新規の感染経路:
タナイス目甲殻類を利用する吸虫類幼虫の初報告

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A novel transmission pathway: first report of a larval trematode in a tanaidacean crustacean

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Abstract. This study reports on the first trematode parasite observed in any species in the crustacean order Tanaidacea. Metacercariae occupying the body cavity of the host (*Longiflagrum nasutus*) were detected in 13 of 37 tanaidaceans collected from Manko Wetland, a Ramsar site on Okinawa Island, Japan. The morphology of the trematode and analyses of its 18S rRNA, 28S rRNA, and ITS1 nucleotide sequences suggest that it belongs in the family Microphallidae. Definitive hosts are not highly specific in microphallids, and are chiefly birds. Tanaidaceans have been reported in stomach contents from wading birds; *L. nasutus* was abundant at Manko Wetland, and is a likely prey for shorebirds there. This circumstantial evidence suggests an avian definitive host for the trematode in *L. nasutus*.

Introduction

The life cycles of most digenean trematodes are unknown, as they involves multiple hosts. In the typical life cycle, digeneans utilise two intermediate hosts and a definitive host. The first intermediate host is usually a gastropod, although a few species' life cycles involve a bivalve, scaphopod, or polychaete worm (Cribb et al. 2001). Second intermediate hosts are more diverse, including crustaceans, annelids, molluscs, fishes, amphibians, and other groups (cf., Lefebvre & Poulin 2005). The discovery of representatives of additional higher taxa acting as second intermediate hosts will facilitate solving the life cycles of digeneans that remained intractable because the second intermediate host has been unknown.

Most species in the crustacean order Tanaidacea are marine, although a few occur in brackish habitats or fresh water. Tanaidaceans are typically a few millimetres long, and primarily inhabit bottom sediments. They can occur at high densities (e.g., 146,000 individuals/m^2; Delille et al. 1985) and appear to be important prey items in marine food webs, where they are consumed by other crustaceans, fishes, and migratory birds (Larsen 2005). Curiously, though tanaidaceans are abundant, there are few records of their endoparasites: only nematodes and acanthocephalan larvae have been reported (Larsen 2005).

During qualitative sampling for benthic animals at Manko Wetland, a Ramsar wetland site on Okinawa Island in subtropical southern Japan, I collected many specimens of the tanaidacean *Longiflagrum nasutus* (Nunomura, 2005), some of which contained trematode metacercariae. In this study, I examined the morphology of these metacercariae and attempted to identify them through molecular phylogenetic analyses.

Materials and methods

Host sampling. Tanaidaceans were collected from muddy bottom sediment in shallow water on Manko Wetland, Okinawa Island, Japan (26°11'41.50"N 127°40'56"E) on 20 November 2013, by washing sediment through a sieve (0.5 mm mesh). Thirty-seven individuals were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) and preserved in PBS. Two infected animals found among captive tanaidaceans in the laboratory on 13 January 2014 were fixed in absolute ethanol and preserved at –20°C.

Morphological observation. Tanaidaceans were examined for parasite infection by ventral observation under a Nikon SMZ-10 stereoscopic microscope. Infected tanaidaceans stored in PBS were dehydrated in an ethanol series. Two specimens were transferred into dimethyl sulfoxide, frozen at 4°C, cut transversely with a hand-held razor, mounted in glycerine, and observed using autofluorescence with a Zeiss LSM 510 confocal laser scanning microscope (CLSM) under the default setting, the DPSS 561 nm laser. Another six specimens were dissected with chemically sharpened tungsten needles to extract parasites. The parasites recovered were mounted in glycerine and observed with an Olympus BX51 light microscope (LM) equipped with Nomarski interference optics. The eight dissected and five intact tanaidacean specimens harbouring the parasite were deposited in the Hokkaido University Museum, Sapporo, Japan (catalogue number ZIHU 4929) and in the University Museum Fujukan, University of the

Ryukyus (catalogue number RUMF-ZC-3678).

Molecular phylogenetic analyses. One of the two infected tanaidaceans stored in absolute ethanol was dissected to extract the parasite. The parasite was cleaned by brief sonication and pierced with a needle before DNA extraction with a DNeasy Blood & Tissue Kit (Qiagen GmbH). Table 1 lists the primers used for PCR and cycle sequencing. DNA amplification conditions were 95°C for 1 min; 30 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 3 min (18S) or 1 min (28S and ITS1); and 72°C for 7 min. All nucleotide sequences were determined by direct sequencing with a BigDye Terminator Kit ver. 3.1 and a 3730 DNA analyzer (Life Technologies).

Three phylogenetic analyses were conducted to identify the parasite. In the first, a combined 18S+28S dataset was analysed by maximum-likelihood (ML) to place the parasite in a superfamily. This analysis utilised the aligned dataset of Olson et al. (2003), which included 170 trematode terminal taxa (available at http://ftp.ebi.ac.uk in directory/pub/databases/embl/align: ALIGN_00525 and _00526; see also table 1 in Olson et al. 2003), sequence data for *Collyriclum faba* (Heneberg & Literák 2013), and data for unidentified species. Alignments were initially carried out independently for the 18S and 28S datasets, as follows. First, a pre-alignment was performed with MAFFT version 7 (Katoh & Standley 2013) with the "Auto" strategy (FFT-NS-i method; Katoh et al. 2002). Ambiguous sites were then removed by using trimAl (Capella-Gutiérrez et al. 2009) with the option "automated1." Finally, the aligned sequences were trimmed in MEGA 5.2 (Tamura et al. 2011) to match the shortest length. Concatenation of the aligned datasets and selection of the optimal substitution model were performed with Kakusan4 version 4.0.2012.12.14 (Tanabe 2011). The ML analysis was conducted in RAxML version 8.0.0 (Stamatakis 2014), assisted by phylogears2 version 2.0.2013.10.22 (Tanabe 2008), and nodal support values were obtained through ML analyses of 1000 bootstrap pseudoreplicates (Felsenstein 1985).

A second ML analysis was conducted with a 28S dataset that included representatives of the superfamily identified in the first analysis and appropriate outgroup taxa (50 terminal taxa; listed in Appendix 1), to determine the family identity of the trematode. All procedures were as detailed for the first analysis above.

A third analysis utilized an ITS1 dataset (17 terminal taxa; listed in Appendix 1) to identify the trematode's phylogenetic position within the family indicated by the second analysis. Sequence alignment was performed as above. Model selection, the ML analysis, and bootstrap analysis of 1000 bootstrap pseudoreplicates were carried out with TREEFINDER, March 2011 version (Jobb 2011).

The optimal substitution models for the three

Table 1. List of PCR and cycle sequencing (CS) primers used in this study.
表 1. PCR 反応とサイクルシーケンシング (CS) で用いたプライマー一覧.

Marker	Primer	Sequence (5′ to 3′)	Reaction	Source
18S	SR1	TACCTGGTTGATCCTGCCAG	PCR & CS	Nakayama et al. (1996)
	SR6	GTCAGAGGTGAAATTCTTGG	CS	Nakayama et al. (1996)
	SR8	GGATTGACAGATTGAGAGCT	CS	Nakayama et al. (1996)
	SR9	AACTAAGAACGGCCATGCAC	CS	Nakayama et al. (1996)
	SR10	AGGTCTGTGATGCCCTTAGA	CS	Nakayama et al. (1996)
	SR11	CGCTTACTAGGAATTCCTCG	CS	Nakayama et al. (1996)
	SR12	CCTTCCGCAGGTTCACCTAC	PCR & CS	Nakayama et al. (1996)
	EU60F	GAAACTGCGAATGGCTCATT	CS	Puitika et al. (2007)
	EU929R	TTGGCAAATGCTTTCGC	CS	Puitika et al. (2007)
	18S554f	AAGTCTGGTGCCAGCAGCGCG	CS	Maraun et al. (2009)
	18S614r	TCCAACTACGAGCTTTTTAACC	CS	Maraun et al. (2009)
28S	U178	GCACCCGCTGAAYTTAAG	PCR & CS	Lockyer et al. (2003)
	300F	CAAGTACCGTGAGGGAAAGTTG	CS	Lockyer et al. (2003)
	900F	CCGTCTTGAAACACGGACCAAG	CS	Lockyer et al. (2003)
	U1148	GACCCGAAAGATGGTGAA	CS	Lockyer et al. (2003)
	L1642	CCAGCGCCATCCATTTTCA	PCR & CS	Lockyer et al. (2003)
ITS1	1780F	ACACCGCCCGTCGCTACTA	PCR & CS	Galaktionov et al. (2012)
	M5-8	GGCTGCGCTCTTCATCGACA	PCR & CS	Galaktionov et al. (2012)

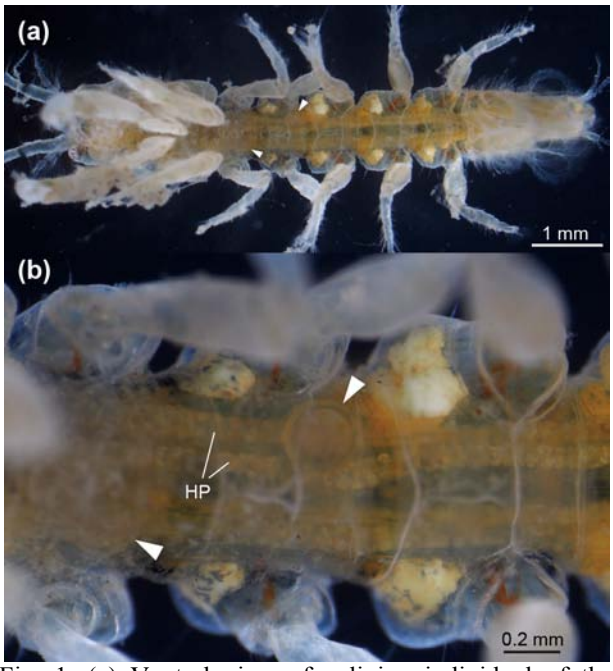

Fig. 1. (a) Ventral view of a living individual of the tanaidacean *Longiflagrum nasutus*, infected with two larval trematodes (arrowheads) in pereonites 2 and 3; stereoscopic microscope image. (b) Enlargement from panel (a). HP, left hepatopancreases.
図 1. (a) ハナダカアプセウデスの第 2, 3 胸節に寄生した 2 個体の吸虫類幼虫 (矢頭); 生時腹側からの実体顕微鏡観察像. (b) 拡大図. HP, 左側の中腸腺.

data sets (18S+28S, 28S, and ITS1) determined by using the Akaike information criterion (Akaike 1974) were GTR+I+G, GTR+I+G, and TVM+G, respectively. No significant nucleotide compositional heterogeneity was detected for any of the data sets (Chi-square test in Kakusan4: P = 0.99995 for 18S+28S combined; P = 0.97865 for 28S; P = 1.00000 for ITS1).

Results

Of the 37 tanaidaceans fixed in the field, 13 individuals harboured trematode parasites, with one to three parasites per host. The parasites occurred in the pereonites and/or the somite that bears the chelipeds (Fig. 1). The parasites occupied the body cavity of the host, with host's hepatopancreas distorted (Figs 1, 2).

The parasites were in the encysted metacercaria stage (Fig. 3), with the body curled up ventrally inside the cyst. In situ, the cysts were 238–259 µm in diameter (n = 5) and 11 µm thick. Under LM observation, two suckers (oral and ventral) were observed. An ovary and a genital atrium were dextral and sinistral to the ventral sucker, respectively; the cirrus sack connected with the

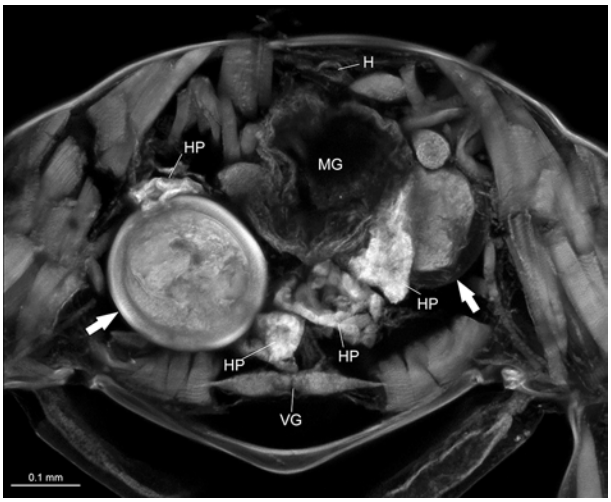

Fig. 2. Trematodes (arrows) in pereonite 2 of a *Longiflagrum nasutus* individual, anterior view, CLSM image. H, heart; HP, hepatopancreas; MG, midgut; VG, ventral ganglion.
図 2. ハナダカアプセウデスの第 2 胸節に寄生した吸虫類 (矢頭); 前方からの共焦点レーザー顕微鏡観察像. H, 心臓; HP, 中腸腺; MG, 中腸; VG, 腹側神経節.

latter. There were two symmetrical clusters of vitellaria. Other structures observed (not shown in Fig. 3) were a pair of testes in the hindbody, the digestive tract, and the pharynx.

The three aligned datasets (18S+28S, 28S, and ITS1) were 2991, 1114, and 379 b.p. long, respectively. The 18S+28S ML tree (Appendix 2) placed the tanaidacean parasite in a clade corresponding to the superfamily Microphalloidea, with moderately high bootstrap (BP) support (94.3%). Within this clade, the parasite comprised a clade (BP = 80.6%) with the three representatives of family Microphallidae (*Microphallus primas*, *M. fusiformis*, and *Maritrema oocysta*). The 28S ML tree (Fig. 4a) again placed the parasite in the family Microphallidae (BP = 87.8%), within a clade comprising representatives of the genus *Microphallus* (BP = 77%). In the ITS1 ML tree (Fig. 4b), the parasite was basal in a highly supported clade (BP = 99.1%) comprising *Microphallus*.

Discussion

This study used a DNA barcoding approach to identify the metacercaria found in a tanaidacean. The 18S+28S, 28S, and ITS1 trees were congruent in placing this parasite in a *Microphallus* clade. Support for the node grouping the unidentified parasite with *Microphallus* was high for the 18S+28S (98.6%) and ITS1 (99.1%) trees. However, sequence data are lacking for several microphalloid

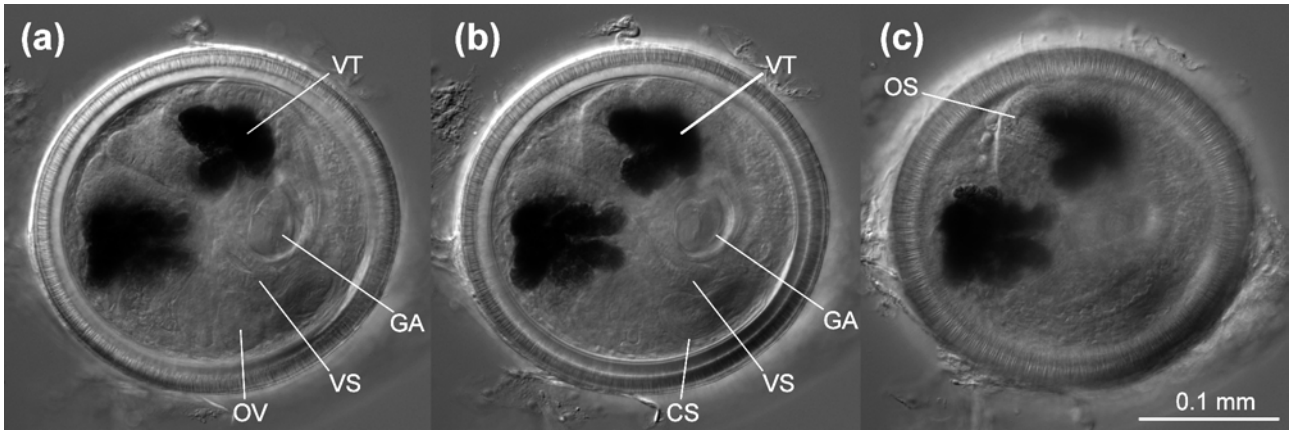

Fig. 3. Encysted metacercaria extracted from *Longiflagrum nasutus*, LM images, taken in different focus planes (a–c, relatively shallow, middle, and deep, respectively). CS, cirrus sack; GA, genital atrium; OS, oral sucker; OV, ovary; VS, ventral sucker; VT, vitellarium.
図 3. ハナダカアプセウデスより摘出した被嚢幼虫; 焦点面の異なる光学顕微鏡観察像 (a–c, それぞれ浅い, 中間, 深い焦点面像). CS, 陰茎嚢; GA, 生殖腔; OS, 口吸盤; OV, 卵巣; VS, 腹吸盤; VT, 卵黄腺.

families and many microphallid genera (Bray et al. 2008), and in both these trees the unidentified parasite could represent a sister group (sister genus) to *Microphallus*.

As all my specimens were encysted metacercariae, I was unable to observe their morphology in detail. The various structures I did observe—a pair of testes in the hindbody, the digestive tract, two symmetrical clusters of vitellaria, and a genital atrium—are compatible with identification as a species in the family Microphallidae.

The definitive hosts for microphallid trematodes are not highly specific, but are chiefly birds (Bray et al. 2008), which are good candidates as the definitive host(s) for the parasite in *Longiflagrum nasutus*. Manko Wetland is a wintering and migration-staging area for shorebirds and other water birds. Furthermore, *L. nasutus* is abundant on the mudflat, with observed densities of up to 523 individuals per 0.0625 m^2 (Naha Nature Conservation Office 2009). Although the role of *L. nasutus* in the food web is unknown, the congener *L. koyonense* has been found in the stomach of catfishes (Angsupanich 2004), and Băcescu & Guţu (1975) reported two confamilial species, *Discapseudes surinamensis* and *Halmyrapseudes spaansi*, to be common in the stomach contents of migratory wading birds on a mudflat. Considering the abundance and size (up to about 8 mm) of *L. nasutus*, this tanaidacean might well be a prey item for fishes and/or birds.

This is the first report of a trematode parasite in tanaidaceans, which thus constitute a novel transmission pathway for trematodes. Tanaidaceans comprise around 1200 described species worldwide (Anderson 2013), often occur at high densities, inhabit sandy/muddy habitats from mudflats to the deep sea, and are preyed upon by many fish and bird species (Larsen 2005). Tanaidaceans could thus potentially be involved in many trematode life cycles.

Acknowledgements

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Fig. 4. ML trees from analyses of sequences of a) the 28S gene (1114 b.p.) and b) ITS1 (379 b.p.) from an encysted metacercaria in *Longiflagrum nasutus*; bootstrap values > 50% given near nodes; black circles indicate nodes with 100% bootstrap support.

図 4. 28S rRNA 遺伝子 (a; 1114 塩基) と ITS1 遺伝子 (b; 379 塩基) に基づく最尤系統樹. 50%より高いブートストラップ値を分岐点に示す. 黒丸は 100%のブートストラップ値を表す.

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新規の感染経路: タナイス目甲殻類を利用する吸虫類幼虫の初報告

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要旨. 吸虫の被嚢幼虫をタナイス目甲殻類 (ハナダカアプセウデス *Longiflagrum nasutus*) から初めて発見した. 寄生部位は胸節体腔内であり, 宿主 37 個体中 13 個体に感染が認められた. 形態的特徴および 3 遺伝子 (18S rRNA, 28S rRNA, ITS1) の配列情報から, 本吸虫は Microphallidae 科の 1 種だと判断された. Microphallidae 科吸虫の終宿主特異性は高くないが, 主として鳥類だと考えられている. タナイス類は鳥類の胃内容物から見出されることがあり, ハナダカアプセウデスは採集地, ラムサール条約登録湿地である漫湖干潟に多産する種類である. このことから, 今回見つかった吸虫類幼虫の終宿主は鳥類であることが考えられる.

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Appendix 1. Digenean species included in the 28S and ITS1 analyses in this study. *Tkatch et al. (2003) synonymised Floridatrema with Maritrema. †1, Tkach et al. (2003); 2, Olson et al. (2003); 3, Heneberg & Literák (2013); 4, Galaktionov et al. (2012); 5, Pina et al. (2011a); 6, Pina et al. (2011b); 7, Pina et al. (2007); 8, Al-Kandari et al. (2011); 9, Lockyer et al. (2003).
附録 1. 28S rRNA 遺伝子, ITS1 遺伝子に基づいた系統解析に用いた二生吸虫類の種一覧. * *Floridatrema* 属は Tkatch et al. (2003) により *Maritrema* 属の新参異名とされている. †1, Tkach et al. (2003); 2, Olson et al. (2003); 3, Heneberg & Literák (2013); 4, Galaktionov et al. (2012); 5, Pina et al. (2011a); 6, Pina et al. (2011b); 7, Pina et al. (2007); 8, Al-Kandari et al. (2011); 9, Lockyer et al. (2003).

Classification / Species	GenBank accession number			References†
	18S	28S	ITS1	
Metacercaria in this study	AB974359	AB974360	AB974361	This study
MICROPHALLOIDEA				
LECITHODENDRIIDAE				
Lecithodendrium linstowi	AY222147	AF151919	-	1
Prosthodendrium longiforme	AY222148	AF151921	-	1
Ophiosacculus mehelyi	-	AF480167	-	1
Pycnoporus heteroporus	-	AF151918	-	1
EUCOTYLIDAE				
Tanaisia fedtschenkoi	AY222154	AY116870	-	2
PACHYPSOLIDAE				
Pachypsolus irroratus	AJ287554	AY222274	-	2
COLLYRICLIDAE				
Collyriclum faba	JQ231122	JQ231122	JQ231122	3
PROSTHOGONIMIDAE				
Prosthogonimus ovatus	AY222149	AF151928	-	1
Prosthogonimus cuneatus	-	AY220634	-	1
PLEUROGENIDAE				
Allassogonoporus amphoraeformis	-	AY220620	-	1
Brandesia turgida	-	AY220622	-	1
Candidotrema loossi	-	AY220621	-	1
Loxogenes macrocirra	-	AY220624	-	1
Parabascus joannae	-	AY220619	-	1
Parabascus duboisi	-	AY220618	-	1
Pleurogenes claviger	AY222152	AF151925	-	1
Pleurogenoides medians	AY222151	AF433670	-	1
Prosotocus confusus	-	AY220623	-	1
MICROPHALLIDAE				
Maritrema arenaria	-	AY220629	HM584144	1, 4
Maritrema oocysta	AJ287534	AY220630	HM584143	1, 4
Maritrema subdolum	-	AF151926	HM584145	1, 4
Maritrema neomi	-	AF151927	-	1
Maritrema prosthometra	-	AY220631	-	1
Maritrema portucalensis	-	-	HQ993044	5
*Maritrema heardi**	-	AY220632	-	1
Maritrema cf. *eroliae*	-	JF826247	-	8
Microphallus abortivus	-	AY220626	HM584159	1, 4
Microphallus basodactylophallus	-	AY220628	-	1
Microphallus primas	AJ287541	AY220627	HM001303	2, 6
Microphallus similis	-	AY220625	HM584156	1, 4

Microphallus fusiformis	AJ287531	AY220633	-	1
Microphallus kurilensis	-	HM584140	HM584168	4
Microphallus sp.	-	HM584142	HM584161	4
Microphallus calidris	-	HM584125	HM584151	4
Microphallus piriformes	-	HM584122	HM584154	4
Microphallus pseudopygmaeus	-	HM584126	HM584147	4
Microphallus pygmaeus	-	HM584133	HM584153	4
Microphallus triangulatus	-	HM584139	HM584162	4
Gynaecotyla longiintestinata	-	-	DQ118021	7
RENICOLIDAE				
Renicola sp.	AY222155	AY116871	-	2
ZOOGONIDAE				
Lepidophyllum steenstrupi	AJ287539	AY157175	-	9
Deretrema nahaense	AJ287498	AY222273	-	2
Zoogonoides viviparus	AJ287590	AY222271	-	2
Diphterostomum sp.	AY222153	AY222272	-	2
FAUSTULIDAE				
Antorchis pomacanthi	AJ287476	AY222268	-	2
Bacciger lesteri	AJ287482	AY222269	-	2
Trigonocryptus conus	AJ287584	AY222270	-	2
PLAGIORCHIOIDEA				
OMPHALOMETRIDAE				
Rubenstrema exasperatum	AJ287572	AY222275	-	2
BRACHYCOELLIDAE				
Brachycoelium salamandrae	AY222160	AF151935	-	2
PLAGIORCHIIDAE				
Glypthelmins quieta	AJ287517	AY222278	-	2
MACRODEROIDIDAE				
Macroderoides typicus	AY222158	AF433673	-	2

Appendix 2. (a) ML tree from an analysis of the 18S+28S dataset (2999 b.p.), including sequences from the encysted metacercaria in *Longiflagrum nasutus*. (b) Enlargement from panel (a). Bootstrap values > 50% given near nodes; black circles indicate nodes with 100% bootstrap support.
付録 2. (a) 18S rRNA 遺伝子と 28S rRNA 遺伝子の結合配列情報 (2999 塩基) に基づく最尤系統樹. (b) 一部拡大図. 50%より高いブートストラップ値を分岐点に示す. 黒丸は 100%のブートストラップ値を表す.