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Identification and profiling of microRNAs in two developmental stages of the model cestode parasite *Mesocestoides corti*

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ABSTRACT

MicroRNAs (miRNAs), a class of small non-coding RNAs, are key regulators of gene expression at post-transcriptional level and play essential roles in fundamental biological processes such as metabolism and development. The particular developmental characteristics of cestode parasites highlight the importance of studying miRNA gene regulation in these organisms. Here, we performed a comprehensive analysis of miRNAs in two developmental stages of the model cestode *Mesocestoides corti*. Using a high-throughput sequencing approach, we found transcriptional evidence of 42 miRNA loci in tetrathyridia larvae and strobilated worms. Tetrathyridium and strobilated worm-specific miRNAs were found, as well as differentialy expressed miRNAs between these developmental stages, suggesting miRNA regulation of stage-specific features. Moreover, it was shown that uridylation is a differential mechanism of post-transcriptional modification of *M. corti* miRNAs. The whole set of *M. corti* miRNAs represent 33 unique miRNA families, and confirm the remarkable loss of conserved miRNA families within platyhelminth parasites, reflecting their relatively low morphological complexity and high adaptation to parasitism. Overall, the presented results provide a valuable platform to studies aiming to identify and characterize novel miRNA-based molecular mechanisms of post-transcriptional gene regulation in cestodes, necessary for the elucidation of developmental aspects of the complex biology of these parasites.

1. Introduction

Tapeworms (phylum Platyhelminthes, class Cestoda) are obligate internal parasites of vertebrates that display a wide range of body forms and host associations. These parasites have evolved their own strategy for achieving enormous reproductive capability by increased fecundity through the serial repetition of their reproductive organs (proglottization). The strobilated condition, being strobilation the formation of strobila or segments [1], is believed to have evolved through a stepwise pattern in which proglottization and external segmentation were independent evolutionary events [2,3].

Despite several studies in the field of cestode biology, little is known about the molecular mechanisms underlying the developmental processes of these organisms, including strobilation. An increasing number of studies have been conducted to characterize genes and proteins involved in cestode development [4–7], but the current knowledge is still limited to define molecular pathways involved in the strobilation and other typical cestode developmental processes [8].

Mesocestoides corti is an endoparasitic platyhelminth used as experimental model to study the class Cestoda [9]. It is easily cultivated, is regarded as non-infective for humans, and lacks some of the experimental limitations associated with the work with other

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cestode parasites, including those concerning the availability of biological material and *in vitro* development capacity. The *in vitro M. corti* culture system allows monitoring of the whole strobilation process, from the larval metacestode stage (tetrathyridium) to the adult segmented worm, under controlled experimental conditions [10].

Small noncoding RNAs belonging to the microRNA (miRNA) class have emerged as important regulators of metazoan gene expression [11], being involved in many different biological processes, including development [12]. miRNAs are endogenous ~23 nt-long RNAs that play their gene-regulatory roles by pairing to target mRNAs to direct their post-transcriptional repression [13]. In the canonical biogenesis pathway, miRNAs are transcribed by RNA polymerase II into long primary miRNAs (pri-miRNAs) that are processed in the nucleus by a ribonuclease III (Drosha), generating the characteristic small hairpin known as pre-miRNA or precursor (~70 nt). In the cytoplasm, Dicer, another RNAse III protein, cleaves the pre-miRNA producing a short duplex of ~21–22 bp. In this duplex, the mature sequence is the functional one, while the complementary strand is called the miRNA star (miRNA*) strand [14]. This miRNA-miRNA* duplex is loaded into a RNA-induced silencing complex (RISC), in which the core RNA-binding protein is a member of the Argonaute family (AGO) [15]. Then, the star sequence is degraded and the mature sequence guides the AGO proteins to complementary mRNA sequences to repress their expression. The sequence comprising nucleotides 2–7 within the 5′-end of the mature miRNA is known as the 'seed' sequence, and is key for the miRNA-mRNA target interaction [16,17]. Mature miRNAs are subject to active regulation by editing, which could influence its stability or function. One mechanism of miRNA editing involves tailing (3′ addition of non-templated nucleotides, like uridine) and possibly induces degradation [18].

With the exception of some unicellular organisms, the key RISC machinery has been shown to be conserved across eukaryotes [19]. Within the phylum Platyhelminthes, the RISC machinery has been identified *in silico* in the free living turbellarian species [20], as well as in cestode and trematode parasite species [5,20–22], indicating that gene expression regulation by miRNAs is present in this phylum. Due to the increasingly affordable costs of next-generation sequencing (NGS), and from the rapid development of reliable quantitative methods for miRNA detection, data regarding miRNA profiles in parasitic helminths and host (tissue or circulating) miRNAs in response to helminth infection have expanded considerably in recent years [23]. For tapeworms, these studies have been largely limited to species of medical or economic importance such as *Echinococcus* spp. and *Taenia* spp. In fact, the first report of miRNAs in cestodes was performed for *E. granulosus* [24]. The miRNA population of *Echinococcus granulosus, Echinococcus multilocularis* and *Echinococcus canadensis* has been updated [25–27], but the data available for other tapeworms, such as *Taenia saginata* and *Taenia multiceps*, is still fragmented or absent [28,29]. Identification of the miRNAs expressed in *M. corti* may shed light on the mechanisms involved in the development of cestode parasites and provide information about the mechanism underlying the regulation of strobilation. In addition, gaining knowledge of the miRNA repertoire of a model species from the class Cestoda would provide support for comprehensive miRNA comparative analyses, and to phylogenetic and evolutionary studies within the phylum Platyhelminthes.

In this study, we performed for the first time a comprehensive analysis of miRNAs in the tetrathyridia larvae and strobilated worm of the model cestode *M. corti* using a high-throughput approach. A differential expression analysis was performed in order to identify regulated miRNAs between life cycle stages, which might suggest a role in maintaining the features of each developmental stage. The conservation analysis of miRNA families among related species was performed in order to address the reported loss of conserved miRNA families in platyhelminth parasites [30], which correlates to their low morphological complexity and high adaptation to parasitism. This study will provide valuable information for better understanding the complex biology of this parasite and other cestodes, and shed light on the molecular mechanisms underpinning the strobilation process.

2. Materials and methods

2.1. Parasite material

Mesocestoides corti larvae (tetrathyridia, TT) were maintained by alternate, serial passages in Wistar female rats and BALB/c female mice as previously described [9]. Experimental hosts were infected by intraperitoneal inoculation and, after 3 months, larvae were collected and used for experiments. Only TT from up to the third serial passage in mice were used for the experiments. Biological triplicates were used; each replicate produced using TT obtained from a single mouse host.

Larvae freshly collected from mice were washed six times in PBS plus streptomycin-penicillin (5 U/ml) and used immediately for culture. TT (n = 125) were kept in culture for 24 h in modified RPMI 1640 medium with 10% FBS (McRPMI) [9] at 37 °C and 5% CO_2. Then, larvae were washed three times with PBS, homogenized in Trizol reagent (Life Technologies) and stored at −80 °C until use for RNA extraction.

Strobilated worms (ST) were obtained from TT (n = 60) cultured under strobilation-inducing conditions as previously described [10]. Briefly, TT (n = 60) were incubated with trypsin 0.662% (w/v) in McRPMI for 24 h at 39 °C and 5% CO_2 for strobilation induction. Induced TT were then cultured in McRPMI without trypsin at 39 °C and 5% CO_2 until complete strobilation, which took around 10–12 days. The culture medium was replaced by fresh McRPMI every 48–72 h and the worms were inspected daily under a Zeiss inverted microscope to follow TT strobilation process and, eventually, to assess the percentage of those that reached the ST stage (which undergone both proglottization and segmentation). Only worm batches with a strobilation rate above 90% were used for experiments. ST were then washed three times with PBS, homogenized in Trizol reagent and stored at −80 °C until used for RNA extraction.

All experimental procedures for *in vivo* maintenance of *M. corti* TT in mouse and rat hosts were previously approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul (Project no. 25726).

2.2. Small RNA isolation

RNA preparations enriched in small RNAs (<200 nt, sRNA) were obtained from TT and ST. RNA extractions were carried out using Trizol according to the manufacturer's instructions and accordingly to [24]. The aqueous phase obtained after the organic phase separation step was enriched in sRNAs (RNAs < 200 nt) with the mirVana miRNA Isolation Kit (Ambion). Resulting RNA samples enriched in sRNAs were precipitated overnight at −20 °C with 0.1 vol of 3 M sodium acetate (pH 5.2) and 2.5 vols of 100% ethanol, RNA was centrifuged at 14,000*g* for 30 min at 4 °C, air dried at room temperature, and resuspended in nuclease-free water. RNA concentration and integrity were determined using a Nanodrop 2000 (Thermo) and an Agilent 2100 Bioanalyzer (Agilent Technologies), respectively.

2.3. sRNA library construction and sequencing

For each sRNA library construction, 100–200 ng of an RNA sample enriched in sRNAs were used as starting material. For each

parasite developmental stage, TT and ST, three libraries were constructed from independent samples (biological replicates). Libraries were constructed with the TruSeq Small RNA Sample Preparation Kit (Illumina) according to the manufacturerís instructions. Library size selection was performed in order to recover 18–30 nt-long sRNAs. The sRNA libraries were sequenced in an Illumina HiSeq 2000 sequencer in the same lane for 50 cycles. Library construction and sequencing were performed at Macrogen.

2.4. Source of genome assemblies and annotation

The *M. corti* draft genome assembly version 1.0.4 and CDS annotation was downloaded from the WormBase ParaSite (http://parasite.wormbase.org, Helminth Genomes Consortium). *M. corti* rRNA and tRNA sequences were downloaded from NCBI (http://www.ncbi.nlm.nih.gov). Additional rRNA sequences from flatworms [31] were also retrieved from NCBI. Metazoan mature miRNA and precursor sequences were obtained from miRBase (release 21) [32]. *Echinococcus* spp. mature miRNA and precursor sequences were retrieved from two recently published high throughput analyses [25,26]. All annotated sequences, along with the new miRNA precursor sequences identified in this study, were used to construct an in-house database for sRNA library data classification (sRNA in-house database).

2.5. Bioinformatics analysis of M. corti small RNAs

Small RNA library data were pre-processed using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) prior to its mapping to *M. corti* genome. After adapter trimming, low quality reads and reads <18 nt were removed to obtain clean reads. Identical clean reads were then collapsed into unique sequences with associated read counts. The processed reads were initially mapped to the *M. corti* genome with Bowtie (version 1.1.0) [33]. Then, to classify all sRNA library sequences as miRNAs, rRNA, tRNA, reads mapping in sense orientation to coding sequences (CDS/sense) or reads mapping in antisense orientation to CDS (CDS/antisense), all processed and mapped reads were analysed by BLASTN (with a <0.01 e-value cut off) against an in house database that included all miRNAs identified in this study (as described in "miRNA identification" section) and classified into the above mentioned categories. Reads with no match were grouped into a "no hit" category. A length distribution analysis of total mapped reads was also performed.

2.6. miRNA identification

The miRDeep2 (v 0.0.5) software package [34] was used to identify miRNAs from the sRNA libraries. Unique sequences were mapped to the *M. corti* genome (version 1.0.4) as described in [26]. For miRNA predictions with the miRDeep2 core algorithm, all metazoan mature miRNAs and precursor sequences (retrieved from the miRBase 21) were included. *E. granulosus* s.s., *E. canadensis* and *E. multilocularis* mature miRNA and precursor sequences were also used as input, as these cestode parasites are the closest phylogenetically related species to *M. corti* with available sequences from high throughput analyses [25–27]. The initial miRDeep2 output list of new miRNA precursors of each library was manually curated to generate a final high confidence set of miRNAs retaining only candidate new precursors with i) miRDeep2 score $\geq$5; ii) significant randfold *p*-value; iii) mature reads in the three replicate libraries of a given sample type, and; iv) presence of at least one star read. The secondary structures of putative precursors, with minimum free energy (MFE) less than −20 kcal/mol and with a mature miRNA located in the stem, were predicted using RNAfold (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

The candidate new precursor sequences were then analysed using BLASTN (with a <0.01 e-value cut-off) against sets of rRNAs, tRNAs, and CDS. Predictions that overlapped with these sequence categories were removed.

miRNA names were assigned by miRBase database manager and will be uploaded in the next miRBase version.

2.7. miRNA annotation, identification of families and conservation analysis

To identify orthologous sequences of *M. corti* miRNAs, the full-length mature *M. corti* miRNA sequences were compared to the mature miRNA sequences present in miRBase 21, and with *Echinococcus* spp. mature miRNA sequences retrieved from [25–27] using SSEARCH (http://www.biology.wustl.edu/gcg/ssearch.html). Only sense matches were allowed, with a 70% nucleotide identity cut-off and according to the following seed match criteria: identical nucleotides 1–7 or 2–8 from the 5′ end of the mature miRNA. Those miRNAs that did not meet the above-mentioned requirements were considered new miRNAs. To identify *M. corti* miRNA families, all-against-all pairwise BLAST sequence alignments were performed and all sequences sharing the seed region (nt 1–7 or nt 2–8) were considered to belong to the same family. To analyse evolutionary conservation of *M. corti* miRNA families, mature miRNA sequences were compared to those present in miRBase 21 for selected phyla covering metazoan diversity: Cnidaria, Arthropoda, Annelida and Nematoda and to the subphylum Vertebrata using only a seed match criteria. The absence and presence of *M. corti* miRNA families in selected phyla were registered. In addition, for sequence conservation analysis, the full length mature miRNA sequences of conserved *M. corti* miRNAs identified in this study were aligned against a set of homologous full-length mature sequences of three selected model species: *Mus musculus*, *Drosophila melanogaster*, and *Caenorhabditis elegans*, and against some related species of the Phylum Platyhelminthes: *Schmitdea mediterranea*, *Gyrodactylus salaris*, *Schistosoma mansoni, Schistosoma japonicum*, *E. granulosus* and *E. multilocularis*, using ClustalX [35].

2.8. miRNA abundance and differential expression analysis

For analysis of miRNA abundance levels, the read counts of each individual miRNA within a given sample were normalized to the total number of mature miRNA read counts in that sample [25,26]. Then, normalized miRNA read counts from biological replicates of the same sample type were averaged. A correlation analysis between independent biological replicates from each sample type (TT or ST) was performed. For this purpose, miRNA read counts within a replicate were plotted against miRNA read counts in the other replicate. All miRNAs identified in this study where considered for this analysis. Differential expression analysis of miRNAs between TT and ST was performed using DESeq [36] using raw reads as input. miRNAs expressed in both developmental stages that showed $-1\geq$ log2 fold change ≥ 1 and *p*-adjusted < 0.05 were considered differentially expressed. In addition, a miRNA was considered expressed in TT or ST stages when mature reads were identified in at least two biological replicates of each sample type.

Arm usage was determined by analysing product ratios of the 5′ (5p) and 3′ (3p) arms. When the minor product of a pre-miRNA showed $\geq$50% of reads with respect to the major product, miRNAs produced from both arms were considered mature miRNAs [37]. In the arm usage conservation analysis, data available in miRBase 21 was used for comparative purposes.

2.9. Editing and post-transcriptional modification analysis

miRDeep2 outputs were manually inspected to determine the presence of editing and/or post-transcriptional modifications in mature miRNA sequences. For this analysis, only those sequences with at least 150 read counts [38] and representing more than 2.5% of the total reads for the corresponding mature miRNA in each library were considered. When more than one sequence with the same type of modification for a given miRNA reached these criteria, the read count numbers of the modified sequences were counted. A miRNA was considered to be under the effect of editing or post-transcriptional modifications when the read count number of the modified sequence(s) was ≥10% of the total read counts of the corresponding miRNA [39].

2.10. Experimental validation of miRNAs expression by poly(T) RT-qPCR

Selected miRNAs from TT and ST samples were experimentally validated by poly(T) reverse transcription followed by quantitative PCR (poly(T) RT-qPCR)-based expression analyses [40]. Prior to the reverse transcription reaction, 1 μg of the small RNA fraction was treated with DNase I (Sigma), and then polyadenylated with *Escherichia coli* Poly(A) Polymerase (New England Biolabs) for 60 min at 37 °C in a 20 μl reaction volume, according to the manufacturer's protocols. cDNA was synthesized from 100 ng of polyadenylated small RNAs from either TT or ST using SuperScript III Reverse Transcriptase (Life Technologies) and 0.5 μg of poly(T) adapter in a 20 μl reaction volume according to the manufacturer's protocol. Controls without reverse transcriptase were included for each sample. Reverse transcription was performed by using a 60 min at 50 °C–15 min at 70 °C program. For each RT-qPCR, 5 μl of diluted cDNA (1:100) was mixed with 0.5 μl of each primer (10 μM final concentration), 4 μl 5 × HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne) and 10 μl sterile water in a final volume of 20 μl. RT-qPCRs were performed using a 7500 Fast Real-Time PCR system (Life Technologies). Cycling conditions were: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15, 60 °C for 20 s and 72 °C for 32 s. Dissociation curve analysis was carried out at the end of each qPCR run to verify amplification specificity for each target sequence.

The relative quantification miRNAs was performed using the $2^{-\Delta\Delta CT}$ method [41] using an endogenous constitutive control. Four biological replicates were used and no template controls were included for each primer pair. qPCR reactions were carried out in duplicate and the statistical significance assessed by the Student's *t*-test. The baseline and Cq were manually inspected and determined using the 7500 Software version 1.3.0 (Applied Biosystems). The primer sequences are listed in Supplementary Table 1.

To establish an endogenous control for qPCR experiments, the four most stable miRNAs between TT and ST samples according to sRNA-seq data (let-7-5p, miR-61-3p, miR-71-5p and miR-4989-3p) were selected along with one differentially expressed miRNA (mi*R*-31-5p), used as an expression variability control. The RefFinder tool [42] (Supplementary Table 2) indicated miR-4989 as that with the most stable expression between biological replicates.

2.11. Data access

The small RNAseq data from this study have been deposited in NCBI's Gene Expression Omnibus [43] and are accessible through GEO Series accession number GSE85058 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE85058).

3. Results

3.1. Small RNA sequencing

sRNA libraries were generated from the tetrathyridia larvae (non strobilated) and strobilated worm stages (TT and ST samples, respectively) of *M. corti*'s life cycle in order to define the miRNA repertoire of each stage and to identify miRNAs involved in *M. corti* strobilation. Three biological replicates from each stage (TT or ST) were sequenced, and the sequencing results are summarized in Table 1. The total numbers of raw reads ranged from 31.7 to 35.9 million per sample, and, after removal of low quality reads and adapter sequences, the numbers of clean reads ranged from 16.7 to 24.9 million (50–74% of total reads) per sample. Percentages of reads mapping to the *M. corti* genome ranged from 73.5 to 88.1%, for TT samples, and from 67.9% to 83.7%, for ST samples.

Sequence classification (Fig. 1A) of mapped reads showed that TT sRNA libraries contained, on average, 56% of miRNAs, 6% of rRNA, 3% of reads mapping to CDS/sense, 3% of reads mapping to CDS/antisense, 2% of tRNA, and 29% of sequences with no hit. ST sRNA libraries, in turn, contained on average 74% of miRNAs, 4% of rRNA, 3% of reads mapping to CDS/sense, 3% of reads mapping to CDS/antisense, 3% of tRNA, and 13% of sequences with no hit.

Length distribution analysis (Fig. 1B) showed that the sRNA profile of total mapped reads was similar for both TT and ST samples. The most frequent read length was 22 nt, as expected for Dicer-derived products, thus supporting the presence of mature miRNA molecules in *M. corti*.

3.2. Identification of conserved and new miRNAs from M. corti

After the removal of rRNAs, tRNAs, and reads that mapped to CDS, the remaining reads were used to search for both conserved and new miRNAs using miRDeep2. The conserved and candidate new mature miRNAs that were identified in *M. corti* samples are shown in Table 2.

We considered that both arms of the same hairpin produced two mature miRNAs when the number of read counts of the minor product represented 50% or more of the read counts of the major product, originated from the opposite arm. By doing this, we observed that three miRNAs precursors showed expression from both arms in TT samples, namely mir-153, mir-190, and mir-12068, while other two, mir-153 and mir-12068 showed expression from both arms in ST samples (Supplementary Table 3).

The repertoire of *M. corti* miRNAs is encoded by 42 loci (37 conserved and 5 new miRNAs, Table 2), grouped in 33 families. Out of these miRNAs, 36 conserved and 6 new mature miRNAs were identified in TT samples, while 38 conserved and 5 new mature miRNAs were identified in ST samples. Therefore, the total number of mature miRNAs is 42 in TT samples and 43 in ST samples, while the number of pre-miRNAs is 39 and 41 for TT and ST samples, respectively (Table 2, Supplementary Table 4).

Based on stage specific expression pattern, one new miRNA, miR-12071, was considered TT-specific, and 3 conserved miRNAs, namely miR-2b, miR-7a, and mi*R*-3479b, were considered ST-specific.

Regarding the features of the *M. corti* miRNAs, the mature miRNA sequences from both stages had lengths between ~22-23 nt and most of them (around 73% for both stages) started with an uracil, while precursor sequences had a medium length of 67 nt (Supplementary Table 4). In addition, they present high percentage of identity with mature and precursor sequences of the related cestode parasite *E. multilocularis* (Table 2). Furthermore, all miRNAs identified form stable hairpin structures (minimum free energy ≤ − 17 kcal/mol), which is essential for the processing of pre-miRNA transcripts into mature miRNAs (Supplementary Fig. 1).

Table 1
–Summary of sequencing results obtained from small RNA libraries of *Mesocestoides corti* tetrathyridia (TT) and strobilated worms (ST).

	TT samples			ST samples		
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
Raw reads	33,477,647	33,263,853	33,391,571	31,707,701	32,548,060	35,961,076
Clean reads	16,703,698	17,923,438	24,910,387	21,969,990	23,133,658	20,757,428
Clean reads (≥18nt)	8,743,910	7,947,066	13,640,395	14,232,891	15,785,899	12,573,010
Mapped reads	6,663,322	5,844,848	12,018,162	11,841,006	13,213,856	8,542,583
Percentage of mapped reads[a]	76.2%	73.5%	88.1%	83.2%	83.7%	67.9%
Unique reads	1,302,902	1,286,675	1,656,001	1,309,141	1,383,638	1,227,070

[a] Relative to clean reads.

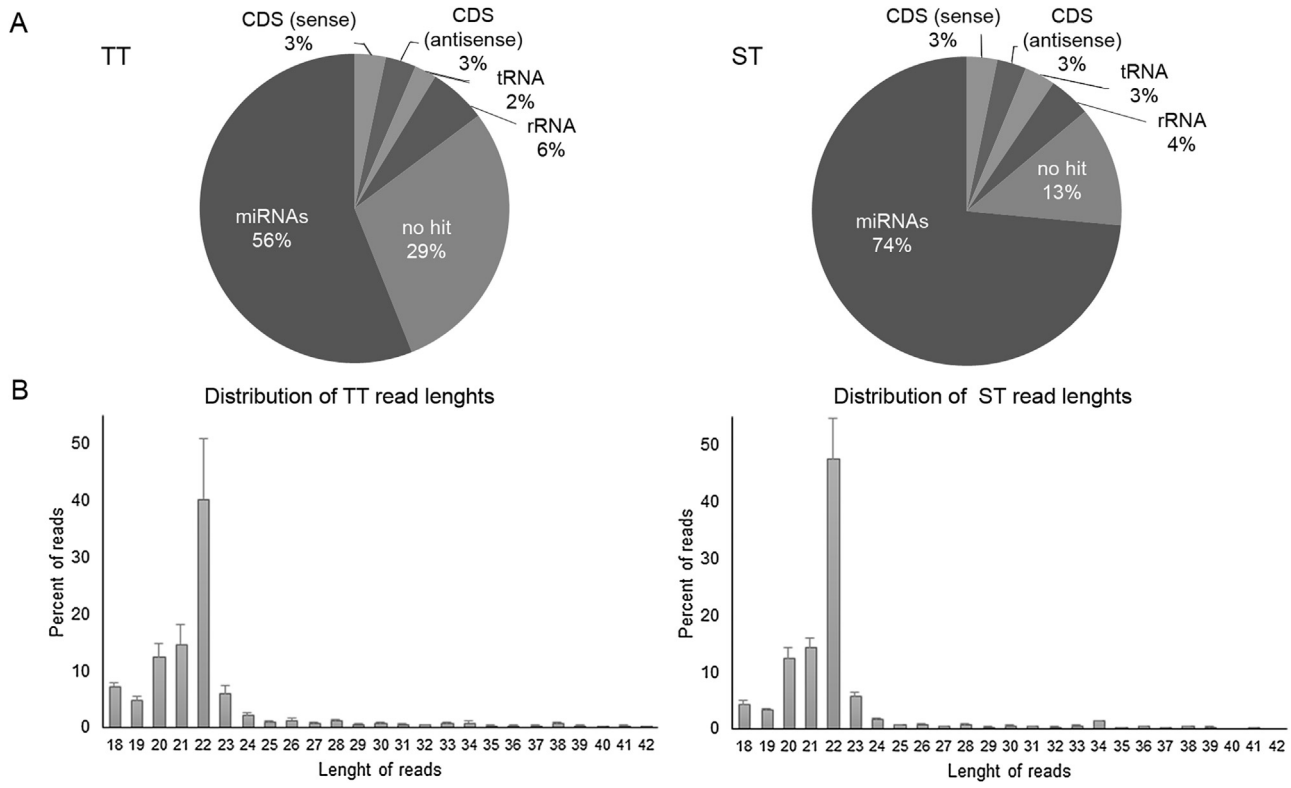

Fig. 1. – Small RNA library composition and length profiles of *Mesocestoides corti* sequencing data. A) Classification of small RNAs of *Mesocestoides corti* tetrathyridia (TT) and strobilated worm (ST): miRNA: microRNAs, rRNA: ribosomal RNAs, tRNA: transfer RNAs, CDS: protein coding sequences, no hit correspond to those not annotated. B) Length distribution analysis of sequenced reads of TT and ST samples; the average proportion in each replicate is displayed.

Seven conserved *M. corti* miRNAs with particularly long hairpins: bantam, mir-7b, mir-36b, mir-87, mir-96, mir-277 and miR-12066 could only be identified after changing the miRDeep2 algorithmic parameters for the excision of hairpins. Star sequence reads were identified for almost all *M. corti* miRNAs adding confidence to the miRNA predictions. Let-7 was one of the first discovered miRNAs and has been described as highly expressed in other platyhelminth parasites [25–27,44,45] indicating the importance of this miRNA in the parasite biology and metabolism. In this work, we detected a high number of reads corresponding to let-7 mature sequence (15–19% of all miRNA reads).The *M. corti* let-7 (mco-let-7) precursor sequence was manually detected by a BLAST search against *M. corti* genome using the *E. multilocularis* let-7 precursor sequence as query. Fig. 2A shows the predicted secondary structure for the identified putative mco-let-7. Its stem-loop structure, characteristic of pre-miRNAs, further confirmed the presence of a let-7 precursor in *M. corti*.

To investigate the possible occurrence of miRNA gene clustering, the genomic arrangement of the *M. corti* identified miRNAs was assessed (Supplementary Table 4). Seven miRNAs were found to be organized in three clusters: mir-1, mir-2b, mir-2c, mir-71, mir-133, mir-277 and mir-4989. Two clusters are formed by two miRNAs (mir-1/133, and mir-277/4989), and one cluster is composed by three miRNAs (mir-71/2c/2b). Fig. 2B and C shows the predicted secondary structures of the mir-277/4989 and mir-71/2c/2b clusters. The predicted secondary structure of the mir-277/4989 cluster forms two main hairpin stem-loop structures and comprises a 208 bp region (contig0000263), while the predicted secondary structure of the mir-71/2c/2b cluster forms the three hairpin stem-loop structure and comprises a 273 bp region (scaffold0000096). In the mir-1/133 cluster, on the other hand, the two encoded miRNAs are located approximately 18 kb apart from each other.

3.3. Expression profiles of M. corti TT and ST miRNAs

The TT and ST miRNAs abundance was assessed by counting the relative number of sequenced reads in each sample (Table 2). The top five predominant miRNAs in TT and in ST samples are shown in Fig. 3. Altogether, they accounted for ~90% of the total miRNA reads in each sample, and, interestingly, they were the same for both life stages (bantam-3p, let-7-5p, miR-10-5p, miR-71-5p, and miR-4989-3p). From these, miR-10 and let-7 accounted for ~75% of the

Table 2
–Catalog of mature microRNAs (miRNAs) of *Mesocestoides corti* TT and ST life cycle stages.

microRNA[a]	miRNA mature sequence[b]	Normalised read counts[c]		miRNA length (nt)	miRNA family[d]	*E. multilocularis*	
		TT	ST			% Identity mature	% Identity precursor
mco-bantam-3p	UGAGAUCGCGAUUAAAGCUGAU	50,967	56,167	22	bantam	95.5	68.9
mco-let-7-5p	UGAGGUAGUGUUUCGAAUGUCU	188,408	150,271	22	let-7	100.0	96.0
mco-miR-1-3p	UGGAAUGUUGUGAAGUAUGU	88	55	20	mir-1	100.0	87.1
mco-miR-2a-3p	UAUCACAGCCCCGCUUGGAACU	2566	3835	22	mir-2	95.0	76.4
mco-miR-2b-3p	UAUCACAGCCCUGCUUGGGAC	ND	8030	21	mir-2	100.0	81.8
mco-miR-2c-3p	UCACAGCCAAUAUUGAUGAAC	8792	6832	21	mir-2	100.0	89.1
mco-miR-7a-5p	UGGAAGACUGGUGAUAUGUUGU	ND	6	22	mir-7	100.0	79.4
mco-miR-7b-3p	CAACUGUCACGGUCUUCCAAGU	44	45	22	mir-7	NA	82.1
mco-miR-9-5p	UCUUUGGUUAUCUAGCUGUGU	3984	1943	21	mir-9	100.0	83.1
mco-miR-10-5p	CACCCUGUAGACCCGAGUUUGA	535,863	604,117	22	mir-10	100.0	81.8
mco-miR-31-5p	UGGCAAGAUACUGGCGAAGCUGA	2	60	23	mir-31	100.0	80.0
mco-miR-36a-3p	CCACCGGGUAGACAUUCAUCCACU	98	12	24	mir-36	94.4	77.8
mco-miR-36b-3p	UCACCGGGUAGCGAUUACGCUU	38	14	22	mir-36	86.4	67.4
mco-miR-61-3p	UGACUAGAAAGUGCACUCACAUC	15,194	10,437	23	mir-279	95.7	78.9
mco-miR-71-5p	UGAAAGACGAUGGUAGUGAGAU	73,359	63,320	22	mir-71	100.0	73.3
mco-miR-87-3p	GUGAGCAAAGUUUCAGGUGUGC	9776	7898	22	mir-87	100.0	79.8
mco-miR-96-5p	AUUGGCACUUUUGGAAUUGU	1882	2073	20	mir-96	100.0	72.2
mco-miR-124a-3p	UAAGGCACGCGGUGAAUGCC	146	131	20	mir-124	100.0	68.5
mco-miR-124b-3p	UAAGGCACGCGGUGAAUAC	153	175	19	mir-124	100.0	89.1
mco-miR-125-5p	UCCCUGAGACCCUAGAGUUGUC	3465	5500	22	mir-125	100.0	82.4
mco-miR-133-3p	UUGGUCCCCAUUAACCAGCCGCU	58	32	23	mir-133	100.0	82.8
mco-miR-153-5p	AUGCUUAUGUGACGUGCACUC	269	165	21	mir-153	ND	81.1
mco-miR-153-3p	UUGCAUAGUCUCAUAAGUGCCA	359	253	22	mir-153	100.0	81.1
mco-miR-184-3p	GGGACGGAAGUCUGAAAGGGUUU	2255	1541	23	mir-184	95.5	91.1
mco-miR-190-5p	AGAUAUGUUUGGGUUACUUGGUG	1798	2545	23	mir-190	100.0	80.0
mco-miR-190-3p	CCAGUGACCGAACAUAUUCACA	1362	ND	22	mir-190	ND	80.0
mco-miR-219-5p	UGAUUGUCCAUUCGCAUUUCUUG	6439	3821	23	mir-219	100.0	86.1
mco-miR-277-3p	UAAAUGCAUUUUCUGGCCCGUAA	10,197	5557	23	mir-277	100.0	83.3
mco-miR-277b-3p	UAAAUGCAAAAUAUCUGGUUAUG	330	187	23	mir-277	100.0	87.7
mco-miR-281-3p	UGUCAUGGAGUUGCUCUCUAU	2395	1849	21	mir-281	100.0	94.3
mco-miR-307-3p	UCACAACCUACUUGAUUGAGGGG	3459	3761	23	mir-67	100.0	74.7
mco-miR-745-3p	UGCUGCCUGGUAAGAGCUGUGA	4279	2623	22	mir-22	100.0	86.0
mco-miR-2162-3p	UAUUAUGCAACAUUUCACUCA	856	612	21	mir-1993	95.0	73.7
mco-miR-3479a-3p	UAUUGCACUUUCUUUCGCCUUC	5468	2410	22	mir-92	90.9	84.2
mco-miR-3479b-3p	GAUUGCACUACCCAUCGCCCUCU	ND	188	23	mir-92	95.5	73.5
mco-miR-4989-3p	AAAAUGCACCAACUAUCUGAGA	62,840	50,555	22	mir-277	100.0	80.0
mco-miR−10293-3p[e]	UAAUUCGAGUCAACAGGGUCGUU	22	5	23	mir−10293[d]	100.0	86.2
mco-miR-12065-3p	UGUGCGUAGUUGCAAUGACCU	389	148	21	mir-210	ND	ND
mco-miR-12066-3p	UUGUGCGUCGUUUCAGUGACCGACA	1828	1434	25	mir-210	ND	ND
mco-miR-12067-3p	UAUGGAUAAGGCGUAUGACUCC	122	97	22	novel	NA	NA
mco-miR-12068-5p	UACCCUCACCGGUCGUGCAAGG	50	54	22	novel	NA	NA
mco-miR-12068-3p	UUGUACUACUGGCGAGGGUAGU	42	56	22	novel	NA	NA
mco-miR-12069-3p	UGUACUACAACAGGCCGUGGCU	277	130	22	novel	NA	NA
mco-miR-12070-3p	GCUACCGAAACGACUUAACUU	63	35	21	novel	NA	NA
mco-miR-12071-5p	GAGGGCCAUUGACAGUCGUUG	18	ND	21	novel	NA	NA

ND, not detected; NA, not aplicable.

[a] miRNA names were assigned by the miRBase database manager and will be uploaded in the next version.

[b] The canonical (100% identical to the reference genome) most frequent read from both biological replicates is reported. When the minor product of a precursor (pre)-miRNA showed a read count number ≥50% with respect to the major product, it was considered as mature miRNA.

[c] miRNA read counts were normalized to the total number of mature miRNAs in each library, multiplied per 1×10^6 and averaged between biological replicates.

[d] Novel families were defined for new miRNAs identified, which didnít show seed conservation.

[e] First reported in *Echinococcus multilocularis* and *E. canadensis* by Cucher et al. (2015, supplementary table S7). The names emu-miR-10293-3p and egr- miR-10293-3p were assigned by the miRBase database manager and will be uploaded in the next version.

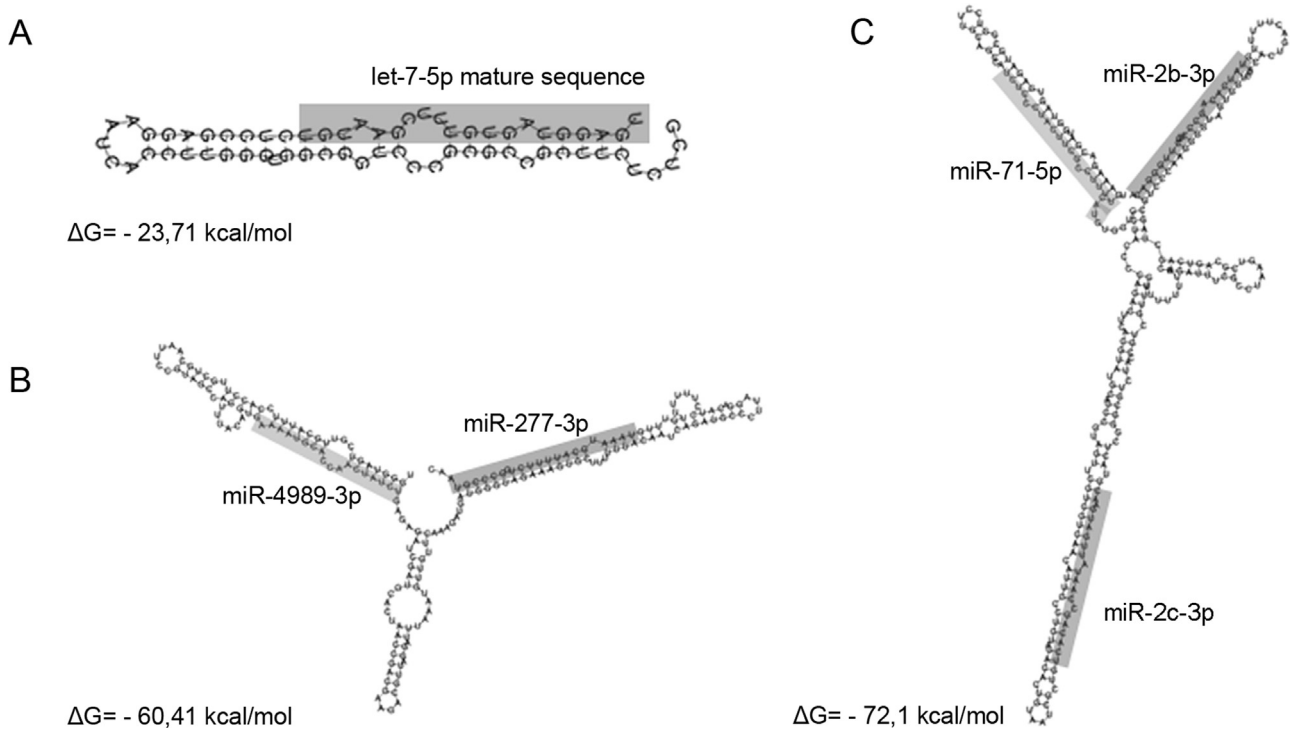

Fig. 2. – Predicted secondary structure of *Mesocestoides corti* miRNA precursors. A) *Mesocestoides corti* miRNA-let-7 precursor secondary structure predicted with RNA fold. B and C) The predicted secondary structures of the *M. corti* miRNA-4989/277 (B) and miRNA-71/2b/2c (C) clusters. The corresponding mature miRNA sequences are highlighted in grey and the free energy (ΔG) is shown.

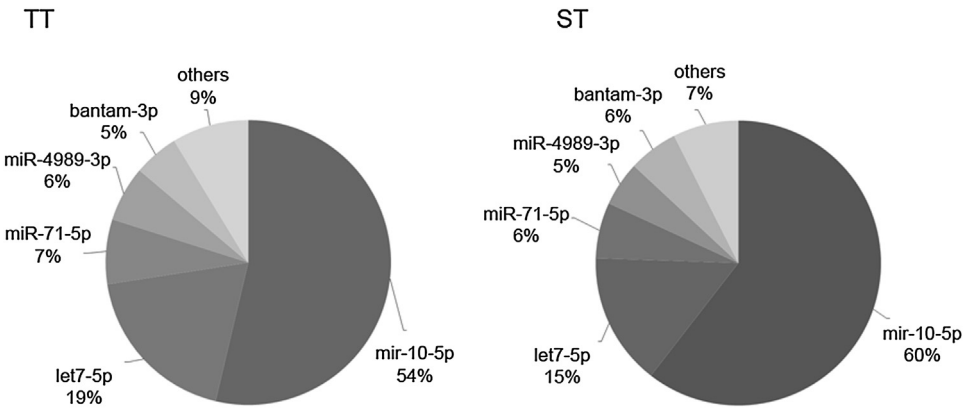

Fig. 3. – Circle chart with the percentages of the five most abundant miRNAs in the two life-stages of *Mesocestoides corti*: tetrathyridia (TT) and strobilated worm (ST). Results are shown as average percentages (± S.D.) of biological replicates.

total miRNA expression in both TT and ST samples. The high expression levels of these five miRNAs in two different life stages suggests their role in constitutive functions, highly relevant for parasite's survival and development. The miRNAs found in *M. corti* TT and ST samples are expressed at very different levels. For instance, most of the miRNAs identified: 83% (35/42) and 86% (37/43) in TT and ST samples, respectively, showed low expression levels, accounting for less than 1% (<100 thousand reads) of the expression levels detected (Table 2, Supplementary Table 4).

Interestingly, some of the miRNAs grouped in clusters showed an asymmetrical transcription pattern. For instance, miR-71-5p is one of the top five most expressed miRNAs, while miR-2b-3p and miR-2c-3p are at least ten fold less expressed (Supplementary Table 4). A very similar pattern is observed regarding the mir-277/4989 cluster, were miR-4989-3p is six fold more expressed than miR-277-3p (Supplementary Table 4). This might suggest the presence of a post-transcriptional mechanism affecting the stability of mature miRNAs organized in the same cluster, since two of *M. corti* miRNA clusters comprises a small genomic region and could be part of a single transcriptional unit.

We also analysed whether each mature miRNA product preferentially originated from one arm of the hairpin. As shown in Supplementary Table 3, around 70% of the mature miRNAs in *M. corti* are processed from the 3p arm of the hairpin, while around 23% are processed from the 5p arm of the hairpin. Three pre-miRNAs precursors showed expression from both arms, namely mir-153, mir190, and mir-12068 in TT samples, and only mir-153 and mir-12068 in ST samples (Supplementary Table 3).

The expression of 6 mature miRNAs, namely bantam-3p, let-7-5p, miR-7a-5p, miR-10-5p, miR-87-3p and miR-4989-3p, was confirmed by RT-qPCR in TT and ST samples (Supplementary Table 5). Furthermore, the high expression levels of let-7-5p and miR-10-

5p detected in the sRNA-seq data were validated in both ST and TT samples.

3.4. Differential expression analysis between TT and ST miRNAs

After the analysis of miRNA abundance, we conducted a comparative expression analysis for those miRNAs identified in both TT and ST samples. First, a correlation analysis was performed between independent biological replicates from each sample type, which showed high reproducibility of the data (Supplementary Fig. 2) thus enabling differential expression analysis. Then, differential expression of miRNAs between TT and ST was assessed using DESeq algorithm (Fig. 4A). We found that mi*R*-36b-3p and the candidate miR-12065-3p were significantly up-regulated in TT, while miR-2a-3p, mi*R*-31-5p, miR-125-5p, and miR-190-5p were up-regulated in ST samples. mi*R*-36b-3p showed the most noticeable up-regulation in the TT stage, with its expression approximately 4-fold higher than in the ST stage, while mi*R*-31-5p presented the most noticeable up-regulation in the ST stage, with an almost 32-fold difference in comparison to that in the TT stage. We also identified some stage-specific miRNAs, expressed only in one developmental stage, namely miR-12071-5p, specific for TT (Fig. 4B), and miR-2b-3p, miR-7a-5p, and mi*R*-3479b-3p, specific for ST (Fig. 4C). These stage-specific miRNAs could be involved in regulating specific features, of the developmental stage in which they are expressed. However, the number of miR-7a-5p and miR-12071-5p reads was very low, implying that stage specificity should be confirmed with higher coverage approach.

Differential expressions of two stage biased miRNAs in TT samples (mi*R*-36b-3p and miR-12065-3p) and three selected stage biased miRNAs in ST samples (mi*R*-31-5p, miR-125-5p, miR-190-5p) was validated by RT-qPCR (Supplementary Table 6). Among them, two miRNAs: mi*R*-31-5p and miR-125-5p were concordant between RNA-seq and RT-qPCR, thus confirming the RNA-seq differential expression data.

3.5. miRNA editing

In order to identify miRNA isoforms (isomiRs), generated by the non-template additions in *M. corti* miRNAs, the presence of uridine (U) at the 3′ end was queried in our datasets. miRNA processing by addition of a single 3p terminal U was found for 13 TT miRNAs and for 15 ST miRNAs (Fig. 5, and Supplementary Table 7). The isomiRs identified in this study were detected in all biological replicates of each sample, and, when identified in both TT and ST samples (11 miRNAs), they corresponded to the same sequence.

In most cases, for both TT (11 out of 13) and ST (13 out of 15), the unmodified form was more abundant. For miR-184-3p and miR-7b-3p, however, the isomer-U presented the same abundance (or even slightly higher) that the unmodified form in both life stages. miR-184-3p presented isomer-U abundances of 53% and 49% in TT and ST, respectively, while miR-7b-3p presented isomer-U abundances of 56% and 46% in TT and ST, respectively. Interestingly, there were differential uridylation patterns between life stages, mi*R*-36b-3p (20%) uridylated only in TT, and mi*R*-31-5p (11%) uridylated only in ST samples. Furthermore, different levels of uridylation were also observed among miRNAs organized in cluster. In one of the identified *M. corti* miRNA clusters (mir-71/2c/2b) only miR-2b-3p was uridylated. In the mir-1/133 cluster, only miR-133-3p was uridylated. Such differential patterns of uridylation are suggest that a possible tailing mechanism of post-transcriptional regulation through the parasite's life cycle is present in *M.* corti.

Primary miRNA editing by adenosine modification into inosine was also investigated in our *M. corti* data sets. We found no significant evidence of adenosine modification to iosine in TT or ST samples.

3.6. Sequence conservation analysis of M. corti miRNAs

All 45 *M. corti* miRNAs identified in this study grouped in 33 miRNA families, based in a seed match criteria. In order to identify conserved and new miRNA families present in *M. corti*, we compared the mature miRNA sequences identified in this study against all miRBase v21 database using only a seed match criteria. This analysis allowed the identification of 28 conserved and 5 new *M. corti* miRNAs families. From the conserved miRNA families, one present in all eumetazoans, 18 in bilaterians, 8 in protostomes and one so far only described in the genus *Echinococcus* (miR-10293).The conserved *M. corti* miRNAs families, shared with selected phyla Cnidaria, Nematoda, Arthropoda, Annelida and with the subphylum Vertebrata are shown in Fig. 6. From the 33 miRNA families identified in *M. corti*, 7 families presented multiple members. The largest ones were the mir-2 family, integrated by miR-2a, miR-2b, and miR-2c, and the mir-277 family, integrated by miR-277, miR-277b and miR-4989. The other *M. corti* miRNA families with multiple members were mir-7 (miR-7a, miR-7b), mir-36 (mi*R*-36a and 36b), mir-124 (miR-124a and 124b), mir-92 (mi*R*-3479a and 3479b) and mir-210 (miR-12065 and miR-12066).

Among the five *M. corti* candidate new miRNAs identified, only one, miR-12067 shared seed sequence (but not 70% of nucleotide identity in the full sequence) with a conserved miRNA, mir-1262, reported in *Homo sapiens* (Fig. 7A). As mentioned before, we identified two members of the mir-210 family (miR-12065 and miR-12066), already described in *S. mediterranea* (phylum Platyhelminthes) and in vertebrates (Fig. 7A), but so far never described in cestode or trematode platyhelminths.

The degree of conservation of *M. corti* miRNAs belonging to conserved families, was assessed comparing their full-length mature sequences against selected species present in miRBase v21, with seed match and ≥70% homology criteria. This comparative analysis was performed against three model species, namely *M. musculus*, *D. melanogaster*, and *C. elegans*, and against some related species of the Phylum Platyhelminthes, namely *S. mediterranea*, *G. salaris*, *S. mansoni, S. japonicum*, *E. granulosus* and *E. multilocularis* (Supplementary Fig. 3). As expected, the majority of the conserved *M. corti* miRNAs (36) have homologs with ≥70% identity in the cestodes *E. multilocularis* and *E. granulosus* (34/36; 94%). On the other hand, the majority of *M. corti* miRNAs (31/36; 86%) have homologs with ≥70% identity in the free living *S. mediterranea* (Turbellaria). Interestingly, a lower number of *M. corti* miRNAs (26 and 23, respectively) are shared with the ectoparasite *G. salaris* (Monogenea), and *S. japonicum* (Trematoda), which also belong to the Neodermata clade. Additionally, 19 (53%), 24 (66%) and 16 (44%) of *M. corti* miRNAs have homologs in *C. elegans* (Nematoda), *D. melanogaster* (Arthropoda), and *M. musculus* (Chordata), respectively. Among all the species analysed, a high conservation was observed among the full-length mature sequences of miR-71 orthologs (Fig. 7B), suggesting functional conservation. On the other hand, some miRNAs, like let-7 and miR-125, seem to be conserved within cestodes, but are highly divergent from their counterparts from other organisms. Other miRNAs, like bantam, showed more conservation among platyhelminth parasites, being more divergent from the other analysed species.

4. Discussion

In this work, we provided experimental evidence of miRNA expression in *M. corti* describing, for the first time, miRNAs reper-

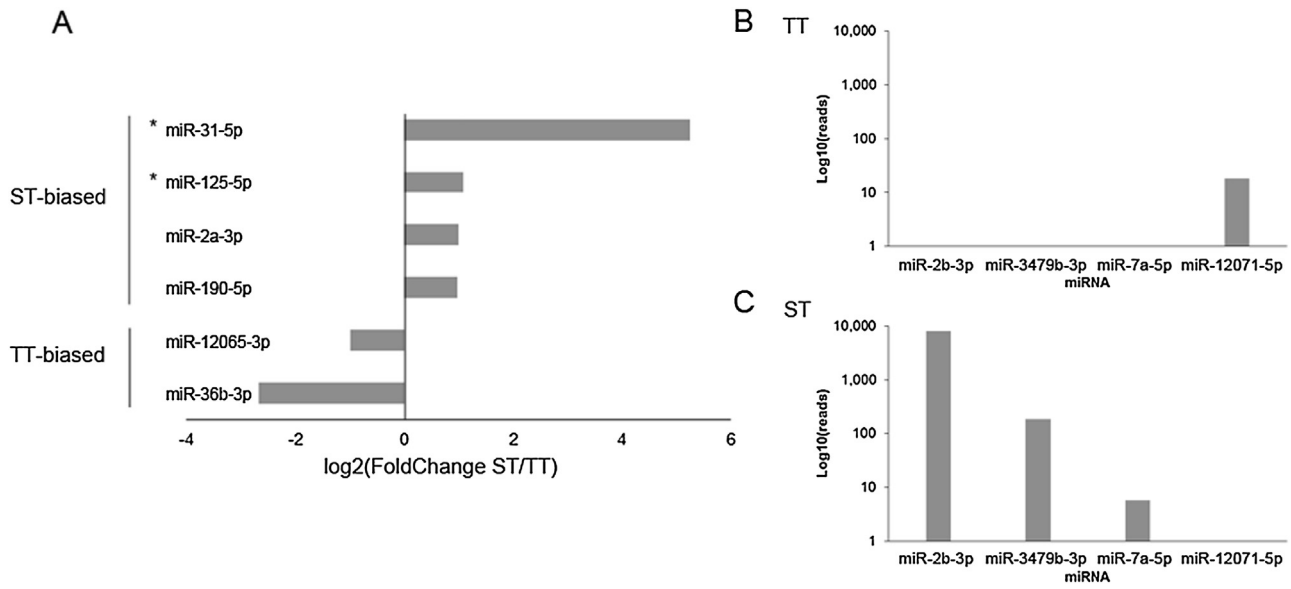

Fig. 4. – Differential expression of microRNAs between tetrathyridia (TT) and strobilated worm (ST) of *Mesocestoides corti*. A) Fold change analysis using DESeq algorithm. miRNA with Log_2 fold change $\geq \pm 1$ and *p*-adjusted < 0.05 are displayed; an asterisk (*) marks stage-biased microRNAs validated by real time PCR (*p*-value < 0.0001, based on *t*-test). B and C) Normalized expression levels of miRNAs detected exclusively in TT (B) and ST samples (C); read counts of each miRNA were normalized to the total number of mature miRNA read counts in that sample.

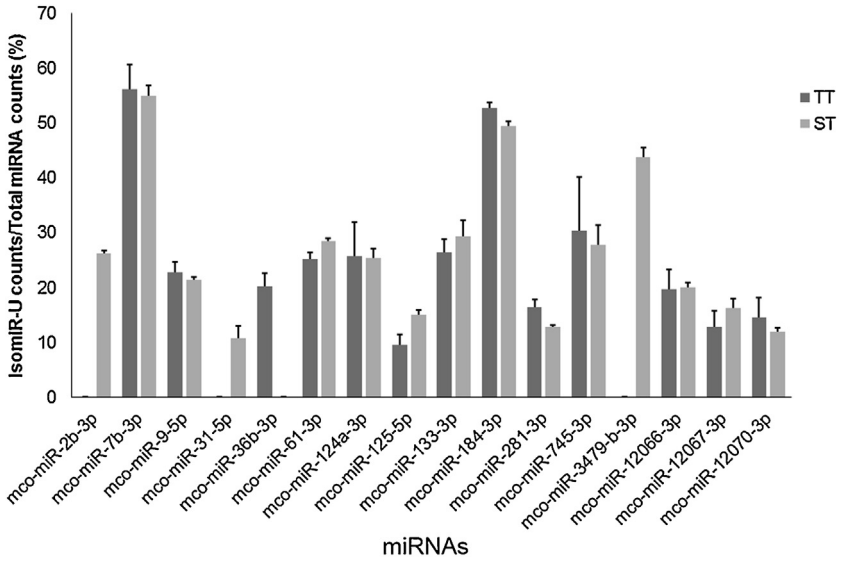

Fig. 5. – IsomiR-U expression profiles in *Mesocestoides corti*. Results are shown as average percentages (± S.D.) of biological replicates. TT (Tetrathyridia), ST (Strobilated worm).

toires of the tetrathyridia larvae and of the strobilated worm, two developmental stages of this model cestode parasite.

miRNAs are the most abundant type of small RNAs in *M. corti*, and the percentages of reads corresponding to miRNAs in TT and ST (56 and 76%, respectively), are in agreement with previous high-throughput analyses in *Echinococcus* [25,26]. We hypothesize that the fraction of miRNAs is larger in ST samples as a reflex of the adult increased morphological and physiological complexity, in comparison to the mostly undifferentiated TT [46–48], since the other assigned categories did not show significant differences between TT and ST samples whatsoever. In agreement with this, a higher fraction of miRNAs was identified in protoscoleces (pre-adult stage) than in cyst wall (larval stage) in *E. canadensis* [26].

The miRNAs identified for *M. corti* in this study share some features with other related organisms, such as a similar sequence length (for both pre-miRNAs and mature miRNAs), a high percentage of mature miRNAs sequences beginning with uracil (U), a bias towards 3′ arm processing, and miRNA tailing, conserved among parasitic platyhelminths [25,44,45] as well as in nematodes [49]. Some of the uridylated isomiRs found in *M. corti*, namely miR-2b, miR-9, miR-61, miR-124a, miR-125, miR-184, miR-745, and miR-3479b, were also found in *E. canadensis* and *E. multilocularis* [25]. This conservation pattern suggests that regulation of these miRNAs by uridylation could be a mechanism shared by cestodes to regulated miRNA stability and expression.

The number of new miRNAs reported in different platyhelminths is quite variable, and may vary as function the stringency of the pipelines of analysis. The *M. corti* sRNA-seq data analysis yielded a smaller number of new miRNAs (6) in comparison to those reported for *E. granulosus* (94), *S. japonicum* (38) and *S. mediterranea* (48) [27,45,50]. This lower number of new miRNAs in the *M. corti* repertoire could be due, at least in part, to the more strin-

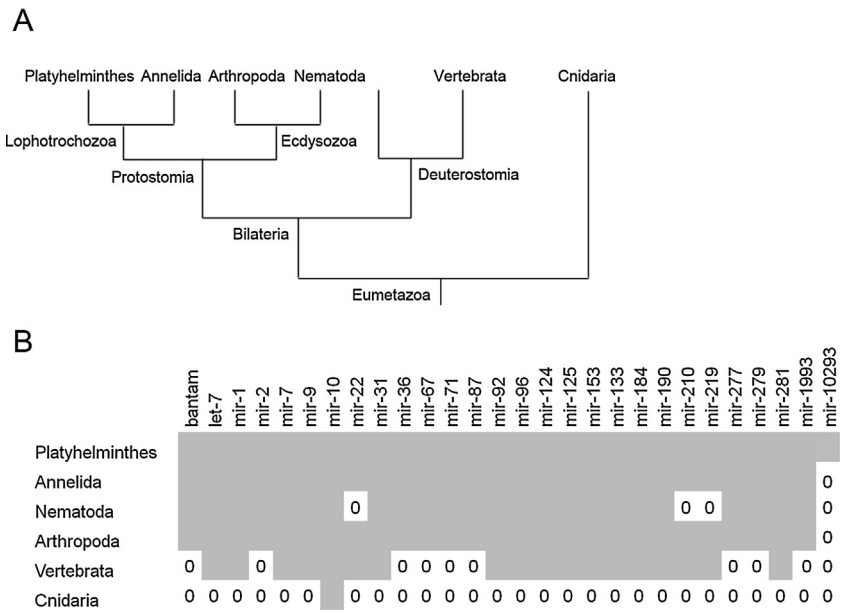

Fig. 6. – Conservation of *Mesocestoides corti* miRNA families. A) Simplified tree of Eumetazoa. B) Conservation analysis between phyla. miRNA belonging to phyla Annelida, Nematoda, Arthropoda, Cnidaria and subclass Vertebrata were compared using seed match criteria. Light grey rectangles show presence of the miRNA family in the clade, 0 represents absence of the miRNA family.

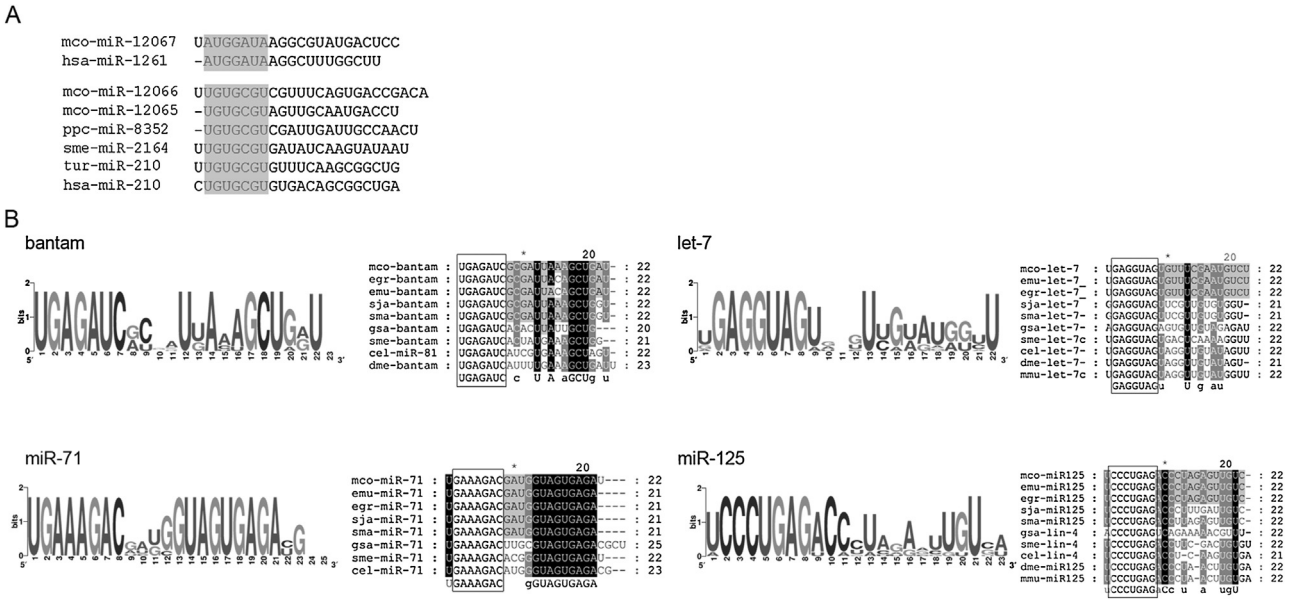

Fig. 7. – Sequence alignments and logos of selected *Mesocestoides corti* miRNAs. A) Seed conservation from two of the new candidates found in *M. corti*: *Homo sapiens (hsa), Pristionchus pacificus (ppc), Tetranychus urticae (tur)*. Alignments were made using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The seed regions are marked with a black rectangle. The identical nucleotides are marked in black and different shades of grey, according to the number of species which share them. B) Sequence logos and alignments of selected *Mesocestoides corti* miRNAs with ortholog miRNAS from *E. multilocularis* (emu), *E. granulosus* (egr), *S. japonicum* (sja), *S. mansoni* (sma), *G. salaris* (gsa), *S. mediterranea* (sme), *C. elegans* (cel), *D. melanogaster* (dme), *M. musculus* (mmu).

gent annotation pipeline used in this work, compared to that used for the close related *E. granulosus* and the trematode *S. japonicum* [27,45]. In line with that, the number of new miRNAs identified in *M. corti* is similarly low as those found in the cestodes *E. canadensis* (5) and *E. multilocularis* (3), and in the trematode *F. hepatica* (5) [25,26,44]. Regarding the comparison with the more distantly related *S. mediterranea* (Turbellaria versus Neodermata), the lower number of new miRNAs found in *M. corti* (6 versus 45) reflects once again lower complexity characteristic of the parasitic way of life.

Despite of the 3p arm processing bias, usual among parasitic platyhelminths, a lack of conservation in arm processing was observed for some *M. corti* miRNAs, particularly those that presented expression from both arms of the hairpin. For instance, the pattern of arm processing in miR-153 and miR-190 was not conserved in most organisms, as mir-190 displays a 5p arm usage pattern in *S. japonicum* and *E. granulosus* [27,51], while miR-153 displays a 3p arm usage pattern in *E. multilocularis* [25]. Interestingly, the pattern of arm expression of *M. corti* miR-153 was also reported in *E. canadensis* and *E. granulosus* [25,27], suggesting that it could be conserved among some cestodes. Another miRNA that did not show conserved arm expression pattern was mir-7b, which displayed a 5p arm usage in *E. canadensis* and *E. granulosus* [26] while

in *M. corti* the 3p arm was the more expressed. The absence of arm usage conservation regarding miR-153, miR-190 and miR-7b in *M. corti* could imply different roles in the biology of these cestodes, since each mature miRNA from the same precursor would target different genes.

We analysed miRNA abundance and found that few miRNAs were expressed at high levels. The highly expressed *M. corti* miRNAs are shared between both stages, and represent ~90% of total miRNA expression. High expression of let-7-5p, miR-10-5p, miR-71-5p and bantam-3p was also observed in other cestodes as *E. multilocularis* and *E. canadensis* [25], and other platyhelminths, like *F. hepatica* adults [52] and *S. japonicum* (in adults and infective larval stages) [45]. In line with that, the mir-10 family, which is highly conserved across metazoans, has been implicated with *Hox* gene regulation, embryonic development, and even cancer [53], and let-7 has been described as a key regulator to ensure the normal development during larval to adult transition both in worms and flies [54]. miR-71, in turn, increases life span via insulin-receptor/phosphatidyl inositol 3-kinase pathway in *C. elegans* [55]. This function may be conserved in *M. corti*, as human insulin is capable of stimulating *M. corti* survival, asexual reproduction, and tyrosine-phosphorylation status in culture [56] and deserves further investigation.

M. corti life cycle involves a number of different life stages and the parasite's development is a complicated and dynamic process [9,57,58], in which miRNA gene regulation could have an important role. We found several miRNAs differentially expressed between the TT and ST stages, which may be functionally involved in regulating stage-specific features. miRNAs up-regulated in the larval stage (TT), could act as repressors of development of the reproductive tissues or controlling reproductive fission, as TT are able to multiply asexually in the intermediate host, being important cell growth and cell division processes for reproduction [58]. We found mi*R*-36 up-regulated in TT. The mir-36 family has been shown to be enriched in planarian neoblasts [59] and is up-regulated in *E. multilocularis* with respect to *E. canadensis* [25], which is in line with the higher regenerative capacity of planarians and the faster proliferation of *E. multilocularis* metacestodes. Therefore, mi*R*-36b in the TT stage of *M* corti, could be regulating proliferation by asexual reproduction. Conversely, miRNAs up-regulated in the ST stage could enhance the development of testes and ovaries or repress asexual reproduction [50]. We found miR-125, up-regulated in the ST stage. In vertebrates, miR-125 regulates somitogenesis through the Notch signaling pathway [60]. The Notch signaling pathway, involved in bilaterian development is present in cestodes [5], so it would be interesting to further investigate the role of this miRNA in *M. corti* development through prediction of assessing miR-125 targets and/or functional studies. On the other hand, antisense-mediated inactivation of miR-2 in *Drosophila* produces embryos with defects in head and posterior abdominal segments [61]. Therefore, miR-125 and miR-2 family in the ST stage of *M. corti*, could be mediating processes like correct strobilum formation (miR-2 via regulation of pro-apoptotic pathways) and signal transduction for sexual maturation (miR-125 via Notch pathways).

Interestingly, miR-190, miR-125, mi*R*-31 were also recently reported as up-regulated in the *E. granulosus* adult stage, while the mir-2 family was more expressed in the pre-adult stage [27], meaning that these miRNAs could be regulating similar processes involved in both *E. granulosus* and *M. corti*. In addition, miRNAs exclusively expressed in *M. corti* TT or ST were identified, suggesting stage-specific roles. Two of these stage-specific miRNAs, miR-7a-5p and miR-12071-5p, showed low expression levels, implying that stage specificity should be confirmed with higher coverage approach.

Recent studies have described that several miRNA families are missing in platyhelminths, especially in the parasitic lineages [30,44]. Our results confirm a reduction of the miRNA complement in *M. corti*. However, we found that the loss of conserved miRNA families in this endoparasitic platyhelminth is smaller than that reported by [30] for *S. japonicum* and *E. granulosus*. This difference in the loss of conserved miRNAs is due to the finding in *M. corti* of mir-92, mir-184, mir-279 and mir-281, along with mir-36 and mir-67, reported as lost in cestodes. Therefore, the proportion of conserved miRNA losses in this cestode parasite seems to be lower than previously reported.

In agreement with this previous studies, a loss of conserved miRNA families among platyhelminths parasites was confirmed in *M. corti*, and could be related to a loss of targets [62] or reduced morphological and metabolic complexity [63], associated with the parasitic way of life. This correlates with the low morphological complexity and gene repertoire reduction, given by the absence of loss of the gut and also the loss of several conserved gene families, for example, homeobox gene families, genes essential for peroxisomes, and fatty acid biosynthesis [5].

A recent study in *E. granulosus* [27] reported mir-96 and mir-184 as absent in this species. In this study we have identified both miRNAs expressed by *M. corti* TT and ST samples. In addition, these authors described mir-92 as exclusive of the free-living flatworm *S. mediterranea*, and absent in Neodermata but we found two members of the mir-92 family expressed in *M. corti*: mi*R*-3479a and mi*R*-3479b. These findings agree with two recent studies in *E. multilocularis, E. canadensis* and *E. granulosus* [25,26]. On the other hand, members of the mir-8 and mir-1992 families were not identified in *M. corti*, although they were reported as present in *Echinococcus* spp [25–27]. Moreover, miR-210 was identified for the first time in platyhelminth parasites and this should be from now on considered in miRNA evolutionary studies.

In this study, three miRNA clusters were reported. However, the number of miRNA clusters may be larger than that, due to the fragmented nature of the assembled draft genome sequence. New clusters may be identified upon completion of the *M. corti* genome sequence assembly. Regarding miRNA cluster conservation, we confirmed that mir-71/2 cluster is widely conserved among protostomes (Ecdysozoan and Lophotrochozoan) as previously described [64], while mir-277/4989 is conserved among cestodes, as it was previously identified in *Echinococcus* spp. and *Hymenolepis microstoma* [24,26,27,65]. There is only one copy of the mir-71/2 cluster in *M. corti*, in contrast with other platyhelminths like *G. salaris*, *S. japonicum*, and *S. mansoni*, where two copies of this cluster have been reported [30]. This might suggest functional shrinking in cestodes as proposed by [65]. On the other hand, while the mir-1/133 cluster is widely conserved across metazoan species [66], its miRNA components seem to be far apart from each other, since the average lengths for platyhelminth miRNA clusters is between 200 bp and 500 bp [67]. However, it has been reported that this particular cluster dramatically varies in length between species, for example in *Echinococcus*, comprises 12 kb [65], and in *D. melanogaster*, the miRNA genes are separated by 50 kb or more [66]. Whether both miR-1 and miR-133 are part of a single transcriptional unit in *M. corti* is an issue that remains to be investigated.

Finally, the divergence of the *M. corti* miRNA sequences found at the nucleotide level with respect to those from other organisms, including other platyhelminths, may likely indicate an accelerated evolution of these miRNAs in the *M. corti* lineage. This could imply specific roles for these miRNAs in development, survival and/or host-parasite interaction. In addition, this may reflect the more complex life cycles of parasitic species and their ability to adapt to different environments. Those miRNAs conserved among cestodes could share functional conservation, and *M. corti* could represent an ideal model for the study of the role of this miRNAs in the biology of this parasites.

In this work, we provide detailed information on the *M. corti* miRNA repertoire, including the first experimental report on the

miRNA expression profiles of the tetrathyridia and strobilated worm. The results reported here show that both life stages display some differentially expressed miRNAs, including some stage specific. In addition, we performed a systematic analysis on the post-transcriptional modifications of each identified miRNA. Conservation of *M. corti* miRNA features, repertoires and expression levels among other cestodes further validate this parasite as a model for the study of biological aspects of cestode parasites and will allow functional studies of miRNAs involved in strobilation process that are difficult to perform in zoonotic parasites such as *Echinococcus* spp. The data obtained from this study will open a way for deep investigation of the parasite biology and host parasite interaction. The findings of differential expression in *M. corti* will allow a deeper understanding more about the biology of development of the parasite.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2016.08.004.

References

[1] B. Fried, Points in question, Int. J. Parasitol. 20 (1990) 721, http://www.ncbi.nlm.nih.gov/pubmed/2242954.
[2] P.D. Olson, D.T.J. Littlewood, R.A. Bray, J. Mariaux, Interrelationships and evolution of the tapeworms (Platyhelminthes: cestoda), Mol. Phylogenet. Evol. 19 (2001) 443–467, http://dx.doi.org/10.1006/mpev.2001.0930.
[3] J.P. Couso, Segmentation, metamerism and the Cambrian explosion, Int. J. Dev. Biol. 53 (2009) 1305–1316, http://dx.doi.org/10.1387/ijdb.072425jc.
[4] J.A. Debarba, K.M. Monteiro, H. Moura, J.R. Barr, H.B. Ferreira, A. Zaha, Identification of newly synthesized proteins by echinococcus granulosus protoscoleces upon induction of strobilation, PLoS Negl. Trop. Dis. 9 (2015) e0004085, http://dx.doi.org/10.1371/journal.pntd.0004085.
[5] I.J. Tsai, M. Zarowiecki, N. Holroyd, A. Garciarrubio, A. Sanchez-Flores, K.L. Brooks, A. Tracey, R.J. Bobes, G. Fragoso, E. Sciutto, M. Aslett, H. Beasley, H.M. Bennett, J. Cai, F. Camicia, R. Clark, M. Cucher, N. De Silva, T.A. Day, P. Deplazes, K. Estrada, C. Fernández, P.W.H. Holland, J. Hou, S. Hu, T. Huckvale, S.S. Hung, L. Kamenetzky, J.A. Keane, F. Kiss, U. Koziol, O. Lambert, K. Liu, X. Lio, Y. Luo, N. Macchiaroli, S. Nichol, J. Paps, J. Parkinson, N. Pouchkina-Stantcheva, N. Riddiford, M. Rosenzvit, G. Salinas, J.D. Wasmuth, M. Zamanian, Y. Zheng, X. Cai, X. Soberón, P.D. Olson, J.P. Laclette, K. Brehm, The genomes of four tapeworm species reveal adaptations to parasitism, Nature 496 (2013) 57–63, http://dx.doi.org/10.1038/nature12031.
[6] H. Zheng, W. Zhang, L. Zhang, Z. Zhang, J. Li, G. Lu, Y. Zhu, Y. Wang, Y. Huang, J. Liu, H. Kang, J. Chen, L. Wang, A. Chen, S. Yu, Z. Gao, L. Jin, W. Gu, Z. Wang, L. Zhao, B. Shi, H. Wen, R. Lin, M.K. Jones, B. Brejova, T. Vinar, G. Zhao, D.P. McManus, Z. Chen, Y. Zhou, S. Wang, The genome of the hydatid tapeworm Echinococcus granulosus, Nat. Genet. 45 (2013) 1168–1175, http://dx.doi.org/10.1038/ng.2757.
[7] A. Laschuk, K.M. Monteiro, N.M. Vidal, P.M. Pinto, R. Duran, C. Cerveñanski, A. Zaha, H.B. Ferreira, Proteomic survey of the cestode Mesocestoides corti during the first 24 hours of strobilar development, Parasitol. Res. 108 (2011) 645–656, http://dx.doi.org/10.1007/s00436-010-2109-2.
[8] P.D. Olson, M. Zarowiecki, F. Kiss, K. Brehm, Cestode genomics—progress and prospects for advancing basic and applied aspects of flatworm biology, Parasite Immunol. 34 (2012) 130–150, http://dx.doi.org/10.1111/j.1365-3024.2011.01319.x.
[9] M.M. Markoski, C.V. Bizarro, S. Farias, I. Espinoza, N. Galanti, A. Zaha, H.B. Ferreira, In vitro segmentation induction of Mesocestoides corti (Cestoda) tetrathyridia, J. Parasitol. 89 (2003) 27–34, http://dx.doi.org/10.1645/0022-3395(2003)089[0027:IVSIOM]2.0.CO;2.
[10] M.M. Markoski, E.S. Trindade, G. Cabrera, A. Laschuk, N. Galanti, A. Zaha, H.B. Nader, H.B. Ferreira, Praziquantel and albendazole damaging action on in vitro developing Mesocestoides corti (Platyhelminthes: cestoda), Parasitol. Int. 55 (2006) 51–61, http://dx.doi.org/10.1016/j.parint.2005.09.005.
[11] E. Berezikov, Evolution of microRNA diversity and regulation in animals, Nat. Rev. Genet. 12 (2011) 846–860, http://dx.doi.org/10.1038/nrg3079.
[12] K.N. Ivey, D. Srivastava, microRNAs as developmental regulators., cold spring harb, Perspect. Biol. 7 (2015) a008144, http://dx.doi.org/10.1101/cshperspect.a008144.
[13] D.P. Bartel, R. Lee, R. Feinbaum, MicroRNAs: genomics biogenesis mechanism function genomics, miRNA Genes 116 (2004) 281–297.
[14] S.M. Hammond, An overview of microRNAs, Adv. Drug Deliv. Rev. 87 (2015) 3–14, http://dx.doi.org/10.1016/j.addr.2015.05.001.
[15] M. Faller, F. Guo, MicroRNA biogenesis: there's more than one way to skin a cat, Biochim. Biophys. Acta. 1779 (2008) 663–667, http://dx.doi.org/10.1016/j.bbagrm.2008.08.005.
[16] R.C. Friedman, K.K.-H. Farh, C.B. Burge, D.P. Bartel, Most mammalian mRNAs are conserved targets of microRNAs, Genome Res. 19 (2009) 92–105, http://dx.doi.org/10.1101/gr.082701.108.
[17] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136 (2009) 215–233, http://dx.doi.org/10.1016/j.cell.2009.01.002.
[18] S.L. Ameres, M.D. Horwich, J. Hung, J. Xu, M. Ghildiyal, Z. Weng, P.D. Zamore, Target RNA-directed trimming and tailing of small silencing RNAs, Science 328 (2010) 1534–1539, http://dx.doi.org/10.1126/science.1187058 (Target.).
[19] H. Cerutti, J.A. Casas-mollano, On the origin and functions of RNA-mediated silencing: from protists to man, Curr. Genet. 50 (2008) 81–99, http://dx.doi.org/10.1007/s00294-006-0078-x.On.
[20] Y. Zheng, Phylogenetic analysis of the Argonaute protein family in platyhelminths, Mol. Phylogenet. Evol. 66 (2013) 1050–1054, http://dx.doi.org/10.1016/j.ympev.2012.11.014.
[21] M.S. Gomes, F.J. Cabral, L.K. Jannotti-Passos, O. Carvalho, V. Rodrigues, E.H. Baba, R.G. Sá, Preliminary analysis of miRNA pathway in Schistosoma mansoni, Parasitol. Int. 58 (2009) 61–68, http://dx.doi.org/10.1016/j.parint.2008.10.002.
[22] Y. Zheng, X. Cai, J.E. Bradley, microRNAs in parasites and parasite infection, RNA Biol. 10 (2013) 371–379, http://dx.doi.org/10.4161/rna.23716.
[23] P. Cai, G.N. Gobert, D.P. Mcmanus, MicroRNAs in parasitic helminthiases: current status and future perspectives, Trends Parasitol. 32 (2015) 71–86, http://dx.doi.org/10.1016/j.pt.2015.09.003.
[24] M. Cucher, L. Prada, G. Mourglia-Ettlin, S. Dematteis, F. Camicia, S. Asurmendi, M. Rosenzvit, Identification of Echinococcus granulosus microRNAs and their expression in different life cycle stages and parasite genotypes, Int. J. Parasitol. 41 (2011) 439–448, http://dx.doi.org/10.1016/j.ijpara.2010.11.010.
[25] M. Cucher, N. Macchiaroli, L. Kamenetzky, L. Maldonado, K. Brehm, M. Rosenzvit, High-throughput characterization of Echinococcus spp. metacestode miRNomes, Int. J. Parasitol. 45 (2015) 253–267, http://dx.doi.org/10.1016/j.ijpara.2014.12.003.
[26] N. Macchiaroli, M. Cucher, M. Zarowiecki, L. Maldonado, L. Kamenetzky, M.C. Rosenzvit, microRNA profiling in the zoonotic parasite Echinococcus canadensis using a high-throughput approach, Parasite Vectors 8 (2015) 83, http://dx.doi.org/10.1186/s13071-015-0686-8.
[27] Y. Bai, Z. Zhang, L. Jin, H. Kang, Y. Zhu, L. Zhang, X. Li, F. Ma, L. Zhao, B. Shi, J. Li, D.P. McManus, W. Zhang, S. Wang, Genome-wide sequencing of small RNAs reveals a tissue-specific loss of conserved microRNA families in Echinococcus granulosus, BMC Genomics. 15 (2014) 736, http://dx.doi.org/10.1186/1471-2164-15-736.
[28] X. Wu, Y. Fu, D. Yang, Y. Xie, R. Zhang, W. Zheng, H. Nie, N. Yan, N. Wang, J. Wang, X. Gu, S. Wang, X. Peng, G. Yang, Identification of neglected cestode Taenia multiceps microRNAs by illumina sequencing and bioinformatic analysis, BMC Vet. Res. 9 (2013) 162, http://dx.doi.org/10.1186/1746-6148-9-162.
[29] L. Ai, M.J. Xu, M.X. Chen, Y.N. Zhang, S.H. Chen, J. Guo, Y.C. Cai, X.N. Zhou, X.Q. Zhu, J.X. Chen, Characterization of microRNAs in Taenia saginata of zoonotic significance by Solexa deep sequencing and bioinformatics analysis, Parasitol. Res. 110 (2012) 2373–2378, http://dx.doi.org/10.1007/s00436-011-2773-x.
[30] B. Fromm, M.M. Worren, C. Hahn, E. Hovig, L. Bachmann, Substantial loss of conserved and gain of novel MicroRNA families in flatworms, Mol. Biol. Evol. 30 (2013) 2619–2628, http://dx.doi.org/10.1093/molbev/mst155.
[31] J. Mallatt, C.W. Craig, M.J. Yoder, Nearly complete rRNA genes from 371 Animalia: updated structure-based alignment and detailed phylogenetic analysis, Mol. Phylogenet. Evol. 64 (2012) 603–617, http://dx.doi.org/10.1016/j.ympev.2012.05.016.
[32] A. Kozomara, S. Griffiths-Jones, miRBase: annotating high confidence microRNAs using deep sequencing data, Nucleic Acids Res. 42 (2014) D68–D73, http://dx.doi.org/10.1093/nar/gkt1181.
[33] B. Langmead, C. Trapnell, M. Pop, S.L. Salzberg, Ultrafast and memory-efficient alignment of short DNA sequences to the human genome, Genome Biol. 10 (2009) R25, http://dx.doi.org/10.1186/gb-2009-10-3-r25.
[34] M.R. Friedländer, S.D. MacKowiak, N. Li, W. Chen, N. Rajewsky, MiRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades, Nucleic Acids Res. 40 (2012) 37–52, http://dx.doi.org/10.1093/nar/gkr688.
[35] M.A. Larkin, G. Blackshields, N.P. Brown, R. Chenna, P.A. Mcgettigan, H. McWilliam, F. Valentin, I.M. Wallace, A. Wilm, R. Lopez, J.D. Thompson, T.J.

Gibson, D.G. Higgins, Clustal W and Clustal X version 2.0, Bioinformatics 23 (2007) 2947–2948, http://dx.doi.org/10.1093/bioinformatics/btm404.
[36] S. Anders, W. Huber, Differential expression analysis for sequence count data, Genome Biol. 11 (2010) R106, http://dx.doi.org/10.1186/gb-2010-11-10-r106.
[37] B. Fromm, T. Billipp, L.E. Peck, M. Johansen, J.E. Tarver, B.L. King, J.M. Newcomb, L.F. Sempere, K. Flatmark, E. Hovig, K.J. Peterson, A uniform system for the annotation of vertebrate microRNA genes and the evolution of the human microRNAome, Annu. Rev. Genet. 49 (2015) 213–242, http://dx.doi.org/10.1146/annurev-genet-120213-092023.
[38] S.L. Fernandez-Valverde, R.J. Taft, J.S. Mattick, Dynamic isomiR regulation in Drosophila development, RNA 16 (2010) 1881–1888, http://dx.doi.org/10.1261/rna.2379610.
[39] T.A. Farazi, M. Brown, P. Morozov, J.J. Ten Hoeve, I.Z. Ben-Dov, V. Hovestadt, M. Hafner, N. Renwick, A. Mihailović, L.F.A. Wesselsv, T. Tuschl, Bioinformatic analysis of barcoded cDNA libraries for small RNA profiling by next-generation sequencing, Methods 58 (2012) 171–187, http://dx.doi.org/10.1016/j.ymeth.2012.07.020.
[40] R. Shi, V.L. Chiang, Facile means for quantifying microRNA expression by real-time PCR, Biotechniques 39 (2005) 519–524, http://dx.doi.org/10.2144/000112010.
[41] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408, http://dx.doi.org/10.1006/meth.2001.1262.
[42] F. Xie, P. Xiao, D. Chen, L. Xu, B. Zhang, miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs, Plant Mol. Biol. 80 (2012) 75–84, http://dx.doi.org/10.1007/s11103-012-9885-2.
[43] T. Barrett, S.E. Wilhite, P. Ledoux, C. Evangelista, I.F. Kim, M. Tomashevsky, K.A. Marshall, K.H. Phillippy, P.M. Sherman, M. Holko, A. Yefanov, H. Lee, N. Zhang, C.L. Robertson, N. Serova, S. Davis, A. Soboleva, NCBI GEO. Archive for functional genomics data sets—Update, Nucleic Acids Res. 41 (2013) 991–995, http://dx.doi.org/10.1093/nar/gks1193.
[44] S. Fontenla, N. Dell'Oca, P. Smircich, J.F. Tort, M. Siles-Lucas, The miRnome of Fasciola hepatica juveniles endorses the existence of a reduced set of highly divergent micro RNAs in parasitic flatworms, Int. J. Parasitol. (2015), http://dx.doi.org/10.1016/j.ijpara.2015.06.007.
[45] L. Hao, P. Cai, N. Jiang, H. Wang, Q. Chen, Identification and characterization of microRNAs and endogenous siRNAs in Schistosoma japonicum, BMC Genomics. 11 (2010) 55, http://dx.doi.org/10.1186/1471-2164-11-55.
[46] F. Kawamoto, H. Fujioka, S. Mizuno, N. Kumada, Studies on the post-larval development of cestodes of the genus Mesocestoides: shedding and further development of M. lineatus and M. corti tetrathyridia in vivo, Int. J. Parasitol. 16 (1986) 323–331.
[47] R.C.A. Thompson, L.P. Jue Sue, S.J. Buckley, In vitro development of the strobilar stage of Mesocestoides corti, Int. J. Parasitol. 12 (1982) 303–314, http://dx.doi.org/10.1016/0020-7519(82)90033-9.
[48] N.J. Barrett, J.D. Smyth, S.J. Ong, Spontaneous sexual differentiation of Mesocestoides corti tetrathyridia in vitro, Int. J. Parasitol. 12 (1982) 315–322 (http://www.ncbi.nlm.nih.gov/pubmed/7118374).
[49] A.D. Winter, W. Weir, M. Hunt, M. Berriman, J.S. Gilleard, E. Devaney, C. Britton, Diversity in parasitic nematode genomes: the microRNAs of Brugia pahangi and Haemonchus contortus are largely novel, BMC Genomics 13 (2012) 4, http://dx.doi.org/10.1186/1471-2164-13-4.
[50] Y.-C. Lu, M. Smielewska, D. Palakodeti, M.T. Lovci, S. Aigner, G.W. Yeo, B.R. Graveley, Deep sequencing identifies new and regulated microRNAs in Schmidtea mediterranea, RNA 15 (2009) 1483–1491, http://dx.doi.org/10.1261/rna.1702009.
[51] H. Han, J. Peng, Y. Hong, Z. Fu, K. Lu, H. Li, C. Zhu, Q. Zhao, J. Lin, Comparative characterization of microRNAs in Schistosoma japonicum schistosomula from Wistar rats and BALB/c mice, Parasitol. Res. 114 (2015) 2639–2647, http://dx.doi.org/10.1007/s00436-015-4468-1.
[52] B. Fromm, M. Trelis, M. Hackenberg, F. Cantalapiedra, D. Bernal, A. Marcilla, The revised microRNA complement of Fasciola hepatica reveals a plethora of overlooked microRNAs and evidence for enrichment of immuno-regulatory microRNAs in extracellular vesicles, Int. J. Parasitol. 45 (2015) 697–702, http://dx.doi.org/10.1016/j.ijpara.2015.06.002.
[53] D. Tehler, N.M. Høyland-Kroghsbo, A.H. Lund, The miR-10 microRNA precursor family, RNA Biol. 8 (2011) 728–734, http://dx.doi.org/10.4161/rna.8.5.16324.
[54] J.-L. Su, P.-S. Chen, G. Johansson, M.-L. Kuo, Function and regulation of let-7 family microRNAs, MicroRNA E. 1 (2012) 34–39, http://dx.doi.org/10.2174/2211536611201010034.
[55] K. Boulias, H.R. Horvitz, The C. elegans MicroRNA mir-71 acts in neurons to promote germline-Mediated longevity through regulation of DAF-16/FOXO, Cell Metab. 15 (2012) 439–450, http://dx.doi.org/10.1016/j.cmet.2012.02.014.
[56] L. Canclini, A. Esteves, In vivo response of Mesocestoides vogae to human insulin, Parasitology 136 (2009) 203–209, http://dx.doi.org/10.1017/S003118200800526X.
[57] I. Espinoza, M. Galindo, C.V. Bizarro, H.B. Ferreira, a Zaha, N. Galanti, Early post-larval development of the endoparasitic platyhelminth Mesocestoides corti: trypsin provokes reversible tegumental damage leading to serum-induced cell proliferation and growth, J. Cell. Physiol. 205 (2005) 211–217, http://dx.doi.org/10.1002/jcp.20382.
[58] C.V. Bizarro, M.H. Bengtson, F.K. Ricachenevsky, A. Zaha, M.C. Sogayar, H.B. Ferreira, Differentially expressed sequences from a cestode parasite reveals conserved developmental genes in platyhelminthes, Mol. Biochem. Parasitol. 144 (2005) 114–118, http://dx.doi.org/10.1016/j.molbiopara.2005.07.002.
[59] M.R. Friedländer, C. Adamidi, T. Han, S. Lebedeva, T.A. Isenbarger, M. Hirst, M. Marra, C. Nusbaum, W.L. Lee, J.C. Jenkin, A. Sánchez Alvarado, J.K. Kim, N. Rajewsky, High-resolution profiling and discovery of planarian small RNAs, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 11546–11551, http://dx.doi.org/10.1073/pnas.0905222106.
[60] D.J. Moloney, V.M. Panin, S.H. Johnston, J. Chen, L. Shao, R. Wilson, Y. Wang, P. Stanley, K.D. Irvine, R.S. Haltiwanger, T.F. Vogt, Fringe is a glycosyltransferase that modifies Notch, Nature 406 (2000) 369–375, http://dx.doi.org/10.1038/35019000htt://dx.org/.
[61] A. Boutla, C. Delidakis, M. Tabler, Developmental defects by antisense-mediated inactivation of micro-RNAs 2 and 13 in Drosophila and the identification of putative target genes, Nucleic Acids Res. 31 (2003) 4973–4980, http://dx.doi.org/10.1093/nar/gkg707.
[62] E.A. Sperling, K.J. Peterson, Micrornas and Metazoan Phylogeny: Big Trees from Little Genes, in: Anim Evol., Oxford University Press, Oxford, 2009, http://dx.doi.org/10.1093/acprof:oso/9780199549429.003.0015.
[63] D.H. Erwin, M. Laflamme, S.M. Tweedt, E.A. Sperling, D. Pisani, K.J. Peterson, The cambrian conundrum: early divergence and later ecological success in the early history of animals, Science 334 (80-) (2011) 1091–1097 (http://science.sciencemag.org/content/334/6059/1091. abstract).
[64] M. De Souza, M.T.A. Gomes, M. Donoghue, R.V. Muniyappa, R. Guerra-Sá, C. Spillane, Computational identification and evolutionary relationships of the microRNA gene cluster miR-71/2 in protostomes, J. Mol. Evol. 76 (2013) 353–358, http://dx.doi.org/10.1007/s00239-013-9563-2.
[65] X. Jin, L. Lu, H. Su, Z. Lou, F. Wang, Y. Zheng, G.T. Xu, Comparative analysis of known miRNAs across platyhelminths, FEBS J. 280 (2013) 3944–3951, http://dx.doi.org/10.1111/febs.12395.
[66] F. Campo-Paysaa, M. Sémon, R.A. Cameron, K.J. Peterson, M. Schubert, microRNA complements in deuterostomes: origin and evolution of microRNAs, Evol. Dev. 13 (2011) 15–27, http://dx.doi.org/10.1111/j.1525-142X.2010.00452.x.
[67] J. Wang, B. Czech, A. Crunk, A. Wallace, M. Mitreva, G.J. Hannon, R.E. Davis, Deep small RNA sequencing from the nematode Ascaris reveals conservation, functional diversification, and novel developmental profiles, Genome Res. 21 (2011) 1462–1477, http://dx.doi.org/10.1101/gr.121426.111.