Molecular and morphological characterization of the cercariae of *Lecithodendrium linstowi* (Dollfus, 1931), a trematode of bats, and incrimination of the first intermediate snail host, *Radix balthica*

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SUMMARY

Lecithodendrium linstowi is one of the most prevalent and abundant trematodes of bats, but the larval stages and intermediate hosts have not been identified. We present the first molecular and morphological characterization of the cercariae of *L. linstowi* based on a phylogenetic analysis of partial fragments of LSU and ITS2 rDNA. The first intermediate host was incriminated as *Radix balthica* by DNA barcoding using cox1 and ITS2 sequences, although the snail morphologically resembled *Radix peregra*, emphasizing the requirement for molecular identification of lymnaeids as important intermediate hosts of medical and veterinary impact. The application of molecular data in this study has enabled linkage of life cycle stages and accurate incrimination of the first intermediate host.

Key words: *Lecithodendrium linstowi*, xiphidiocercariae, *Radix balthica*, bats, LSU, ITS2, cox1.

INTRODUCTION

Trematode life cycles are complex, usually employing multiple hosts, and often with low specificity in the definitive vertebrate host. Resolution of their life cycles is therefore challenging, requiring direct linkage of morphologically distinct larval stages such as cercariae with adults (Brant *et al*. 2006). Furthermore, life cycle elucidation in the laboratory can be technically and ethically problematic. DNA sequencing and the development of databases with species-specific reference DNA sequence data have enabled larval and adult trematodes to be matched and hosts accurately incriminated, thus informing taxonomy, biodiversity and epidemiology (Brant *et al*. 2006).

The Lecithodendriidae (Digenea: Plagiorchiida) are a prime example of taxonomic uncertainty due to missing links between larval and adult stages. These parasites infect insectivorous vertebrates and typically use prosobranch molluscs as first intermediate hosts. The emergent cercariae encyst as metacercariae in aquatic insect larvae, which are later ingested as adult insects by foraging definitive hosts. More detailed life cycle elucidation exists for only a few species (reviewed in Kudlai *et al*. 2015) making it difficult to assess the diversity of these parasites and their contribution to trematode communities in host populations. In addition, identification to species level is important as lecithodendriids are common hosts of intracellular endosymbiotic *Neorickettsia* bacteria (Rickettsiales, Anaplasmataceae), which can cause debilitating and sometimes fatal diseases in vertebrates, including humans (Greiman *et al*. 2017).

Published reports on Lecithodendriidae in the UK are limited to early morphological studies (e.g. Nicoll, 1923; Brown, 1933) and a detailed study of gastrointestinal *Lecithodendrium* spp. in pipistrelle bats by Lord *et al*. (2012) who used molecular analysis to revise phylogenetic relationships between lecithodendriid species. Otherwise, there are morphological reports of xiphidiocercariae under provisional names such as *Cercaria helvetica* XII Dubois, 1928 (Nasir and Erasmus, 1964), now known to be phylogenetically close to, but not identical to, *Lecithodendrium linstowi* (Kudlai *et al*. 2015), illustrating the importance of molecular confirmation. Here, we report the first identification of the cercariae of *L. linstowi* using molecular and morphological approaches and molecular incrimination of the snail intermediate host. The cercariae were collected during the UK Digenean Diversity Project, a molecular study of digeneans infecting freshwater snails in the UK.

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MATERIALS AND METHODS

Collection and screening of snails

Eighty-three *Radix* sp. (Lymnaeidae) snails were collected by hand net from the Queen's River, Bushy Park, Surrey, England (51°24′42″N; 0°20′27″W) in September 2013. This artificial river was created in the 17th century to bring water from the Colne River to Hampton Court Palace (Bushy Park Management Plan, The Royal Parks, 2014, unpublished). Snails were individually placed in 50 mL glass beakers containing filtered, dechlorinated water and were screened for emergent cercariae by microscopy in the laboratory. Only one snail, identified as *Radix peregra* based on shell morphology, was observed to shed xiphidiocercariae.

Morphological description of cercariae

Cercariae were fixed in 4% formaldehyde solution and stored in 70% ethanol prior to processing. Cercariae examined by light microscopy were stained with acetocarmine, dehydrated in a graded ethanol series, cleared in HistoChoice (Sigma-Aldrich, UK) and mounted in Canada balsam. Image capture and measurements of cercariae were made using a Nikon Eclipse NiE microscope and NIS-Elements BR (Nikon Instruments, UK) software. Cercariae examined by scanning electron microscopy were dehydrated in a graded ethanol series, dried in hexamethyldisilazane, attached to stubs using double-sided tape, sputter coated with gold palladium and examined under a Zeiss EVO 50 scanning electron microscope. Upon confirmation of species, parasite reference material was deposited at the Natural History Museum, London, UK (accession numbers NHMUK 2017·6·15·1-2).

Molecular analysis

Total genomic DNA was isolated from a pool of ten 96% ethanol-fixed morphologically identical cercariae using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc.) following the manufacturer's protocol. PCR was performed to amplify partial fragments of the large ribosomal subunit (LSU) using primers LSU28S (forward; TAGGTC GACCCGCTGAAYTTAAGCA) and 1500R (reverse; GCTATCCTGAGGGAAACTTCG) as described by Olson *et al*. (2003). The internal transcribed spacer region (ITS) was amplified using primers; p1 (forward; GTCGTAACAAGGTTT CCGTAGGTG) and p2 (reverse; TATGCTTA AATTCAGCGGGTAATC) according to Wang *et al*. (2009).

In order to accurately identify the *Radix* species acting as an intermediate host, DNA was also extracted from a tissue snip from the foot of the infected snail using the same methods described above, but with an extended 24 h initial digest. A partial fragment of the mitochondrial cytochrome c oxidase 1 gene (cox1) was amplified with PCR using primers LCO1490 (GGTCAACAAATCA TAAAGATATTGG) and HCO2198 (TAAACT TCAGGGTGACCAAAAAATCA) using protocols described by Folmer *et al*. (1994) and the ITS2 region was amplified using primers NEWS (TGTGTCGATGAAGAACGCAG) and RIXO (TTCTATGCTTAAATTCAGGGG) (Almeyda-Artigas *et al*. 2000).

PCR amplicons generated from both the cercariae and the snail were visualized in 1% agarose gels stained with gel red (Bioline™) prior to sequencing using the same PCR primers with Fluorescent Dye Terminator Sequencing Kits (Applied Biosystems™) run on an Applied Biosystems™ 3730XL automated sequencer. Resultant sequences were assembled in BioEdit (Hall, 1999) and corrected manually to resolve ambiguous base calls. BLASTn searches were performed at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to provide initial identification and to ensure no contamination and sequences were submitted to GenBank (accession numbers: MF498820- MF498823).

Phylogenetic analysis

The MUSCLE algorithm (http://www.ebi.ac.uk) was used to align the generated sequences with retrieved GenBank sequences: (i) for lecithodendriid spp., *Maritrema* spp., *Microphallus* spp. and *Collyriclum faba* were used as out-groups; (ii) for *Radix* spp., *Lymnaea stagnalis* was used as the out-group. Since most of the available lecithodendriid sequences on GenBank were ITS2, the complete ITS sequence from this study and other retrieved sequences were trimmed to the ITS2 fragment prior to analysis.

Neighbour-joining (NJ) and maximum-likelihood (ML) methods were employed to perform phylogenetic reconstruction for the parasite and the snail species using MEGA v6 (Tamura *et al*. 2013). For the xiphidiocercariae, NJ trees based on ITS2 and LSU were constructed under the conditions of the Kimura 2 parameter model (K2P). Based on the lowest Bayesian information criterion, MEGA6 identified that the K2P model with a gamma distribution best fit the ITS2 and LSU data; thus, both ML analyses were performed under the conditions of this model. For *Radix* spp., the NJ analysis for both the ITS2 and cox1 were performed under the conditions of the K2P model, but the ML analysis was performed using the Tamura 3 parameter with gamma distribution for ITS 2 and the Hasegawa–Kishino–Yano with gamma distribution for cox1. In all analyses, nodal support values were estimated using 1000 bootstrap replicates.

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Table 1. Comparison of morphometric features of *Lecithodendrium linstowi* and *Lecithodendrium* sp. [syn. *Cercaria helvetica* XII Dubois, 1928 (Kudlai *et al*. 2015)] cercariae

Morphological feature	*Lecithodendrium linstowi* from *Radix balthica* $n = 15$	*Lecithodendrium* sp. from *Bithynia tentaculata* $n = 27$
Body length	78–116 (88)	125 –158 (138)
Body width	27–43 (32)	50–95 (62)
Tail length	38–89 (54)	100–137 (126)
Tail width	07–12 (09)	15–23 (19)
Oral sucker diameter	09–14 × 09–15 (11 × 11)	20– 33 × 23–35 (26 × 28)
Ventral sucker diameter	10–13 × 08–14 (11 × 09)	20–23 (21)

Measurements (μm) are given as the range followed by the mean value in parentheses.

RESULTS

Morphological description of L. linstowi *cercariae*

The body was oval-elongate and very contractile, usually longer than the tail (Table 1, Fig. 1A–C). The oral sucker was sub-terminal, round-oval with a small central stylet (Fig. 1B and D). The ventral sucker was round-oval, located posterior to the mid-body (Fig. 1C and E). Fine spines and type 1 sensory papillae with tegumental collars covered the body tegument (Fig. 1D–F). The tail was simple with indented margins, without a finfold, spines or sensory papillae (Fig. 1G). Three pairs of penetration gland cells filled with granules were located anterior to the ventral sucker with ducts opening anteriorly either side of stylet. The pharynx was small and the intestinal tract was indistinct. The v-shaped excretory vesicle was thin-walled ending in a sub-terminal excretory pore. Numerous cystogenous cells and refractile granules obscured structures in the body (Fig. 1B).

Molecular and phylogenetic analysis

The xiphidiocercariae sequences were 930 base pairs (bp) long for the complete ITS (365 bp after trimming to the ITS2 fragment) and the partial fragments of the LSU were 1110 bp. BLAST searches on GenBank and pairwise *p*-distance comparisons of ITS2 and LSU sequences demonstrated that the cercariae were an exact match to *L. linstowi*. Phylogenetic analyses based on the ITS2 and LSU alignments for NJ and ML showed that the novel sequences clustered with *L. linstowi* adult sequences from bats and formed a clade with *Lecithodendrium* sp. cercariae (syn. *C. helvetica* XII Dubois, 1928) (Fig. 2A). Comparison of uncorrected pairwise genetic distance (*p*-distance) between both species using MEGA v6 revealed greater genetic divergence in ITS2 (0·014, 1·4%) than LSU (0·006, 0·6%).

The generated ITS2 and cox1 sequences for the snail were 440 and 570 bp, respectively. Phylogenetic analysis based on both molecular markers and NJ/ML methods (Fig. 2B) produced congruent hypotheses regarding the placement of the novel sequences from this study. Three main sub-clades of *Radix* spp. were observed: *R. ampla* + *R. lagotis*; *R. labiata* + *R. auricularia* and *Radix balthica* in the ITS2 tree and *R. ampla* + *R. labiata; R. auricularia* and *R. balthica* in the cox1 tree. In both trees the sequence from this study clustered within the *R. balthica* clade (Fig. 2B).

DISCUSSION

We report the first molecular and morphological identification of the cercariae of *L. linstowi* and incrimination of *R. balthica* as the molluscan first intermediate host. The rDNA LSU and ITS2 data confirm that the xiphidiocercariae in this study were *L. linstowi* based on 100% sequence similarity to adults from *Nyctalus noctula* (common noctule) in the Ukraine (Tkach *et al*. 2000) and *Pipistrellus pipistrellus* in the UK (Lord *et al*. 2012). Phylogenetic analysis of ITS2 and cox1 identified the snail host of *L. linstowi* as *R. balthica*, although it morphologically resembled *R. peregra,* and therefore further supports synonymy of *R. balthica* with *R. peregra* as proposed by Bargues *et al*. (2001) and Lawton *et al*. (2015). The data emphasize the need for molecular identification of lymnaeid snails to determine their role as intermediate hosts in the life cycles of digeneans, particularly those of medical and veterinary importance.

Lecithodendrium linstowi is a generalist trematode species that is one of the most prevalent and abundant helminths of Eurasian bats (Esteban *et al*. 2001; Lord *et al*. 2012) and also infects the Hungarian harvest mouse (*Micromys minutus pratensis*) (Matskási, 1971). Its prevalence can be partly explained by the ubiquity of *R. balthica*. Adults of *L. linstowi* were first reported in the UK by Lord *et al*. (2012) from the duodenum and upper jejunum of pipistrelle bats (*P. pipistrellus* and *P. pygmaeus*). Bushy Park is an important bat habitat with nine bat species recorded since 2004 (Bushy Park Management Plan, The Royal Parks, 2014 unpublished) so further lecithodendriid species are likely

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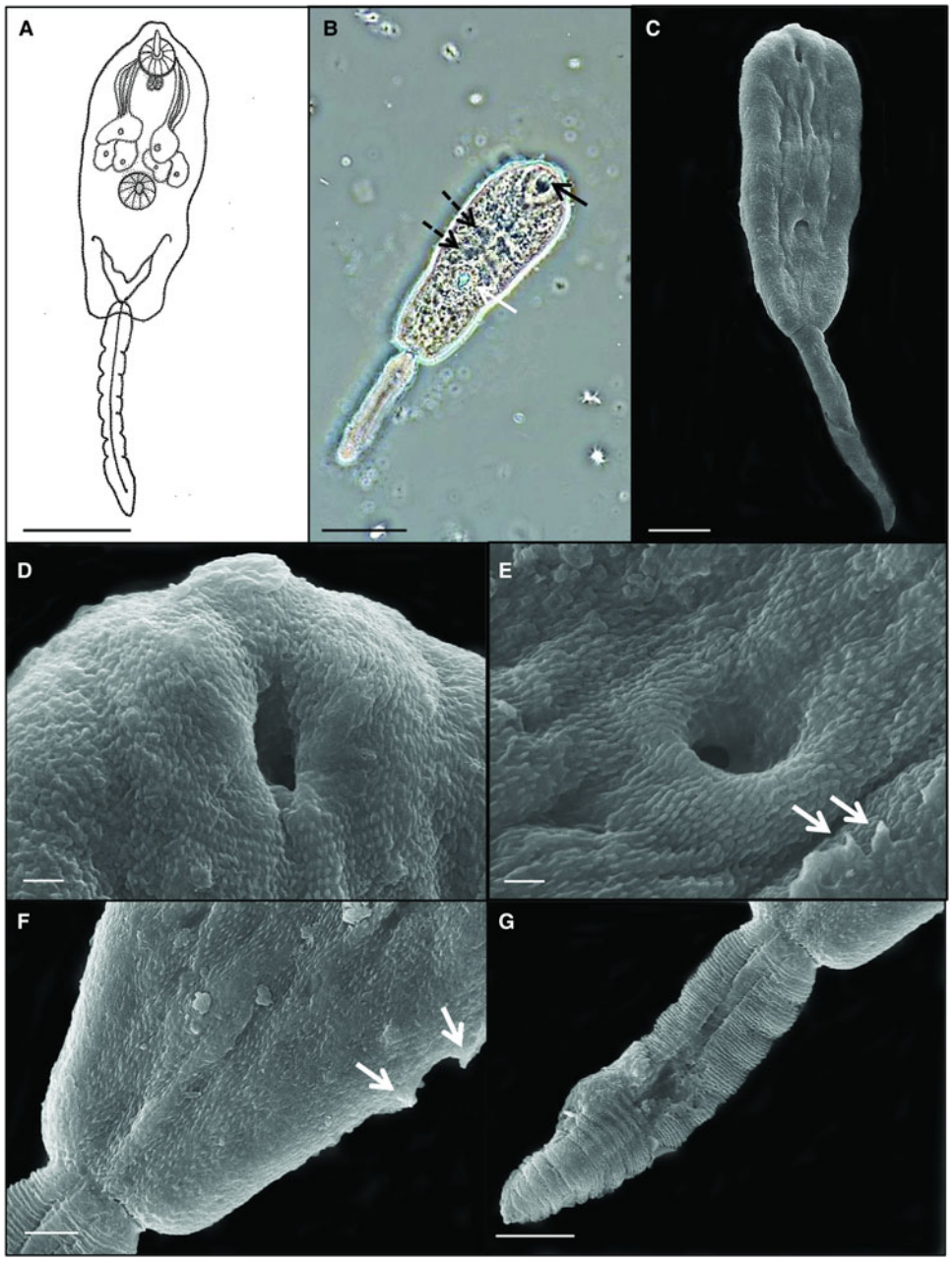

Fig. 1. Cercariae of *Lecithodendrium linstowi* from *Radix balthica*. (A–C) Entire cercaria. (A) Line drawing, scale bar = 25 μm. (B) Photomicrograph, stylet (black arrow), penetration glands (stippled arrows), ventral sucker (white arrow), scale bar = 25 μm. (C) Scanning electron micrograph, scale bar = 20 μm. (D–G) Scanning electron micrographs showing characteristic features, including spinose body tegument. (D) Subterminal oral sucker, stylet detached during processing, scale bar = 2 μm. (E) Ventral sucker, sensory papillae (arrows), scale bar = 2 μm. (F) Junction of body with tail, sensory papillae (arrows), scale bar = 5 μm. (G) Simple tail, scale bar = 10 μm.

to exist at this location, particularly since *L. linstowi* is commonly associated with *L. spathulatum*, which probably shares aquatic insect larvae hosts (Lord *et al*. 2012). There is no evidence available for negative health impacts of lecithodendriid species on bat hosts.

Phylogenetic reconstruction illustrates a well-supported relationship between cercariae and adults of *L. linstowi* (Tkach *et al*. 2000; Lord *et al*. 2012) and confirms the separate *Lecithodendrium* clade proposed by Lord *et al*. (2012). Analysis of *p*-distance estimates of divergence verify that *L. linstowi* and *Lecithodendrium* sp. (syn. *C. helvetica* XII Dubois, 1928) from *Bithynia tentaculata* (Kudlai *et al*. 2015) are closely related separate species. The observed differences were within levels usually recorded among closely related congeneric species such as *Echinostoma caproni* and *E. paraensei* (Vilas *et al*. 2005). Both species have non-virgulate, morphologically similar xiphidiocercariae, although *L. linstowi* is smaller (Table 1). The lack of a virgula organ in *L. linstowi* demonstrates that this

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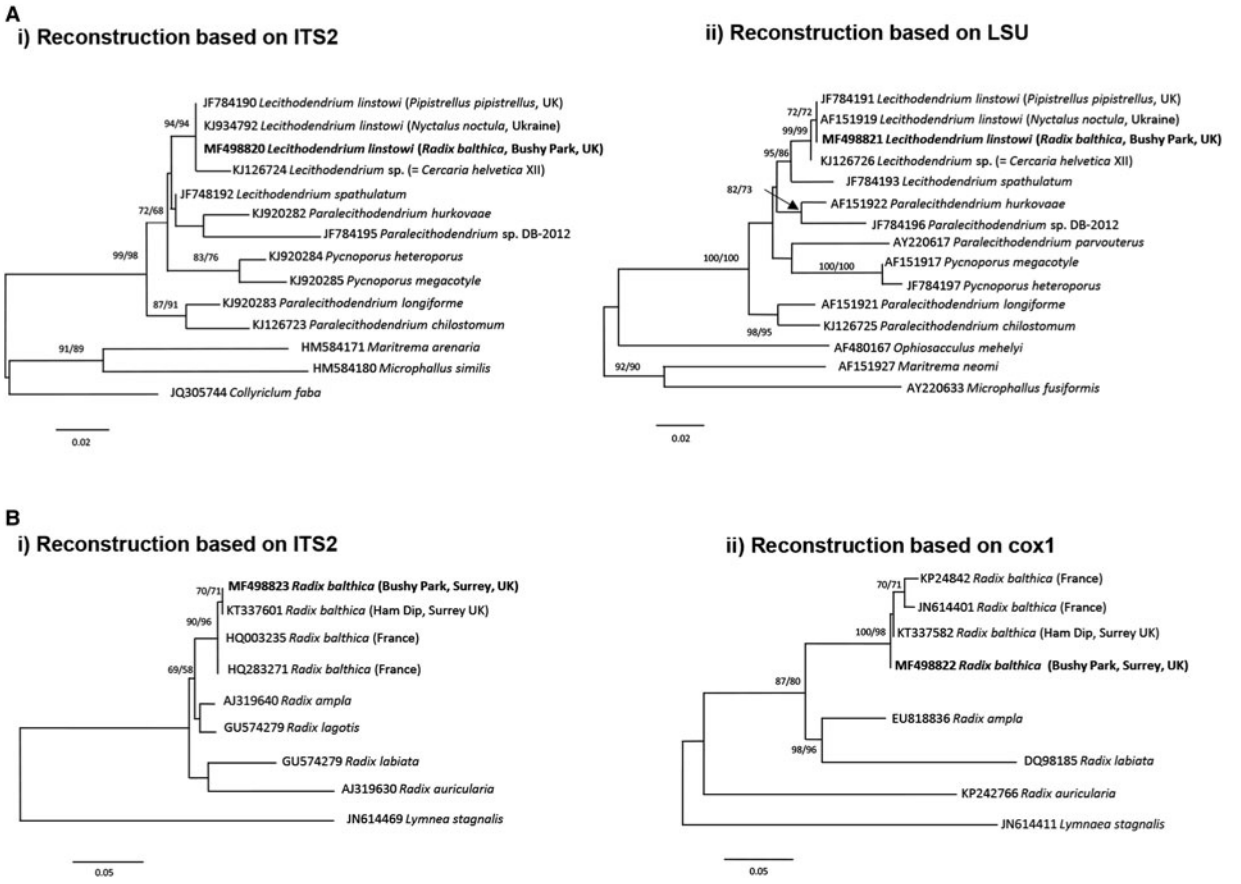

Fig. 2. Phylogenetic identification of *Lecithodendrium linstowi* and *Radix balthica*. (A) Phylogenetic reconstructions based on (i) ITS2 and (ii) LSU sequences of Lecithodendriidae used for the identification of xiphidiocercariae infecting *R. balthica* from Bushy Park, Surrey, UK. (B) Phylogenetic reconstructions based on (i) ITS2 and (ii) cox1 sequences used for identification of *R. balthica* from Bushy Park, Surrey, UK. Trees were constructed using the ML method. The scale shows the number of nucleotide substitutions per site between sequences. The nodal support is given in NJ and ML bootstraps respectively and shows values >50%. Sequences from this study are indicated in bold.

trait is not an absolute synapomorphy for lecithodendriids (Lotz and Font, 2008) and cannot be used as a broad phylogenetic characteristic.

The application of molecular approaches in the current study has enabled taxonomic linkage of cercariae of *L. linstowi* to adult stages without attempting life cycle elucidation, and accurate incrimination of the snail host, thus emphasizing the essential role of DNA sequencing in understanding digenean life cycles. Future molecular studies will be required to identify the second intermediate hosts of *L. linstowi* to achieve resolution of its life cycle. The intermediate host species for many bat parasites are unknown and the lack of reference material and DNA sequence data hinders an understanding of parasite biodiversity in bats. As highlighted by Lord and Brookes (2014), protected species status in the UK means that bats, unless dead or euthanized due to injury, cannot be directly examined. Molecular-based surveys of first and second intermediate hosts are therefore important for long-term monitoring of parasitic infections in endangered bat populations and other vertebrates and the identification of emerging zoonoses. Lecithodendriidae in bats have been identified as hosts of *Neoricketssia* in Egypt, the Philippines, Thailand, North and South America. *Neoricketssia* are vertically transmitted through the parasite life cycle, but can be horizontally transmitted to vertebrate hosts and cause disease (Greiman *et al*. 2017). *Lecithodendrium* sp. harbours *Neoricketssia risticii*, which causes the debilitating and sometimes fatal disease equine monocytic ehrlichiosis (Potomac horse fever) in the Americas. Horses are probably infected through inadvertently consuming metacercariae in insect hosts, while grazing or drinking (reviewed in Vaughan *et al*. 2012). It is therefore important to screen accessible intermediate hosts for both digeneans and their endosymbiont bacteria to provide new insights into neorickettsial-digenean epidemiology.

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