More parasitic myositis cases in humans in Australia, and the definition of genetic markers for the causative agents as a basis for molecular diagnosis

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ABSTRACT

Since 1998, there have been six reported human cases of myositis in Australia, attributable to infection with the nematode *Haycocknema perplexum*. However, an unequivocal diagnosis of *H. perplexum* infection and associated disease has been seriously compromised by a lack of molecular markers for this nematode. Here, we report new cases of disseminated myositis in two male patients from the states of Queensland and Tasmania in Australia, respectively; genetically characterize the causative agent from each case; and, also establish a PCR-based sequencing approach as a tool to support the diagnosis of future cases and to underpin epidemiological studies.

*Keywords:* Disseminated myositis, human, Australia, nematode, *Haycocknema perplexum*
1. Introduction

Myositis is an inflammation of muscle(s), characterized by pain, swelling, and/or weakness. There are many aetiologies of myositis, including injury, medicines, toxins, excessive exercise/exertion, chronic diseases (involving the immune or endocrine system), electrolyte imbalance and infections. Infectious myositis can involve one or more species of viruses, bacteria, fungi and/or parasites (Crum-Cianflone, 2008, 2010). For parasitic myositis, clinical diagnosis is often assisted by the use of complementary laboratory tests that detect and attempt to microscopically identify the agent in histological sections of muscle biopsies from patients. However, diagnosis is often not reliable without the genetic identification of the agent using molecular methods.

Parasitic myositis is mainly reported to be associated with protists, flatworms or roundworms (nematodes), the latter of which often involves *Toxocara* and *Trichinella* species (Crum-Cianflone, 2008). However, some unusual myositis cases involving nematodes have been reported in the literature. For instance, since 1998, there have been six human cases of disseminated myositis (inflammation of multiple muscle groups) in the States of Queensland and Tasmania of Australia (McKelvie et al., 2013), attributed to the nematode *Haycocknema perplexum* (Spratt et al., 1999). In these cases, clinical diagnosis was made on the basis of progressive muscle weakness, weight loss and/or dysphagia, complemented by the microscopic detection/identification of the nematode in muscle biopsies (Dennett et al., 1998; Spratt et al., 1999; Basuroy et al., 2008; McKelvie et al., 2013), elevated eosinophil and creatine kinase levels in blood and an exacerbation of clinical signs following anti-inflammatory (usually corticoid steroid) treatment against the myositis. Parasitological diagnosis from muscle biopsies has relied on the microscopic identification of the non-encysted, intra-fibrillar nematode, which is ~ 350 µm in length and 20 µm in width, and has a rounded cephalic end and a sharply tapered tail; female worms within the muscle are often gravid (Basuroy et al., 2008). However, the diagnosis of *H. perplexum* infection, and associated disease, is consistently challenging and has been seriously compromised by a lack of molecular markers for this nematode. In the present study, we report two new cases of disseminated myositis in patients from Queensland and Tasmania, from where all previous *H. perplexum* cases had been reported (cf. McKelvie et al., 2013). Both of these cases were consistent with previous cases, in terms of symptomatology and the presence of characteristic non-encysted nematodes in muscle biopsy material. Importantly, here, for the first time, we genetically characterized these agents, and established PCR-based DNA sequencing as a laboratory-based tool for future suspected cases and epidemiological studies.

2. Materials and methods

2.1. Human case histories

2.1.1. Case in Queensland

In December 2014, a 30 year-old indigenous male Australian presented with a progressive proximal muscle weakness, weight loss and muscle wasting. In 2012, he first noticed muscle weakness in both arms and legs, and had difficulty in getting up
from the sitting position. There was no other symptomatology. In particular, he had no loss of appetite, fever, rash or pain in joints, change in sensation, muscle cramps, or bladder or bowel dysfunction. From 2012 to 2014, the muscle weakness gradually progressed distally.

This patient had had no medical history of diabetes, hypertension, hyperlipidaemia or ischaemic heart disease, but had been treated against depression with desvenlafaxine. This patient had lost his job because of muscle weakness, and became bed-bound, requiring assistance for daily tasks and walking. He had only travelled in the Mackay region, Toowoomba and Townsville. He had no exposure to wild animals, and had never consumed raw or undercooked “bush-meat” from native wildlife.

In September 2013, clinical biochemistry analyses revealed elevated levels of serum alanine transaminase (ALT; 142 U/l; normal: <45 U/l) and aspartate transaminase (AST; 81 U/l; normal: <35 U/l). ALT and AST remained elevated over time, with peaks of 291 U/l and 118 U/l, respectively, in April 2015. Blood examination showed an elevated eosinophil count of 1.24 x 10^9 cells/l (normal range: 0.1-0.6 x 10^9/l). Serological testing detected anti-Strongyloides antibody, but stool examination for nematode larvae was test-negative. Nonetheless, the patient had been treated with ivermectin against Strongyloides infection. Microscopic examination of a liver biopsy did not reveal any pathological findings.

In December 2014, neurological examination revealed a proximal weakness in both the upper and lower limbs, with a Grade 3-4 muscle weakness, but no detectable weakness distally. There was no detectable impairment of sensation or reflexes. Although he had a significant wasting of the muscles, there was no detectable joint or cardio-respiratory involvement. Nerve conduction was normal, and electromyography revealed low amplitude, polyphasic potentials, consistent with myopathy. Serum creatine kinase levels were elevated at 2840 to 3400 U/l (normal range: 46-171 U/l). No auto-antibodies associated with liver or collagen vascular disease were detected.

In February 2015, histopathological examination of a biopsy from the quadriceps femoralis muscle showed a severe, active chronic inflammatory myopathy with prominent plasma cell infiltration. Based on this presumptive diagnosis, the patient was given immunoglobulins intravenously and put on an oral course of prednisolone and ivermectin for the presumed Strongyloides infection. Following this treatment regimen, muscle weakness decreased significant over the next 1-2 months. However, the patient developed bulbar symptoms, with dysphagia for solids, dysphonia and nasal regurgitation of food being prominent signs. His extra-ocular and neck muscles were demonstrably weak. He had also lost ~ 20 kg over the previous two years, and had occasional episodes of night sweats. The computer tomographic (CT) scans of the chest, abdomen and pelvis revealed no abnormal findings. Serological testing for anti-Strongyloides antibody remained positive, but stool examination for nematode larvae was test-negative.

In May 2015, due to continued muscle weakness, the examination of another muscle biopsy revealed parasitic myositis, with scattered myocytes containing single elongate nematodes (an average of 50 per transverse section). Many of the intracellular nematodes had dark brown, haemazoin-like pigmentation. In addition, there was evidence of muscle fibrosis necrosis and regeneration as well as patchy interstitial inflammation involving mainly mononuclear cells and eosinophils. The morphological features of this nematode were consistent with those of H. perplexum (Fig. 1). Following the detection of the nematodes in musculature, immunoglobulin and immunosuppressive therapy ceased immediately, and the patient was
administered albendazole (400 mg twice a day) for a period of four months. After this treatment, the patient’s muscle weakness, dysphagia and dysphonia had ceased. Importantly, he gained significant weight, mobilized independently and was able to resume his job. He remains under surveillance as an outpatient for any further clinical symptoms.

2.1.2. Case in Tasmania

In 2014, a 72 year-old male resident from Kingston, Tasmania, who was retired for some years from working in Western Australia, Queensland and Tasmania, presented with a myositis linked to gradual muscle weakness over a period of several years, was a recreational hunter and liked to consume bush-meat (e.g., wallaby) jerky. Serological evaluations revealed no evidence of viral (HIV, HCV and HBV) infections, but did suggest the presence of (possibly cross-reactive) antibodies against *Strongyloides*. Coproscopic examinations showed no evidence of parasitic (worm or protistan) infections. From April to December 2014, a series of clinical and laboratory assessments were undertaken. During this time, the patient exhibited a persistent blood eosinophilia (2.0-2.4 x 10⁹ cells/l) and elevated creatine kinase (2068-2082 U/l) levels in blood. In June 2014, a histopathological examination of a muscle biopsy from the right lateral vastus muscle revealed a chronic, moderately severe myopathy, with active fibre necrosis and florid inflammatory infiltrate, including lymphocytes, plasma cells and eosinophils within the fascicle. As these findings were initially interpreted as a rheumatoid process, the patient was treated with methyl-prednisolone and methotrexate at therapeutic doses. Subsequently, on 11 November 2014, he was admitted to hospital after a deterioration of his condition, and was administered therapeutic doses of methyl-prednisolone intravenously for three days as well as cyclophosphamide. Thereafter, his muscle weakness decreased, and he was discharged by mid November 2014. While his blood eosinophil count was low (0.01-0.2 x 10⁹ cells/l), creatine kinase remained high (1607-1968 U/l). Finally, at the end of December 2014, a histopathological examination of a muscle biopsy from the left lateral vastus muscle detected nematodes that appeared to be consistent in morphology with *H. perplexum* (cf. Fig. 1). The patient was treated with therapeutic doses of albendazole (400 mg twice daily) and prednisolone for some weeks; he improved clinically and was then discharged in March 2015. He remains on surveillance as an outpatient for any further clinical developments.

2.2. Microscopic methods

A portion of the muscle biopsy from the Queensland case (see subsection 2.1.1.) was snap-frozen in liquid nitrogen and transverse sections (5 µm thickness) were prepared using Leica CM1850 cryostat (Leica Microsystems Pty Ltd, Australia). The sections were stained with Harris’ haematoxylin & eosin (Sheenan and Hrapchak, 1980) and examined by routine light microscopy. A second portion of the biopsy was fixed overnight in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Longitudinal sections were prepared using the Leica EMTP automated tissue processor (Leica Microsystems Pty Ltd, Australia). Specifically, the tissue was post-fixed in 1% modified osmium tetroxide (containing 1.5% potassium ferrocyanide) (Proscitech, Australia), stained *en bloc* with 2% uranyl acetate (Proscitech, Australia), and then infiltrated and embedded in Procure 812 resin (Proscitech, Australia). Ultra-thin sections of 70-100 nm thickness were cut with the Leica UC7 Ultratome (Leica...
Microsystems Pty Ltd, Australia), mounted on grids, and post-stained with 2% lead citrate (Leica Microsystems Pty Ltd, Australia). Ultrastructural examination was undertaken using a JEOL 1011 electron microscope (JEOL Australasia Pty Ltd, Australia) at 100 kV. Images were acquired with the Gatan Orius SC1000, model 832 CCD camera (Thompson Scientific Instruments Pty Ltd, Australia).

2.3. Molecular methods

Genomic DNA was extracted separately from individual frozen (Queensland case) and ethanol-fixed (Tasmanian case) muscle biopsies using the Powersoil® kit (MoBio, USA). Muscle biopsies were taken from both patients by registered medical surgeons, and were donated (with consent from the patients) to the investigators at the University of Melbourne for molecular analyses.

Two gene regions were amplified by PCR and the amplicons directly sequenced. First, the small subunit of nuclear ribosomal RNA gene (SSU) was amplified in two overlapping fragments using primer pair 1096F (forward: 5’-GGTAATTCTGGAGCTAATAC-3’) and 1912R (reverse: 5’-TTTACGGTCAGAACTAGGG-3’) (~815 bp), and primer pair 1813F (forward: 5’-CTGCCGTGAGGTTGAAAT-3’) and 2646R (reverse: 5’-GCTACCTTGTACGACTTTT-3’) (~830 bp) (Holterman et al., 2006). For both amplifications, the cycling protocol was 94 °C/5 min (initial denaturation), followed by 5 cycles of 94 °C/30 sec (denaturation), 45 °C/30 sec (annealing) and 72 °C/70 sec (extension), followed by 35 cycles of 94 °C/30 sec, 54 °C/30 sec, and 72 °C/70 sec, with a final extension at 72 °C/5 min. Second, the cox-1 gene was amplified using the primer pair RhigoCoxF (forward: 5’-TTTTTTGGACATCCTGAGGTGTAT-3’) and RhigoCoxR (reverse: 5’-CAGACTCAACACATAATGAAAATG-3’) (~450 bp), designed for this study by aligning conserved regions of the cox-1 gene of Rhigonema thysanophora (rhigonematid), Gongylonema pulchrum (spirurid) and Philometroides sanguineus (camallanid), represented by accession nos. KF534714, KM264298 and KM111526, respectively. The cycling protocol was 94 °C/5 min (initial denaturation), followed by 35 cycles of 94 °C/30 sec (denaturation), 47 °C/30 sec (annealing) and 72 °C/30 sec (extension), with a final extension at 72 °C/5 min.

The size and intensity of the amplicons were assessed by electrophoresis (7 V/cm) in 1.5% agarose gels using TBE (65 mM Tris-HCl, 27 mM boric acid, 1 mM EDTA, pH 9; Bio-Rad, USA) as the buffer. Following electrophoresis, gels were stained with ethidium bromide and their size estimated by comparison to ΦX174-HaeIII (Promega, USA) markers. Aliquots (5 µl) of individual amplicons (undigested) were treated with ExoSAP-IT (Affymetrix, USA), according to the manufacturer’s instructions, and then subjected to direct, automated sequencing (BigDye Terminator v.3.1 chemistry, Applied Biosystems, USA) in both directions using the same primers as employed for PCR amplification.

2.4. Phylogenetic analyses

SSU sequences (consensus alignment length: 2258 bp) were aligned using R-Coffee (Moretti et al 2008) (which takes into account predicted secondary structures for noncoding sequences) and Mesquite v 3.03 (Maddison and Maddison 2015). The cox-1 sequences were aligned (consensus alignment length: a 248 bp) using the program ClustalW (Larkin et al., 2007) in Mesquite. For SSU, a preliminary tree was constructed using available sequences representing the major clades of the
Nematoda (cf. Blaxter et al., 2008) (Supplementary Fig. 1). This placed *H. perplexum* within the secernentean nematodes. In order to gain a finer phylogenetic resolution while maintaining a reasonable alignment, representative sequences from neighbouring clades from the extensive study by van Megen et al., (2009) were included in the final alignment of SSU sequences. Outgroups used in the SSU analyses were *Axonolaimus* sp. and *Cylindrolaimus communis* (representing Clade 5A; van Megen et al., 2009). For *cox-1*, a 248 bp alignment (limited to the size of the available *Riouvgolvania kapakpamui* sequence) was used to construct a tree including representative nematodes from GenBank, and *Trichinella spiralis* and *Trichuris trichiura* as outgroups. The number and range of *cox-1* sequences available on GenBank for nematodes is far less compared to those available for SSU therefore SSU was selected to provide finer phylogenetic resolution.

Bayesian inference (BI) (SSU and *cox-1*) and maximum likelihood (ML) (for SSU only) analyses of sequence data were conducted using MrBayes v3.2.3 (Ronquist and Huelsenbeck, 2003) and Randomized Axelerated Maximum Likelihood for High Performance Computing (RAxML HPC v. 8.2; Stamatakis, 2006). The likelihood parameters set for the BI analysis of sequence data were based on the Akaike Information Criteria test in jModeltest v.2.1.7 (Posada 2008). For the analyses of SSU and *cox-1* sequence data sets, the number of substitutions was set at six, with a gamma-distribution and a proportion of invariable sites. The aligned COX-1 amino acid sequence data were run, with the rate matrix set to ‘mixed’. For each tree, posterior probability (pp) values were calculated by running 2,000,000 generations with four simultaneous tree-building chains. Trees were saved every 100th generation. At the end of each run, the standard deviation (SD) of split frequencies value was ~0.01, and the potential scale reduction factor approached one. For each analysis, a 50%-majority rule consensus tree was constructed based on the final 75% of trees produced by BI. Analyses were run three times to ensure convergence and insensitivity to priors. For RAxML the general time reversible (GTR) with gamma parameter was applied using 200 bootstraps; the topologies of trees constructed using BI and ML were almost identical.

3. Results

From the genomic DNAs from two muscle biopsy samples representing the human cases from Queensland and Tasmania, we sequenced 1,603 bp and 852 bp of the SSU gene, respectively, and found a single base difference between the sequences over the corresponding 852 bp, interpreted to represent intraspecific variability. A comparison with publicly available sequences for nematodes showed at least 10% difference from the best-matching sequences representing *Carnoya filipjevi* (Rhigonematomorpha), *Cyrnea seurati* (Spiruromorpha) and *Pseudonymus islamabadi* (Oxyuridomorpha). The consensus phylogenetic tree constructed using SSU data places *H. perplexum* between members of Clades 8A (Oxyuridomorpha) and 8B (Ascaridomorpha) (cf. van Megen et al. 2009) (see Fig. 2) and within the Secerentea rather than the Chromadorea (cf. Spratt et al., 1999; Spratt 2014).

We also sequenced 440 bp of the *cox-1* gene from the same two genomic DNAs, and calculated 5.7% nucleotide difference between the two sequences, relating to 24 synonymous and one non-synonymous mutations. The one amino acid change over 146 positions of the COX-1 sequence linked to the 5.7% nucleotide difference in *cox-1* between the two samples is suggested to represent intraspecific variability, a statement supported by the single nucleotide difference in the partial SSU sequence
The consensus phylogenetic tree constructed using available cox-1 data (consensus alignment length: 240 bp) (Fig. 3) shows a clustering of the two sequences representing *H. perplexum* from the two localities to the exclusion of sequences representing a range of other nematode taxa (n = 69) (Fig. 3). In this tree, *H. perplexum* groups most closely with oxyurid nematodes (although cox-1 should not be used to infer relationships among distantly related nematodes). Interestingly, the level of sequence difference in cox-1 between *H. perplexum* and the muspiceoid *R. kapapkmui* was 25.3% (Fig. 3), showing considerable genetic distinctiveness. Additional trees constructed using a subset of sequences (consensus alignment length: 440 nucleotides or inferred amino acid sequence data) had similar topologies and support values at the nodes (not shown).

4. Discussion

The two cases of parasitic myositis described here were consistent clinically with those reported previously to be linked to *H. perplexum* infection (Dennett et al., 1998; Basuroy et al., 2008; McKelvie et al., 2013) and also clustered with them geographically, suggesting a particular association among the parasite, geographic loci and humans. PCR-based sequencing was able to define, for the first time, genetic markers for the causative agent, and phylogenetic analyses allowed us to infer that this nematode belongs to the Secernentea, and is positioned between the Oxyuridomorpha and Ascaridomorpha. Although the nematodes from the two disparate locations (Tasmania and Queensland) were shown to be genetically distinct, present evidence suggests that this distinctiveness likely represents within-species genetic variation in the nematode rather than difference between species. Nonetheless, these two genotypes or haplotypes of nematode might differ in their biology (e.g., life cycle and host animal/s) and/or their epidemiology (host range/s and transmission). Irrespective of this variability, we have been able to define genetic markers for this nematode, which we consider very likely to represent *H. perplexum* based on its morphological features, as observed in transverse sections of muscle biopsies, the location of the human cases and also the expression of disease.

In contrast to the molecular findings here, *H. perplexum* had been assigned to the family Robertdollfusidae Chabaud and Campana, 1950, of the order Muspiceida Bain and Chabaud, 1959, and class Chromadorea (Spratt et al., 1999; Spratt 2014). Other muspiceoids are found in subcutaneous connective tissue of mice, skin cysts of bats, eyes and brains of corvids, circulatory system of marsupials and ear capillaries of reindeer (Spratt, 2014). *Riouxgolvania kapapkmui* (found in bats) is the only muspiceoid previously characterized molecularly; the molecular evidence suggested that this taxon muspiceoid belongs to the Secernentea based on the mitochondrial cytochrome oxidase subunit 1 gene (*cox-1*) sequence (Hasegawa et al., 2012), consistent with the nematodes characterized genetically in the present study. The considerable genetic difference (25.3%) in *cox-1* between *H. perplexum* and *R. kapapkmui* also suggests that the Muspiceoidea are not a monophyletic group. However, further molecular evidence is needed for other muspiceoid nematodes to elucidate their phylogenetic relationships, because the present results using nuclear and mitochondrial gene data contrast the findings obtained using morphological data sets (Bain and Chabaud, 1968, 1979; Spratt and Nicholas, 2002).

As *H. perplexum* is distantly related (at least 10% sequence difference in SSU) to the other nematodes for which sequence data are presently available, it is not possible to make major inferences about its life cycle based on the neighbouring taxa.
However, some generalizations can be made. *H. perplexum* falls amongst members of the Oxyurida, Ascaridida and the Rhigonematida, all of which have monoxenous (direct) life cycles. The Oxyurida have an ancestral history with arthropods, as do the Rhigonematida, which are found in millipedes (Sudhaus, 2010). The oxyurids most closely related to *H. perplexum* are *Thelastoma krausi* and *Leidynema portentosae*, both found in millipede and cockroach, respectively (van Waerebeke, 1978; Carreno, 2007). The ascarids are thought to have switched hosts, from insects to tetrapods (Sudhaus, 2010). Larval forms of other muspineoid nematodes have been found in insects. For instance, *Durikainema* sp. (found in tree kangaroos) was recovered from the abdomen of the biting midge *Culicoides victoriae*. In addition, an undescribed Robertdollfusidae was found in the gut of the blackfly, *Simulium damnosum*, in Cameroon (Spratt et al., 1999). With these related nematodes in mind, it could be proposed that *H. perplexum* uses an arthropod as an intermediate host and has accidentally found its way into humans. Another possibility is that *H. perplexum* is transmitted via the consumption of undercooked bush-meat from a native marsupial, as hypothesized for the first human case of disease caused by *H. perplexum*, initially misdiagnosed as *Trichinella pseudospiralis* (see Andrews et al., 1994; Spratt, 2005). However, thus far, only two of eight cases were proposed to relate to the consumption of bush-meat on one occasion (cf. Basuroy et al., 2008; McKelvie et al., 2013).

In conclusion, as seen from the clinical cases presented here, the diagnosis of parasitic myositis is a major clinical challenge. Although considered as rare, disease caused by *H. perplexum* in humans in Australia might be an occult or emerging issue, possibly affecting some people by subclinical infection/s in what presently appear to be two endemic foci (Queensland and Tasmania). The definition of nuclear and mitochondrial genetic markers for nematodes that we presently consider to represent *H. perplexum* (Spratt et al., 1999) now provides a sound basis for routine molecular diagnosis of parasitic myositis in human patients suffering from debilitating and progressive muscle wastage and associated complications, in combination with conventional clinical, biochemical and immunological tests. Moreover, the PCR-based sequencing approach established here would be a useful research tool to undertake surveys of potential reservoir animals or vectors for the occurrence of the present and related nematode genotypes in Australia or elsewhere, and to support studies of the biology and transmission of these/this enigmatic worm/s.

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References


FIGURES

Fig. 1. Light (panels A-B) and electron microscopic (panels C-D) images from sections through skeletal muscle biopsies from an adult male patient (affected by myositis) from Queensland, Australia, showing evidence of an intrafibrillar nematode morphologically consistent with *Haycocknema perplexum*. Panel A: Transverse section (haematoxylin & eosin) through five developing larvae within a female nematode within a muscle cell. Panel B: Transverse sections of three nematodes in different muscle cells. Panel C: End of nematode, showing fine transverse cuticular striation (S). Panel D: Section through vulva (V) of female nematode, and faint transverse cuticular striation (S).
Fig. 2. Phylogenetic tree constructed from nuclear small subunit ribosomal RNA gene (SSU) sequence data (~1600 nucleotides) for selected nematodes representing Clades 5 to 8 (cf. van Megen et al., 2009) using the Bayesian inference method. The longest SSU sequence from the causative agent from an adult male patient (affected by polymyositis) from Queensland, Australia (see text), and inferred to represent *Haycocknema perplexum* (bold), is positioned between the Oxyuridomorpha and Ascaridomorpha. Nodal support values are given as posterior probabilities (Bayesian), followed by bootstrap support (maximum likelihood). Infraorders and superfamilies are labelled with vertical bars. GenBank accession numbers precede the nematode taxa. A scale bar indicates the number of substitutions per site.
Fig. 3. Phylogenetic tree constructed from mitochondrial cytochrome c oxidase subunit 1 gene (cox-1) sequence data (~240 nucleotides) from selected nematodes representing major nematode clades. The two sequences from the causative agents originating from male human patients from Queensland and Tasmania, respectively, and inferred to represent *Haycocknema perplexum* (bold) are genetically distinctive from the morphologically similar muspiceoid nematode *Riouxgolvania kapakamui*. Nodal support is given as a posterior probability. GenBank accession numbers precede the nematode taxa. The scale bar indicates the number of substitutions per site.
Supplementary Fig. 1. Phylogenetic tree constructed from nuclear small subunit ribosomal RNA gene (SSU) sequence data (~1600 nucleotides) for representing the major clades of the Nematoda (cf. Blaxter et al., 2008) using the Bayesian inference method. The SSU sequence inferred to represent *Haycocknema perplexum* (bold), is positioned between within the Secernentea. Nodal support values are given as posterior probabilities (Bayesian). Nematode classes are labelled with vertical bars. GenBank accession numbers precede the nematode taxa. The invertebrates *Priapulus caudatus* and *Chordodes morgani* were used as outgroups. A scale bar indicates the number of substitutions per site.
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