Molecular signatures of the rediae, cercariae and adult worm stages in the complex life cycles of parasitic flatworms (Psilostomatidae, Trematoda).

Maksim A. Nesterenko^{1*}, Viktor V. Starunov^{1,2}, Sergei V. Shchenkov^{1,3}, Anna R. Maslova¹, Sofia
A. Denisova¹, Andrey I. Granovich¹, Andrey A. Dobrovolskij¹ and Konstantin V. Khalturin⁴

¹ Department of Invertebrate Zoology, St-Petersburg State University. St-Petersburg 199034, Russia

² Zoological Institute Rus. Acad. Sci., St-Petersburg 199034, Russia

³ The A.O.Kovalevsky Institute of Marine Biological Research of RAS, Sevastopol 299011, Russia

⁴ Marine Genomics Unit, OIST, 1919-1 Tancha, Onna-son, Kunigami-gun, Okinawa, 904-0495
Japan;

* Corresponding author

Email addresses:

MAN : maxnest.research@gmail.com

VVS: starunov@gmail.com

SVS: sergei.shchenkov@gmail.com

ARM: shuvalova.a.r@gmail.com

SAD: dersteppenwolf1608@gmail.com

AIG: a.granovich@spbu.ru

AAD: a.dobrovolsky@spbu.ru

KVK: konstantin.khalturin@oist.jp

[Abstract]

Trematodes are one of the most remarkable animals with complex life cycles with several generations. Life histories of a parasitic flatworms include several stages with disparate morphological and physiological characteristics follow each other and infect hosts ranging from mollusks to higher vertebrates. How does one genome regulate the development of various life forms and how many genes are needed to the functioning of each stages? How similar are molecular signatures of life stages in closely related species of parasitic flatworms? Here we present the comparative analysis of transcriptomic signatures of the rediae, cercaria and adult worm stages in two representatives of the family Psilostomatidae (Echinostomata, Trematoda) - *Psilotrema simillimum* and *Sphaeridiotrema pseudoglobulus*. Our results indicate that the transitions between the stages of the complex life cycle are associated with massive changes in gene expression with thousands of genes being stage-specific. In terms of expression dynamics, the adult worm is the most similar stage between *Psilotrema* and *Spaeridiotrema*, while expression patterns of genes in the rediae and cercariae stages are much more different. This study provides transcriptomic evidences not only for similarities and differences between life stages of two related species, but also for cryptic species in *Sphaeridiotrema*.

[Introduction]

Life cycles with morphologically distinct stages occur in various clades of the animal kingdom¹. They are typical for free-living Cnidaria² and Aphiodioidea³ as well as for parasitic Apicomplexa⁴ and Trematoda⁵. In contrast to simple life cycle that include only one ontogeny, the complex life cycles are characterized by alteration of several generations and each of them has its own ontogeny⁶.

Trematoda is a clade of parasitic flatworms that possess one of the most striking examples of complex life cycles⁷. In the most common variant, the trematode life cycle include three hosts: an invertebrate animals (usually a gastropod mollusk) and a vertebrate as intermediate and definitive hosts, respectively⁵. Adult worm that inhabits definitive host is usually hermaphroditic and lays eggs from which a free-living larva of the next parthenogenetic generation, called the miracidium, hatches. The main goal of miracidia is to find and infect the first intermediate host. Inside of the intermediate host it transforms into an individual of the next generation. In “rediod” species it turns into a rediae, and in “sporocystoid” species it gives rise to the sporocysts which grow through the tissues of a host. After several cycles of self-reproduction, the individuals of the parthenogenetic generation produce cercariae – the free-living larvae of the amphimictic generation. Cercaria leave the first intermediate host and spread around in search for a new host. If cercariae manages to infect the right definitive host it transforms into an adult worm that again produces eggs with future miracidia, thereby completing the life cycle.

Trematodes of medical or veterinary importance include *Paragonimus westermani* (human lung fluke), *Clonorchis sinensis* (human liver fluke), *Fasciola hepatica* (cattle liver fluke) and, most importantly, the schistosomes (blood flukes)⁸. *Schistosoma japonicum*⁹ and *Schistosoma mansoni*¹⁰ were the first representatives of trematodes with the sequenced genomes. Availability of genomic data allowed to study many aspects of Trematode biology at the molecular level, including differential gene regulation and epigenetics¹¹. Nevertheless, the blood flukes possess numerous traits that make them very different from the rest of Trematoda, for instance the presence of a schistosomula stage and two separate sexes in their life cycle suggest their highly specialized and evolutionary derived state.

The development of the New Generation Sequencing (NGS) has led to increase of transcriptomic data from different trematode species^(12,13,22,23,14–21). However, the majority of studies describes only one stage of the life cycle, mostly adult worms^(12–20). Only few studies provide the comparative analysis of the different stages of the same generation^(22,23) or the same stage in different species^(19–21). Comparative transcriptomic analysis of different generations within one life cycle has not been performed so far in any species other than schistosomatides. Such experiment are

impeded by a number of the technical challenges. It is prohibitively difficult to maintain complex life cycles in laboratory conditions as that requires cultivation of multiple intermediate and definitive hosts. However, comparative data of that kind are critically important for the understanding of the origin and evolution of trematode life cycles and could provide new insights for treatment and prevention of human and animal trematodosis.

The representatives of the family Psilostomatidae have a dixenous life cycle which appear to be the closest to the “archetypical” life cycle according to modern ideas about the digeneans evolution⁵. The free-swimming cercariae of these parasites are encysted outside the body of their intermediate host, but in close connection with it (on the mollusk shells or under their mantle fold). The adult psilostomatid worms are typical histiophages and hermaphrodites that parasitize in the intestines of birds. The adult worm body does not have any secondary modifications and possesses a shortened uterus. This feature is useful for experimental work since it allows to reduce the level of adult worm tissue contamination by developing miracidiae. Free-swimming miracidiae actively infect the first intermediate host and give rise to a typical microhemipopulation of rediae. The rediae have a well-developed gut and an extensive brood cavity with developing cercariae. Psilostomatidae use the same species of prosobranch snails, *Bithynia tentaculata*, as the first and the second intermediate hosts, which facilitates their maintenance in the laboratory.

In this paper, we present the comparative transcriptomic analysis of the rediae, cercariae and adult worms of the two species of Trematoda, belonging to the Psilostomatidae family – *Psilotrema simillimum* (Mühling, 1898) and *Sphaeridiotrema pseudoglobulus* (McLaughlin, Scott, Huffman, 1993). The aim of our research was to identify genes with stage-associated expression which most probably contribute to disparate anatomical and physiological characteristics of the life stages. Another goal was to determine similarities and differences in gene expression patterns within one life cycle and between two related species of trematodes.

[Results]

Animal cultivation and library preparation

Sphaeridiotrema pseudoglobulus (McLaughlin, Scott, Huffman, 1993) and *Psilotrema simillimum* (Mühling, 1898) have dixenous life cycles (only two hosts). The mollusk *Bithynia tentaculata* (Linnaeus, 1758) is an intermediate host, and waterfowl birds represent the definitive hosts (Fig.1). Naturally infected mollusks were collected from water parts of plants and stones of the Kristatelka pond (Peterhof, Russia) and maintained at the Dept. of Invertebrate Zoology SPbSU. The identification of the cercariae species was carried out using a light microscope according to the

1 morphological features. Rediae and cercaria stages were obtained from cultivated mollusks and the
2 adult worms were cultivated in the experimentally infected chicken.

3 For both *Psilotrema simillimum* and *Sphaeridiotrema pseudoglobulus* at least 23.3 million pairs of
4 reads were obtained for each of the two biological replicates of the redia, cercaria, and adult worm
5 stages (Tab.1). Contamination with host-derived sequences did not exceed 6.3% from the total
6 number of reads in each library. More than 70% of reads remained in all the libraries after the
7 removal of potential host-derived sequences, adapters and poor-quality reads.

8 **Quality and completeness of de novo assembled transcriptomes**

9 From BUSCO database representing single-copy orthologs of Metazoa 89.4% (Complete: 83.6%;
10 Fragmented: 5.8%) and 90.7% (Complete: 86.6%; Fragmented: 4.1%) are present in *P.simillimum*
11 and *S.pseudoglobulus* reference transcriptomes, respectively (Tab.2). Among the 79 orthologs
12 missing in our assemblies, 67 sequences were also not found in the previously published genomes
13 and transcriptomes of Trematoda. Thus, most probably these genes represent lineage-specific gene
14 losses most probably associated with the parasitic life style. The complete list of the “common”
15 missing genes with their annotations are presented in Table S2.

16 TransRate score of assemblies were equal to 0.237 for *P.simillimum* and 0.2095 for
17 *S.pseudoglobulus*. 117130 and 146563 contigs with highest scores were used in the downstream
18 analysis, which comprise 72% of the total number of assembled sequences from *P.simillimum* and
19 *S.pseudoglobulus*, respectively.

20 **Annotation and orthologues searching**

21 According to ESTScan prediction, 21075 (18%) and 49626 (34%) of transcripts in the assemblies of
22 *P.simillimum* and *S.pseudoglobulus* had open reading frames (ORFs) with more than 70 amino
23 acids, respectively. 62% of predicted protein in both species match to the sequences from Digenea
24 or did not have any matches in NR NCBI protein database. Latter category most probably
25 represents the novel sequences of Trematoda that are strongly derived in Psilostomatidae.

26 Among assembled transcripts of *P.simillimum* and *S.pseudoglobulus* 17225 and 39346 sequences,
27 respectively, have expression levels above 1 transcript per million (TPM), which constitute 85%
28 and 74% of the reference transcripts of the corresponding species. In both species, the open reading
29 frame was found in approximately 70% of the sequences with expression levels equal to or greater
30 than 1TPM. Gene Ontology annotations could be assigned to 9947 and 22554 proteins in
31 *P.simillimum* and *S.pseudoglobulus*, respectively (Table S12-13). KOBAS 3.0 results showed that
32 8125 proteins of *P.simillimum* had matches in the metabolic pathways of *H.sapiens* and 9978 had

1 matches in *S.mansoni*. In *S.pseudoglobulus*, 17769 proteins could be assigned to human pathways
2 and 22763 proteins had clear counterparts among the proteins of *S.mansoni*.

3 **Gene sets in Plathelminthes**

4 Comparison with *Schmidtea mediterranea*, *Opisthorchis viverrini*, *O.felineus*, *Clonorchis sinensis*,
5 *Fasciola hepatica*, *Schistosoma mansoni*, *Trichobilharzia regenti* proteomes allowed to identify
6 11138 groups of orthologous proteins (Table S3). As shown in Fig.1H, three clusters of species are
7 obvious based on the orthologs number shared among them. One cluster contains three species of
8 Opisthorchidae: *O.felineus*, *C. sinensis* and *O. viverrini*; second one unites schistosomatides: *S.*
9 *mansoni* and *T.regenti*. *Fasciola hepatica* groups together with *P.simillimum* and *S.pseudoglobulus*.
10 Only 3075 groups of orthologous proteins (OGs) include sequences from all nine species analyzed
11 and 121 OGs are species-specific (Fig. 1H, Table S3). Complete statistics for all the species are
12 presented in Table S4 (see Supplementary materials).

13 The comparison of the orthologous groups (OGs) among four species - *S.mediterranea*, *S.mansoni*,
14 *P.simillimum* и *S.pseudoblogulus* revealed that 3946 OGs are common for all studied flatworm
15 species. 2057 OGs are specific for trematodes, and 1244 OGs are specific to the family
16 Psilostomatidae (Fig. 1I). *P.simillimum* has 79 species-specific OGs and in 870 OGs are specific for
17 *S.pseudoglobulus*. *S.mansoni* also has a lot of species-specific proteins (510 OGs), but their number
18 in *S.pseudoglobulus* clearly stands out. It was an unexpected observation that the number of
19 species-specific OGs in *P.simillimum* or *S.pseudoglobulus* differs more than 9-fold (Fig. 1E).

20 **Genes with stage-specific expression in rediae, cercariae and adult worms**

21 Each stage of the trematode life cycle is characterized by structures and functions that are not
22 present in the other stages. Thus, different sets of genes must be activated in order to allow these
23 differences. How many genes in the genomes of the trematodes are utilized for generation of stages-
24 specific trait? In order to answer this question, we identified genes with preferential expression in
25 the redia, cercaria and adult worm stages in *Psilotrema* and *Sphaeridiotrema*.

26 According to the Jongeneels criterion (see Materials and Methods), approximately 15.5% of genes
27 in *P.simillimum* have rediae-specific expression, 24.5% are specific for the cercariae, and 26.9% -
28 for the adult worm. Differences between *S.pseudoglobulus* life cycle stages in terms of gene sets
29 utilized are more pronounced: 10.8% of genes have rediae-specific expression, 11.5% are rediae-
30 specific, and 66.1% of the genes are active mainly on the adult worm stage. In both species
31 examined the majority of genes with stage-specific expression are associated with the adult worms
32 (fig.1J-K) and this tendency is most prominent in *Spaeridiotrema*.

1 Comparison of gene sets with stage-specific expression revealed that in contrast to *P. simillimum*,
2 where stage-specific sets are most similar to themselves only (Fig.2A), in *S. pseudoglobulus* there is
3 a significant overlap between the genes specifically expressed in rediae and cercariae stages (Fig.
4 2B). This observation might be explained by the fact that inside of the redia the next generation
5 (cercaria) is developing. This can be clearly observed in Fig.2E where numerous round-shaped
6 structures visible inside of the *Psilotrema* redia are the developing cercaria larvae.

7 Next, we compared how similar are the transcriptomic signatures of the life stages between
8 *Psilotrema* and *Sphaeridiotrema*. As a measure of similarity we used expression levels of
9 orthologous genes in each of the stages (TROM score²⁴). Our analysis revealed that the
10 transcriptomes of adult worm demonstrate the greatest similarity (TROM-score = 12.53), whereas
11 for cercariae and rediae, the similarity score is almost two times lower and equal to 6.49 and 6.72,
12 respectively. A weak “overlap” is also present between *S.pseudoglobulus* rediae and *P.simillimum*
13 cercariae (TROM-score = 0.82). In total 681 pairs of orthologous genes demonstrate similar
14 expression dynamics in the course of the life cycle progression: 118 pairs have similar expression in
15 the cercariae, 140 in the rediae, and 423 in the adult worms.

16 In order to obtain a global view of metabolic and signaling pathways that are most important in
17 each of the stages we mapped the sets of stage-specific genes onto KEGG pathways of human
18 (Tables S5-S11). The most “enriched” pathways in *P.simillimum* rediae are those regulating
19 pluripotency of stem cells, Lysosome function, Wnt signaling (see Fig.2D), Axon guidance,
20 Neuroactive ligand-receptor interactions, Adherens junctions, Hippo signaling, Calcium signaling,
21 Cholinergic synapse and Focal adhesion.

22 Cercaria-specific genes in *P.simillimum* are mainly the members of Metabolic pathways, Calcium
23 signaling, Neuroactive ligand-receptor interaction, cAMP signaling, Lysosome function, Oxytocin
24 signaling, Renin secretion, cGMP-PKG signaling, Adrenergic signaling in cardiomyocytes and
25 Vascular smooth muscle contraction. Last two pathways are rather expectable because a cercaria is
26 the only stage with striated muscles an active locomotion.

27 The most enriched pathways in *P. simillimum* adult worm are Metabolic pathways, Lysosome, Gap
28 junction, Oocyte meiosis, Galactose metabolism, cGMP-PKG signaling pathway, Oxytocin
29 signaling pathway, Vascular smooth muscle contraction, Apoptosis and Gastric acid secretion.

30 In general, differentially expressed genes in *Psilotrema* and *Sphaeridiotrema* belong to the similar
31 pathways which is not surprising taking into consideration close phylogenetic relations of these two
32 species (Tables S5-9, 11). However, sets of enriched pathways in both species are overlapping not
33 completely: 32.2% are common in adult worms, 48.6% - in rediae and 65.6% - in cercariae.

One interesting observation is the staggered expression of the homeobox-containing transcription factors during the life cycle of *Psilotrema* and *Sphaeridiotrema* (Fig.2F). It is obvious that some of the homeobox genes are expressed in redia-, cercaria- or adult worm-specific manner. It is remarkable that the majority of homeobox genes are more active in parthenogenetic generations and their expression is down-regulated in the adult worms. This trend might be explained by active morphogenetic processes that take part in during the development of the redia and cercaria. Alternatively, this might indicate that in Trematoda some homeobox genes are utilized as molecular switches regulating transition between different life cycle stages.

[Discussion]

Both species selected for our study are characterized by the life cycles with two hosts and the presence of a rediae stage. Due to their phylogenetic positions and well retained ancestral traits these species can be regarded as good models to study the molecular basis the life cycle regulation in the Trematoda. Successful maintenance of *P.simillimum* and *S.pseudoglobulus* under laboratory conditions allowed us to obtain sufficient amounts of material for transcriptomic analysis of all individual stages of their life cycles except for miracidia and metacercaria.

In the absence of genomic references for *P.simillimum* and *S.pseudoglobulus*, their transcriptomes were assembled *de novo* and quality controls suggest that both assemblies are of high quality and completeness (Table 2). The absence of similar sets of the metazoa-specific single-copy orthologs (BUSCOs) in our species and NCBI datasets strongly of other flatworms suggests the evolutionary trend towards considerable gene losses in Trematoda. Probably, observed gene loss is the direct consequence of a parasitic lifestyle. Another explanation for the similar set of “missing” orthologs between our transcriptomes and genomic data may be caused by the absence of their expression in the analyzed stages of the life cycle. In that case, the results of the genome analyses may be compromised by inaccurate *ab initio* identification of coding sequences by gene predicting software. This scenario is corroborated by the observation that on average approximately 23.3% of the BUSCO orthologs from the metazoa_odb9 are “missing” in the analyzed genomes, while only 10.825% of BUSCOs on average are not found in the transcriptomes (Table S1).

The results of the comparative gene expression analysis within the life cycles indicate that the majority of genes in trematodes are characterized by a stage-specific expression: 66% in *P.simillimum* and 88% in *S.pseudoglobulus* belong to this category. The absence of significant overlap between the sets of genes with stage-specific expression is clearly noticeable when analyzing *P.simillimum* data (fig.2A). Thus, our approach allows the identification of distinct molecular markers for future analysis of evolutionary trends in each of the stages. In our opinion, the similarities between the gene sets associated with the rediae and cercariae in *S.pseudoglobulus*

(fig.2B) are caused by the presence of developing embryos of the cercaria stage inside of the rediae. It is almost impossible to separate them from each other (see Fig.2E). Detected rediae-specific expression of *Wnt* and homeobox-containing genes (fig.2D, F) and results of enrichment analysis (Table S10) may also suggest the active cercariae embryogenesis inside the specimens of the parthenogenetic generation.

Comparison of the expression dynamics throughout the life cycles of two species indicate clear similarities (Fig.2C) but their degree is lower than would be expected from the close phylogenetic relationships of *Psilotrema* and *Spaeridiotrema* within Psilostomatidae. Only 681 pairs of orthologous genes from the total of 7593 OGs found *P.simillimum* and *S.pseudoglobulus* show identical patterns of stage-specific expression indicating that after the separation from the common ancestor a lot of genes have changed their expression patterns.

Two-fold differences between TROM-scores between the adult worms and parthenogenetic stages have several possible implications. Most probably in the course of evolution the expression machinery and gene sets, necessary for amphimictic adult worm formation have been subjected to less changes. As a result, in terms of their transcriptomic signatures, the adult worm of *Psilotrema* and *Sphaeridiotrema* remained much more similar than their corresponding rediae and cercaria stages. What might be the reason for that? It is possible that their definitive hosts (birds) are much more similar than their intermediate hosts (mollusks) and it took much less changes in order to adjust the physiology and morphology of the worms to their hosts.

In both *P.simillimum* and *S.pseudoglobulus*, the majority of genes are transcriptionally active in the adult worms. In *P.simillimum*, the number of sequences associated with this stage differs less than twice from those related with rediae or cercariae. At the same time in *S.pseudoglobulus*, this difference is larger more than 5.5 times. Such striking differences between the adult worms and other stages of *S. pseudoglobulus* life cycle can reflect the diversity and intensity of the processes taking place in the adult worm body as well as may be a result of the presence of several transcriptional activity “sources” in the collected material. We can suggest that the miracidium eggs that are formed in the body of an adult worm as well can be regarded as candidates for these “sources”. Another explanation of this phenomenon may be the presence of cryptic species. Sympatric populations of two morphologically similar species - *S. globulus* and *S. pseudoglobulus*, have already been reported earlier in the study of McLaughlin²⁵ and Bergmame et al.²⁶. The presence of two cryptic species in the *S. pseudoglobulus* population would be a good explanation of the fact that the majority of the orthologues sequences (81.6%, Table 2) from the “metazoan-odb9” database are reported as duplicated in this transcriptome.

1 Complex life cycle of the trematodes is a system of adaptations ⁵. Every stage has distinct
2 functional contribution to the implementation of the life cycle. Results of the transcriptomic
3 analysis clearly demonstrate the concept of functional complementation among different stages. The
4 major role of the parasitic stage is the increase in the number of parasite individuals at the expense
5 of the host resources. Accordingly, the rediae stage is associated with the pathways involved in
6 protein and RNA synthesis, cell proliferation and cell differentiation (see Wnt signaling pathway,
7 Fig.2D), size regulation during organogenesis (Hippo signaling pathway) and the control of the
8 nervous system formation (Axon guidance). These pathways are also important for the processes
9 associated with cercariae embryogenesis. In the mature worms active assimilation of host resources
10 (Endocytosis; Phagosome; Gastric acid secretion; Ubiquitin mediated proteolysis) and preparation
11 for reproduction (Cell cycle; Oocyte meiosis) are the most important processes. Free-living larvae
12 need to locate the suitable place for encystment. The increased activity of the pathways associated
13 with signal transduction (Neuroactive ligand-receptor interaction; Calcium signaling pathway,
14 cAMP signaling pathway; cGMP-PKG signaling pathway) and muscle contraction (Adrenergic
15 signaling in cardiomyocytes; Vascular smooth muscle contraction), at the cercaria stage in both
16 species could be directly related with the active locomotion and environmental scan.

17 **[Conclusion]**

18 Established culturing methods and the availability of transcriptomes provide the basis for future
19 comparative and experimental research and make *P.simillimum* and *S.pseudoglobulus* perspective
20 models to study the evolution of trematode life cycles. While from the current dataset the adult
21 worm stage seems to be the most conserved in term of gene expression profile between two species,
22 comparative data of the miracidium and metacercaria stages as well as qualitative and quantitative
23 differences in the cellular composition of life cycle stages would be important in the future.

24 **[Material and Methods]**

25 **RNA isolation and sequencing**

26 Cercariae and parasitic stages (rediae and adult worms), recovered from the hosts, were fixed and
27 stored in the IntactRNA (Eurogene, Moscow, Russia) according to the manufacturer instructions.
28 The stage-specific samples contained approximately 7 adult worms, 100 rediae or 300 cercariae
29 respectively. Prior to RNA isolation, the intactRNA-fixed samples were rinsed in 0.1M phosphate-
30 buffered saline (PBS). The total RNA isolation was done using Quick-RNA™ Microprep kit
31 (R1050, Zymo Research, Irvine, California, USA). For every life cycle stage, two biological
32 replicates were made. The libraries were synthesized using NEBNext Ultra Directional RNA
33 Library Prep Kit for Illumina (E7760, New England BioLabs, Ipswich, Massachusetts, USA).

1 Paired-end sequencing was carried out using Illumina HiSeq 2500 instrument (Illumina, San-
2 Diego, California, USA).

3 **Libraries analysis**

4 The quality of paired-end read data was manually assessed using FastQC (v0.11.5). Sequencing
5 error correction was performed by Karet (v1.0) (--celltype=diploid --matchtype=hamming)²⁷. All
6 libraries were checked for human tissue contamination (Encode: GRCh38, primary assembly) using
7 BBTools packages (v37.02). Additionally, the reference transcriptome of *Bithynia siamensis*
8 *goniomphalos*²⁸ and *Gallus gallus* (Encode: *Gallus gallus* v5.0) genome were used for verification
9 of rediae and adult worms libraries host tissue contamination. Sequencing adaptors and nucleotides
10 with a Phred quality score below of 20 removing was performed by Trimmomatic (v0.36)²⁹.

11 **De novo assembly**

12 For each species the prepared RNA-seq data were pooled together and used to *de novo* assembly of
13 the reference transcriptomes using Trinity (v2.3.2)³⁰. From both transcriptomes only the contigs
14 longer than 200 nucleotides were classified as “good” by the TransRate software (v1.0.1)³¹ and
15 were selected for farther analysis. Isoform clustering was carried out with CDHIT-EST (-c 0.95)
16 (v4.6)³². The presence of the Metazoan single-copy orthologues in both reference transcriptomes
17 was verified using BUSCO (v3.0.1) (metazoan-odb9, evaluate = 1e-3, sp = schistosoma)^{33,34}.

18 The sequences from the latest database may be missing in transcriptomes, both due to the lack of
19 their expression on the analyzed stages and the Platyhelminthes parasitic way of life. Taking this
20 into account, the Metazoan single-copy orthologues searching was also carried out using BUSCO
21 pipeline with same parameters for publicly available *Trichobilharzia regenti*²³ and *Opisthorchis*
22 *felineus*²² transcriptomes, as well as *Schistosoma mansoni* (version 7)³⁵, *S.haematobium*
23 (SchHae_1.0)³⁶, *S.japonicum* (ASM15177v1)⁹, *Opisthorchis viverrini* (*OpiViv1.0*)³⁷, *Clonorchis*
24 *sinensis* (C_sinensis-2.0)³⁸ and *Fasciola hepatica* (Fasciola_10x_pilon)³⁹ genomes.

25 **Aminoacid sequences annotation and orthologues groups searching**

26 The nucleotide sequences were compared with NCBI non-redundant protein database using
27 Diamond BLASTx (v0.9.22; evaluate = 1e-5)⁴⁰. Only contigs, that had no match with database or
28 had similarity with sequences from Digenea, were included in further analysis. Their aminoacid
29 sequences identified by the ESTscan⁴¹ were re-compared to the NR database using Diamond
30 BLASTp (v0.9.22; evaluate = 1e-5).

31 PANNZER2 (Protein ANnotation with Z-scoRE) service⁴² was used for the function description
32 and Gene Ontology classes prediction. Only hits of the first rank were taken for further analysis.

1 The functional annotation of the predicted amino acid sequences was carried out with
2 KOBAS3.0^{43,44}. *Homo sapiens* and *Schistosoma mansoni* were chosen as references.

3 Reference proteomes of *Schistosoma mansoni* (UP000008854), *Trichobilharzia regenti*
4 (UP000050795), *Opisthorchis viverrini* (UP000054324), *Clonorchis sinensis* (UP00008909),
5 *Fasciola hepatica* (UP000230066) from UniProt database together with predicted amino acid
6 sequences of *Opisthorchis felinus*²², *Psilotrema simillimum* and *Sphaeridiotrema pseudoglobulus*
7 transcriptomes as well as *Schmidtea mediterranea* (Rhabditophora: Dugesiida) proteins (SmedGD;
8 genome annotations ver.4.0)⁴⁵ were used as input for the OrthoFinder (v2.2.6)⁴⁶.

9 **Expression level quantification**

10 Salmon (v0.8.2)⁴⁷ was used for expression level quantification (-l A -gcBias). The analysis was
11 performed on the average of two biological replicates TPM (Transcripts Per Million) values,
12 normalized using the “network centrality analysis” method⁴⁸. The genes with expression level
13 below 1 TPM in all analyzed stages were excluded from subsequent analysis.

14 **Life cycle stages comparison**

15 The Jongeneel’s specificity measure⁴⁸ was used to determine stage-specific genes. Only the genes
16 with positive value (i.e. the gene activity was observed in one stage at a level higher than the sum of
17 all other stages combined) were considered as stage-specific.

18 The Transcriptome Overlap Measure (TROM) analysis (v1.3)²⁴ was performed to find the
19 similarity between stage-associated genes sets within life cycles and between two species. The
20 stage-associated genes set include stage-specific sequences according to the Jongeneel’s criterion as
21 well as that are active on analyzed, but not all life cycle stages.

22 The enrichment analysis of the reconstructed pathways with stage-specific genes was carried out
23 using the KOBAS resource (v3.0). The *Homo sapiens* pathways were chosen as the background,
24 and the Fisher’s exact test with Benjamini and Hochberg FDR correction method was used for the
25 statistical analysis. We considered the pathway as “enriched” only when its corrected p-value was
26 below 0.05.

27 **Immunolabeling and confocal scanning microscopy**

28 For immunostaining studies, the samples were fixed in a paraformaldehyde 4% solution in 0,1 M
29 phosphate buffered saline (PBS) for 8 hours at +4°C, then washed in 0,1 M PBS, incubated in 5%
30 Triton X100 solution in PBS for 24 hours, and blocked in 1% solution of bovine serum albumin in
31 PBS for 6 hours. The blocked specimens were incubated in mixture of rabbit anti-5-HT (S5545,
32 Sigma-Aldrich, St. Louis, USA, diluted 1:1000) and rabbit anti-FMRamide antibodies (AB15348,

1 EMD Millipore, Burlington, Massachusetts, USA, diluted 1:1000). Rediae were incubated in
2 primary antibody for 24 hours, then washed in PBS with 0,1% Triton X100 and incubated in the
3 secondary anti-rabbit CF488 antibody (SAB4600044, Sigma-Aldrich, diluted 1:1000) for 8 hours.
4 After antibody incubations the samples were washed in PBS and mounted in glycerol. The
5 specimens were examined under Leica TCS SP5 MP confocal laser scanning microscope (Leica
6 Microsystems, Wetzlar, Germany) and analyzed using Fiji software⁴⁹.

7 **Scanning electron microscopy**

8 For scanning electron microscopy, the animals were fixed in a 2,5% glutaraldehyde solution in 0,05
9 M sodium cacodilate buffer with a postfixation in 2% osmium tetroxide in 0,05 M sodium
10 cacodilate buffer. The fixed samples were dehydrated in ethanol series of increasing concentration,
11 transferred to anhydrous acetone, critical point dried, sputtered with platinum, and examined using
12 Tescan MIRA3 LMU scanning electron microscope (Tescan, Brno, Czech Republic).

13 **Availability of data**

14 BioProject has been deposited at NCBI under accession PRJNA516017. *Sphaeridiotrema*
15 *pseudoglobulus* and *Psilotrema simillimum* Transcriptome Shotgun Assembly projects have been
16 deposited at DDBJ/EMBL/GenBank under the accession GHGK000000000 and GHGL000000000,
17 respectively. The versions described in this paper are the first versions, GHGK010000000 and
18 GHGL010000000, respectively.

19 **[Ethical approval]**

20 All applicable international, national, and institutional guidelines for the care and use of animals
21 were followed. We neither used endangered species nor were the investigated animals collected in
22 protected areas.

23 **[Consent for publication]**

24 Not applicable

25

26 **[Competing interests]**

27 The authors declare that they have no competing interests.

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[Authors' contributions]

MAN, VVS, SAD and SVS collected the experimental material. MAN and VVS made the RNA isolations. ARM prepared libraries for RNA-seq and performed the run of the instrument. SAD and SVS carried out the confocal and electron scanning microscopy. MAN and KVK conducted the data analysis. MAN, VVS and KVK wrote the manuscript draft, AIG and AAD contributed substantially to the interpretation of data and to the writing of the manuscript. All authors read and approved the final manuscript.

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[Tables]

Table 1: Libraries preparation

	<i>Psilotrema simillimum</i>					
Metrics\Samples	Rediae (1)	Rediae (2)	Cercaria (1)	Cercaria (2)	Adult worm (1)	Adult worm (2)
Number of raw reads pairs	29876923	30629840	30613174	24004011	23397182	23760802
Human contamination (%)	0.91%	0.88%	0.93%	1.47%	0.65%	0.71%
Mollusk contamination (%)	4.43%	5.40%	X	X	X	X
Chicken contamination (%)	X	X	X	X	0.99%	0.29%
Trimmomatic survived (%)	85.96%	86.31%	71.59%	92.74%	90.91%	92.90%
	<i>Sphaeridiotrema pseudoglobulus</i>					
Metrics\Samples	Rediae (1)	Rediae (2)	Cercaria (1)	Cercaria (2)	Adult worm (1)	Adult worm (2)
Number of raw reads pairs	28192806	24510459	32082446	24737749	29331490	25717702
Human contamination (%)	0.49%	0.51%	0.85%	0.85%	0.61%	0.41%
Mollusk contamination (%)	4.36%	4.28%	X	X	X	X
Chicken contamination (%)	X	X	X	X	0.70%	0.22%
Trimmomatic survived (%)	75.94%	88.93%	70.45%	92.79%	80.22%	93.16%

(1|2) – first and second biological replicates, respectively; Human|Mollusk|Chicken – the proportion of potential Human|Mollusk|Chicken-derived read pairs in sample; Trimmomatic survived – the proportion of read pairs remaining after trimming step

Table 2: General Statistics of reference transcriptomes quality and completeness measure

Metrics \ Assemblies	<i>P.simillimum</i>	<i>S.pseudoglobulus</i>
Number of sequences	163266	203659
Number of sequences over 1 kilobase	28900	38618
ESTScan: Number of ORF	21075	49626
N50 (nt)	917	958
BUSCO: Complete and Single (%)	62.7	6.5
BUSCO: Complete and Duplicated (%)	24.4	81.6
BUSCO: Fragmented (%)	5.1	4.0
BUSCO: Missing (%)	9.0	9.3
Fragments mapped (%)	92	89
Good mapping (%)	81	75
Bases uncovered (%)	4	4
Contigs uncovbase (%)	49	50
Contigs uncovered (%)	5	4
Contigs lowcovered (%)	64	50
Contigs segmented (%)	10	13
TransRate assembly score	0.237	0.2095
TransRate optimal score	0.4566	0.4183
Good contigs (%)	72	72

BUSCO: Complete and Single | Complete and Duplicated | Fragmented | Missing – the proportion of complete and single-copy | complete and duplicated | fragmented | missing orthologues from Metazoa-odb9 in transcriptomes; Fragments mapped – the total number of read pairs mapping; good mapping – the number of read pairs mapping in a way indicative of good assembly (both members of the pair are aligned; in the correct orientation; on the same contig; without overlapping either end of the contig); Bases uncovered – the propotion of bases that are not covered by any reads; Contigs uncovbase – the proportion of contigs that contain at least one base with no read coverage; Contigs uncovered – the proportion of contigs that have a mean per-base read coverage of < 1; Contigs lowcovered – the proportion of contigs that have a mean per-base read coverage of < 10; Contigs segmented – the proportion of contigs that have >= 50% estimated chance of being segmented; TransRate Assembly score – the geometric mean of all contig scores multiplied by the proportion of input reads that provide positive support for the assembly; TransRate optimal score – TransRate score of the subset of well-assembled contigs; Good contigs – the proportion of contigs with high TransRate scores in transcriptomes.

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2 [Figure legends]

3 **Figure 1: Complex life cycle, similarities and differences between life cycle stages and**
4 **Trematodes species. (A)** The realization of Psilostomatidae complex life cycle in wild (yellow
5 arrows) and laboratory (green arrows) conditions; 1a – Definitive host in the wild (waterfowl birds),
6 1b – Definitive host in the lab (chicken), 2 and 3 – first and second intermediate hosts (mollusk,
7 *Bithynia tentaculata*). **(B-G)** Scanning electron microscope of *Psilotrema simillimum* **(B-D)** and
8 *Sphaeridiotrema pseudoglobulus* **(E-G)** life cycle stages; **(B, E)** – rediae (parasitic stage of
9 parthenogenetic generation), **(C, F)** – cercariae (free-living larvae of amphimictic generation), **(D,**
10 **G)** – adult worms of amphimictic generation. **(H)** Number of shared orthogroups between
11 *Schmidtea mediterranea*, *Opisthorchis felinus*, *O.viverrini*, *Clonorchis sinensis*, *Schistosoma*
12 *mansoni*, *Trichobilharzia regenti*, *Fasciola hepatica*, *Psilotrema simillimum* and *Sphaeridiotrema*
13 *pseudoglobulus*. Based of gene sets there are three clusters corresponding to the Opisthorchiidae,
14 Schistosomatidae and Echinostomata. **(I)** Venn diagram shows relation between sets of
15 orthogroups, including *Schistosoma mansoni*, *Schmidtea mediterranea*, *Psilotrema simillimum* and
16 *Sphaeridiotrema pseudoglobulus*. **(J-K)** Venn diagram shows the number of genes with stage-
17 specific expression in *P.simillimum* **(J)** and *S.pseudoglobulus* **(K)**.

18 **Figure 2: Similarities and differences between molecular signatures of complex life cycles**
19 **stages. (A,B)** Overlap measure between *Psilotrema simillimum* **(A)** and *Sphaeridiotrema*
20 *pseudoglobulus* **(B)** stage-associated gene sets within one complex life cycle. In contrast to
21 *P.simillimum* where stage-associated sets are similar to themselves only **(A)**, in *S.pseudoglobulus*
22 there is a significant overlap between the genes specifically expressed in rediae and cercariae stages
23 **(B)**. **(C)** Overlap measure between *Psilotrema simillimum* and *Sphaeridiotrema pseudoglobulus*
24 stage-associated gene sets. The two-fold differences between Transcriptomes Overlap Measure
25 (TROM) scores obtained for *P.simillimum* and *S.pseudoglobulus* adult worms and for the rediae and
26 cercariae stages of both species is present. A weak “overlap” is also present between
27 *S.pseudoglobulus* rediae and *P.simillimum* cercariae. **(D)** Expression of Wnt family genes in
28 *P.simillimum* life stages. **(E)** Confocal microscopy of *P.simillimum* rediae with developing cercariae
29 embryos. **(F)** Expression of homeobox-containing genes in complex life cycle stages.

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2 **[Supplementary materials]**

3 Table S1. BUSCO Metazoa_odb9 results

4 Table S2. BUSCO Common missing

5 Table S3. OrthoFinder_Statistics

6 Table S4. OrthoFinder_StatisticPerSpecies

7 Table S5. Psilotrema_simillimum_rediae_KEGG_enrichment

8 Table S6. Psilotrema_simillimum_cercariae_KEGG_enrichment

9 Table S7. Psilotrema_simillimum_adult_worm_KEGG_enrichment

10 Table S8. Sphaeridiotrema_pseudoglobulus_rediae_KEGG_enrichment

11 Table S9. Sphaeridiotrema_pseudoglobulus_cercariae_KEGG_enrichment

12 Table S10. Sphaeridiotrema_pseudoglobulus_rediae_and_cercariae_overlap_KEGG_enrichment

13 Table S11. Sphaeridiotrema_pseudoglobulus_adult_worm_KEGG_enrichment

14 Table S12. Psilotrema_simillimum_combined_results

15 Table S13. Sphaeridiotrema_pseudoglobulus_combined_results