re-evaluating the basic steps of intra-oligochaete development. Recent evidence has suggested that studies using invertebrate systems should consider possible adverse effects that co-infections can have on experimental outcomes. The discovery of *N. aurantiae* highlights the need for careful screening of experimental animals to help circumvent erroneous results.

1. Introduction

The Myxozoa is a group of obligate parasites affecting aquatic hosts. Infections within fish hosts can result in significant economic losses to commercial enterprises such as fisheries and aquaculture. The higher taxonomy of the group is controversial but they are generally considered to be either cnidarians or basal bilaterians (Siddall et al., 1995; Schlegel et al., 1996; Evans et al., 2008).

The Myxozoa are split into two classes: the Malacosporea and the Myxosporea (Canning et al., 2000). The Myxosporea, representing the vast majority of described myxozoans (>2,100 species), has been further divided into several clades based on phylogenetic analysis in conjunction with comparative developmental studies. The largest of these, in terms of described species numbers, is referred to as the freshwater clade in reference to the host's environment (Fiala, 2006; Morris and Adams, 2008). Where life cycles have been determined for these freshwater species, they are indirect with the parasites cycling between a fish and an oligochaete (Wolf and Markiw, 1984; Kent et al., 2001). While numerous taxonomic studies have been conducted describing freshwater species, very few describe either the intra-piscine or intra-oligochaete development in detail. Of these, the studies of El-Matbouli et al. (1995) and El-Matbouli and Hoffmann (1998) are the most complete, examining both the intra-piscine and intra-oligochaete development of *Myxobolus cerebralis*, respectively, a species that is regarded as a substantial risk to the salmonid fisheries of the United States (Gilbert and Granath, 2003). These two studies are regarded as the model system when referring to myxosporean development (Kent et al., 2001; Canning and Okamura, 2004; Feist and Longshaw, 2006).

Like the Myxozoa, the phylum Microsporidia is also composed entirely of obligate parasitic species, some of which infect aquatic hosts including fish and oligochaetes. Some microsporidians can also act as hyperparasites, with a few reports from representatives of the Copepoda, Digenea, Monogenea, Insecta and Myxozoa (Canning et al., 1979; Cable and Tinsley, 1992; Freeman et al., 2003; Frazen et al., 2005). All of the species described from myxozoans have been detected within fish and are deleterious to the parasite host, affecting sporogony (Kudo, 1944; Diamant and Paperna, 1985; Dyková and Lom, 1999; Tun et al., 2000).

While initiating a study to examine the development of a myxozoan infection of an oligochaete, we unexpectedly discovered a novel microsporidian species, *Neoflabelliforma aurantiae* n. gen. n. sp. Here we characterise this parasite, describe its effects on the

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host(s) together with aspects of its ecology and discuss implications regarding the examination and understanding of myxosporean developmental cycles.

2. Materials and methods

2.1. Oligochaete collection

Sediment was collected from an earth pond stocking brown trout, *Salmo trutta*, at a fish farm in central Scotland, United Kingdom (UK), using a kick net. Sampling was conducted once in July 2006 and once in June 2007 from exactly the same location on the farm. On both occasions, the sediment was transported to Stirling University (UK) in large plastic bags and passed through graded sieves (5 mm, 1 mm, 500 μm) 24 h after collection to remove silt. The material caught on the sieves was placed into a bucket, covered in tap water with gentle aeration at ambient room temperature (~21 °C). Over the following 2 days, the material was transferred to three shallow dishes in ~250 mL batches, examined under a lighted magnifier, and the oligochaetes transferred to a beaker containing distilled water using a plastic pipette. After the bucket was emptied, the oligochaetes were placed into seven 96 cell-well plates (one oligochaete per well), covered with a couple of drops of distilled water, and maintained at ambient room temperature and light conditions. The cell-well water was changed on a weekly basis using distilled water.

2.2. Examination for actinospore releasing oligochaetes

Over the following 4 weeks the oligochaetes were examined with an inverted microscope using a combination of bright field and phase contrast microscopy at different magnifications (40–400×). Oligochaetes releasing actinospores (collective name for myxosporean spores released from an invertebrate host) were identified and transferred to a 96-well plate with fresh distilled water.

2.3. Measurement of spores

Released actinospores were transferred in a drop of water to a glass slide, placed under a coverslip, photographed using a KS300 image analyser (Zeiss) and measured using ImageJ (Abramoff et al., 2004). The categorisation of spore morphology and measurement parameters followed the recommendations of Lom et al. (1997a). Fresh microsporidian spores were collected from the bottom of the cell well and examined on glass slides. Some spores were also stained using 0.1% v/v Blankophor (4′4′-bis[(4-anilino-6-substituted 1,3,5-triazine-2-yl) amino]stilbene-2,2′disulphonic acid (ICN Biomedicals) in distilled water, and precisely measured using a Zeiss TCS SP2 AOBS confocal microscope. Oligochaetes were identified using published keys (Brinkhurst, 1963; Kathman and Brinkhurst, 1999). Prior to the oligochaetes being used for molecular and ultrastructural studies, a Varley microcompressor was used to non-destructively identify structures of taxonomic relevance (e.g. structure/form of chaete and penile sheath).

2.4. Preparation/storage and ultrastructural examination of infected oligochaetes

Infected oligochaetes were bisected with the anterior half being placed in 99% ethanol and stored in a −20 °C freezer. The posterior halves of the oligochaetes were processed immediately for electron microscopy. These pieces were cut into 1 mm lengths, fixed in 2.5% glutaraldehyde solution overnight followed by rinsing in cacodylate buffer, post-fixed in 1% osmium tetroxide for 1 h and embedded in Spurr's resin. Semi-thin sections were taken, transferred to glass slides and stained with toluene blue. Ultrathin sections were taken, transferred to formvar coated grids and post-stained in uranyl acetate/lead citrate to be examined at 120 kv using a Tecnai Spirit G^2 electron microscope. The frozen tissues were used for molecular characterisation.

2.5. Molecular characterisation

Infected oligochaetes were homogenised with a plastic pestle and digested overnight at 56 °C in 0.4 mL high concentration urea buffer containing 100 μg/mL proteinase K. DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN Inc.) following the manufacturer's tissue protocol and used as the template for PCRs. Small subunit (ss), internal transcribed spacer (ITS) and partial large subunit (ls) regions of the rRNA gene were amplified using previously described oligonucleotide PCR primers (Vossbrinck et al., 1993; Pomport-Castillon et al., 1997). An additional internal primer 870rev (Freeman et al., 2004), and its complementary forward primer 870fwd were used in pairs with the forward ssrRNA and reverse lsrRNA primers, respectively, to allow a sufficient overlap for sequence confirmation. PCRs were performed according to their original descriptions and sequencing reactions were performed using BigDyeTM Terminator Cycle Sequencing chemistry utilising the same oligonucleotide primers that were used for the original PCRs. DNA sequencing was performed on both sense and antisense strands for all PCR products and consensus sequences were obtained manually using CLUSTAL-X (Thompson et al., 1997) and BioEdit (Hall, 1999). CLUSTAL-X was used for the initial sequence alignments with the settings for gap opening/extension penalties being adjusted manually to achieve optimum alignments. Regions of ambiguous sequence alignments were manually edited using the BioEdit sequence alignment editor. Alignment files of 60 taxa consisting of 1,515 characters of ssrRNA sequence of which 977 were parsimony informative, were used in the phylogenetic analyses.

Phylogenetic analyses were done using maximum parsimony (MP) in PAUP*4.0 beta10 (Swofford, 2002) implementing heuristic searches using a tree–bisection–reconnection swapping algorithm. Clade support was assessed with bootstrapping of 1,000 replicates; gaps were treated as missing data. Bayesian inference (BI) analyses were conducted using MrBayes v. 3.0 (Ronquist and Huelsenbeck, 2003). Models of nucleotide substitution were evaluated for the data using MrModeltest v. 2.2 (Nylander et al., 2004). The most parameter-rich evolutionary model based on the Akaike information criterion (AIC) was the general time-reversible, GTR+I+G model of evolution. Therefore, the settings used for the analysis were nst = 6, with the gamma-distributed rate variation across sites and a proportion of invariable sites (rates = invgamma). The priors on state frequency were left at the default setting (Prset statefreqpr = dirichlet (1,1,1,1)). Posterior probability distributions were generated using the Markov Chain Monte Carlo (MCMC) method with four chains being run simultaneously for 1,000,000 generations. Burn in was set at 2,500 and trees were sampled every 100 generations making a total of 7,500 trees used to compile the consensus trees.

3. Results

3.1. Description of the actinospore

In the 2006 sample, six worms were identified as releasing an aurantiactinomyxon-type actinospore (672 worms examined, mixed species). In 2007, two worms released aurantiactinomyxon-type actinospores, while one worm released a triactinomyxon-type