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Exploring the evolutionary potential of parasites: Larval stages of pathogen digenic trematodes in their thiarid snail host *Tarebia granifera* in Thailand

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Abstract

Minute intestinal flukes from several distinct families of endoparasitic platyhelminths are a medically important group of foodborne trematodes prevalent throughout Southeast Asia and Australasia. Their lifecycle is complex, with freshwater snails as primary intermediate hosts, with infecting multiple species of arthropods and fish as second intermediate hosts, and with birds and mammals including humans as definitive hosts. In Southeast Asian countries, the diversity of snail species of the Thiaridae which are frequently parasitized by trematode species is extremely high. Here, the thiarid *Tarebia granifera* in Thailand was studied for variation of trematode infections, by collecting the snails every two months for one year from each locality during the years 2004–2009, and during 2014–2016 when snails from the same localities were collected and new localities found. From ninety locations a total of 15,076 *T. granifera* were collected and examined for trematode infections. With 1,577 infected snails the infection rate was found to be 10.46 %. The cercariae were categorized into fifteen species from eight morphologically distinguishable types representing several distinct families, viz. (i) virgulate xiphidiocercariae (*Loxogenoides bicolor*, *Loxogenes liberum* and *Acanthatrium histaense*), (ii) armatae xiphidiocercariae cercariae (*Maritreminoides caridinae* and *M. obstipus*); (iii) parapleurophocercous cercariae (*Haplorchis pumilio*, *H. taichui* and *Stictodora tridactyla*); (iv) pleurophocercous cercariae (*Centrocestus formosanus*); (v) megarulous cercariae (*Philophthalmus gralli*); (vi) furcocercous cercariae (*Cardicola alseae*, *Alaria mustelae* and *Transversotrema laruei*); as well as (vii) echinostome-type cercariae, and (viii) gymnocephalous-type cercariae. In addition, a phylogenetic marker (internal transcribed spacers 2, ITS2) was employed in generic and infrageneric level classifications of these trematodes, using sequences obtained from shed cercariae isolated from *T. granifera* specimens of the second study period collected in various regions in Thailand. We obtained ITS2 sequences of cercariae from nine species (of seven types): *Loxogenoides bicolor*, *Loxogenes liberum*, *Maritreminoides obstipus*, *Haplorchis taichui*, *Stictodora tridactyla*, *Centrocestus formosanus*, *Philophthalmus gralli*, as well as from one species each of echinostome cercariae and gymnocephalous cercariae. Thus, this analysis combines the parasites' data on morphology and geographical occurrence with molecular phylogeny, aiming to provide the groundwork for future studies looking into more details of the parasite-snail evolutionary relationships.

Introduction

Trematodes (or flatworms) are endoparasitic platyhelminths that not only infect fishes, birds and other wildlife worldwide but also mammals as well as humans. As foodborne parasites they are of medical importance resulting in significant morbidities and mortalities worldwide. For example, the disability adjusted life years (also known as DALYs) for the foodborne trematodiases including *Fasciola* spp., *Clonorchis* spp., *Opisthorchis* spp., *Paragonimus* spp. and the minute intestinal trematodes such as *Fasciolopsis buski*, *Heterophyes* spp. and *Metagonimus* spp., are estimated to be 2.02 million worldwide (Torgerson et al. 2015).

Especially as liver flukes and intestinal flukes human infecting parasites are highly prevalent in Southeast Asian countries (Wongratanacheewin et al. 2001, Chai et al. 2005, 2013, Krailas et al. 2014). Infections caused by these flukes have a major public health impact and are also of economic importance in veterinary medicine. Humans or domestic animals become infected when they eat raw, salted, pickled or smoked fish containing the infective metacercariae (e.g. *Opisthorchis* spp.,) or contaminated to raw or uncooked vegetables (e.g. *Fasciola* spp.) (see e.g. Krailas et al. 2011, Krailas et al. 2014). Examples include the liver fluke *Opisthorchis viverrini*, which can cause cholangiocarcinoma, a kind of cancer in the bile ducts. The intestinal fluke *Haplorchis taichui* is a possible agent of irritable bowel syndrome-like symptoms, and *Centrocestus formosanus* may cause epigastric pain and indigestion accompanied by occasional diarrhea (Watthanakulpanich et al. 2010, Sripa et al. 2010, Chai et al. 2013). The prevalence of human trematode infections of the mentioned species was found to be the highest in the northern and northeastern regions of Thailand (Srisawangwong et al. 1997, Pungpak et al. 1998, Radomyos et al. 1998, Sukontason et al. 1999). In Northeast Thailand alone, for example, about six million people are infected with the liver fluke, *O. viverrini* (Shin et al. 2010). As Thailand has the highest incidence of cholangiocarcinoma associated with *O. viverrini* (Sripa et al. 2007), opisthorchiasis received greater attention for research than infection with the minute intestinal flukes, such as *Haplorchis taichui*, for which no such associations have been documented. Nevertheless, Thai people have considerably underestimated these trematodes in the past by continually eating traditional Thai food prepared from raw freshwater fish (Chuboon et al. 2005). Hence, the prevalence of trematodes in Thailand remains a continuous problem (Krailas et al. 2014).

Trematodes often have very complex life cycles involving at least one, sometimes two or four, but usually three different hosts, of which the first is almost always a mollusc (Galaktionov and Dobrovolskij 2003). Eggs are released by the definitive host and either the first larval stage, i.e. the miracidium, hatches from the egg in a suitable medium (usually water) being adapted for actively recognizing and penetrating the first intermediate host; or the miracidium remains embryonated within the egg and infects the first intermediate host through passive uptake and subsequent hatching and penetration within the host. The miracidium develops directly into a (mother) sporocyst that may produce daughter sporocysts or rediae (sometimes rediae also produce a second generation of rediae). Another larval form, i.e. the cercariae, then develops either within the sporocyst or within the redia in the first intermediate host and is typically released into the environment where it either actively searches and penetrates the host or is passively taken up. Within the second host cercariae encyst and develop into metacercariae. Through predation metacercariae are taken up by the definitive host and then develop into the adult trematode completing the life cycle. Deviations from this typical life cycle occur either in the number of different life cycle stages that actually develop or in the number of hosts involved in the development (for a detailed overview, see Galaktionov and Dobrovolskij 2003).

The occurrence of trematodes depends on the presence of first and second intermediate host species, as well as the eating habit of local people (Radomyos et al. 1998). In Thailand, medically-important freshwater snails have been investigated since 1980 for trematode infections (Upatham et al. 1980, Nithiuthai et al. 2002, Krailas et al. 2003, 2008, 2014, Sri-aroon et al. 2005, Dechruksa et al. 2007, 2013, 2017, Ukong et al. 2007). For example, the liver fluke *Opisthorchis viverrini* (Family: Opisthorchiidae) is found in Thailand in freshwater snails *Bithynia funiculata*, *B. siamensis goniomphalos* and *B. siamensis siamensis* (Bithyniidae). However, despite the importance of the snail intermediate host(s) to the lifecycle of trematodes, the faunistic and biosystematic knowledge of these limnic molluscs is scarce in general. In particular, among the Cerithioidea which is ecologically and phylogenetically a highly important caenogastropod group of molluscs (Glaubrecht 1996, 2009, 2011; Strong et al. 2011), several freshwater gastropods are known especially in the Thiaridae Gill, 1871 to be important first intermediate hosts of trematodes. For example, species of the intestinal lung fluke *Paragonimus* have been identified in paludomids and/or thiarids, such as e.g. species of *Paludomus* as well as in *Melanoides tuberculata* and *Tarebia granifera*. Pinto and de Melo (2011) list 37 species of cercariae and another 81 trematode larval forms for *Melanoides tuberculata* Müller, 1774. For Thailand Brandt (1974) lists five snail species, viz. *Melanoides tuberculata*, *M. jugicostis* Hanley & Theobald, 1876, *Sermyla riqueti* Grateloup, 1840, *Neoradina prasongi* Brandt, 1974 and *Tarebia granifera* Lamarck, 1816 (see Lamarck 1816), that are currently assigned to the Thiaridae (Glaubrecht 1996, 1999, 2011, Lydeard et al. 2002, Glaubrecht et al. 2009, Strong et al. 2011). Most recently, Krailas et al. (2014) and Dechruksa et al. (2017) investigated the cercarial fauna of *M. tuberculata* and *M. jugicostis* populations from Thailand in detail, reporting 18 different cercariae from the former and four from the latter; among them *C.*

formosanus, *H. taichui*, *Haplorchis pumilio* Looss, 1896 and *Stictodora tridactyla* Martin & Kuntz, 1955 that are known to be human pathogen (Watson 1960, Malek and Cheng 1974, Upatham et al. 1995, Pointier and Jourdane 2000, Dechruksa et al. 2007, Ukong et al. 2007).

In the present study, the cercarial fauna of *Tarebia granifera* populations from Thailand is investigated. This thiarid species is widespread in the Oriental region, with an autochthonous range including South and Southeast Asia, South China and numerous islands of the Western Pacific (Brandt 1974, Glaubrecht 1996). The species has been introduced to Africa, the Near East, North and Central America as well as to the Caribbean region and is considered to be invasive there (Abbott 1952, Chaniotis et al. 1980, Prentice 1983, Vargas et al. 1991, Férnandez et al. 1992, Gutierrez et al. 1997, Pointier et al. 1998, Appleton 2002, Mukaratirwa et al. 2005, Facon and David 2006, Appleton et al. 2009, Miranda et al. 2010, 2011, Miranda and Perissinotto 2012). A parallel study on *Tarebia granifera* (also published in this journal; see Veeravechsukij et al. 2018) shows this species to be widely distributed throughout Thailand, with several named and described congeneric constituent populations, as is revealed by respective collections carried out in the North, Northeast, South, East, and Central region, and morphological documentation conducted detailing the biometrical parameters of the adult shells. In addition, molecular phylogenies using fragments of the mitochondrial cytochrome c oxidase subunit 1 (*cox1*) and 16 S rRNA genes have been constructed, as well as the reproductive strategy documented (i.e. the various stages of embryos and juveniles in the brood pouch) and analysed as to the effect of cercariae infection in female snails.

Here we apply, aside from traditional morphological methods, molecular genetic techniques in order to delimit species of cercariae; i.e. sequencing parts of the nuclear ribosomal RNA gene cluster that have been shown to be efficient for the identification of species of trematodes from their distinct life stages (Skov et al. 2009, Prasad et al. 2011, Davies et al. 2015, Anucherngchai et al. 2016, 2017). With this combination of molecular phylogeny and the parasites' data on morphology and geographical occurrence, we attempt to provide the groundwork for future studies determining the parasite's evolutionary potential within the complex snail-host relationship.

Materials and methods

Sampling

Specimens of *Tarebia granifera* were collected in streams, ponds, rivers, brooks, trenches and mountain creeks in all major regions of Thailand (North, South, East, Central and Northeast). Geographic coordinates (WGS84 datum) of sampling sites were determined with the global positioning system (GPS) (Garmin PLUS III, Taiwan). Where GPS data for sampling sites were unavailable, coordinates were determined as accurately as possible from a map. Sampling sites were mapped on a dot-by-dot basis on a public domain map (ArcGIS, Esri, Redlands, California, USA) and then compiled using Photoshop CS6 (Adobe Systems, San Jose, California, USA).

Collection methods and determination of snails

Snail collections were done during two periods. In the first period, from 2004 to 2009, the snails were collected every two months for one year from each of all the locations. During the second period, from 2014 to 2016, the same localities were visited again, but additional samples were also taken at several new localities, this time collected once only from each location. The snails were collected using the counts per unit of time sampling method (Olivier and Schneiderman 1956). Five researchers collected samples by handpicking and scooping every 10 minutes at each sampling site. The snails were transferred and studied in the laboratory of the Parasitology and Medical Malacology Research Unit, Silpakorn University, Nakhon Pathom, Thailand (PaMaSU: code SUT). The snails were identified according to their shell morphology , following essentially Brandt (1974), and subsequently examined for trematode infections.

Cercarial study

Collected snails were investigated for trematode infections by using shedding and crushing methods. Descriptions of their morphology were based on living cercariae that had escaped from the snails. The emerged cercariae were studied unstained or vitally stained with 0.5% neutral red. Details of the cercariae were drawn using a camera lucida and identified according to Schell (1970), Yamaguti (1975), Ito (1980) and Krailas et al. (2014). Sample measurements (average size) in micrometers were taken, using an ocular micrometer, from 10 specimens fixed in 10% formalin. Some cercariae (c. 20 specimens from each location) belonging to identified trematode species were then preserved in 95% ethanol for further DNA analysis.

Molecular study of cercariae

The preserved cercariae were processed for molecular identification at the Department of Animal Diversity, Zoological Museum of the Center for Natural History (CeNak), Universität Hamburg, Germany. Genomic DNA from the cercariae was extracted using the DNeasy blood and animal tissue kit (QIAGEN, Venlo, The Netherlands). Amplification by polymerase chain reaction (PCR) of the nuclear internal transcribed spacer 2 (ITS2) region were performed with the following primers ITS2-F (5'-CTT GAACGC ACA TTG CGG CCA TGG G-3') and ITS2-R: (5'-GCG GGT AAT CACGTC TGA GCC GAG G-3') (Sato et al. 2009). Reactions were set up in 20 µl volumes containing 1.0 µl dNTPs (2 mM each), 2.0 µl 10× mM DreamTaq

Green buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.3 µl GreenTaq DNA polymerase (5 U/µl, Thermos Fisher Scientific), 1.0 µl of each primer (10 µM) and 14.7 µl ddH_2O. The DNA samples were initially denatured at 94 °C for 4 min followed by 35 cycles (denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, and elongation at 72 °C for 2 min; see Sato et al. 2009) and a final elongation step at 72 °C for 7 min. The PCR products were purified according to the protocol for enzymatic PCR product clean-up with exonuclease I (20 U/µl, Thermo Fisher Scientific) and FastAP thermosensitive alkaline phosphatase (1 U/µL, Thermo Fisher Scientific). Purified PCR products were sequenced at Macrogen Europe Lab. (Amsterdam, The Netherlands). Alignments of forward and reverse strands were conducted using Geneious 10.1.3 (Biomatters Ltd., Auckland, New Zealand). The ITS2 consensus sequences were aligned in MEGA 7 (Kumar et al. 2016) using MUSCLE (Edgar 2004) under default settings. A Neighbor joining (NJ) analysis was performed based on p-distances with 1,000 bootstrap replicates. For details on sequences used for this study, see Table 1.

Results

Geographical origin of collected snails

Specimens of *Tarebia granifera* were found at 90 sampling sites in five regions of Thailand (Fig. 1). During the first sampling period (2004–2009), infected snails were reported from 18 sampling sites. In the second period (2014–2016), infected snails were reported from 51 sampling sites. At a total of 58 localities in four regions of Thailand snails with cercarial infections were found. For information on sampling sites including geographic coordinates and the number of infected snails, see Table 2.

Occurrence of trematodes obtained from *Tarebia granifera* in Thailand

The various trematode cercariae (distinguished and described in more detail below) exhibit a certain geographical pattern within the various water bodies in Thailand. Only two among the fifteen trematode species found in the thiarid *T. granifera*, viz. *Loxogenoides bicolor* and *Stictodora tridactyla*, were recorded in the present study from almost all major river systems in Thailand (Fig. 2).

In contrast, several species exhibit a more restricted distribution. For example, *Haplorchis taichui* was only detected in *T. granifera* samples from the Nan River (Chao Phraya river system) and the Loei River (Mekong river system), whereas *Philophthalmus gralli* and gymnocephalous cercaria were only detected in the Phachi River (Mae Klong river system). Echinostome cercaria were only present in the *T. granifera* population from the Khek River (Chao Phraya river system).

Cercariae of *Loxogenes liberum*, *Centrocestus formosanus* and *Maritreminoides obstipus* had again a somewhat wider distribution in Thai *T. granifera* populations, being present in several rivers of the Chao Phraya, Mae Klong and Gulf of Thailand drainages (Fig. 2).

Cercarial diversity and infection rates

A total of 15,076 snails of *T. granifera* were collected and examined for trematode infections. With 1,577 parasitized snails the overall infection rate was found to be 10.46 %. The obtained cercariae were classified into a total of fifteen species from eight morphologically distinguishable types representing at least seven distinct trematode families, viz. (i) virgulate xiphidiocercariae (*Loxogenoides bicolor*, *Loxogenes liberum* and *Acanthatrium histaense*), (ii) armatae xiphidiocercariae (*Maritreminoides caridinae* and *Maritreminoides obstipus*), (iii) parapleurophocercous cercariae (*Haplorchis pumilio*, *Haplorchis taichui* and *Stictodora tridactyla*), (iv) pleurophocercous cercariae (*Centrocestus formosanus*), (v) megarulous cercariae (*Philophthalmus gralli*), (vi) furcocercous cercariae (*Cardicola alseae*, *Alaria mustelae* and *Transversotrema laruei*), as well as (vii) echinostome cercariae, and (viii) gymnocephalous cercariae. The virgulate xiphidiocercariae were the dominant cercarial type infecting snails (5.10%), while infections with other cercarial types were found at rates of (ii) 0.15%, (iii) 3.73%, (iv) 1.14%, (v) 0.02%, (vi) 0.25%, (vii) 0.07%, (viii) 0.01%, respectively; see Table 3 for details.

In this study, neither double trematode infections nor triple trematode infections of collected *Tarebia granifera* were found.

Morphology of infecting cercariae

The cercariae were categorized by their morphology and organ characters, using as reference previous morphological descriptions (e.g. Schell 1970, Yamaguti 1975, Frandsen and Christensen 1984, Krailas et al. 2014). They are described as follows for the eight distinct morphological cercarial types known and found to date, attributable to at least seven distinct trematode families.

Type 1. Virgulate xiphidiocercariae cercariae

Lecithodendriidae Lühe, 1901 (sensu Odhner 1910)

1.1 *Loxogenoides bicolor* (Krull, 1933) (sensu Kaw 1945) (Fig. 3)

Body oval; throughout with granules. Oral sucker bigger than ventral sucker; globular in shape and with stylet. Virgulate organ in the anterior part of the body. Pharynx small; an esophagus was not observed. Three pairs of penetration glands present located at about two thirds of the body, two anterior pairs with fine granules and a posterior pair with rather coarse, dark granules. Genital primordial C-shaped; excretory bladder U-shaped. Tail shorter than body; spinose at its tip.

Table 1. List of ITS2 sequences used for the phylogenetic analysis. For SUT numbers, see the material lists in the main part of the text.

Species of cercariae	Type of cercariae	Locality	GenBank accession number	Reference
Angiostrongylus cantonensis	–	–	HQ540551	C. Y. Liu (unpubl.)
Lecithodendrium spathulatum	Xiphidiocercariae	–	JF784192	Lord et al. (2012)
Lecithodendrium linstowi	Xiphidiocercariae	–	KJ934792	Kudlai et al. (2015)
Loxogenoides bicolor	Xiphidiocercariae	SUT 0515066 B	MH991970	This study
		SUT 0515067 B	MH991981	This study
		SUT 0515077 B	MH991985	This study
		SUT 0515079 C	MH991978	This study
		SUT 0515087 B	MH991983	This study
		SUT 0515090 B	MH991976	This study
		SUT 0516106 A	MH991982	This study
		SUT 0516109 B	MH991977	This study
		SUT 0516118 B	MH991984	This study
		SUT 0516121 A	MH991974	This study
		SUT 0516125 A	MH991980	This study
		SUT 0516128 B	MH991972	This study
		SUT 0516129 B	MH991979	This study
		SUT 0516130 B	MH991971	This study
		SUT 0516139 B	MH991973	This study
		SUT 0516145 B	MH991975	This study
Loxogenes liberum	Xiphidiocercariae	SUT 0516109 B	MH991986	This study
		SUT 0516143 B	MH991987	This study
Maritreminoides obstipus	Xiphidiocercariae	SUT 0516124 A	MH991988	This study
		SUT 0516138 B	MH991989	This study
Haplorchis pumilio	Parapleurophocercous cercariae	–	KP165437	Mei et al. (2015)
		–	KX815125	Le et al. (2017)
Haplorchis taichui	Parapleurophocercous cercariae	SUT 0515090 B	MH991968	This study
		SUT 0516125 A	MH991969	This study
		–	KX815126	Le et al. (2017)
Stictodora tridactyla	Parapleurophocercous cercariae	SUT 0515058 A	MH991962	This study
		SUT 0515059 B	MH991960	This study
		SUT 0515071 A	MH991958	This study
		SUT 0515072 B	MH991953	This study
		SUT 0515074 B	MH991959	This study
		SUT 0515075 B	MH991954	This study
		SUT 0515078 B	MH991963	This study
		SUT 0515086 A	MH991957	This study
		SUT 0516138 B	MH991961	This study
		SUT 0516139 B	MH991956	This study
		SUT 0516142 B	MH991955	This study
Centrocestus formosanus	Pleurophocercous cercariae	SUT 0516102 B	MH991964	This study
		SUT 0516109 B	MH991966	This study
		SUT 0516125 A	MH991967	This study
		SUT 0516142 B	MH991965	This study
Centrocestus sp.	Pleurophocercous cercariae	–	JQ390547	M. Karamian, S. M. Sadjjadi and B. Farhangmehr (unpubl.)
Centrocestus sp.	Pleurophocercous cercariae	–	AY245699	Dzikowski et al. (2004)
Opisthorchis viverrini	Pleurophocercous cercariae	–	AY584735	Parvathi et al. 2008
Opisthorchis felineus	Pleurophocercous cercariae	–	EF688141	Katokhin et al. (2008)
Philophthalmus gralli	Megarulous cercariae	SUT 0515058 A	MH991965	This study
		–	KF986200	Heneberg et al. (2014)
Echinostome cercariae	Echinostome cercariae	SUT 0515086 A	MH991991	This study
Gymnocephalous cercariae	Gymnocephalous cercariae	SUT 0515059 B	MH991990	This study
Fasciola hepatica	Gymnocephalous cercariae	–	AM900370	Ali et al. (2008)
Fasciola gigantica	Gymnocephalous cercariae	–	AJ853848	M. D. Bargues and S. Mas-Coma (unpubl.)
		–	AM850108	Ali et al. (2008)

The cercariae develop within sporocysts.

The infection rate was 3.84% (579/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	53–88 μm (mean: 72 μm) × 105–138 μm (mean: 117 μm)
Stylet	5–8 μm (mean: 6 μm) × 20–40 μm (mean: 30 μm)
Oral sucker	23–40 μm (mean: 33 μm) × 23–33 μm (mean: 29 μm)
Pharynx	8–12 μm (mean: 10 μm) × 5–8 μm (mean: 8 μm)
Ventral sucker	13–25 μm (mean: 18 μm) × 8–20 μm (mean: 16 μm)
Excretory bladder	18–55μm (mean: 33 μm) × 10–35 μm (mean: 20 μm)
Tail	10–28 μm (mean: 21 μm) × 25–88 μm (mean: 44 μm)

1.2 *Loxogenes liberum* Seno, 1907
(Fig. 4)

Body oval. Oral sucker at the anterior end of body, with stylet. Virgulate organ present. Ventral sucker roundish, smaller than oral sucker. Pharynx very small, a prepharynx, an esophagus and ceca were not observed. Four pairs of penetration glands present, located near the middle of the body; the two anterior pairs with fine granules and the two posterior pairs with coarse granules. Excretory bladder V-shaped. Tail shorter than body, rather slender and spinose at its tip.

The cercariae develop within sporocysts.

The infection rate was 0.15% (23/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	65–93 μm (mean: 81 μm) × 95–120 μm (mean: 108 μm)

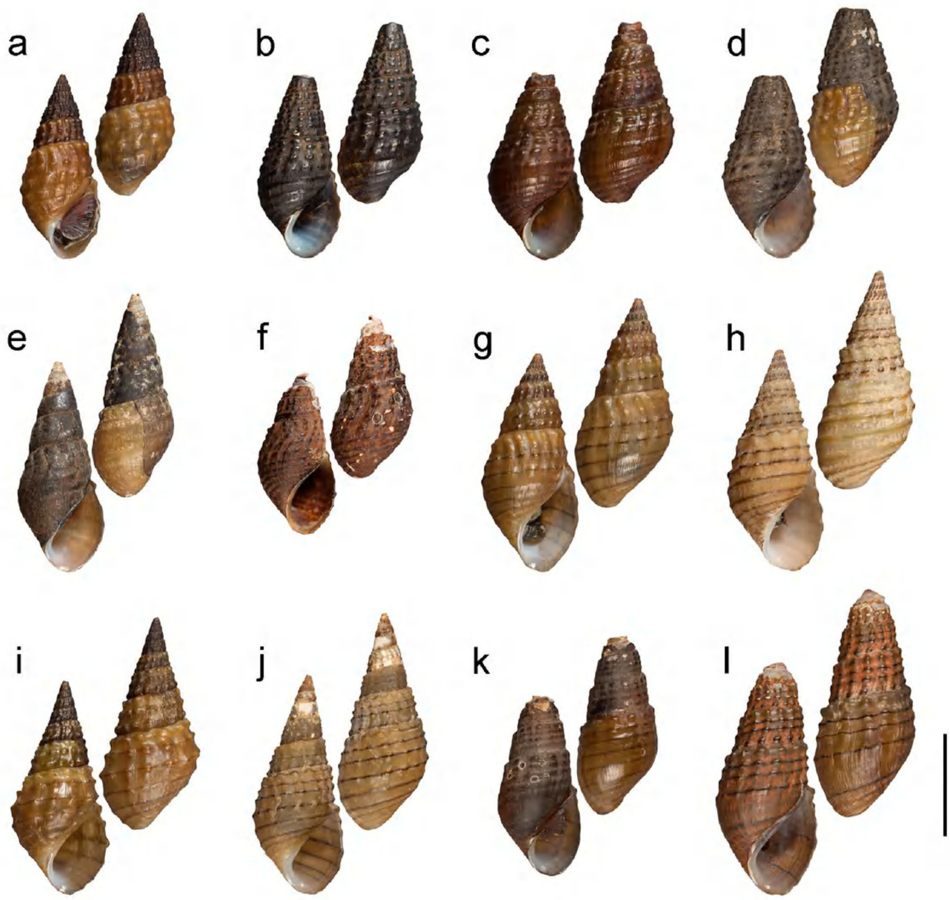

Figure 1. Shells of *Tarebia granifera* (Lamarck, 1816) from representative populations in Thailand. **a**. Ban Thung Hang stream, Lampang Province (SUT 0514044); **b**. Huai Sa Dao Pong, Phetchabun Province (SUT 0516123); **c**. Kaeng Bang Ra Chan, Phetchabun Province (SUT 0515088); **d**. Pla Ba waterfall, Loei Province (SUT 0515068); **e**. Ban Nong Phai, Kanchanaburi Province (SUT 0515059); **f**. Khlong Sathing Mo, Songkhla Province (SUT 0516144); **g**. Huay Nam Kong, Mae Hong Son Province (SUT 0515081); **h**. Huay MaeYuak, Lampang Province (SUT 0514046); **i**. Sam Sip Khot waterfall, Phetchabun Province (SUT 0516129); **j**. Sai Yok Yai waterfall, Kanchanaburi Province (SUT 0515092); **k**. Khlong Palian, Trang Province (SUT 0515095); **l**. Khlong Cham Rai reservoir, Songkhla Province (SUT 0516143). For more details on locality data, see Table 2. Scale bar: 10 mm.

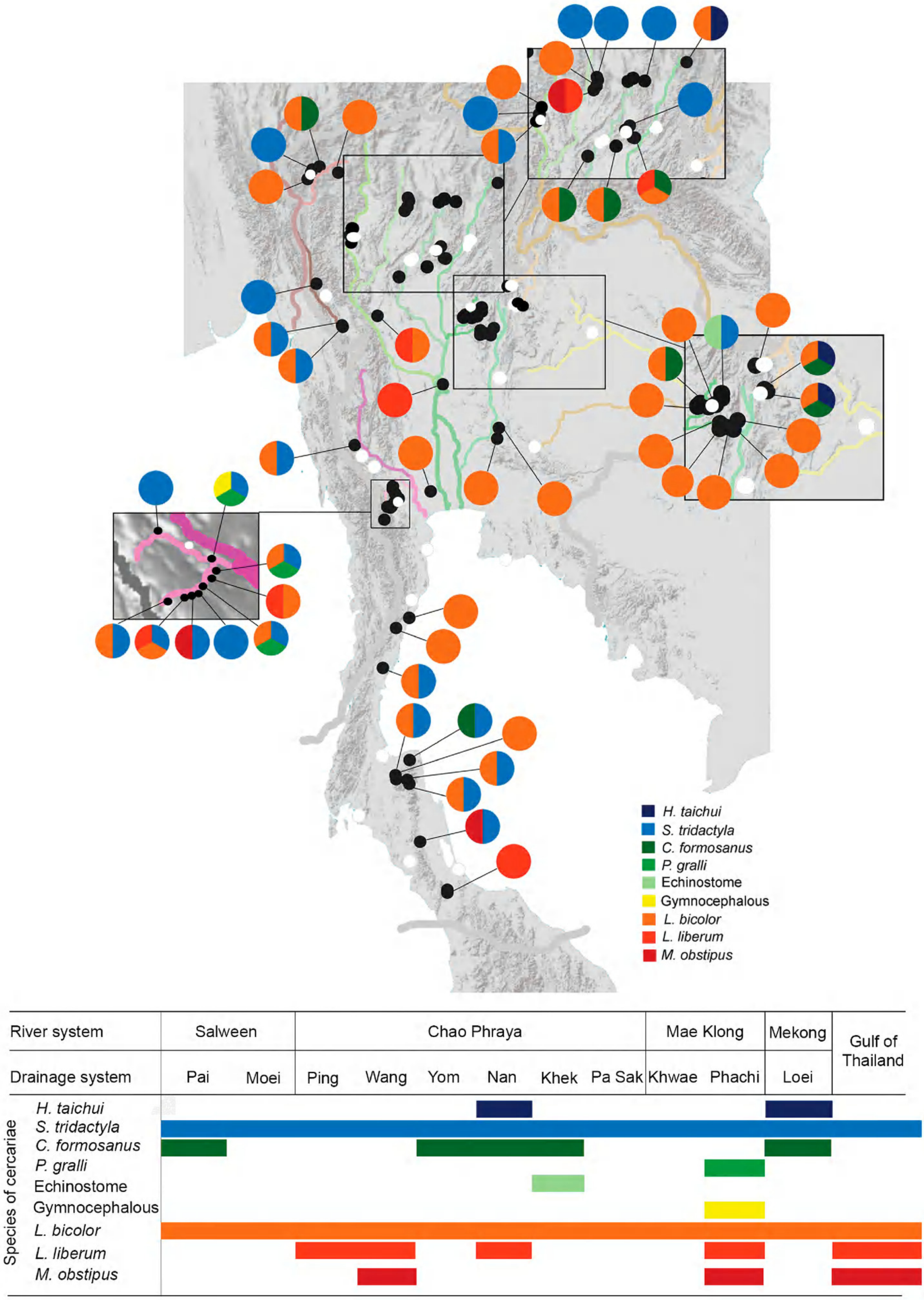

Figure 2. Distribution of *Tarebia granifera* and trematodes in different river systems in Thailand. **a**. Distribution map. **b**. Comparative table of the occurrence of trematode cercariae in different river systems in Thailand. Black dots with attached pie charts in the map represent sampling sites where trematode infected specimens of *T. granifera* were found; white dots represent sampling sites where no infections were observed. Colors in the pie charts and the comparative table refer to trematode species/types (see legend inset).

Table 2. Localities, number of collected snails, number of infected snails and trematodes obtained from collected snails; sampling periods: 2004–2009 and 2014–2016.

NO.	VOUCHER NUMBER	LOCATION	GPS	2004–2009			2014–2016		
				No. of collected snails	No. of infected snails	Infection rates (%)	No. of collected snails	No. of infected snails	Infection rates (%)
THE NORTH									
N1	SUT 0515083	Huai Pa Hung (Pai drainage, Salween river system), Pang Mapha District, Mae Hong Son Province	19°22‘19.6”N, 098°26‘35.9”E, Altitude 437 m	*	*	*	179	1: *L. bicolor* (1)	0.56
N2	SUT 0515081	Huay Nam Kong (Salween river system), Muang District, Mae Hong Son Province	19°28‘33.6”N, 098°07‘02.4”E, Altitude 425 m	*	*	*	24	0	0
N3	SUT 0515077	Tham Pla (Pai drainage, Salween river system), Muang District, Mae Hong Son Province	19°25‘31.1”N, 097°59‘27.2”E, Altitude 300 m	185	144: *L. bicolor* (34), *A. hitaense* (25), *H. pumilio* (68), *C. formosanus* (7), *C. alseae* (5), *T. laruei* (5)	77.84	179	8: *L. bicolor* (3), *H. pumilio* (5)	4.47
N4	SUT 0515078	Pai river (Pai drainage, Salween river system), Muang District, Mae Hong Son Province	19°21’54.8”N, 097°58’10.7”E, Altitude 217 m	*	*	*	64	1: *S. tridactyla* (1)	1.56
N5	SUT 0515079	Huay Sua Tao (Pai drainage, Salween river system), Muang District, Mae Hong Son Province	19°15’31.6”N, 097°54’44.6”E, Altitude 237 m	574	98: *L. bicolor* (52), *A. hitaense* (38), *H. pumilio* (5), *T. laruei* (3)	17.07	153	2: *L. bicolor* (2),	1.31
N6	SUT 0514052	Ban Mai Saraphi (Ping drainage, Chao Phraya river system), Chom Thong District, Chiang Mai Province	18°16’26.1”N, 098°38’54.0”E, Altitude 277 m	*	*	*	162	11: *L. bicolor* (6), *S. tridactyla* (5)	6.79
N7	SUT 0514051	Ban Mae Suai Luang (Ping drainage, Chao Phraya river system), Chom Thong District, Chiang Mai Province	18°17’04.4”N, 098°39’15.0”E, Altitude 268 m	*	*	*	23	2: *S. tridactyla* (2)	8.70
N8	SUT 0514054	Mae Soy bridge (Ping drainage, Chao Phraya river system), Chom Thong District, Chiang Mai Province	18°17’23.0”N, 098°39’3.6”E, Altitude 271 m	*	*	*	70	5: *L. bicolor* (5)	7.14
N9	SUT 0514050	Ban Huay Phang (Ping drainage, Chao Phraya river system), Chom Thong District, Chiang Mai Province,	18°17’08.5”N, 098°39’16.9”E, Altitude 263 m	*	*	*	103	0	0
N10	SUT 0516119	Thansawan waterfall (Yom drainage, Chao Phraya river system), Chiang Muan District, Phayao Province	18°51’22.2”N, 100°11’09.1”E, Altitude 415 m	219	2: *A. hitaense* (1), *A. mustelae* (1)	0.91	17	1: *S. tridactyla* (1)	5.88
N11	SUT 0516117	Yom river (Yom drainage, Chao Phraya river system), Chiang Muan District, Phayao Province	18°54’39.7”N, 100°16’27.7”E, Altitude 266 m	*	*	*	30	0	0
N12	SUT 0516108	Mae Nam Saai kg 9 +457 bridge (Yom drainage, Chao Phraya river system), Muang District, Phrae Province	18°05’03.1”N, 100°13’00.1”E, Altitude 171 m	*	*	*	143	0	0
N13	SUT 0516113	Mae Marn reservoir (Yom drainage, Chao Phraya river system), Sung Men District, Phrae Province	18°00’50.6”N, 100°08’22.6”E, Altitude 205 m	*	*	*	52	0	0
N14	SUT 0514045	Wang river (Wang drainage, Chao Phraya river system), Chae Hom District, Lampang Province	18°56’00.5”N, 099°38’54.6”E, Altitude 376 m	*	*	*	49	12: *S. tridactyla* (12)	24.49
N15	SUT 0514044	Ban Thung Hang stream (Wang drainage, Chao Phraya river system), Chae Hom District, Lampang Province	18°52’47.5”N, 099°40’01.0”E, Altitude 373 m	*	*	*	165	11: *S. tridactyla* (11)	6.67
N16	SUT 0514046	Huay MaeYuak (Wang drainage, Chao Phraya river system), Chae Hom District, Lampang Province	18°46’39.8”N, 099°38’38.7”E, Altitude 352 m	*	*	*	44	1: *L. bicolor* (1)	2.27
N17	SUT 0516124	km. 40+075 bridge (Wang drainage, Chao Phraya river system), Chae Hom District, Lampang Province	18°42’14.8”N, 099°35’31.7”E, Altitude 330 m	*	*	*	59	4: *L. liberum* (3), *M. obstipus* (1)	6.78
N18	SUT 0515090	Wa river (Nan drainage, Chao, Phraya river system), Bo Kluea District, Nan Province	19°11’30.4”N, 101°12’13.2”E, Altitude 713 m	*	*	*	159	16: *L. bicolor* (6), *H. taichui* (10)	10.06
N19	SUT 0516114	Huay Si Pun reservoir (Nan drainage, Chao Phraya river system), Ban Luang District, Nan Province	18°51’45.1”N, 100°28’37.1”E, Altitude 430 m	*	*	*	108	0	0

NO.	VOUCHER NUMBER	LOCATION	GPS	2004–2009 No. of collected snails	2004–2009 No. of infected snails	2004–2009 Infection rates (%)	2014–2016 No. of collected snails	2014–2016 No. of infected snails	2014–2016 Infection rates (%)
N20	SUT 0516109	Mae pool waterfall (Nan drainage, Chao Phraya river system), Laplae District, Uttaradit Province	17°43'42.3"N, 099°58'49.6"E, Altitude 123 m	137	43: *L. bicolor* (29), *A. hitaense* (5), *H. pumilio* (6), *C. formosanus* (3)	31.39	91	10: *L. bicolor* (4), *L. liberum* (4), *C. formosanus* (2)	10.99
N21	SUT 0516112	Kaeng Sai Ngam (Nan drainage, Chao Phraya river system), Tha Pla District, Uttaradit Province	17°52'19.5"N, 100°18'02.1"E, Altitude 257 m	*	*	*	32	0	0
N22	SUT 0513019	Kaeng Wangwua (Nan drainage, Chao Phraya river system), Tha Pla District, Uttaradit Province	17°52'29.5"N, 100°18'25.6"E, Altitude 231 m	*	*	*	292	4: *S. tridactyla* (4)	1.37
N23	SUT 0513023	Huai Nam Re Noi (Nan drainage, Chao Phraya river system), Tha Pla District, Uttaradit Province	17°52'51.3"N, 100°16'14.9"E, Altitude 269 m	*	*	*	155	0	0
N24	SUT 0516103	Tat Duen waterfall (Yom drainage, Chao Phraya river system), Si Satchanalai District, Sukhothai Province	17°33'16.2"N, 099°29'48.2"E, Altitude 135 m	300	141: *L. bicolor* (71), *A. hitaense* (36), *H. pumilio* (8), *C. formosanus* (19), *A. mustelae* (7)	47	137	0	0
N25	SUT 0516102	Si Satchanalai national park (Yom drainage, Chao Phraya river system), Si Satchanalai District, Sukhothai Province	17°33'07.7"N, 099°29'28.8"E, Altitude 147 m	749	262: *L. bicolor* (85), *A. hitaense* (35), *H. pumilio* (11), *C. formosanus* (116), *A. mustelae* (15)	34.98	147	1: *C. formosanus* (1)	0.68
N26	SUT 0515075	Cheek point near moei river (Moei drainage, Salween river system), Tha Song Yang District, Tak Province	17°13'23.4"N, 098°13'34.2"E, Altitude 130 m	*	*	*	55	9: *S. tridactyla* (9)	16.36
N27	SUT 0515076	Mae Salit Luang harbour (Moei drainage, Salween river system), Tha Song Yang District, Tak Province	17°26'04.8"N, 098°03'33.3"E, Altitude 109 m	*	*	*	25	0	0
N28	SUT 0515073	Ban Wang Takhian (Moei drainage, Salween river system), Mae Sot District, Tak Province	16°42'38.5"N, 098°30'22.2"E, Altitude 196 m	*	*	*	17	0	0
N29	SUT 0515072	Thong Dee harbour (Moei drainage, Salween river system), Mae Sot District, Tak Province	16°41'39.3"N, 098°31'04.4"E, Altitude 206 m	*	*	*	304	21: *L. bicolor* (3), *S. tridactyla* (18)	6.91
N30	SUT 0515074	Ban Huay Muang (Moei drainage, Salween river system), Mae Sot District, Tak Province	16°40'58.4"N, 098°31'06.9"E, Altitude 199 m	*	*	*	300	21: *L. bicolor* (1), *S. tridactyla* (20)	7.00
N31	SUT 0516126	Ban Pak Huay Mae Tho (Ping drainage, Chao Phraya river system), Muang District, Tak Province	16°52'29.3"N, 099°07'13.6"E, Altitude 106 m	*	*	*	150	3: *L. bicolor* (1), *L. liberum* (2)	2.00
N32	SUT 0516121	Kaeng Wang Nam Yen (Khek drainage, Chao Phraya river system), Khao Kho District, Phetchabun Province	16°37'23.8"N, 100°54'0.5"E Altitude 710 m	*	*	*	9	8: *L. bicolor* (8)	88.89
N33	SUT 0516120	Rajapruek resort (Khek drainage, Chao Phraya river system), Khao Kho District, Phetchabun Province	16°36'01.3"N, 100°54'29.9"E, Altitude 707 m	*	*	*	52	28: *L. bicolor* (28)	53.85
N34	SUT 0516123	Huai Sa Dao Pong (Khek drainage, Chao Phraya river system), Khao Kho District, Phetchabun Province	16°34'24.1"N, 100°59'23.6"E, Altitude 322 m	*	*	*	31	0	0
N35	SUT 0515088	Kaeng Bang Ra Chan (Khek drainage, Chao Phraya river system), Khao Kho District, Phetchabun Province	16°32'51.7"N, 100°54'03.2"E, Altitude 599 m	*	*	*	71	6: *L. bicolor* (6)	8.45
N36	SUT 0516129	Sam Sip Khot waterfall (Pa Sak drainage, Chao Phraya river system), Khao Kho District, Phetchabun Province	16°32'25.6"N, 101°04'58.4"E, Altitude 386 m	*	*	*	47	18: *L. bicolor* (18)	38.30
N37	SUT 0514041	Ban Wang Ta Pak Moo 13 (Pa Sak drainage, Chao Phraya river system), Wichian Buri District, Phetchabun Province	15°47'54.2"N, 101°14'8.1"E, Altitude 120 m	*	*	*	312	0	0
N38	SUT 0514042	Huai Leng (Pa Sak drainage, Chao Phraya river system), Wichian Buri District, Phetchabun Province	15°47'52.2"N, 101°13'54.4"E, Altitude 117 m	*	*	*	84	0	0

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				No. of collected snails	No. of infected snails	Infection rates (%)	No. of collected snails	No. of infected snails	Infection rates (%)
N39	SUT 0514040	Ban Wang Tian (Pa Sak drainage, Chao Phraya river system), Wichian Buri District, Phetchabun Province	15°47'29.7"N, 101°13'30.7"E, Altitude 121 m	*	*	*	212	0	0
N40	SUT 0514043	Huay Range reservoir, Ban Wang Ta Pak (Pa Sak drainage, Chao Phraya river system), Wichian Buri District, Phetchabun Province	15°47'19.3"N, 101°15'07.4"E, Altitude 138 m	*	*	*	128	0	0
N41	SUT 0516130	Than Thip waterfall (Pa Sak drainage, Chao Phraya river system), Lom Sak District, Phetchabun Province	16°39'46.3"N, 101°08'09.8"E, Altitude 374 m	*	*	*	41	16: *L. bicolor* (16)	39.02
N42	SUT 0515087	Ban Kaeng Lat (Khek drainage, Chao Phraya river system), Nakhon Thai District, Phitsanulok Province	16°57'21.3"N, 100°55'31.0"E, Altitude 324 m	*	*	*	14	5: *L. bicolor* (5)	35.71
N43	SUT 0516118	Kaeng Sopha (Khek drainage, Chao Phraya river system), Wang Thong District, Phitsanulok Province	16°52'13.1"N, 100°50'17.4"E, Altitude 413 m	282	72: *L. bicolor* (33), *A. hitaense* (24), *C. formosanus* (15)	25.53	30	2: *L. bicolor* (2)	6.67
N44	SUT 0515067	Poi waterfall (Khek drainage, Chao Phraya river system), Wang Thong District, Phitsanulok Province	16°50'36.3"N, 100°45'16.1"E, Altitude 208 m	*	*	*	83	9: *L. bicolor* (6), *M. caridinae* (1), *H. pumilio* (2)	10.84
N45	SUT 0516105	Phunamkej Resort (Khek drainage, Chao Phraya river system), Wang Thong District, Phitsanulok Province	16°51'02.2"N, 100°36'41.1"E, Altitude 208 m	*	*	*	73	0	0
N46	SUT 0516111	Kaeng Nangkoi (Khek drainage, Chao Phraya river system), Wang Thong District, Phitsanulok Province	16°53'09.0"N, 100°38'47.8"E, Altitude 180 m	*	*	*	15	0	0
N47	SUT 0516106	Kaeng Hom (Khek drainage, Chao Phraya river system), Nakhon Thai District, Phitsanulok Province	16°52'20.8"N, 100°50'46.8"E, Altitude 185 m	*	*	*	95	0	0
N48	SUT 0515086	Huai Nam Sai (Khek drainage, Chao Phraya river system), Nakhon Thai District, Phitsanulok Province	17°01'07.6"N, 100°55'36.0"E, Altitude 217 m	*	*	*	93	38: *S. tridactyla* (28), Echinostome (10)	40.86
THE NORTHEAST									
NE1	SUT 0516128	Tat Kok Tup waterfall (Loei drainage, Mekong river system), Phu Luang District, Loei Province	17°03'03.9"N, 101°31'38.7"E, Altitude 688 m	*	*	*	45	12: *L. bicolor* (10), *H. taichui* (1), *C. formosanus* (1)	26.67
NE2	SUT 0515068	Pla Ba waterfall (Mekong river system), Phu Ruea District, Loei Province	17°23'24.7"N, 101°22'27.3"E, Altitude 664 m	53	1: *A. hitaense* (1)	1.89	178	3: *L. bicolor* (3)	1.69
NE3	SUT 0516125	km. 50+350 Loei river (Loei drainage, Mekong river system), Phu Luang District, Loei Province	17°04'38.0"N, 101°29'20.6"E, Altitude 675 m	*	*	*	55	13: *L. bicolor* (9), *H. taichui* (3), *C. formosanus* (1)	23.64
NE4	SUT 0515064	Bueng Thung Sang (Chi drainage, Mekong river system), Muang District, Khon Kaen Province	16°34'45.6"N, 102°50'22.5"E, Altitude 160 m	*	*	*	20	0	0
NE5	SUT 0516131	Lamphraphloeng reservoir (Mun drainage, Mekong river system), Pak Thong Chai District, Nakhon Ratchasima Province	14°35'32.3"N, 101°50'30.1"E, Altitude 259 m	*	*	*	36	0	0
THE EAST									
E1	SUT 0516135	Mae Rumphueng Beach (Mae Rumphueng canal, Gulf of Thailand), Muang Rayong District, Rayong Province	12°37'50.0"N, 101°20'35"E, Altitude 8 m	*	*	*	150	0	0
THE CENTRAL									
C1	SUT 0516127	Bung Boraphet (Chao Phraya river system), Muang District, Nakhon Sawan Province	15°40'59.6"N, 100°14'59.3"E Altitude 32 m	*	*	*	42	1: *L. liberum* (1)	2.38
C2	SUT 0516133	Dong Phaya Yen waterfall (Pa Sak drainage, Chao Phraya river system), Muak Lek District, Sara Buri Province	14°44'06.4"N, 101°11'31.4"E, Altitude 156 m	371	1: *L. bicolor* (1)	0.27	27	1: *L. bicolor* (1)	3.70
C3	SUT 0516132	Suanmaduea waterfall (Pa Sak drainage, Chao Phraya river system), Phatthana Nikhom District, Lop Buri Province	14°55'12.3"N, 101°13'10.9"E, Altitude 136 m	358	5: *L. bicolor* (5)	1.40	48	0	0
C4	SUT 0515055	Pond of Silpakorn University (Tha Chin river system), Muang District, Nakhon Pathom Province	13°49'01.2"N, 100°02'27.9"E, Altitude 79 m	381	2: *L. bicolor* (2)	0.52	30	0	0

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				No. of collected snails	No. of infected snails	Infection rates (%)	No. of collected snails	No. of infected snails	Infection rates (%)
C5	SUT 0515091	Hin dad hot spring (Khwae Noi drainage, Mae Klong river system), Thong Pha Phum District, Kanchanaburi Province	14°37’25.9”N, 098°43’40.5”E, Altitude 159 m	39	5: *L. bicolor* (1), *H. pumilio* (3), *S. tridactyla* (1)	12.82	2	0	0
C6	SUT 0515092	Sai Yok Yai waterfall (Khwae drainage, Mae Klong river system), Sai Yok District, Kanchanaburi Province	14°26’03.0”N, 098°51’14.7”E, Altitude 104 m	*	*	*	49	0	0
C7	SUT 0515093	Sai Yok Noi waterfall (Khwae drainage, Mae Klong river system), Sai Yok District, Kanchanaburi Province	14°14’27.6”N, 099°03’55.9”E, Altitude 116 m	*	*	*	29	0	0
C8	SUT 0515061	Ban Thung Makham Tia (Phachi drainage, Mae Klong river system), Dan Makham Tia District, Kanchanaburi Province	13°54’18.1”N, 099°23’07.8”E, Altitude 45 m	*	*	*	42	1: *S. tridactyla* (1)	2.38
C9	SUT 0515060	Ban Ta Pu (Phachi drainage, Mae Klong river system), Dan Makham Tia District, Kanchanaburi Province	13°51’17.7”N, 099°22’58.9”E, Altitude 56 m	*	*	*	99	0	0
C10	SUT 0515059	Ban Nong Phai (Phachi drainage, Mae Klong river system), Dan Makham Tia District, Kanchanaburi Province	13°46’44.8”N, 099°25’26.7”E, Altitude 72 m	*	*	*	118	5: *S. tridactyla* (3), *P. gralli* (1), Gymnocephalous (1)	4.24
THE SOUTH									
S1	SUT 0515066	Ban Purakom (Phachi drainage, Mae Klong river system), Suan Phueng District, Ratchaburi Province	13°19’29.2”N, 099°14’22.0”E, Altitude 277 m	*	*	*	280	30: *L. bicolor* (29), *S. tridactyla* (1)	10.71
S2	SUT 0515069	Huay Nueng (Phachi drainage, Mae Klong river system), Suan Phueng District, Ratchaburi Province	13°32’52.2”N, 099°17’33.7”E, Altitude 156 m	832	94: *L. bicolor* (30), *S. tridactyla* (64)	11.30	272	23: *L. liberum* (2), *S. tridactyla* (21)	8.46
S3	SUT 0515070	Lum Nam Phachi (Phachi drainage, Mae Klong river system), Suan Phueng District, Ratchaburi Province	13°32’54.2”N, 099°21’42.3”E, Altitude 110 m	*	*	*	242	5: *S. tridactyla* (5)	2.07
S4	SUT 0515057	Ban Dan Thap Tako (Phachi drainage, Mae Klong river system), Chom Bueng District, Ratchaburi Province	13°41’28.1”N, 099°29’08.1”E, Altitude 82 m	*	*	*	240	11: *L. bicolor* (3), *L. liberum* (8)	4.58
S5	SUT 0515058	Phachi river Bridge (Phachi drainage, Mae Klong river system), Chom Bueng District, Ratchaburi Province	13°45’00.5”N, 099°26’27.4”E, Altitude 65 m	*	*	*	292	16: *L. bicolor* (1), *M. caridinae* (10), *S. tridactyla* (4), *P. gralli* (1)	5.48
S6	SUT 0515056	Ban Pa Wai (Phachi drainage, Mae Klong river system), Chom Bueng District, Ratchaburi Province	13°37’0.15”N, 099°24’36.9”E, Altitude 74 m	*	*	*	111	11: *L. bicolor* (3), *M. caridinae* (4), *S. tridactyla* (3), *P. gralli* (1)	9.91
S7	SUT 0515071	Huai Ban Bor (Phachi drainage, Mae Klong river system), Suan Phueng District, Ratchaburi Province	13°32’07.4”N, 099°20’31.8”E, Altitude 137 m	*	*	*	196	21: *M. obstipus* (1), *S. tridactyla* (20)	10.71
S8	SUT 0513032	Khlong Cha-am (Cha-am canal, Gulf of Thailand), Cha-am District, Phetchaburi Province	12°48’02.7”N, 099°58’53.2”E, Altitude 22 m	*	*	*	72	0	0
S9	SUT 0516146	Khlong Bueng reservoir (Bueng canal, Gulf of Thailand), Muang District, Prachuap Khiri Khan Province	11°55’29.1”N, 099°42’40.9”E, Altitude 72 m	*	*	*	92	0	0
S10	SUT 0514037	Khlong Huai Yang (Yang canal), Thap Sakae District, Prachuap Khiri Khan Province	11°36’50.0”N, 099°40’07.9”E, Altitude 53 m	961	1: *L. bicolor* (1)	0.10	22	0	0
S11	SUT 0514038	Kar on waterfall (Nongyaplong canal), Bang Saphan District, Prachuap Khiri Khan Province	11°26’14.4”N, 099°26’33.0”E, Altitude 53 m	685	5: *L. bicolor* (5)	0.73	39	0	0
S12	SUT 0516149	Krapo waterfall (Tha Sae canal), Tha Sae District, Chumphon Province	10°44’28.8”N, 099°12’54.9”E, Altitude 74 m	223	181: *L. bicolor* (32), *S. tridactyla* (149)	81.17	30	0	0
S13	SUT 0516137	Khlong Klai (Nong Noi canal, Ta Pi river system), Ban Na San District, Surat Thani Province	08°48’06.9”N, 099°26’45.1”E, Altitude 108 m	*	*	*	104	4: *L. bicolor* (4)	3.85
S14	SUT 0514048	Dat Fa waterfall (Lumpool canal, Ta Pi river system), Ban Na San District, Surat Thani Province	08°52’18.8”N, 099°25’59.1”E, Altitude 79 m	*	*	*	144	2: *L. bicolor* (1), *S. tridactyla* (1)	1.39

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S15	SUT 0516142	Vibhavadi waterfall (Tha Thong canal), Don Sak District, Surat Thani Province	09°08'07.2"N, 099°40'31.6"E, Altitude 26 m	*	*	*	107	24: *S. tridactyla* (17), *C. formosanus* (7)	22.43
S16	SUT 0516147	Khlong Tha Sai (Takhoei canal, Gulf of Thailand), Tha Chang District, Surat Thani Province	09°12'39.8"N, 099°11'55.7"E, Altitude 8 m	*	*	*	20	0	0
S17	SUT 0516148	Ban Tung Ao (Ta Khoei canal, Gulf of Thailand), Phunphin District, Surat Thani Province	09°12'25.7"N, 099°12'25.7"E, Altitude 7 m	*	*	*	35	0	0
S18	SUT 0516145	Krung Ching waterfall (Klai canal), Nopphitam District, Nakhon Si Thammarat Province	08°43'17.3"N, 099°40'14.8"E, Altitude 195 m	157	12: *L. bicolor* (5), *A. hitaense* (2), *S. tridactyla* (5)	7.64	22	4: *L. bicolor* (4)	18.18
S19	SUT 0516139	Khlong Prong (Klai canal), Nopphitam District, Nakhon Si Thammarat Province	08°47'23.0"N, 099°38'13.2"E, Altitude 98 m	*	*	*	50	11: *L. bicolor* (1), *S. tridactyla* (10)	22.00
S20	SUT 0515097	Khlong Sai (Khlong Sai canal, Andaman sea), Muang District, Krabi Province	08°10'20.8"N, 098°47'37.6"E, Altitude 23 m	*	*	*	5	0	0
S21	SUT 0515098	Wang Than Thip (Wang Than Thip canal, Andaman sea), Muang District, Krabi Province	08°09'49.2"N, 098°47'50.9"E, Altitude 21 m	*	*	*	42	0	0
S22	SUT 0515095	Khlong Palian (Palian canal), Yan Ta Khao District, Trang Province	07°22'11.0"N, 099°40'47.9"E, Altitude 19 m	77	15: *L. bicolor* (2), *S. tridactyla* (11), *C. alseae* (2)	19.48	15	4: *S. tridactyla* (4)	26.67
S23	SUT 0516138	Khlong Tha Leung (Tha Nae canal), Si Banphot District, Phatthalung Province	07°42'48.3"N, 099°51'33.6"E, Altitude 70 m	*	*	*	36	14: *M. obstipus* (5), *S. tridactyla* (9)	38.89
S24	SUT 0516141	Khlong La reservoir (Utaphao canal, Gulf of Thailand), Khlong Hoi Khong District, Songkhla Province	06°52'29.3"N, 100°19'48.4"E, Altitude 60 m	*	*	*	35	0	0
S25	SUT 0516144	Khlong Sathing Mo (Songkhla lake, Gulf of Thailand), Singhanakhon District, Songkhla Province	07°13'36.6"N, 100°31'41.8"E, Altitude 10 m	*	*	*	3	0	0
S26	SUT 0516143	Khlong Cham Rai reservoir (Utaphao canal), Khlong Hoi Khong District, Songkhla Province	06°49'29.5"N, 100°19'49.7"E, Altitude 56 m	*	*	*	139	3: *L. liberum* (3)	2.16
TOTAL				6,583	1,084	16.47	8,493	493	5.80

N = North, NE = Northeast, E = East, C = Central, S = South
* = no record.

Stylet	3–3 µm (mean: 3 µm) × 10–23 µm (mean: 16 µm)
Oral sucker	13–30 µm (mean: 24 µm) × 10–28 µm (mean: 20 µm)
Pharynx	5–15 µm (mean: 10 µm) × 8–10 µm (mean: 8 µm)
Ventral sucker	8–33 µm (mean: 18 µm) × 13–28 µm (mean: 19 µm)
Excretory bladder	13–35 µm (mean: 27 µm) × 13–48 µm (mean: 37 µm)
Tail	15–25 µm (mean: 20 µm) × 40–90 µm (mean: 72 µm)

1.3 *Acanthatrium histaense* Koga, 1953
(Fig. 5)

Body oval. Oral sucker with stylet, virgulate organ near oral sucker. Pharynx round and short, esophagus absent. Ventral sucker smaller than oral sucker. Two pairs of penetration glands present, one anterior pair with fine granules and one posterior pair with coarse granules. Excretory bladder near posterior end of body. Tail short, spinose at its end.

The cercariae develop within sporocysts.

The infection rate was 1.11% (167/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	54–93 µm (mean: 78 µm) × 80–110 µm (mean: 100 µm)
Stylet	9–14 µm (mean: 11 µm) × 12–14 µm (mean: 12 µm)
Oral sucker	26–33 µm (mean: 31 µm) × 35–41 µm (mean: 38 µm)
Pharynx	11–16 µm (mean: 14 µm) × 13–25 µm (mean: 21 µm)

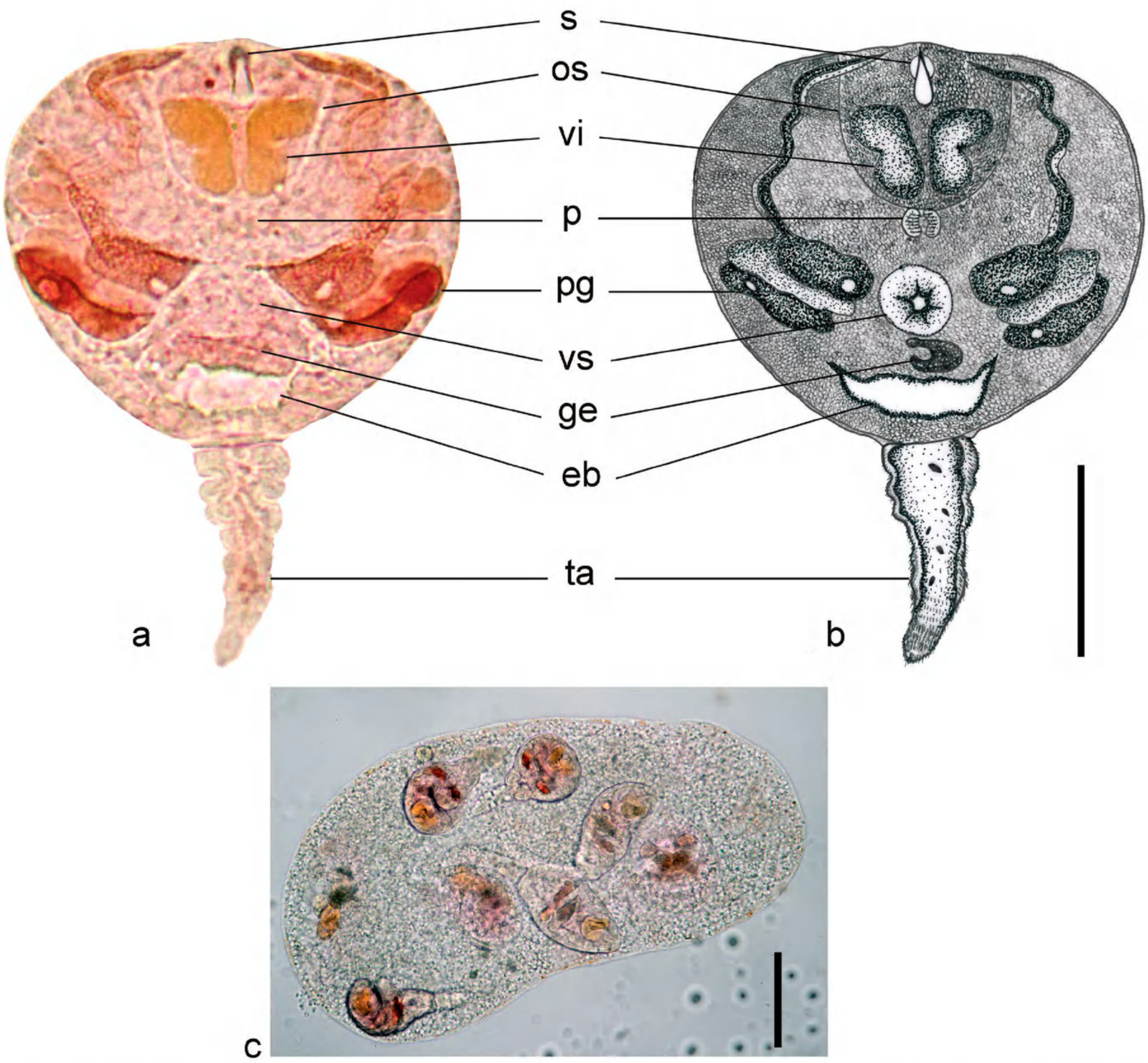

Figure 3. Images of *Loxogenoides bicolor* (Krull, 1933). **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. sporocyst stained with 0.5% neutral red. Abbreviations – eb: excretory bladder; ge: genital primordium; p: pharynx; pg: penetration gland; os: oral sucker; s: stylet; ta: tail; vi: virgulate organ; vs: ventral sucker. Scale bars: 50 μm.

Ventral sucker	15–17 μm (mean: 17 μm) × 16–19 μm (mean: 18 μm)
Excretory bladder	9–13 μm (mean: 10 μm) × 21–47 μm (mean: 39 μm)
Tail	18–26 μm (mean: 24 μm) × 27–76 μm (mean: 69 μm)

Type 2. Armatae xiphidiocercariae cercariae

Microphallidae Ward, 1901 (sensu Travassos 1921)

2.1 *Maritreminoides caridinae* (Yamaguti & Nisimura, 1944) (sensu Chen 1957)

(Fig. 6)

Body oval, rather small. Stylet present, but virgulate organ absent. Pharynx small, esophagus Y-shaped. Ventral sucker poorly developed. Two pairs penetration glands present, located near the middle of the body. Excretory bladder thin-walled, located in the posterior part of the body. Tail long and round.

The cercariae develop within sporocysts.

The infection rate was 0.10% (15/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	78–98 μm (mean: 89 μm) × 105–133 μm (mean: 113 μm)
Stylet	3–3 μm (mean: 3 μm) × 10–18 μm (mean: 15 μm)
Oral sucker	18–30 μm (mean: 25 μm) × 20–30 μm (mean: 23 μm)
Pharynx	5–10 μm (mean: 8 μm) × 5–10 μm (mean: 9 μm)
Ventral sucker	15–20 μm (mean: 19 μm) × 15–20 μm (mean: 18 μm)
Excretory bladder	30–40 μm (mean: 34 μm) × 15–18 μm (mean: 16 μm)

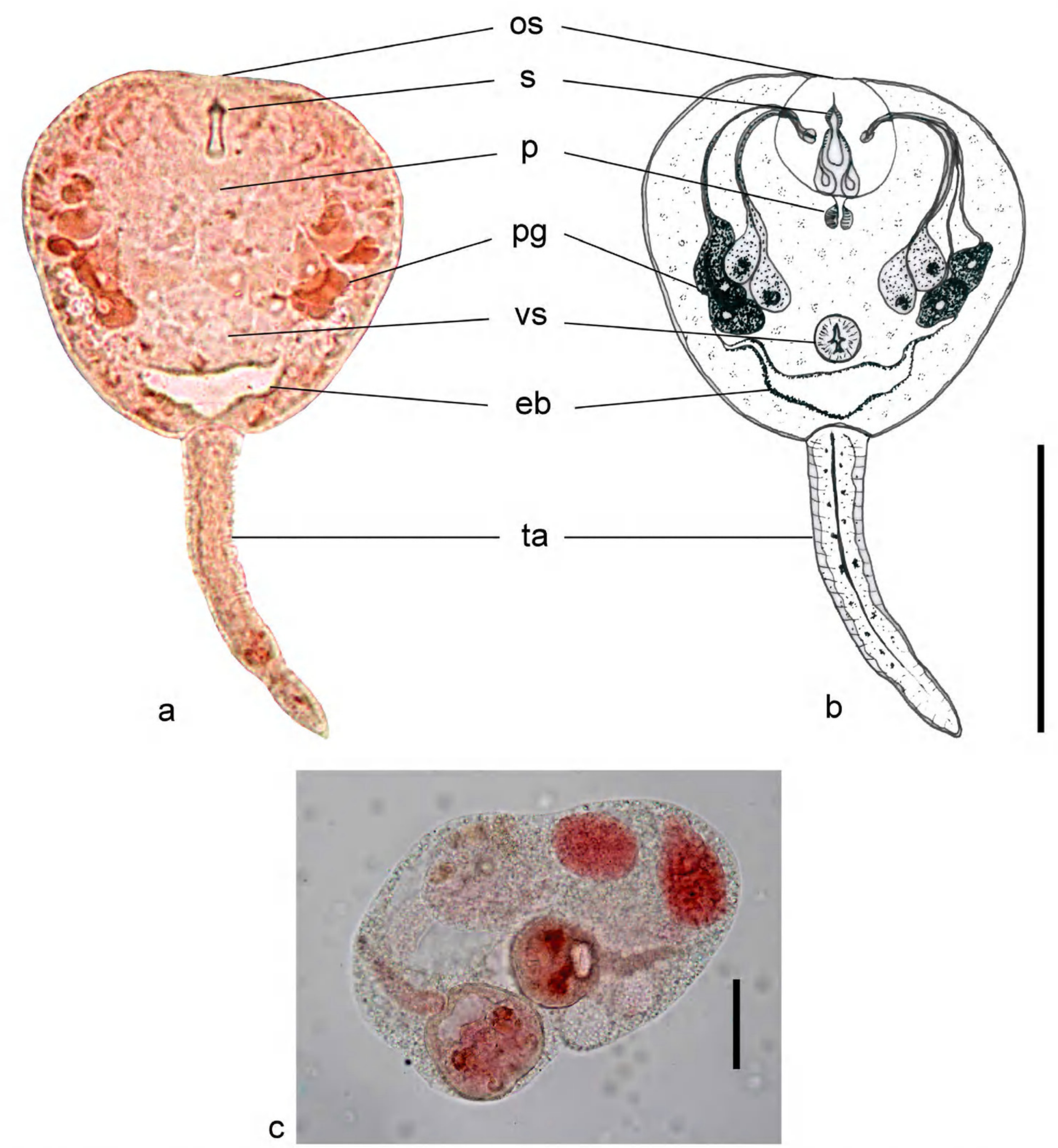

Figure 4. *Loxogenes liberum* Seno, 1907. **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. sporocyst stained with 0.5% neutral red. Abbreviations – eb: excretory bladder; os: oral sucker, p: pharynx, pg: penetration gland, s: stylet; ta: tail; vs: ventral sucker. Scale bars: 50 µm.

Tail	13–20 µm (mean: 16 µm) × 85–125 µm (mean: 106 µm)

2.2 *Maritreminoides obstipus* (Van Cleave & Mueller, 1932) (sensu Rankin 1939)
(Fig. 7)

Body oval, rather small. Oral and ventral sucker of approximately equal in size. Oral sucker with long stylet, virgulate organ absent. Pharynx rather large, esophagus short and slender, bifurcating, located between oral and ventral sucker. Genital primordium located just posterior of ventral sucker. Four pairs of penetration glands grouped together near anterior margin of ventral sucker. Excretory bladder thin-walled. Tail shorter than body and round, not spinose at its tip.

The cercariae develop within sporocysts.

The infection rate was 0.05% (7/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

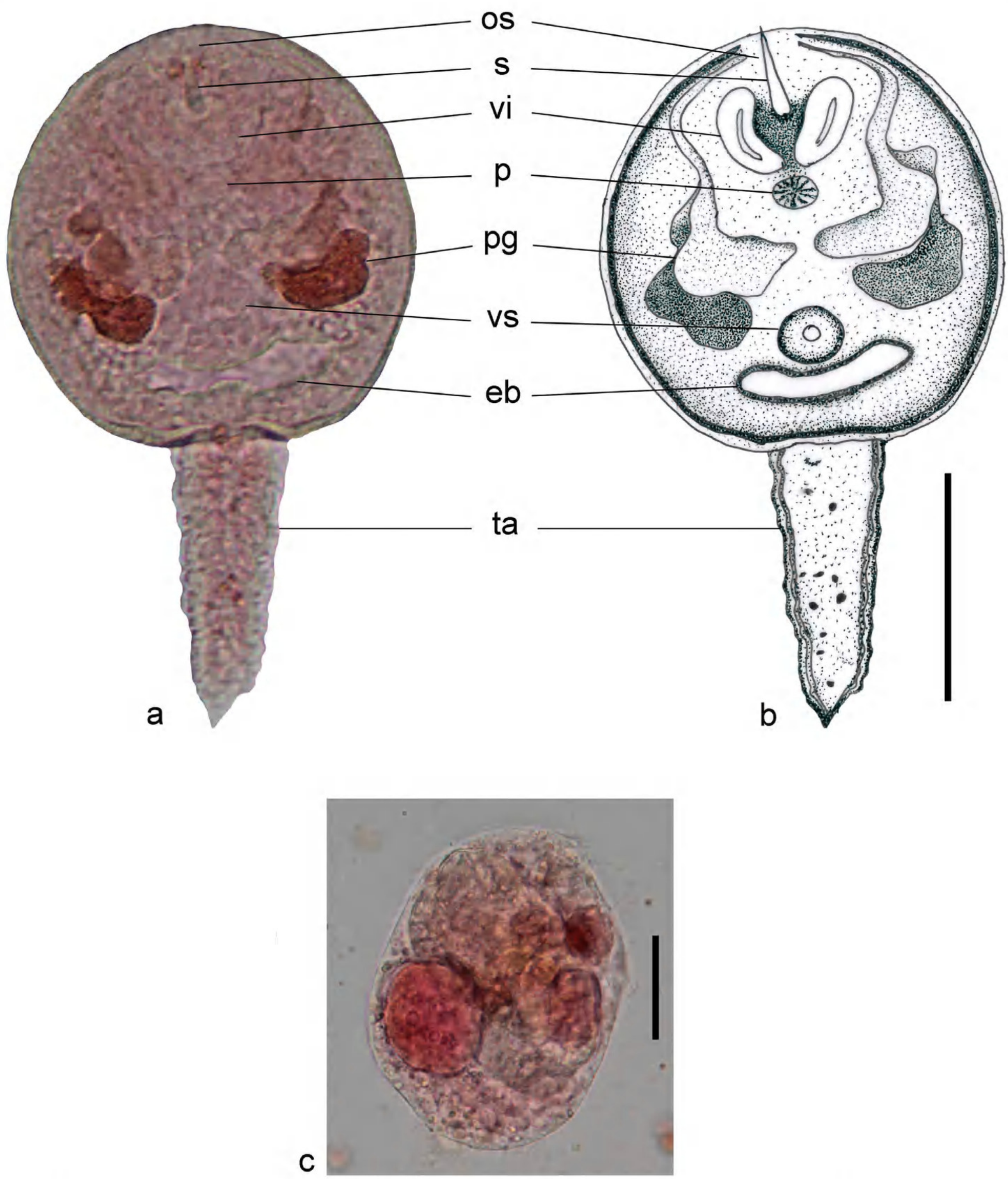

Figure 5. *Acanthatrium histaense* Koga, 1953. **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. sporocyst stained with 0.5% neutral red. Abbreviations – eb: excretory bladder; os: oral sucker; s: stylet; p: pharynx; pg: penetration gland; ta: tail; vi: virgulate organ; vs: ventral sucker. Scale bars: 50 µm.

Body	73–103 µm (mean: 89 µm) × 85–128 µm (mean: 106 µm)
Stylet	3–3 µm (mean: 3 µm) × 13–18 µm (mean: 16 µm)
Oral sucker	20–30 µm (mean: 25 µm) × 13–30 µm (mean: 24 µm)
Pharynx	8–13 µm (mean: 9 µm) × 5–13 µm (mean: 9 µm)
Ventral sucker	13–20 µm (mean: 16 µm) × 10–20 µm (mean: 15 µm)
Excretory bladder	18–35 µm (mean: 28 µm) × 13–23 µm (mean: 16 µm)
Tail	15–28 µm (mean: 20 µm) × 65–113 µm (mean: 82 µm)

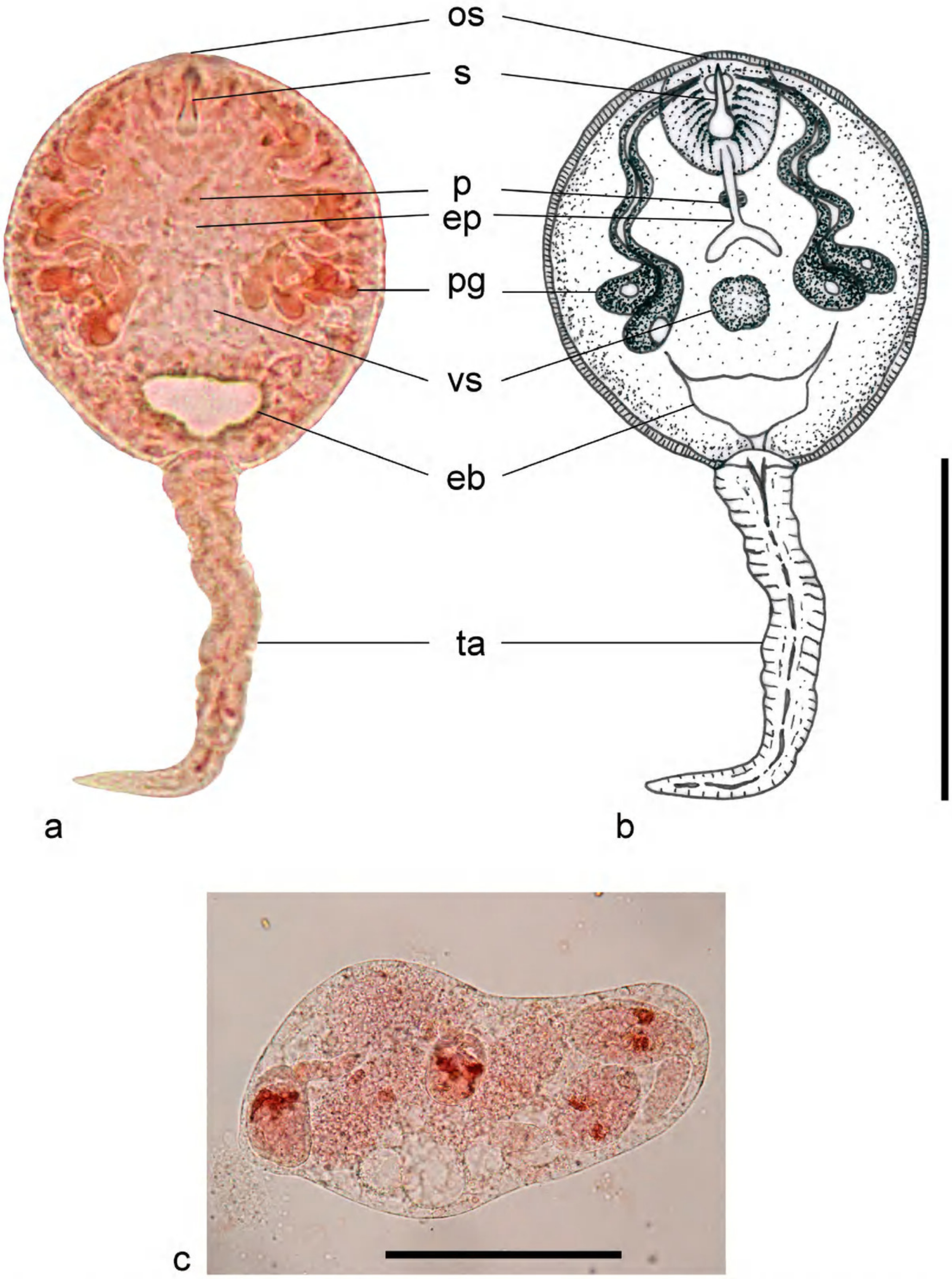

Figure 6. *Maritreminoides caridinae* (Yamaguti & Nisimura, 1944). **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. sporocyst stained with 0.5% neutral red. Abbreviations – eb: excretory bladder; ep: esophagus; os: oral sucker; p: pharynx; pg: penetration gland; s: stylet; ta: tail; vs: ventral sucker. Scale bars: 50 µm.

Type 3. Parapleurophocercous cercariae

Heterophyidae (Leiper, 1909) (sensu Odhner 1914)

3.1 *Haplorchis pumilio* (Looss, 1896) (sensu Looss 1899)

(Fig. 8)

The cercarial body is pear-shaped. It has a circular oral sucker that is located near the proximal end of the body. The mouth is equipped with transverse rows of spines. The small ventral sucker is located approximately at two-thirds of the body length measured from the front. The small pharynx is situated in the anterior part of the body just distal of the oral sucker between the two distinct eyespots; an esophagus is absent. There are seven pairs of penetration glands, which are arranged laterally in two longitudinal rows in the posterior two thirds of the body. The excretory bladder has an oval shape and is

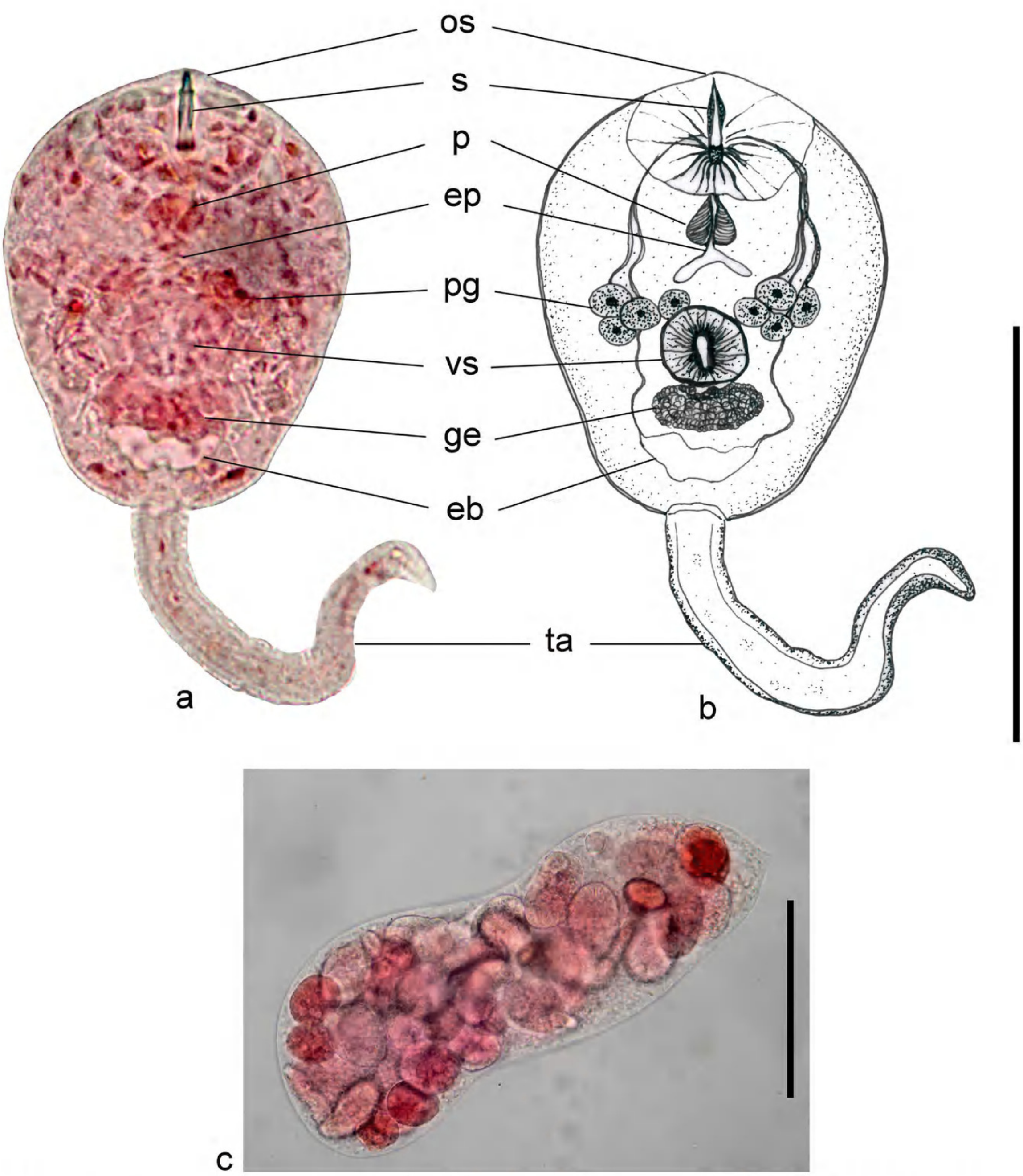

Figure 7. *Maritreminoides obstipus* (Van Cleave & Müller, 1932). **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. sporocyst stained with 0.5% neutral red. Abbreviations – eb: excretory bladder; ep: esophagus; ge: genital primordium; os: oral sucker; p: pharynx; pg: penetration gland; s: stylet; ta: tail; vs: ventral sucker. Scale bars: 50 µm.

dark pigmented. A genital primordium is present, located between the ventral sucker and the excretory bladder. The tail is longer than the body and rather slender, and is equipped with lateral finfolds proximally and a dorsoventral finfold along the longer distal portion.

The cercariae develop within rediae.

The infection rate was 0.72% (108/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	91–141 µm (mean: 125 µm) × 169–296 µm (mean: 258 µm)
Oral sucker	28–49 µm (mean: 37 µm) × 28–49 µm (mean: 36 µm)
Pharynx	9–11 µm (mean: 10 µm) × 13–20 µm (mean: 16 µm)
Ventral sucker	15–25 µm (mean: 19 µm) × 15–24 µm (mean: 18 µm)
Excretory bladder	29–41 µm (mean: 35 µm) × 29–41 µm (mean: 35 µm)
Tail	11–37 µm (mean: 31 µm) × 466–529 µm (mean: 491 µm)
Lateral finfolds	9–18 µm (mean: 14.75 µm) × 70–129 µm (mean: 111 µm)

Table 3. Distribution of trematodes obtained from *Tarebia granifera* (A total of 15,076 snails) in Thailand (N = North, NE = North-east, E = East, C = Central, S = South).

Type and species of trematodes	2004–2009 No. infected snails					2014–2016 No. infected snails					Total	Infection rate (%) (infected snail / no. of the total collected snails = 15,076)
	N	NE	E	C	S	N	NE	E	C	S		
Type 1. Virgulate xiphidiocercariae cercariae												
1. ***Loxogenoides bicolor***	304	0	0	9	75	122	22	0	1	46	579	3.84
2. ***Loxogenes liberum***	0	0	0	0	0	9	0	0	1	13	23	0.15
3. ***Acanthatrium histaense***	164	1	0	0	2	0	0	0	0	0	167	1.11
Total	**468**	**1**	**0**	**9**	**77**	**131**	**22**	**0**	**2**	**59**	**769**	**5.10**
Type 2. Armatae xiphidiocercariae cercariae												
1. ***Maritreminoides caridinae***	0	0	0	0	0	1	0	0	0	14	15	0.10
2. ***Maritreminoides obstipus***	0	0	0	0	0	1	0	0	0	6	7	0.05
Total	**0**	**0**	**0**	**0**	**0**	**2**	**0**	**0**	**0**	**20**	**22**	**0.15**
Type 3. Parapleurophocercous cercariae												
1. ***Haplorchis pumilio***	98	0	0	3	0	7	0	0	0	0	108	0.72
2. ***Haplorchis taichui***	0	0	0	0	0	10	4	0	0	0	14	0.09
3. ***Stictodora tridactyla***	0	0	0	1	229	111	0	0	4	95	440	2.92
Total	**98**	**0**	**0**	**4**	**229**	**128**	**4**	**0**	**4**	**95**	**562**	**3.73**
Type 4. Pleurophocercous cercariae												
1. ***Centrocestus formosanus***	160	0	0	0	0	3	2	0	0	7	172	1.14
Total	**160**	**0**	**0**	**0**	**0**	**3**	**2**	**0**	**0**	**7**	**172**	**1.14**
Type 5. Megarulous cercariae												
1. ***Philophthalmus gralli***	0	0	0	0	0	0	0	0	1	2	3	0.02
Total	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**2**	**3**	**0.02**
Type 6. Furcocercous cercariae												
1. ***Cardicola alseae***	5	0	0	0	2	0	0	0	0	0	7	0.05
2. ***Alaria mustelae***	23	0	0	0	0	0	0	0	0	0	23	0.15
3. ***Transversotrema laruei***	8	0	0	0	0	0	0	0	0	0	8	0.05
Total	**36**	**0**	**0**	**0**	**2**	0	0	0	0	0	**38**	**0.25**
Type 7. Echinostome cercariae												
1. Echinostome cercariae	0	0	0	0	0	10	0	0	0	0	10	0.07
Total	**0**	**0**	**0**	**0**	**0**	**10**	**0**	**0**	**0**	**0**	**10**	**0.07**
Type 8. Gymnocephalous cercariae												
1. Gymnocephalous cercariae	0	0	0	0	0	0	0	0	1	0	1	0.01
Total	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**1**	**0**	**1**	**0.01**

3.2 *Haplorchis taichui* (Nishigori, 1924) (sensu Witenberg 1930)

(Fig. 9)

Body is oval in shape. The oral sucker is located at the anterior of body. The mouth aperture is equipped with transverse rows of spines. A pair of pigmented eyespots and pharynx are present. Seven pairs of penetration glands extend from the pharynx to the posterior end of the body. Cystogenous cells are arranged in lateral fields from the level of the pharynx to the posterior end of the body. The excretory bladder is saccular and thick-walled. The tail is longer than the body. There are lateral finfolds at one-third of tail tunk and a dorso-ventral finfold at the distal portion.

The cercariae develop within rediae.

The infection rate was 0.09% (14/15,076) (Table 3)

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	43–83 μm (mean: 61 μm) × 105–140 μm (mean: 120 μm)
Oral sucker	20–30 μm (mean: 25 μm) × 23–35 μm (mean: 28 μm)
Ventral sucker	15–33 μm (mean: 23 μm) × 18–30 μm (mean: 25 μm)
Pharynx	8–20 μm (mean: 14 μm) × 8–25 μm (mean: 12 μm)
Excretory bladder	10–50 μm (mean: 26 μm) × 20–35 μm (mean: 26 μm)
Tail	20–30 μm (mean: 26 μm) × 263–355 μm (mean: 311 μm)
Lateral finfolds	8–15 μm (mean: 13 μm) × 75–125 μm (mean: 103 μm)
Dorsal finfolds	5–23 μm (mean: 13 μm) × 183–253 μm (mean: 218 μm)

3.3 *Stictodora tridactyla* Martin & Kuntz, 1955

(Fig. 10)

The body is oval in shape. The oral sucker is located at the anterior end of the body. There are three transverse rows of oral spines present. Seven pairs of penetration glands in

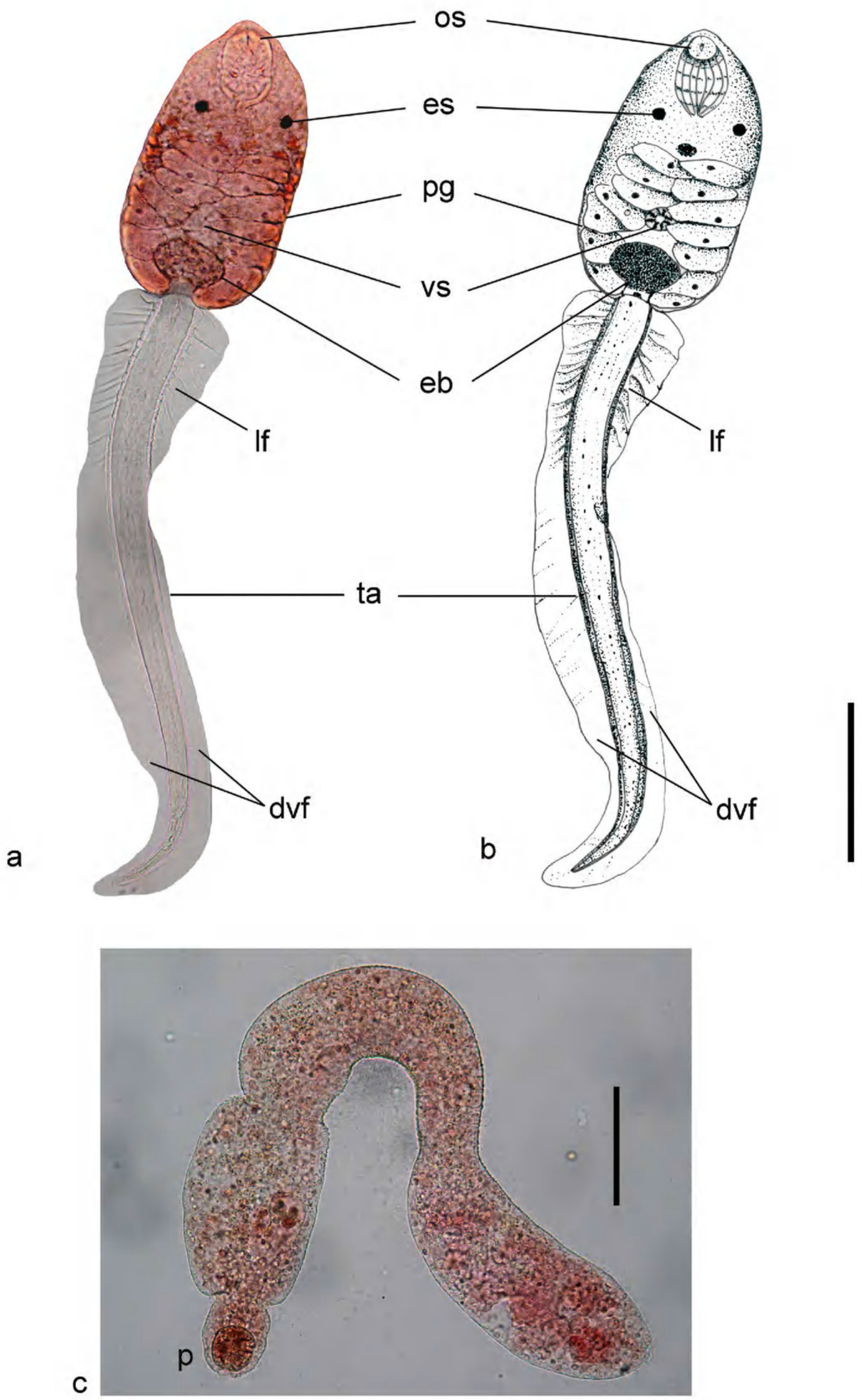

Figure 8. *Haplorchis pumilio* (Looss, 1896). **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. redia stained with 0.5% neutral red. Abbreviations – dvf: dorsoventral finfold; eb: excretory bladder; es: eyespot; lf: lateral finfold; os: oral sucker; p: pharynx; pg: penetration gland; ta – tail; vs: ventral sucker. Scale bars: 50 µm.

four groups of 3:4:4:3 are present that are situated between the pharynx and the excretory bladder. A pair of pigmented eyespots and a pharynx are present. The ventral sucker is poorly developed. The excretory bladder is V-shaped and thick-walled. The tail is longer than the body. There is a bilateral finfold and a dorso-ventral finfold on the tail.

The cercariae develop within rediae.

The infection rate was 2.92% (440/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	80–118 µm (mean: 99 µm) × 168–207 µm (mean: 202 µm)
Oral sucker	28–38 µm (mean: 34 µm) × 30–50 µm (mean: 41 µm)

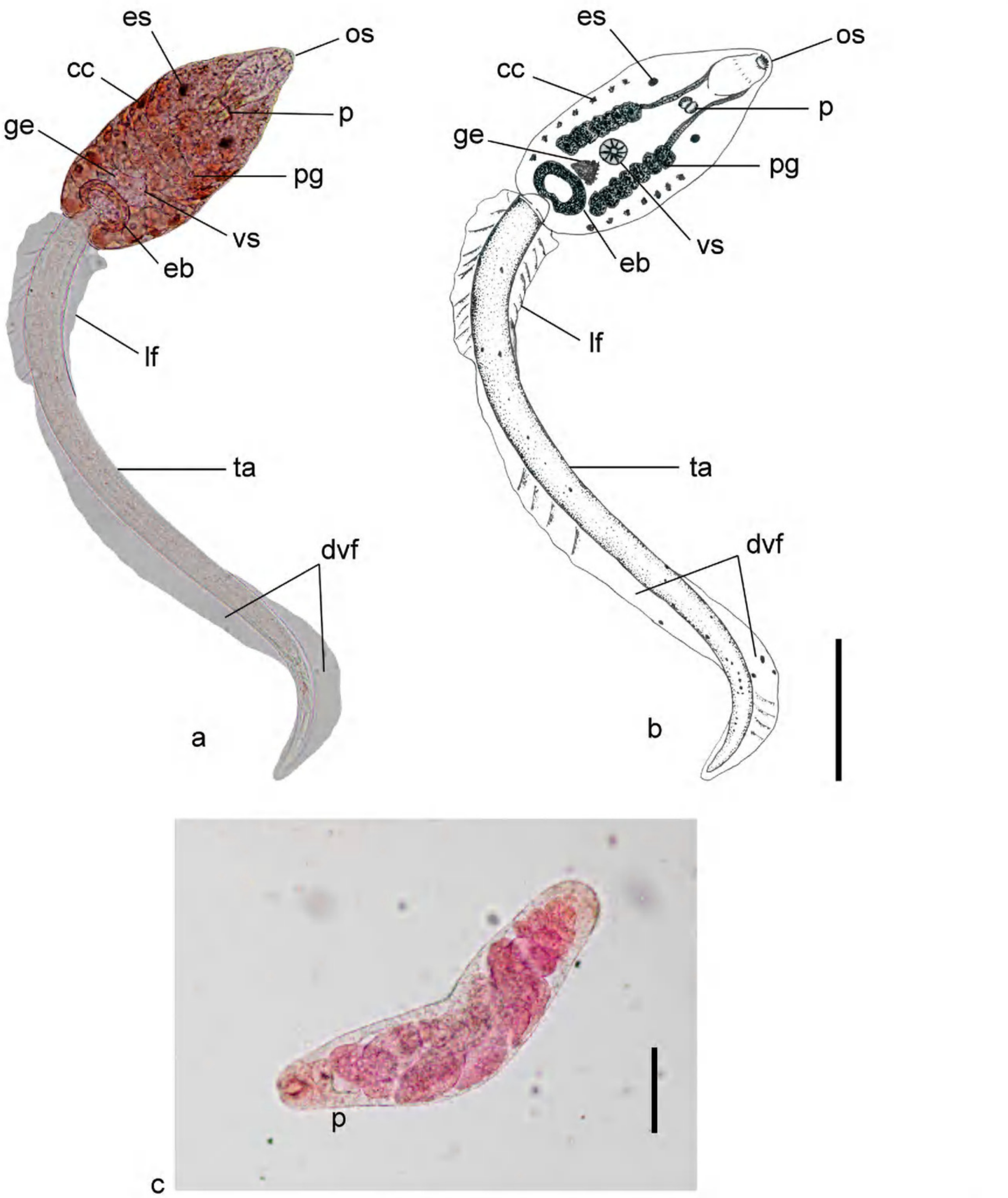

Figure 9. *Haplorchis taichui* (Nishigori, 1924). **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. redia stained with 0.5% neutral red. Abbreviations – cc: cystogenous cells; dvf: dorsoventral finfold; eb: excretory bladder; es: eyespot; ge: genital primordium; lf: lateral finfold; os: oral sucker; p: pharynx; pg: penetration gland; ta: tail; vs: ventral sucker. Scale bars: 50 μm.

Eye spots	5–15 μm (mean: 9 μm) × 5–15 μm (mean: 9 μm)
Pharynx	10–22 μm (mean: 17 μm) × 10–28 μm (mean: 19 μm)
Ventral sucker	13–35 μm (mean: 23 μm) × 15–45 μm (mean: 27 μm)
Excretory bladder	43–90 μm (mean: 64 μm) × 20–55 μm (mean: 39 μm)
Tail	20–33 μm (mean: 26 μm) × 405–495 μm (mean: 458 μm)
Lateral finfold	10–25 μm (mean: 18 μm) × 74–148 μm (mean: 108)

Type 4. Pleurophocercous cercariae

Heterophyidae (Leiper, 1909) (sensu Odhner 1914)

4.1 *Centrocestus formosanus* (Nishigori, 1924) (sensu Price 1932)

(Fig. 11)

The body is oval in shape. The oral sucker has oral spines or rostellar hooks like a tapeworm on the dorsal wall of the mouth aperture. A pair of eyespots is located above the prenetration glands at the same level as the pharynx. There are seven pairs of penetration

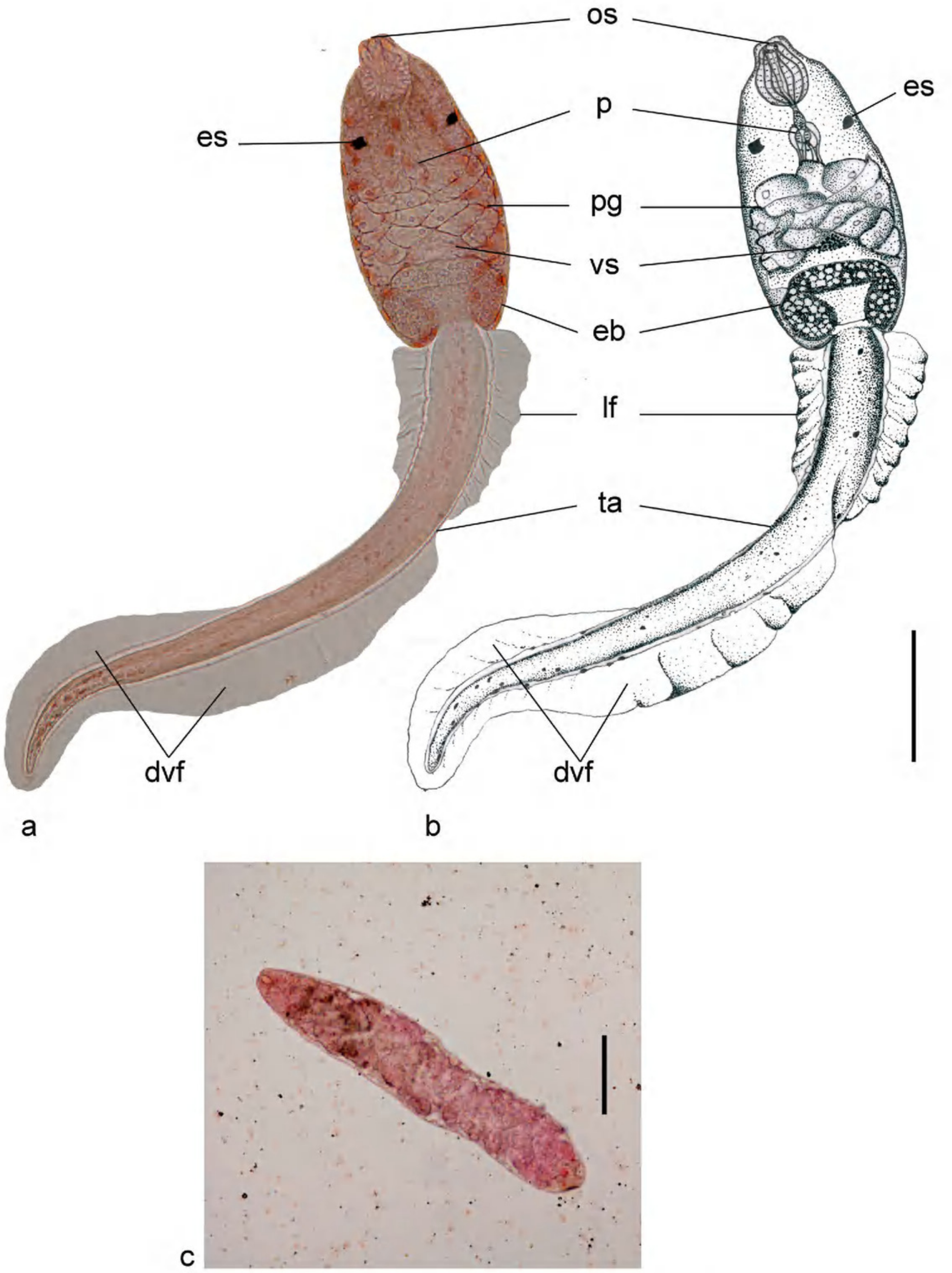

Figure 10. *Stictodora tridactyla* Martin & Kuntz, 1955. **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. redia stained with 0.5% neutral red. Abbreviations – dvf: dorsal finfold; eb: excretory bladder; es: eyespot; lf: lateral finfold; os: oral sucker; p: pharynx; pg: penetration gland; ta: tail; vs: ventral sucker. Scale bars: 50 µm.

glands. The genital primordial is elongated-triangular and located between the ventral sucker and the excretory bladder. The excretory bladder has dark granules and is thin-walled. The tail is slender and longer than the body. It is equipped with very narrow finfolds.

The cercariae develop within rediae.

The infection rate was 1.14% (172/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	45–73 µm (mean: 65 µm) × 83–121 µm (mean: 118 µm)
Oral sucker	17–27 µm (mean: 25 µm) × 18–30 µm (mean: 26 µm)
Pharynx	8–10 µm (mean: 9 µm) × 9–11 µm (mean: 10 µm)
Ventral sucker	13–17 µm (mean: 15 µm) × 14–18 µm (mean: 16 µm)
Excretory bladder	25–31 µm (mean: 29 µm) × 39–53 µm (mean: 46 µm)
Tail	15–18 µm (mean: 15 µm) × 70–93 µm (mean: 83 µm)

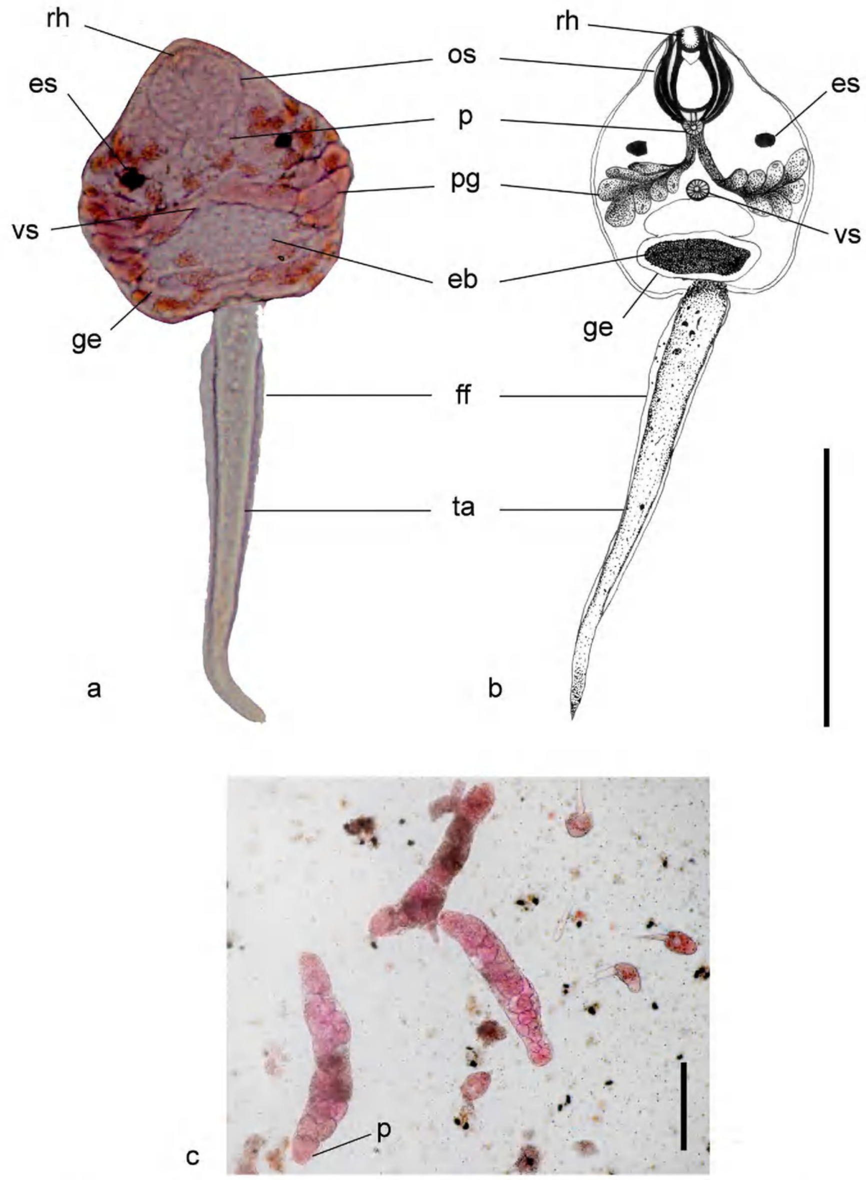

Figure 11. *Centrocestus formosanus* (Nishigori, 1924). **a**. Specimen stained with 0.5% neutral red. **b**. Drawing of cercaria. **c**. Redia stained with 0.5% neutral red. Abbreviations – eb: excretory bladder; es: eyespot; ff: finfold; ge: genital primordium; os: oral sucker; p: pharynx; pg: penetration gland; rh: rostellar hooks; ta: tail; vs: ventral sucker. Scale bars: 50 µm.

Type 5. Megarulous cercariae

Philophthalmidae (Looss, 1899) (sensu Travassos 1918)

5.1 *Philophthalmus gralli* Mathis & Léger, 1910
(Fig. 12)

The body is elongate pear-shaped and distinctly granulose. Eyespots are absent. The pharynx is large and extends into an esophagus that is bifurcating (Y-shape) into two blind ending intestinal caeca that almost reach the posterior end of the body. The ventral sucker is bigger than the oral sucker. The excretory bladder is rather small. The tail is about as long as the body and relatively slender. There is an adhesive gland present at its tip.

The cercariae encyst rapidly after developing within rediae.

The infection rate was 0.02% (3/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	143–175 µm (mean: 153 µm) × 438–470 µm (mean: 453 µm)
Oral sucker	50–68 µm (mean: 60 µm) × 63–73 µm (mean: 68 µm)
Pharynx	15–23 µm (mean: 20 µm) × 28–38 µm (mean: 34 µm)
Ventral sucker	60–78 µm (mean: 67 µm) × 48–80 µm (mean: 6 µm)
Excretory bladder	43–48 µm (mean: 45 µm) × 33–40 µm (mean: 36 µm)
Tail	40–50 µm (mean: 45 µm) × 463–475 µm (mean: 469 µm)

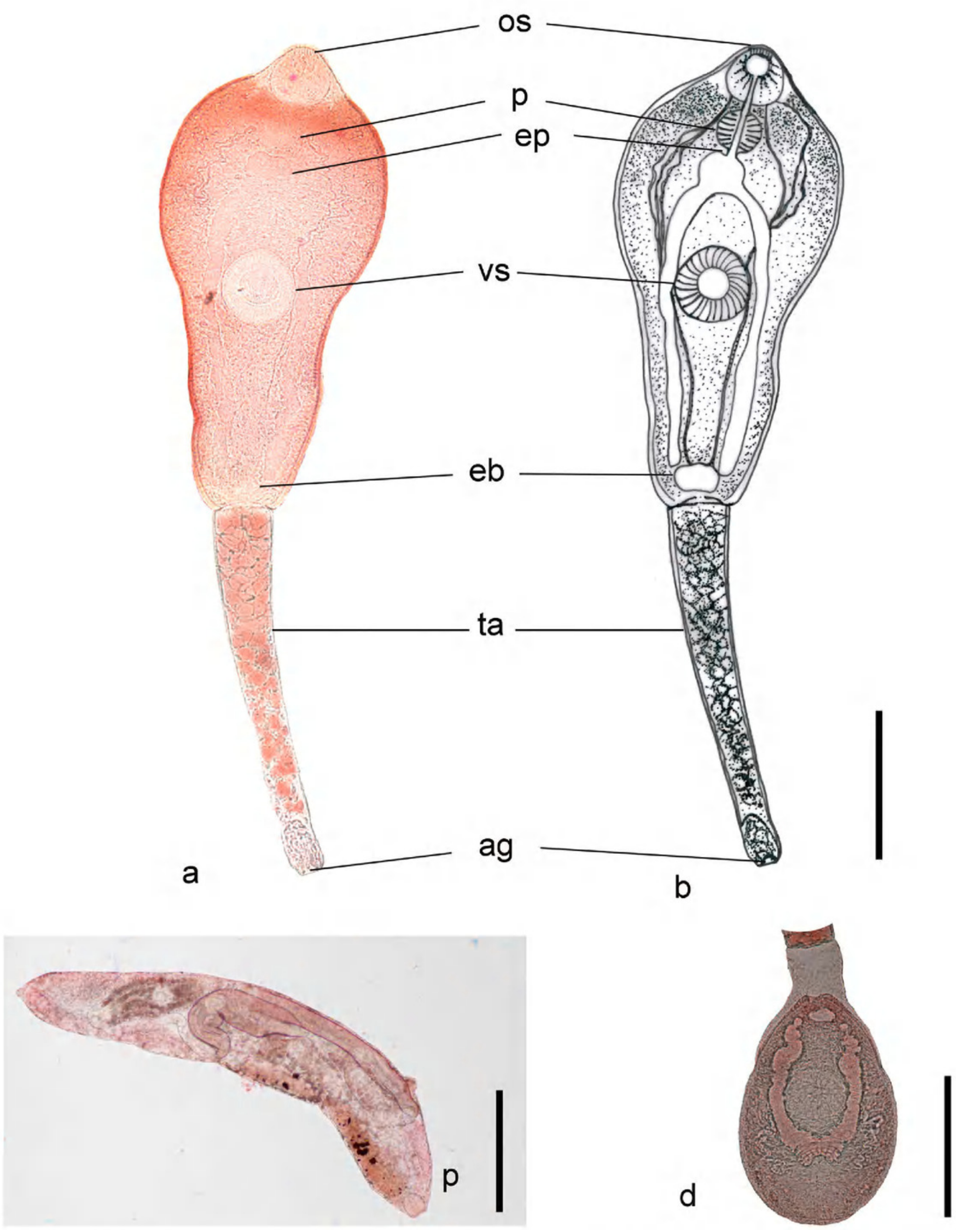

Figure 12. *Philophthalmus gralli* Mathis & Léger, 1910 **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. redia stained with 0.5% neutral red. **d**. metacercaria stained with 0.5% neutral red. Abbreviations – ag: adhesive gland; eb: excretory bladder; ep: esophagus; os: oral sucker; p: pharynx; ta: tail; vs: ventral sucker. Scale bars: 50 µm.

Type 6. Furcocercous cercariae

Sanguinicolidae Graff, 1907

6.1 *Cardicola alseae* Meade & Pratt, 1965

(Fig. 13)

The body is elongate-oval, slightly bent. Eyespots, a pharynx, an esophagus, intestinal caeca and a ventral sucker are absent. There is a narrow dorsal finfold in the middle part of the body. The penetration gland is located in the anterior part of the body. The excretory bladder is small and thin-walled, located at the posterior end of the body. The tail is forked. The stem of the tail is rather thick and longer than the furcae. Finfolds are present along the margins of the furcae.

The cercariae develop within sporocysts.

The infection rate was 0.05% (7/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	19–40 µm (mean: 30 µm) × 73–112 µm (mean: 96 µm)
Anterior organ	12–16 µm (mean: 14 µm) × 15–22 µm (mean: 19 µm)
Excretory bladder	4–8 µm (mean: 6 µm) × 12–37 µm (mean: 23 µm)
Tail stem	16–32 µm (mean: 28 µm) × 155–199 µm (mean: 187 µm)
Tail furcal	8–12 µm (mean: 10 µm) × 29–56 µm (mean: 52 µm)
Dorso-median finfold	6–15 µm (mean: 11 µm)

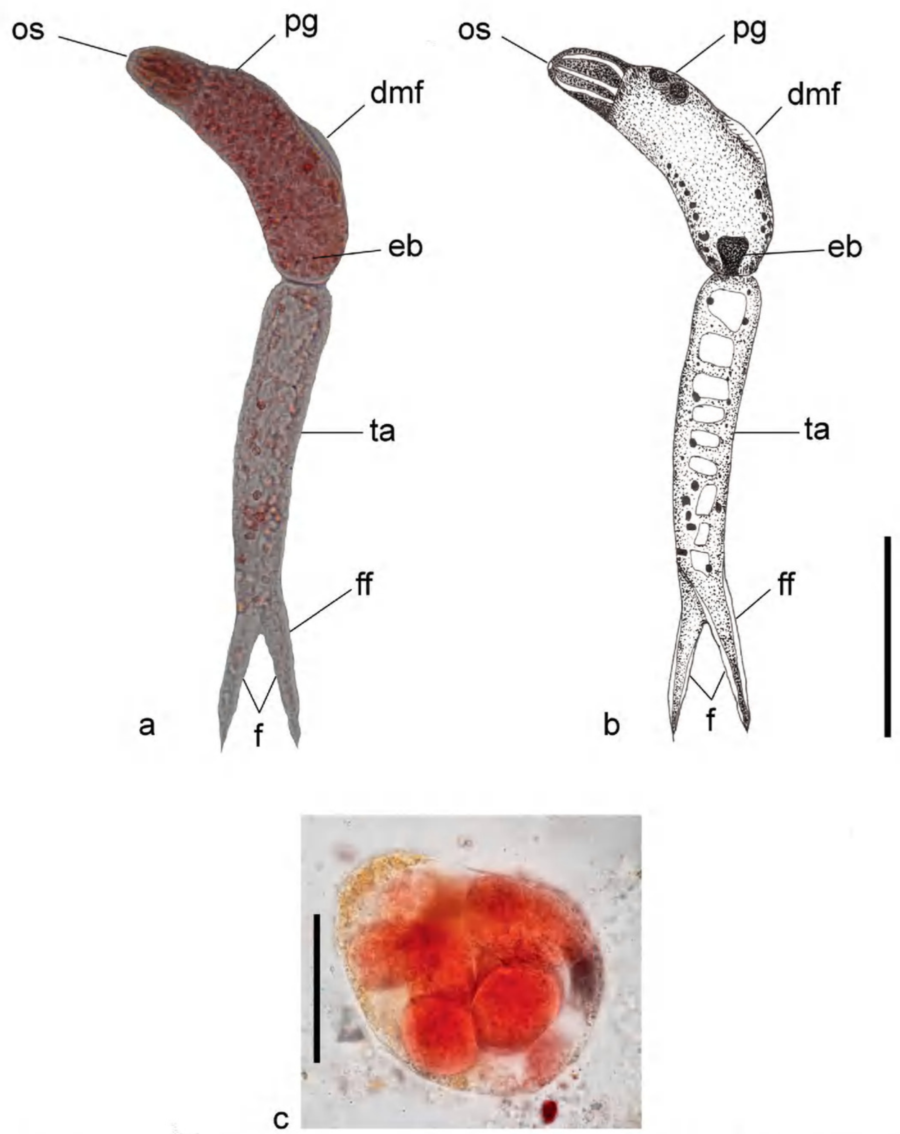

Figure 13. *Cardicola alseae* Meade & Pratt, 1965. **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. sporocyst stained with 0.5% neutral red. Abbreviations – dmf: dorso-median finfold; eb: excretory bladder; f: furca; ff: furcal finfold; os: oral sucker; pg: penetration gland; ta: tail. Scale bars: 50 µm.

Diplostomidae Poirier, 1886

6.2 *Alaria mustelae* Bosma, 1931
(Fig. 14)

The body is elongate-oval in shape. A pair of unpigmented eyespots is present. A prepharynx is present but rather short. The pharynx is small and roundish in shape. The esophagus is long, bifurcating into two intestinal caeca that are shorter than half the length of the esophagus. The oral sucker is larger than the ventral sucker. There are two pairs of penetration glands, filled with dark granules that are located around the ventral sucker. There is a Y-shaped excretory bladder located medially close to the posterior end of the body. The tail is longer than the body and divided into two furcae. The tail stem is slender and about as long as the furcae.

The cercariae develop within sporocysts.

The infection rate was 0.15% (23/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	106–155 µm (mean: 139 µm) × 186–282 µm (mean: 257 µm)
Oral sucker	29–41 µm (mean: 37 µm) × 29–42 µm (mean: 38 µm)
Pharynx	12–16 µm (mean: 14 µm) × 15–20 µm (mean: 17 µm)
Ventral sucker	16–38 µm (mean: 26 µm) × 16–32 µm (mean: 23 µm)
Tail	49–62 µm (mean: 57 µm) × 221–311 µm (mean: 275 µm)
Fork-tail	40–65 µm (mean: 61 µm) × 241–321 µm (mean: 286 µm)

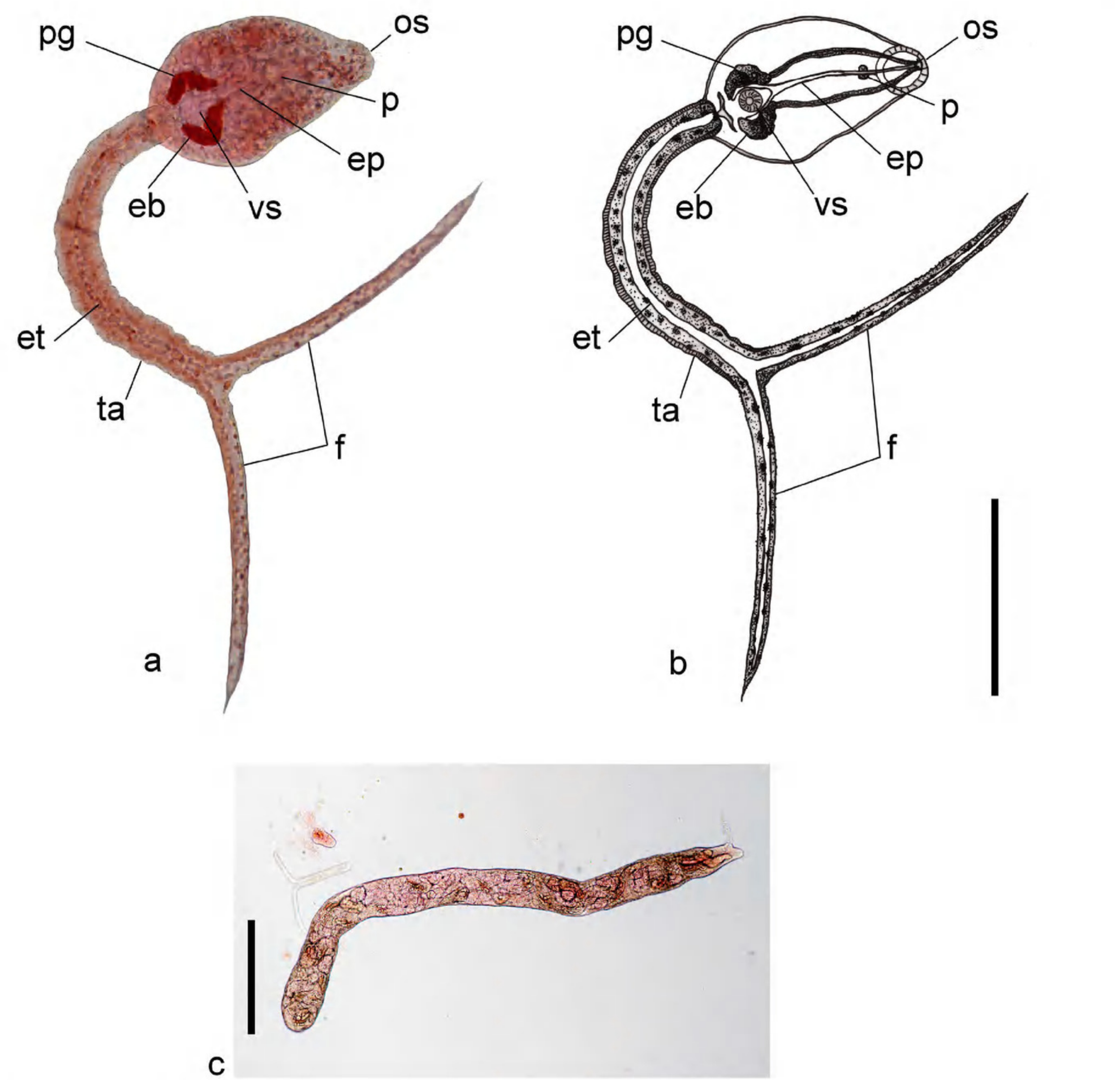

Figure 14. *Alaria mustelae* Bosma, 1931. **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. redia stained with 0.5% neutral red. Abbreviations – eb: excretory bladder; ep: esophagus; et: excretory tubule; f: furca; os: oral sucker; p: pharynx; pg: penetration gland; ta: tail; vs: ventral sucker. Scale bars: 50 µm.

Transversotrematidae Yamaguti, 1954

6.3 *Transversotrema laruei* Velasquez, 1958
(Fig. 15)

The body is of a bowl-like shape. The surface of the body is covered with spines that have the appearance of fish scales. The genital pore of the seminal vesicle is located in the anterior part of the body. Eyespots are present. The mouth is located near the ventral sucker. The esophagus is narrow and the intestinal caeca form a ring. There is one pair of testes present, and an ovary is located anterolateral to the left of the testes. The excretory bladder is small and short, and is situated close to the posterior end of the body. The tail is longer than the body and possesses spatulate furcae. At the base of the tail a pair of bilaterally symmetrical appendages is present, each equipped with an adhesive pad at its distal end.

The cercariae develop within rediae.

The infection rate was 0.05% (8/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	460–600 µm (mean: 533 µm) × 280–430 µm (mean: 362 µm)
Genital pore	20–40 µm (mean: 31 µm) × 20–50 µm (mean: 34 µm)
Ventral sucker	50–110 µm (mean: 76 µm) × 50–120 µm (mean: 77 µm)
Testis	30–120 µm (mean: 88 µm) × 40–120 µm (mean: 85 µm)
Excretory bladder	20–70 µm (mean: 40 µm) × 40–90 µm (mean: 57 µm)
Tail	120–180 µm (mean: 146 µm) × 620–800 µm (mean: 686 µm)

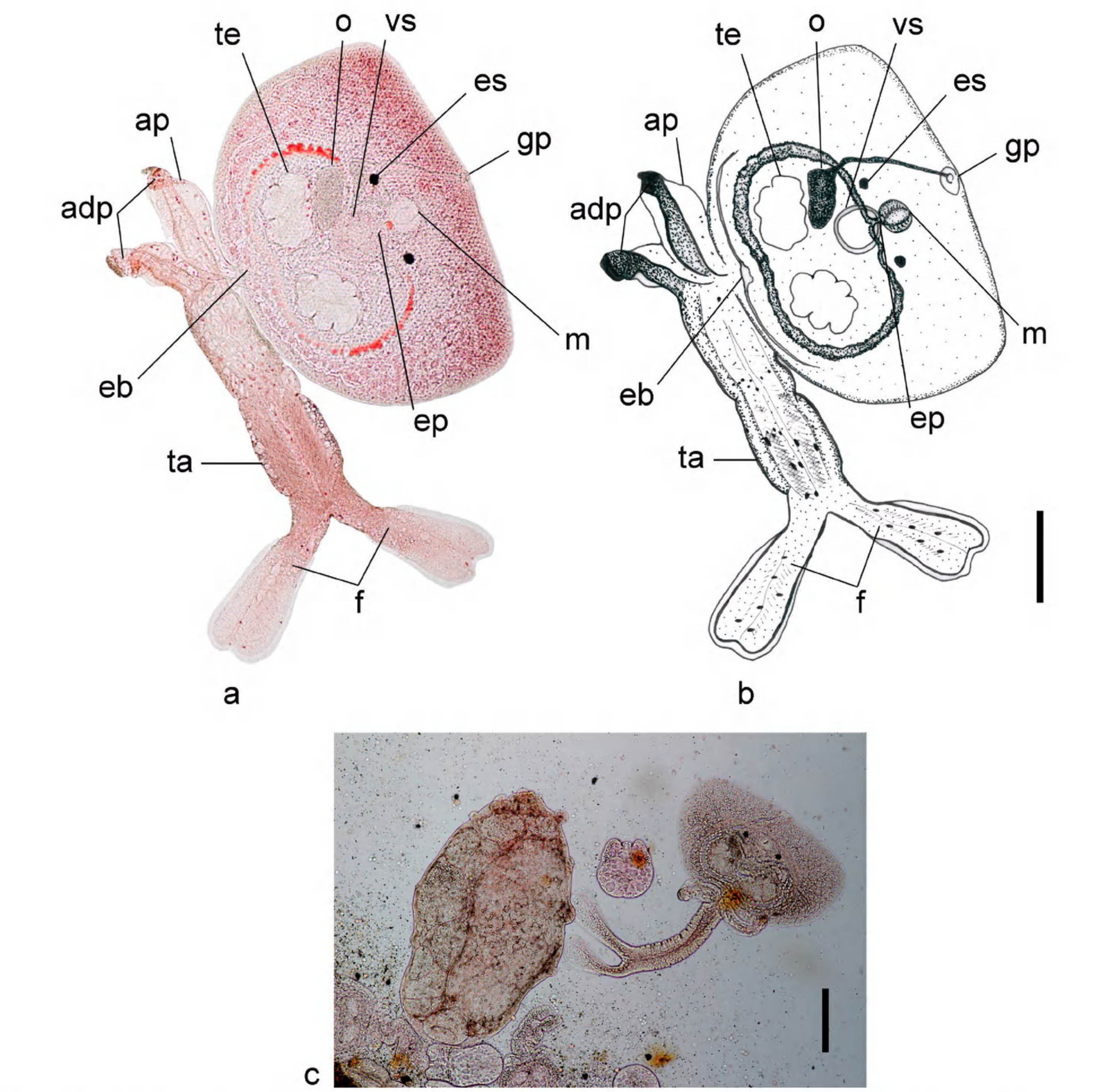

Figure 15. *Transversotrema laruei* Velasquez, 1958. **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. redia (left) and cercaria (right) stained with 0.5% neutral red. Abbreviations – adp: adhesive pad; ap: appendages; eb: excretory bladder; ep: esophagus; es: eyespot; f: furca; gp: genital pore; m: mouth; ov: ovary; ta: tail; te: testes; vs: ventral sucke. Scale bars: 50 μm.

Tail stem	120–180 μm (mean: 146 μm) × 390–530 μm (mean: 467 μm)
Tail furcal	80–150 μm (mean: 111 μm) × 180–290 μm (mean: 219 μm)
Appendages	40–70 μm (mean: 58 μm) × 120–150 μm (mean: 138 μm)

Type 7. Echinostome cercariae
(Fig. 16)

The body is elongate pear-shaped. Eyespots are absent. The oral sucker is circular in shape and is equipped with collar spines. The prepharynx is long. The esophagus is shorter than the prepharynx, bifurcating into two intestinal caeca that almost reach to the posterior end of the body. The relatively large ventral sucker is located approximately at two-thirds of the body length measured from the front. Penetration glands are absent. The excretory bladder is small and triangular in shape, its two main collecting tubes beginning at the level of the esophagus. The tail is slender and almost of the same length as the body.

The cercariae develop within rediae.

The infection rate was 0.07% (10/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	150–163 μm (mean: 151 μm) × 243–325 μm (mean: 270 μm)

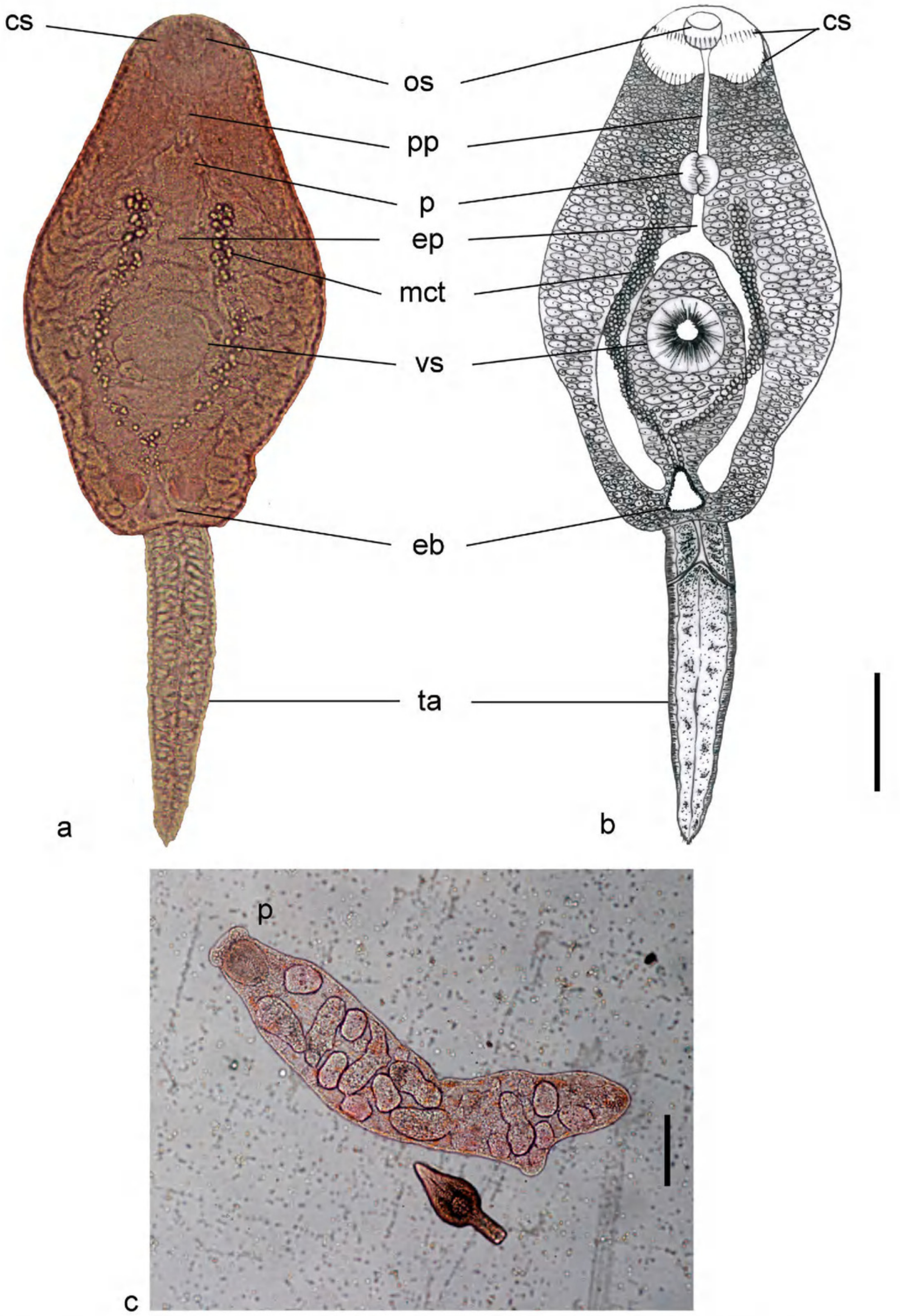

Figure 16. Echinostome cercaria. **a**. specimen stained with 0.5% neutral red. **b**. drawing of cercaria. **c**. redia stained with 0.5% neutral red. Abbreviations – cs: collar spines; eb: excretory bladder; ep: esophagus; mct: main collecting tube; os: oral sucker; p: pharynx; pp: prepharynx; ta: tail; vs: ventral sucker. Scale bars: 50 µm.

Oral sucker	38–48 µm (mean: 44 µm) × 38–48 µm (mean: 44 µm)
Ventral sucker	40–73 µm (mean: 62 µm) × 55–63 µm (mean: 60 µm)
Pharynx	13–18 µm (mean: 14 µm) × 20–30 µm (mean: 24 µm)
Excretory bladder	18–55 µm (mean: 38 µm) × 18–55 µm (mean: 33 µm)
Tail	28–40 µm (mean: 34 µm) × 195–313 µm (mean: 240 µm)

Type 8. Gymnocephalous cercariae
(Fig 17)

The body is oval and covered with spines. The terminal oral sucker is equipped with minute spines. Eyespots are absent. The prepharynx is long and thin. The pharynx is rather large and of a round shape. The esophagus is short but rather wide, bifurcating into two intestinal caeca that extend towards the posterior part of the body. There are 4–5 penetration glands present that are located laterally of the caeca between the

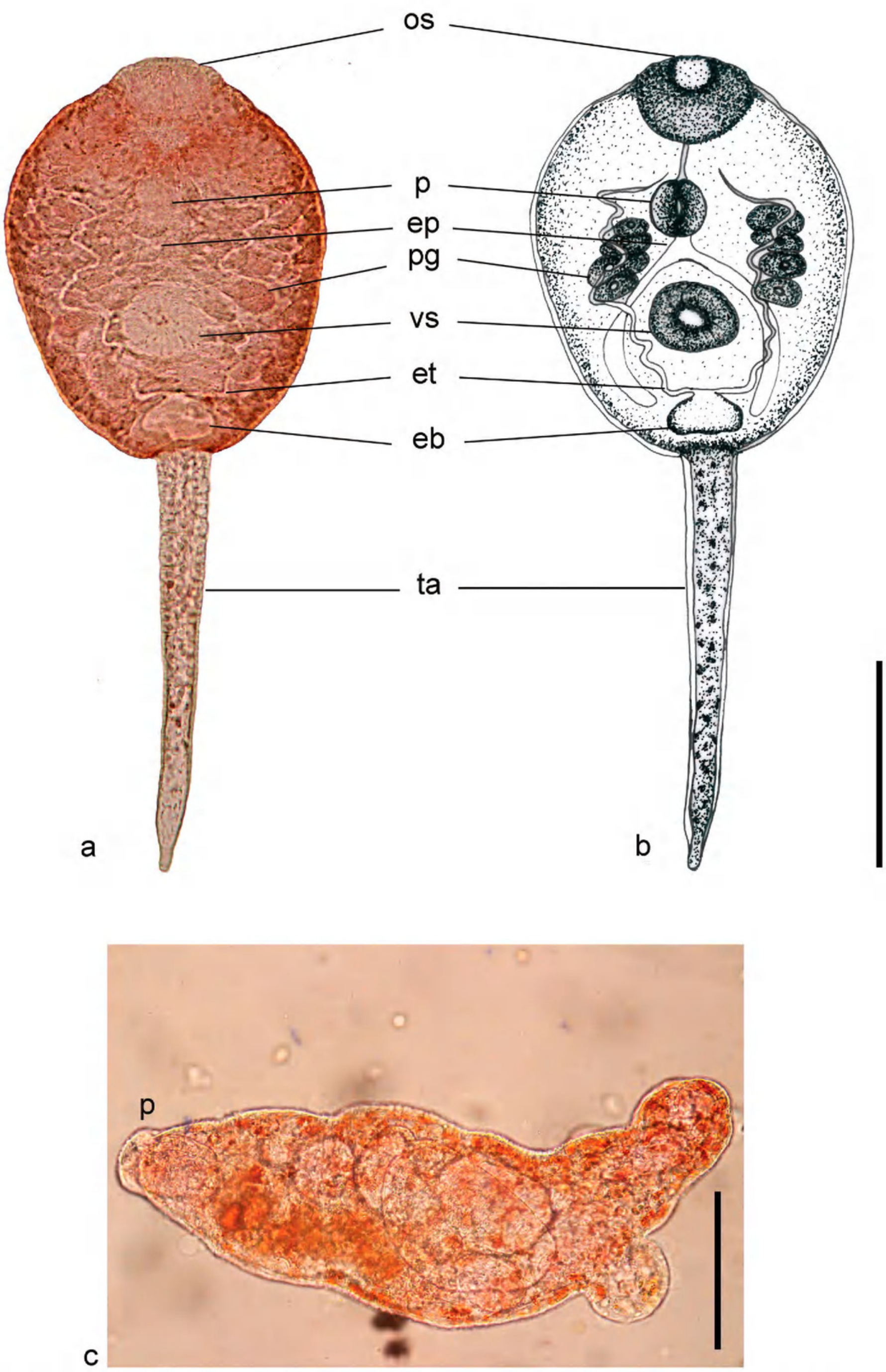

Figure 17. Gymnocephalous cercaria. **a**. Specimen stained with 0.5% neutral red. **b**. Drawing of cercaria. **c**. Redia stained with 0.5% neutral red. Abbreviations – eb: excretory bladder; ep: esophagus; et: excretory tubule; os: oral sucker; p: pharynx; pg: penetration gland; ta: tail; vs: ventral sucker. Scale bars: 50 µm.

level of the pharynx and the ventral sucker. The ventral sucker is of about the same size as the oral sucker. The excretory bladder is roundish, with a thin wall and located medially near the posterior end of the body. Two thin, undulating excretory tubules that begin just anterior of the pharynx insert into the excretory bladder. The tail is longer than the body, with the opening duct of the excretory bladder located at its end. There are groups of of 3–5 distinct pigment granules present in the tail but flame cells could not observed.

The cercariae develop within rediae.

The infection rate was 0.01% (1/15,076) (Table 3).

Size range and average size (in micrometers, calculated from 10 cercariae):

Body	115–160 µm (mean: 134 µm) × 150–195 µm (mean: 176 µm)
Oral sucker	30–40 µm (mean: 33 µm) × 28–40 µm (mean: 36 µm)
Pharynx	8–20 µm (mean: 13 µm) × 13–28 µm (mean: 22 µm)

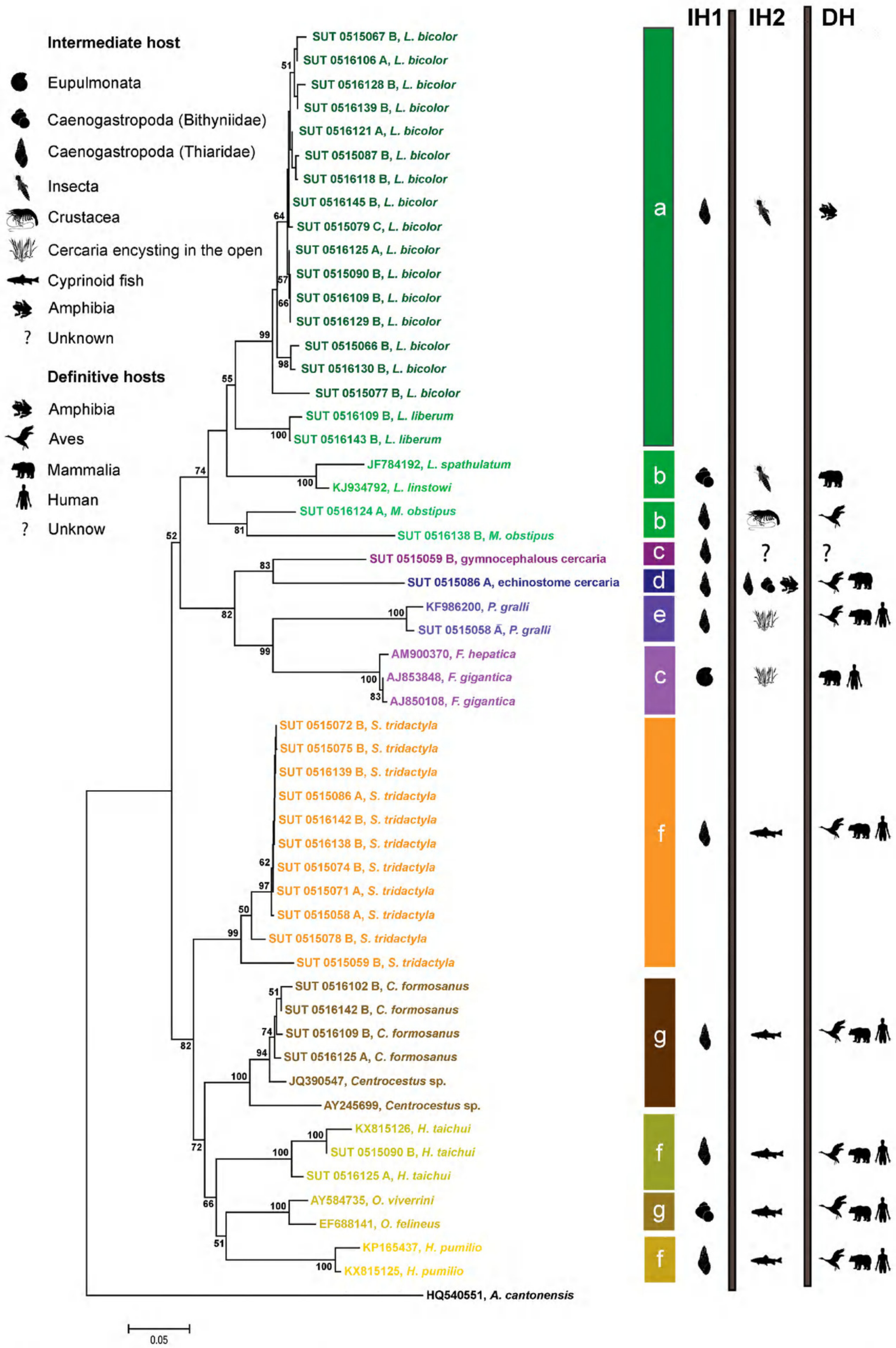

Figure 18. Neighbor-joining tree on the basis of ITS2 sequences of cercarial species obtained from Thai populations of *Tarebia granifera* (Lamarck, 1816) and several published sequences obtained from GenBank. Nodes are annotated with bootstrap support values ≥ 50. Taxon names and voucher or GenBank accession numbers are provided at the tips of the tree (see also Table 1). First and second intermediate hosts and definitive hosts are indicated (see legend). Abbreviations – DH: definitive host: IH1: first intermediate host; IH2: second intermediate host. Cercarial types – **a**: virgulate xiphidiocercariae; **b**: armatae xiphidiocercariae; **c**: gymnocephalous cercariae; **d**: echinostome cercariae; **e**: megarulous cercariae; **f**: parapleurophocercous cercariae; **g**: pleurophocercous cercariae.

Ventral sucker	35–48 µm (mean: 41 µm) × 33–45 µm (mean: 41 µm)
Excretory bladder	28–45 µm (mean: 39 µm) × 25–43 µm (mean: 31 µm)
Tail	23–35 µm (mean: 27 µm) × 183–223 µm (mean: 199 µm)

Molecular analysis

In the present study, ITS2 sequences from nine distinct cercarial types (collected during the second period of this study) of a total of fifteen trematode species found in Thai populations of *Tarebia granifera* could be amplified by PCR and sequenced. The ITS2 sequences of the virgulate xiphidiocercariae and the armatae xiphidiocercariae had a length of approximately 320 bp, while the ITS2 sequences of the parapleurophocercous cercariae and the pleurophocercous cercariae had a length of approximately 380 bp. The ITS2 sequences of the remaining cercarial types, i.e. megarulous cercariae, echinostome cercariae and gymnocephalous cercariae, had a length of approximately 500 bp.

The phylogenetic tree obtained from the neighbor-joining analysis (Fig. 18) was rooted with the nematode *Angiostrongylus cantonensis* (Chen, 1935) (GenBank accession number: HQ540551.1). All trematode species from Thai populations of *T. granifera* that were distinguished on the basis of cercarial morphology and for which more than one sequence was obtained, formed well supported groups in the phylogenetic analysis. These are highlighted in the following:

- Specimens of *S. tridactyla*, *C. formosanus*, *Centrocestus* sp., *H. taichui*, *O. viverrini*, *O. felineus* (Rivolta, 1884) and *H. pumilio*, which all have cyprinoid fish as a second intermediate host, were grouped together with relatively high support.
- The sequences of the echinostome cercaria and the gymnocephalous cercaria obtained from *T. granifera* were grouped together with relatively high support.
- This latter clade in turn formed a well-supported clade together with *P. gralli* and *Fasciola hepatica* Linnaeus, 1758 and *Fasciola gigantica* Cobbold, 1856 (for which we obtained data from previously published sequences).
- A group of species with arthropods as second intermediate hosts, i.e. *L. bicolor*, *L. liberum*, *Lecithodendrium spathulatum* (Ozaki, 1929), *Lecithodendrium linstowi* Dollfus, 1931 and *M. obstipus*, formed a moderately supported group in the phylogenetic analysis. The relationships of species within this clade, however, could not be resolved robustly.

Discussion

Thiarid gastropods, that transmit parasites of native birds, fishes or mammals, have frequently been reported as first intermediate hosts of trematodes affecting the respiratory, intestinal and hepatic systems not only in some domestic animals but also in humans. As outlined in the Introduction, this represents a serious threat to public health. For example, thiarid snails such as *Melanoides tuberculata*, *T. granifera*, *Mieniplotia scabra* and *Sermyla riqueti* have been reported as the intermediate hosts of a wide array of diverse trematodes, such as *Haplorchis pumilio*, *H. taichui*, *Loxogenoides bicolor*, *Centrocestus formosanus*, *Acanthatrium hitaense*, *Haematoloechus similes*, *Cloacitrema philippinum*, *Transversotrema laruei*, *Stictodora tridactyla*, *Apatemon gracilis*, *Mesostephanus appendicalatus*, *Cardicola alseae* and *Alaria mustelae* (Dechruksa et al. 2007, Ukong et al. 2007, Krailas et al. 2011, 2014). Furthermore, the phenotypically highly diverse and, thus, systematically problematic thiarid snails are widely distributed in Southeast Asia and Australasia (e.g. Glaubrecht 1996, 2009, 2011, Glaubrecht et al. 2009). This not only renders them most suitable objects for various systematic, biogeographical and evolutionary studies but also brings them into special focus from a parasitological perspective.

The present study aimed at bringing together the classical parasitological approach of the morphological characterization of the cercariae stages of trematodes obtained from their snail host, with a molecular parasitology approach, presenting a phylogenetic analyses of the minute intestinal flukes identified from their thiarids host, exemplified here for the first time with *Tarebia granifera* from Thailand. This particular snails host is common in many Thai freshwater systems, inhabiting rivers, lakes, streams and ponds (Hyslop 2003). Pillay and Perissinotto (2008) recorded that *T. granifera* was also able to colonize moderately saline habitats (brackish water). Without doubt, therefore, this thiarid is well established as an intermediate host for several species of trematodes.

We here focussed on the larval trematode infections found in this snail collected in various regions in Thailand during two periods of field work. When we started the research in the first period (2004–2009), *T. granifera* was found in 18 sampling sites. In the second period (2014–2016), we not only went back to the same sampling sites but also added samples from new locations. Thus, snails from a total of 72 locations could be analysed from all over Thailand, covering for the first time most of the distributional range of the snail host *T. granifera* in this country. In more than two-thirds of these locations, i.e. in populations at 51 sampling sites, infected snails were found, indicating the wide prevalence of these trematodes in Thailand.

As we mentioned above, only three species of trematodes, viz. *L. bicolor*, *S. tridactyla*, *C. formosanus*, were found to commonly occur in *Tarebia granifera* from most river systems and regions in Thailand. They were also found during all seasons, thus independent of the time of the year the snails were collected. By re-visiting during the years 2014 to 2016 the same locations of the first collecting period five to ten years earlier, and recording infected snails in 18 of these sampling sites, we also found that these trematode infections are apparently long-lasting, in the sense of a permanent phenomenon of these snail host populations, despite seasonal variation in the abundances of plants and animals in general (Shimadzu et al. 2013). Among the total of 15 species from 8 types of cercariae recorded in our study, we found only half of them (i.e. 8 species from 4 types) during the first period; whereas 11 species from 7 types were found in the second period. Thus, with the new study period and with collecting at various other and thus new locations all over Thailand we were able to expand our knowledge with respect to the taxonomical and geographical aspects of this analysis.

In the following we discuss in more details various aspects for the distinct trematode species found in their Thai thiarid snail host *Tarebia granifera*:

Parapleurophocercous cercariae and pleurophocercous cercariae were reported to be commonly found also in other freshwater snails in Thailand, such as e.g. *Melanoides tuberculata* (Krailas et al. 2014). In this study, three species of parapleurophocercous cercariae and one species of pleurophocercous cercariae were found in *T. granifera*.

Various reports have indicated the presence of parapleurophocercous cercariae and some species of pleurophocercous cercariae of the intestinal trematodes Heterophyidae, such as *H. taichui, H. pumilio, S. tridactyla* and *C. formosanus* (e.g. Chontananarth and Wongsawad 2013, Waikagul and Thaekham 2014). These parasites have an aquatic life cycle, using freshwater snails as the first and cyprinid fish as the second intermediate host, with definitive hosts being fish-eating mammals and humans (Nithikathkul and Wongsawad 2008, Krailas et al. 2011, 2014, 2016).

In this study, we found human trematodes, viz. *H. taichui*, *H. pumilio*, *S. tridactyla* and *C. formosanus*. Especially the snail infections by the minute intestinal fluke of *S. tridactyla* (2.92%) and *C. formosanus* (1.14%) showed a high level of prevalence in Thailand. In addition, *H. taichui* is important for public health, as was shown in several studies. For example, Kumchoo et al. (2005) reported high prevalence of fish as being the second intermediate host (91.4%) of *H. taichui* from Mae Taeng district of Chiang Mai province. Also, in the PDR Laos many patients have been infected by *H. taichui*, as cases were reported with mucosal ulceration, chronic inflammation and fibrosis of submucosa (Sukontason et al. 2005, Sohn et al. 2014). Chai et al. (2013) reported for seven patients who were infected by *C. formosanus* in Laos that they had abdominal pain, indigestion and diarrhea. Chung et al. (2011) reported the first case in Korea for patients being infected by *H. pumilio*. This heterophyid trematode is an important and continuing public health problem in many countries, as there are case reports not only from Southeast Asia but also from other Asian countries.

Therefore, it is from this perspective that for the epidemiology of zoonosis in general we recommend the study of snail intermediate hosts of human and animal trematode infections. It would be interesting to study whether there are geographically related higher or lower incidences of human infections, perhaps also correlated to infected fishes in these areas.

In contrast, known as parasites to animals only, xiphidiocercariae can be distinguished by their stylet organ in the mouth part of the cercariae. They can be divided into two morphological types, the first type being the virgulate xiphidiocercariae, and the second type the armatae xiphidiocercariae (see e.g. Frandsen and Christensen 1984). The virgulate xiphidiocercariae have a virgular organ present in the region of the oral sucker. For this group, the present study reported three species of parasites from the Lecithodendriidae, viz. *L. bicolor*, *L. liberum* and *A. histaense*, for which the hosts are amphibians (Brooks et al. 1985). It should be noted that we found *L. bicolor* to have the highest prevalence, with an infection rate of 3.84 %, and to be distributed in every water body, river system and region of Thailand. In the second group, i.e. the armatae xiphidiocercariae, the cercariae do not possess a virgular organ. For this group, we here reported two species, viz. *M. caridinae* and *M. obstipus* of the Microphallidae, which are parasites in birds as definite hosts. For the time being, we refrain from speculating on what might cause these differences before more detailed studies will be done.

Megarulous cercariae have been morphologically characterized as belonging to *Philophthalmus*. This parasite is commonly known as the oriental avian eyefluke and it had been reported in connection with human accidental infections (Waikagul et al. 2006, Derraik 2008). Nollen and Murray (1978) reported that *P. gralli* parasitized the conjunctival sac of various galliform and anseriform birds. This fluke was also found in ostriches, causing conjunctivitis. In earlier studies the cercariae of this trematode were found in the thiarid snail *Melanoides tuberculata* as intermediate host (Kalatan et al. 1997, Pinto and de Melo 2010, Krailas et al. 2014). In this study, we found *P. gralli* now also in the thiarid *Tarebia granifera* from the Phachi River in western Thailand. This river system originates in the Tenasserim mountain range and tributes to the Mae Klong river system to the east of it.

Furcocercous cercariae are generally from trematodes of the Sanguinicolidae; they develop to cercariae in brackish-water and freshwater snails, while the definitive hosts were found in fishes. In this study, we found cercariae of three species, viz. *C. alseae*, *A. mustelae* and *T. laruei*, to parasitize *Tarebia granifera* as intermediate host. Cercariae of all three trematode species were also found in other thiarid snails, as they were reported in *Melanoides tuberculata* (Krailas et al. 2014, Anucherngchai et al. 2017).

Echinostome cercariae are distributed throughout Southeast Asia (Chai 2009). Most species mainly parasitize avian hosts, such as migratory birds, but sometimes also infect mammals including humans. The echinostome trematodes are associated with the ingestion of raw snails and amphibians that transmit metacercariae as the infective stage (Esteban and Antoli 2009). In the present study, echinostome cercariae were found in *Tarebia gran-*

ifera populations from the north of Thailand only; which corroborates the report by Nithikathkul and Wongsawad (2008) that echinostomiasis cases have been commonly found in the north and northeast of Thailand.

Gymnocephalous cercariae are small larval stages of trematodes, in general attributed to the Fasciolidae (e.g. Schell 1970). In this study, we found only one snail infection with cercariae that morphologically are obviously attributable to *Fasciola* cercariae. However, the molecular identification showed that these cercariae were actually neither *F. gigantica* nor *F. hepatica*. Instead, the phylogentic analyses indicate a closer affinity of these sequences to those from cercariae with echinostoma type. By morphology the echinostome cercariae are clearly distinguishable by being elongated spinose with a reniform collar, armed with a single or double row of spines surrounding the dorsal and lateral margins of the oral sucker (Anucherngchai et al. 2016, Ayoub et al. 2017). Thus, our study here revealed one case of obvious conflict between the morphologically based identification and the molecular indication of affinity, which clearly is in need to be studied further. We cannot exclude the possibility of a simple laboratory mix-up, but should also keep in mind e.g. hybrid effects.

In a previous report, the gymnocephalous cercariae were produced by trematodes of the Fasciolidae. They were found in *Biomphalaria* sp., *Bulinus* sp., *Ceratophallus* sp., *Gabbiella* sp., *Gyraulus* sp., *Lymnaea* sp., and in *Melanoides* sp. (Frandsen and Christensen 1984). However, thiarid snails were never reported with fasciolid trematode infections in Thailand. Even though, the morphology of gymnocephalous cercariae was obviously to be *Fasciola* cercariae. The sequence of DNA was shown to match with that from the group of echinostome cercariae.

Molecular analyses of cercaria and their host correlations

In general, morphological as well as molecular studies of cercariae were able to confirm the specific identity and prevalence of various infectious trematodes in Thai freshwater snails of *Tarebia granifera*. In this study, we found that the ITS2 marker allowed to distinguish a total of nine trematode species, with the cercariae attributable to seven of the morphologically distinguishable types, viz. the parapleurophocercous cercariae, pleurophocercous cercariae, the virgulate xiphidiocercariae and armatae xiphidiocercariae, megarulous cercariae, as well as echinostome cercariae and gymnocephalous cercariae; only the furcocercous cercariae were not available for molecular studies. And in one case only we found a conflict insofar, as the sequence data revealed that cercariae of the morphologically distinguished gymnocephalous type grouped closely with those of the echinostome type.

We also used the ITS2 marker for a phylogenetic reconstruction (Fig. 18), in which two characteristics are most noteworthy: First, while the relationships of species within molecular clades found could not be resolved robustly, our analyses revealed, second, two well-supported major molecular clusters or groups. These clusters can be well interpreted in context of their respective zoonotic parasites and human pathogens.

The first group with parapleurophocercous and pleurophocercous cercariae, respectively (marked f and g in Fig. 18), i.e. *S. tridactyla*, *C. formosanus*, *Centrocestus* sp., *H. taichui*, *H. pumilio*, *O. viverrini*, *O. felineus*, all have cyprinoid fish as second intermediate host, while birds and mammals, including in particular humans, are the definite host. Note that the latter two trematode species have a bithyniid instead a thiarid snail as first intermediate host.

In a second group cluster trematode species with virgulate xiphidiocercariae and armatae xiphidiocercariae, respectively (marked a and b in Fig. 18), i.e. *L. bicolor*, *L. liberum*, *Lecithodendrium spathulatum*, *L. linstowi* and *M. obstipus*), which all have arthropods (Insecta or Crustacea) as second intermediate hosts while amphibians, birds and mammals, but with the exclusion of humans, are the definite hosts.

In addition, also the sequences of trematode species with echinostome cercaria and the gymnocephalous cercaria obtained from *T. granifera* grouped together with relatively high support. However, no clear picture as to a correlation with their second intermediate hosts and definitive hosts is visible to date, as we lack knowledge on the latter in particular for the gymnocephalous cercaria. Nevertheless, the latter two form a well-supported clade together with *P. gralli*, *F. hepatica* and *F. gigantica*, which all have gymnocephalous, echinostome or megarulous cercariae (Fig. 18, c,d,e). Note that the latter two trematode species are known to have an eupulmonate instead of a thiarid snail host. Interestingly, in this latter monophyletic clade, formed by *P. gralli* together with *F. hepatica* and *F. gigantica*, only those trematodes are known to be human pathogens (as definite hosts) when the cercaria are encysting in the open instead of parasitizing a second intermediate host; see Fig. 18.

We anticipate that more detailed studies, based on molecular phylogenetic analyses, looking into these and other correlations of intermediate hosts, their regional occurrences and ecological specifics will shed more light on the evolutionary potential of trematode parasites from thiarid snails.

Conclusion and outlook

To date, studies on freshwater snails and their interactions with parasitic trematodes are under-represented worldwide (Adema et al. 2012). There is an urgent need for collaboration bringing together deeper understanding on the basic biology, biodiversity, and evolutionary associations of parasitic trematodes on the one hand and their snail hosts on the other, i.e. those studying parasitology and malacology, taking advantage of their respective expertise in host-parasite interactions and evolutionary systematics.

Accordingly, the aims of this approach presented here were to establish reliable and reproducible data for the morphological identification as well as the methodology for the extraction of high quality DNA from preserved trematode cercariae in specifically known populations of their thiarid snails hosts (including museum samples collected several years ago). It was also the aim to conduct a phylogenetic analysis of the minute intestinal flukes. In addition, the present paper adds to a more in-depth evolutionary systematic analysis with data on reproductive biology, geographical distribution, morphology and molecular phylogenies of *T. granifera*.

Using this combinational approach, it will eventually be possible to identify in more details the host-parasite relationships of thiarid snails as first intermediate host populations not only in Thailand, and also to determine the role of parasitic infections in these gastropods and as human pathogens.

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