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Identification and expression profiling of microRNAs in *Hymenolepis*

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ABSTRACT

Tapeworms (cestodes) of the genus *Hymenolepis* are the causative agents of hymenolepiasis, a neglected zoonotic disease. *Hymenolepis nana* is the most prevalent human tapeworm, especially affecting children. The genomes of *Hymenolepis microstoma* and *H. nana* have been recently sequenced and assembled. MicroRNAs (miRNAs), a class of small non-coding RNAs, are principle regulators of gene expression at the post-transcriptional level and are involved in many different biological processes. In previous work, we experimentally identified miRNA genes in the cestodes *Echinococcus, Taenia* and *Mesocestoides.* However, current knowledge about miRNAs in *Hymenolepis* is limited. In this work we described for the first known time the expression profile of the miRNA complement in *H. microstoma,* and discovered miRNAs in *H. nana.* We found a reduced complement of 37 evolutionarily conserved miRNAs, putatively reflecting their low morphological complexity and parasitic lifestyle. We found high expression of a few miRNAs in the larval stage of *H. microstoma* that are conserved in other cestodes, suggesting that these miRNAs may have important roles in development, survival and for host-parasite interplay. We performed a comparative analysis of the identified miRNAs across the Cestoda and showed that most of the miRNAs in *Hymenolepis* are located in intergenic regions, implying that they are independently transcribed. We found a *Hymenolepis*-specific cluster composed of three members of the mir-36 family. Also, we found that one of the neighboring genes of *mir-10* was a *Hox* gene as in most bilaterial species. This study provides a valuable resource for further experimental research in cestode biology that might lead to improved detection and control of these neglected parasites. The comprehensive identification and expression analysis of *Hymenolepis* miRNAs can help to identify novel biomarkers for diagnosis and/or novel therapeutic targets for the control of hymenolepiasis.

1. Introduction

Tapeworms (cestodes) of the genus *Hymenolepis* are the causative agents of hymenolepiasis, a neglected zoonotic disease transmitted by rodents. Two cosmopolitan species of *Hymenolepis* infect humans, the rat tapeworm *Hymenolepis diminuta* and, particularly, the dwarf tapeworm *Hymenolepis nana* that is the most prevalent human tapeworm worldwide, especially affecting children in temperate areas (Soares Magalhaes et al., 2013). Whereas most *H. nana* infections are asymptomatic, heavy infections contribute to increased morbidity in children and symptoms including severe diarrhea, abdominal pain, decreased appetite and reduced growth (Soares Magalhaes et al., 2013). Although drugs such as praziquantel are available and effective against adult tapeworm infections, they do not prevent re-infection in endemic areas with poor hygiene and sanitation where the frequency of transmission is high (Thompson, 2015). Infections in such areas frequently co-occur with other intestinal helminths (Soares Magalhaes et al., 2013) and diseases such as HIV-AIDS. The latter poses a particular threat as *H. nana* infections in immunocompromised individuals have been shown to give rise to invasive, tapeworm-derived tumors (Olson et al., 2003; Muehlenbachs et al., 2015). Finally, praziquantel resistance could become a problem in large scale deworming campaigns (Olson et al., 2012) and thus novel

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strategies for the control of hymenolepiasis are needed. The mouse bile duct tapeworm *H. microstoma,* that is prevalent in rodents worldwide, is a laboratory model for the human parasite *H. nana* and for other tapeworms causing neglected tropical diseases such as *Echinococcus* and *Taenia* for which complete life cycles cannot be maintained in the laboratory. The genome of *H. microstoma* and other cyclophyllidean cestodes have been recently sequenced and assembled (Olson et al., 2012; Tsai et al., 2013). Importantly, the laboratory strain of *H. microstoma* used for genome sequencing has been previously characterized (Cunningham and Olson, 2010). In addition, the draft genome of *H. nana* is available as part of the Helminth Genomes Initiative, a collaborative project that aims to survey the genomes of parasitic helminths that are either of medical or veterinary importance, or are used as models for those (Coghlan et al., 2018). These unique genomic resources will enable the discovery of novel biomarkers for diagnosis and/or therapeutic targets for control of the infections they cause.

MicroRNAs (miRNAs), a class of small non-coding RNAs, are principle regulators of gene expression at the post-transcriptional level and are involved in many different biological processes (Bartel, 2004; He and Hannon, 2004; Filipowicz et al., 2008). miRNAs are transcribed by RNA polymerase II as long, primary miRNAs (pri-miRNAs) from miRNA genes or from introns of protein coding genes (Filipowicz et al., 2008). The primary miRNA is cleaved by Drosha to produce a ~70-nucleotide (nt) long stem-loop precursor miRNA (pre-miRNA) that is further cleaved by Dicer to generate both the mature miRNA and antisense miRNA products. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC) and binds to complementary sequences of target genes. In most bilaterian animals, target recognition is primarily through Watson-Crick pairing between miRNA nucleotides 2–7 (miRNA seed) and sites located in the 3′ untranslated regions (3′UTRs) of target mRNAs (Bartel, 2018). This promotes the repression of protein translation and/or degradation of the target mRNA (Hutvagner and Zamore, 2002; Filipowicz et al., 2008; Bartel, 2009; Ghildiyal and Zamore, 2009). MiRNAs have been identified in a range of organisms such as viruses, plants and metazoans including free-living and parasitic helminths, with an increase in the number of miRNA families correlated with an increase in morphological complexity (Niwa and Slack, 2007; Berezikov, 2011). Recently, it has been suggested that miRNAs might be potential therapeutic targets for the control of parasitic helminths (Britton et al., 2014). Furthermore, worm-derived miRNAs have shown promise as markers for the early detection of helminth infections (Cai et al., 2016).

The recent availability of the genomes of parasitic helminths of medical and veterinary importance (Howe et al., 2017), including cestodes, has provided a platform for the identification of miRNAs using both computational and experimental approaches. In previous work, we identified miRNA genes through deep sequencing in the cestodes *Echinococcus canadensis* (Cucher et al., 2015; Macchiaroli et al., 2015), *Echinococcus granulosus* s. s. (Macchiaroli et al., 2015), *Echinococcus multilocularis* (Cucher et al., 2015), *Mesocestoides corti* (Basika et al., 2016), *Taenia solium* and *Taenia crassiceps* (Perez et al., 2017). In addition, miRNA genes have been computationally identified in *H. microstoma* by Jin et al. (2013). However, a comprehensive identification of the miRNA repertoire and their expression profile are still lacking in *H. microstoma*, and we found no previous report of miRNAs in *H. nana*. The aims of this study were to analyze the miRNA expression profile in *H. microstoma* larvae and to discover miRNAs in *H. nana* from recently available genome data. The comprehensive identification and expression analysis of *Hymenolepis* miRNAs can help to identify biomarkers for diagnosis and/or novel therapeutic targets for the control of hymenolepiasis.

2. Materials and methods

2.1. Parasite material

miRNA expression profiling was based on larval samples of the mouse bile-duct tapeworm *H. microstoma*. The Nottingham strain (Cunningham and Olson, 2010) of *H. microstoma* was maintained in vivo using flour beetles (*Tribolium confusum*) and laboratory mice (*Mus musculus*) in accordance with project license PPL70/8684 issued by the UK Home Office. To produce mid-metamorphosis larval samples, beetles were starved for 5 days and then exposed to freshly macerated, gravid proglottides of *H. microstoma* for ~6 h. Gravid tissues were removed and the beetles were allowed to feed on flour ad libitum. Beetles were dissected 5 days post-exposure and the resulting larvae collected from the haemocoel into conditioned water. Morphologically, the larvae were elongated and well differentiated at both poles (ie. stage 3 according to Voge's system). All larvae were approximately half way through metamorphosis from the oncosphere to cysticercoid stages, albeit some variation in maturity was seen among individuals and thus the samples included 'stages' 2–4 as defined by Voge (1964) with the majority representing stage 3. Approximately 550 individuals were combined in each of the three biological replicate larval samples. The samples were then transferred live to RNAlater (Ambion, USA) and stored at −80 C until RNA extraction. Additional information about these and other samples is given in (Olson et al., 2018).

2.2. Small RNA isolation, library construction and sequencing

Larval samples were mechanically homogenized in Trizol (Invitrogen, USA) for 10 s. Then, 200 µl of chloroform:isoamyl alcohol (24:1) was added and mixed thoroughly. Phase separation was carried out by centrifugation at maximum speed at 4 °C. Then, 0.5× isopropanol and 4 µl of glycogen (5 mg/ml) were added to the aqueous phase and the RNA was pelleted by centrifugation at maximum speed at 4 °C for 30 min. The resulting pellet was washed with 70% ethanol, air dried, and re-suspended in nuclease-free water. The amount and integrity of total RNA was determined using a 2100 BioAnalyzer (Agilent, USA). RNA was concentrated by ethanol precipitation at −20 °C overnight after elimination of polyadenylated mRNA using oligo-dT dynabeads. The resulting pellet was re-suspended in 6 µl of nuclease-free water and used as the input material.

Small RNA libraries were prepared using the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England BioLabs Inc. (NEB), USA). The 3′ and 5′ adapters were sequentially annealed with the annealing of the reverse transcription (RT) primer prior to 5′ ligation to reduce the frequency of adapter dimer formation. First strand synthesis was performed, followed by PCR enrichment of the libraries during which the index sequences were introduced post-PCR, the small RNA libraries were quantified using the Agilent Bioanalyzer and the libraries pooled based on the concentration of the 147 bp peak (small RNA and adapters). The resulting pool was cleaned up through columns and size selected using Pippin Prep and the settings detailed in the manual. Libraries were paired-end sequenced using an Illumina sequencing platform (HiSeq 2500) for 100 cycles. Small RNA libraries were constructed from three independent samples in order to count with biological replicates. For each sample, three technical replicates were sequenced. A total of nine libraries were sequenced. The small RNAseq data are available in ArrayExpress under accession code **E-ERAD-236** (samples ERS353237, ERS353255 and ERS353262) (https://www.ebi.ac.uk/arrayexpress/experiments/E-ERAD236/samples/?s_page=1%26s_pagesize=25).

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2.3. Source of genome assemblies and annotations

The ~182 Mb *H. microstoma* genome assembly (PRJEB124) and the gene annotations of 12,368 coding genes (Tsai et al., 2013) were downloaded from the WormBase Parasite database (Howe et al., 2017). Also, the ~163 Mb *H. nana* genome assembly (PRJEB508) and the corresponding 13,777 coding genes were retrieved from the WormBase Parasite database (Howe et al., 2017).

2.4. miRNA identification in H. microstoma larvae

To identify conserved and novel miRNAs from the small RNA libraries, the miRDeep2 software package (Friedländer et al., 2012) was used. The unique sequences were mapped to the *H. microstoma* genome and used as input for miRNA prediction as previously described (Cucher et al., 2015; Macchiaroli et al., 2015). The initial miRDeep2 output list of candidate miRNA precursors of each library was manually curated to generate a final high confidence set of miRNAs retaining only candidate precursors with (i) miRDeep2 score ≥ 4; (ii) mature reads in more than one biological sample; (iii) star reads and/or seed conservation; (iv) no match to rRNA, tRNA or mRNA. The secondary structures of putative precursors and clusters and the minimum free energy were predicted using the mfold web server (Zuker, 2003) and RNAfold software (Gruber et al., 2008), respectively. miRNA annotation and classification of the small RNAseq reads into RNA types (miRNAs, rRNA, tRNA and mRNAs) were performed as previously described (Cucher et al., 2015; Macchiaroli et al., 2015). In addition, mature miRNA sequences were compared by BLASTN against an in-house database of all previously reported cestode miRNA sequences obtained by deep sequencing (Bai et al., 2014; Cucher et al., 2015; Macchiaroli et al., 2015; Basika et al., 2016; Perez et al., 2017).

2.5. miRNA discovery in H. nana from genome-wide data

To discover miRNAs in the *H. nana* genome, the miRDeep2 software package (Friedländer et al., 2012) was used. The small RNA-seq reads of *H. microstoma* were mapped to the *H. nana* genome and miRNAs predicted as previously described (Macchiaroli et al., 2015). In addition, an independent approach that required the *H. nana* genome and was based on the combination of three methods, (i) miRNA-SOM (Kamenetzky et al., 2016), (ii) deepSOM (Stegmayer et al., 2017), and (iii) miRNAss (Yones et al., 2018), was used. A unique list of best candidates was obtained as the intersection of the three methods. Briefly, sequences with a minimum free energy threshold of −20 kcal/mol and single-loop folded sequences were selected according to the miRNA biogenesis model (Bartel, 2004). After that, the best candidates to precursor miRNA sequences in *H. nana* were identified as those sequences more similar to high confidence miRNAs of *H. microstoma* in the feature space (Yones et al., 2015). These methods were recently used by our group for genome-wide discovery of miRNA precursor sequences in *E. multilocularis* (Kamenetzky et al., 2016) and *T. solium* (Perez et al., 2017).

2.6. miRNA expression profiling in H. microstoma larvae

The number of reads obtained in a small RNA sequencing experiment can be used as an indicator of the abundance of a given miRNA at a particular life cycle stage (Kato et al., 2009).

To analyze miRNA expression, read counts of each individual miRNA in a sample (biological replicate) were normalized to the total number of mature miRNA read counts in that sample as described in Macchiaroli et al. (2015). Then, normalized miRNA reads were averaged between the three biological replicates and the most expressed miRNAs in *H. microstoma* larvae were determined. Correlation analyses between pairs of independent biological replicates were performed.

2.7. Evolutionary conservation analysis of Hymenolepis miRNAs

To identify miRNA families within *Hymenolepis*, all-against-all pairwise sequence alignments were computed using BLASTN and all sequences sharing the seed region (positions 2–7 of the mature miRNA) were considered to belong to the same *Hymenolepis* miRNA family. To analyze conservation of all expected miRNA families in *Hymenolepis* (Fromm et al., 2013, 2017)) mature miRNA sequences were compared with those previously reported as present in miRBase 22 for selected phyla, Cnidaria, Nematoda, Arthropoda, Annelida and the subphylum Vertebrata, using only a seed match criteria. To analyze conservation of *Hymenolepis* miRNA sequences across Platyhelminthes, the species used for comparative analysis were selected based on the following criteria: (i) genome available; (ii) data deposited in miRBase v22; (iii) the species with a more complete miRNA complement within a same genera; (iv) study based on high-throughput sequencing. Selected species were *E. canadensis*, *E. multilocularis*, *E. granulosus*, *M. corti, T. solium, T. crassiceps, Schistosoma mansoni, Gyrodactylus salaris and Schmidtea mediterranea.*

2.8. Cluster identification and genomic location of Hymenolepis miRNAs

To identify miRNA clusters in both *Hymenolepis* spp., the genomic arrangement of the miRNAs identified in this study was assessed. Precursor miRNA sequences were considered to be grouped in clusters if they were in the same scaffold/contig, less than 10 kb apart and on the same strand. Alignments of precursor miRNA sequences were performed using MUSCLE (Edgar, 2004) followed by RNAalifold (Bernhart et al., 2008) using default parameters. The phylogenetic analysis of the cluster mir-4989/277 was conducted in MEGA7 (Kumar et al., 2016). The phylogenetic trees were inferred by Maximum Likelihood using the Tamura three-parameter model.

The support for the node was assessed using 2000 bootstrap replicates. The genomic location (intronic, exonic and intergenic) of all miRNAs identified in this study and the genomic context of mir-10 was assessed by BLAST searches against current annotations of *Hymenolepis* genomes available in WormBase Parasite database v. WBPS9 (WS258). For intronic miRNAs, only miRNAs located in introns of coding genes with a predicted functional annotation were considered. For analysis of the genomic context of mir-10, only the two neighboring genes were considered (i.e. the closest protein coding gene upstream/downstream with a functional annotation).

3. Results and discussion

3.1. miRNA identification in H. microstoma and miRNA discovery in H. nana from genome-wide data

To identify the repertoire of miRNAs expressed in *H. microstoma* larvae, we sequenced small RNA libraries from three biological replicates of *H. microstoma* larvae. After trimming and filtering, between 8 and 87 million reads per sample were mapped to the *H. microstoma* genome, representing ~97% of reads. The general results of the Illumina deep sequencing are shown in Supplementary Table S1. We predicted a high confidence repertoire of 37 conserved miRNAs in *H. microstoma*, providing for the first known time

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experimental evidence of miRNA expression in *H. microstoma* (Table 1). Of the 37 miRNAs, 26 precursors have previously been bioinformatically predicted from the *H. microstoma* genomic data (Jin et al., 2013) but 11 are new to this study. The latter precursor miRNAs comprised four mir-36 (hmi-mir-36b, hmi-mir-36c, hmi-mir-36d, hmi-mir-36e), two mir-3479 (hmi-mir-3479a and hmi-mir-3479b), hmi-mir-210, hmi-mir-307, hmi-mir-7b, hmi-mir-124a and hmi-mir-277b. The secondary structures of all precursor miRNA sequences identified in *H. microstoma* are shown in Supplementary Data S1. Thus, we expanded the miRNA repertoire of *H. microstoma,* highlighting the potential of the deep sequencing approach for miRNA discovery. The repertoire of precursor miRNA sequences and their genomic location are shown in Supplementary Table S2.

To discover miRNA precursors in the *H. nana* genome by using small RNAseq data of *H. microstoma*, we first mapped the small RNAseq reads of *H. microstoma* to the *H. nana* genome. We obtained a high percentage of genome mapping (average 90%, Supplementary Table S1). Then, we predicted a high confidence repertoire of 37 conserved miRNAs in the *H. nana* genome. All precursor miRNAs identified in *H. nana* were conserved in *H. microstoma,* with 81% (mir-210) to 100% (bantam, mir-71, mir-2162) sequence identity (average 95%). We did not find *H. nana*-specific precursor miRNAs in the genome. The precursor miRNA sequences of *H. nana* and their genomic location are shown in Supplementary Table S3.

In addition, we performed a genome-wide discovery of miRNAs in *H. nana* by using a different approach based on the combination of three methods. We found a unique list of 36 best candidates to miRNA precursors by the intersection of the three methods (Supplementary Fig. S1). All best candidates were previously found by using small RNAseq data of *H. microstoma.* To our knowledge, this is the first time that miRNAs are described in *H. nana*.

3.2. miRNA expression profiling in H. microstoma larvae

To analyze miRNA expression profiling in *H. microstoma* larvae, the normalized reads per million of each mature miRNA were averaged among the three biological replicates. Correlation analyses between pairs of biological replicates indicated high technical reproducibility and low biological variation ($r > 0.83$). The mature miRNA repertoire and larval expression levels for the 37 mature *H. microstoma* miRNAs are shown in Table 1.

For most precursor sequences identified (32/37), we detected the corresponding antisense miRNA sequences consistent with the miRNA biogenesis model, adding confidence to the predictions obtained (Supplementary Data S1). Also, we found that most mature miRNAs in *H. microstoma* (~65%) are processed from the 3′ arm (Table 1, Supplementary Data S1). This bias was also observed in *Echinococcus* (Cucher et al., 2015; Macchiaroli et al., 2015), *T. crassiceps* (Perez et al., 2017) and *M. corti* (Basika et al.,

Table 1
The repertoire and expression profile of the mature microRNAs (miRNAs) identified in *Hymenolepis microstoma* larvae.

miRNA name	Mature sequence (5′-3′)	Length (nt)	Normalized reads per million (average)[a]	Normalized reads % (average)
hmi-bantam-3p	ugagaucgcgauuacagcugau	22	51,279	5.13%
hmi-let-7-5p	ugagguaguguuucgaacgucu	22	39,361	3.94%
hmi-miR-1-3p	uggaauguugugaaguaugua	21	42,250	4.23%
hmi-miR-2a-3p	uaucacagcccugcuugaaaaau	23	6479	0.65%
hmi-miR-2b-3p	uaucacagcccugcuugggac	21	556	0.06%
hmi-miR-2c-3p	ucacagccaauauugaugaacg	22	23,929	2.39%
hmi-miR-7a-5p	uggaagacuggugauauguugca	23	918	0.09%
hmi-miR-7b-5p	uggaagacuugugauuagauuguu	24	1361	0.14%
hmi-miR-9-5p	ucuuugguuaucuagcugugug	22	257,606	25.76%
hmi-miR-10-5p	cacccuguagacccgaguuuga	22	141,117	14.11%
hmi-miR-31-5p	uggcaagauacuggcgaagcuga	23	122	0.01%
hmi-miR-36b-3p	ucaccggguaguuauuacgccu	22	84	0.01%
hmi-miR-36c-3p	ucaccggguugauaauaucuca	22	1067	0.11%
hmi-miR-36d-3p	ccaccggguugauaauagucga	22	600	0.06%
hmi-miR-36e-3p	ucaccggguugauaauggcuca	22	806	0.08%
hmi-miR-61-3p	ugacuagaaagagcacucacauc	23	4587	0.46%
hmi-miR-71-5p	ugaaagacgaugguagugagaua	23	241,743	24.17%
hmi-miR-87-3p	gugagcaaaguuucaggugugc	22	23,844	2.38%
hmi-miR-96-5p	auuggcacuuuuggaauuguca	22	2969	0.30%
hmi-miR-124a-3p	uaaggcacgcggugaaugcc	20	76	0.01%
hmi-miR-124b-3p[b]	uaaggcacgcggugaauacca	21	1190	0.12%
hmi-miR-125-5p	ucccugagaccauagaguuguc	22	4824	0.48%
hmi-miR-133-3p	uuggucccauuaaccagccgc	22	595	0.06%
hmi-miR-153-5p	cugcuuacgagacgugcauuc	21	357	0.04%
hmi-miR-184-3p	gggacggaagucugaaagguuu	22	5773	0.58%
hmi-miR-190-5p	agauauguuuggguuacuuggug	23	16,928	1.69%
hmi-miR-210-3p	uugugcgucguuucagugaccgac	24	333	0.03%
hmi-miR-219-5p	ugauuguccauucgcauuucuug	23	3689	0.37%
hmi-miR-277a-3p	uaaaugcauuuucuggcccgua	22	26,235	2.62%
hmi-miR-277b-3p[c]	uaaaugcaaaauaucugguuauga	24	569	0.06%
hmi-miR-281-3p	ugucauggaguugcucucuaua	22	1595	0.16%
hmi-miR-307-5p	ccucguccuguggguuggggagaug	24	984	0.10%
hmi-miR-745-3p	ugcugccuggguaagagcuguga	22	23,830	2.38%
hmi-miR-2162-3p	uauuaugcaacuuuucacucc	21	2025	0.20%
hmi-miR-3479a-3p	uauugcacauucuuucgccauc	22	26,311	2.63%
hmi-miR-3479b-3p[d]	gauugcacuacucaucgcccgc	22	615	0.06%
hmi-miR-4989-3p	aaaaugcaccaacuaucugaca	22	43,391	4.34%

nt, nucleotides.

[a] MiRNA read numbers were normalized to the total number of mature miRNAs in each library and multiplied per 1×10^6. Normalized miRNA reads were averaged between biological replicates.

[b] Previously reported as miR-124a by Jin et al. (2013).

[c] Previously reported as miR-new-2 by Macchiaroli et al. (2015).

[d] Previously reported as miR-new-3 by Macchiaroli et al. (2015).

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2016). In addition, this bias toward 3′ arm processing was observed in nematodes, fruit fly and plants (de Wit et al., 2009). For three precursor miRNAs (hmic-mir-36b, hmic-mir-210 and hmic-mir-3479b), the antisense miRNA sequence was abundantly expressed (>30% with respect to the mature sequence) (Supplementary Data S1). Since the antisense miRNA sequence has a different seed sequence that is the principal determinant of the interaction between miRNA and mRNA target, it may indicate additional functions for the same miRNA gene.

Regarding the miRNA expression profile, a few miRNAs showed very high expression levels (Table 1). The most expressed miRNAs identified in *H. microstoma* larvae were hmic-miR-9-5p, hmic-miR-71-5p and hmic-miR-10-5p, which accounted for approximately 60% of total miRNA expression. The expression level of these miRNAs was followed by hmic-4989-3p and hmic-bantam-3p (Fig. 1). These top five miRNAs accounted for approximately 70% of the total miRNA expression. The high expression of these miRNAs in *Hymenolepis* larvae is conserved in the larval stages of the cestode parasites *Echinococcus* (Cucher et al., 2015; Macchiaroli et al., 2015), *M. corti* (Basika et al., 2016) and *T. cracisseps* (Perez et al., 2017), suggesting an essential function in the biology of the parasites. Interestingly, miR-9 and miR-10 are highly conserved miRNAs across metazoans with known roles in neural development (Yuva-Aydemir et al., 2011) and Hox regulation (Lund, 2010; Tehler et al., 2011), respectively. MiR-71, a bilaterian miRNA absent in vertebrates, is known to be involved in lifespan regulation and stress response in *Caenorhabditis elegans* (Zhang et al., 2011; Boulias and Horvitz, 2012). Recently, miR-71 and miR-10 were predicted to target developmental pathways such as the mitogen-activated protein kinase (MAPK) pathway and the Wnt pathway in *Echinococcus* (Macchiaroli et al., 2017). In addition, most miRNAs identified in *H. microstoma* larvae (24/37) showed very low expression levels with less than 1% of total miRNA reads (Table 1) and the low the expression is conserved in the larval stages of the cestode parasites *Echinococcus* (Cucher et al., 2015; Macchiaroli et al., 2015), *M. corti* (Basika et al., 2016) and *T. cracisseps* (Perez et al., 2017).

3.3. Evolutionary origin and conservation analysis of Hymenolepis miRNA families

The 37 miRNAs identified in both *Hymenolepis* spp. were classified into 27 miRNA families according to the conservation of their seed regions (positions 2–7 of the mature miRNAs). Among them, we found that six miRNA families had multiple members: miR-2 (miR-2a, miR-2b, miR-2c), miR-7 (miR-7a, miR-7b), miR-36 (miR-36b, miR-36c, miR-36d, miR-36e), miR-124 (miR-124a, miR-124b), miR-277 (miR-277a, miR-277b, miR-4989) and miR-3479 (miR-3479a, miR-3479b). All miRNA families identified in *Hymenolepis* were conserved across evolution and the protostomian-specific miR-36 family is the largest in *Hymenolepis*. Regarding their evolutionary origin, we found one eumetazoan-specific miRNA family, 18 bilaterian-specific miRNA families and eight protostomian-specific miRNA families in *Hymenolepis* (Fig. 2). We did not find either lophotrochozoan-specific miRNA families or platyhelminth-specific miRNA families (Fig. 2). The phylogenetic distribution of all expected miRNA families (Fromm et al., 2013, 2017)) was based on the classification of miRNA families by Wheeler et al. (2009) and Tarver et al. (2013), and was confirmed by homology searches in miRBase v.22. Interestingly, miR-71 is a bilaterian-specific miRNA that is absent in the subphylum Vertebrata (Fig. 2).

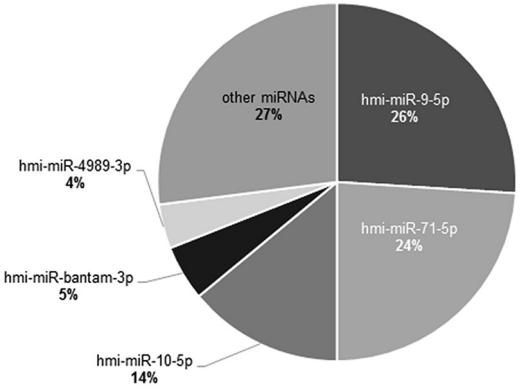

Fig. 1. The top five most expressed miRNAs (miR) identified in *Hymenolepis microstoma* (hmi) larvae. The normalized reads per million of each mature miRNA were averaged among biological replicates. The average proportions of miRNA reads among samples are shown in the pie chart.

We found that the small number of conserved miRNA families identified in *Hymenolepis* (~27) is similar to that found in species of *Echinococcus* and *Taenia*, as well as *M. corti*, the trematode *S. mansoni*, the monogenean *G. salaris* and the planarian *S. mediterranea* (Table 2). These results agree with the loss of flatworm miRNAs proposed by Fromm et al. (2013) and reflect the low morphological complexity of platyhelminths compared with other metazoans, consistent with previous knowledge (Niwa and Slack, 2007; Berezikov, 2011).

However, the total number of conserved miRNAs is ~ two-fold lower in parasitic platyhelminths compared with the free-living *S. mediterranea* (Table 2). This may be due to a reduction in the number of members of almost all miRNA families in parasitic platyhelminths compared with free-living *S. mediterranea* (with the exception of miR-3479 and miR-36). The reduced complement of evolutionarily conserved miRNAs found in both *Hymenolepis* spp. may reflect their parasitic lifestyle as previously described for other cestodes (Macchiaroli et al., 2015). Many microRNA families are deeply conserved in bilaterian animals and display similar tissue specificities between divergent species, suggesting a role in the evolution of tissue identity (Christodoulou et al., 2010). The loss of some of these deeply conserved miRNA families in tapeworms may be related to the loss or reduction of tissues and organs. For example, miRNAs associated with locomotor-related cilia (miR-29), gut (miR-216/miR-283, miR-278) and sensory organs (miR-2001) were specifically lost in tapeworms, probably reflecting the reduction or loss of these cells and organs.

Some differences between the miRNA repertoires of *Hymenolepis* and other parasitic platyhelminths were found. Interestingly, we found four members of the miR-36 miRNA family in *Hymenolepis*, whereas only two members of miR-36 were described in the cestodes *Echinococcus*, *Taenia* and *M. corti* and the trematode *S. mansoni*. We found members of the miR-210 family in *Hymenolepis* that are not found in *Echinococcus* and *Schistosoma*. Recently, two members of the miRNA mir-210 family were identified by us for the known first time in *M. corti:* mco-miR-12065-3p and mco-miR-12066-3p (Basika et al., 2016), and one member in *Taenia* (Perez et al., 2017).

We did not identify miR-8 and miR-1992 orthologs in *Hymenolepis*, consistent with a previous report from *H. microstoma* (Jin et al., 2013) and *M. corti* (Basika et al., 2016). Also, miR-8 was not identified in *Taenia* (Perez et al., 2017). However, these two miRNAs were found to be expressed in *Echinococcus* (Bai et al., 2014; Cucher et al., 2015; Macchiaroli et al., 2015), suggesting that miR-8 and miR-1992 may have been lost during evolution in *H. microstoma* (Table 2). We also failed to find mir-8 and mir-1992 orthologs within the genome of *H. microstoma* using bioinformatics searches. However, we cannot rule out that these miRNAs that are not expressed in the larval stage here analyzed could be present in the genomes.

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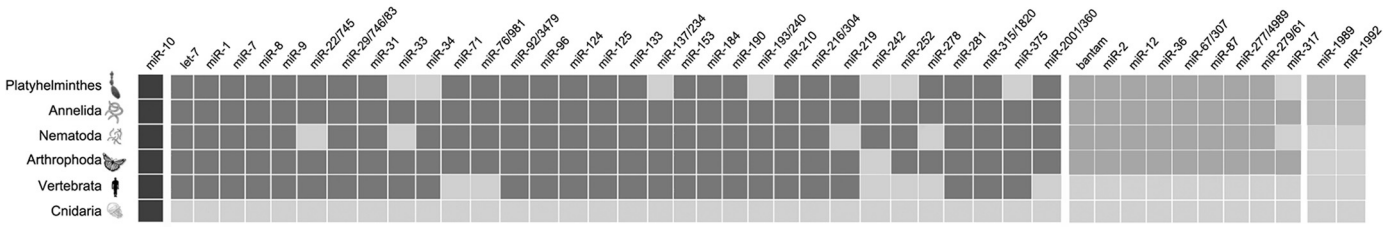

Fig. 2. Evolutionary origin of all expected *Hymenolepis* miRNA families(miR) and their conservation among selected phyla. Blocks from left to right represent the evolutionary origin of all expected miRNA families in *Hymenolepis*: eumetazoan, bilaterian, protostomian and lophotrochozoan. Medium and dark grey blocks represent presence in the corresponding phyla. Light grey blocks represent absence in the corresponding phyla.

Table 2
MicroRNA (miRNA) families conserved in selected platyhelminths and their numbers of members.

miRNA family	Seed	Class Cestoda				Class Trematoda	Class Monogenea	Class Rhabditophora
		Hymenolepis microstoma/ nana[a]	*Echinococcus canadensis/ granulosus/ multilocularis*[b]	*Taenia solium/ crassiceps*[c]	*Mesocestoides corti*[d]	*Schistosoma mansoni*[e]	*Gyrodactylus salaris*[f]	*Schmidtea mediterranea*[g]
bantam	GAGAUCG	1	1	1	1	1	1	4
let-7	GAGGUAG	1	1	1	1	1	1	4
mir-1	GGAAUGU	1	1	1	1	2	1	3
mir-2	AUCACAG	3	3	3	3	6	4	7
mir-7	GGAAGAC	2	2	2	2	1	1	4
mir-8	AAUACUG	-	1	–	–	1	1	2
mir-9	CUUUGGU	1	1	1	1	–	1	2
mir-10	ACCCUGU	1	1	1	1	1	2	2
mir-31	GGCAAGA	1	1	1	1	1	1	4
mir-36	CACCGGG	4	2	2	2	2	1	3
mir-61/279	GACUAGA	1	1	1	1	1	1	2
mir-71	GAAAGAC	1	1	1	1	2	3	4
mir-87	UGAGCAA	1	1	1	1	–	1	4
mir-96	UUGGCAC	1	1	1	1	1	2	2
mir-124	AAGGCAC	2	2	2	2	2	2	6
mir-125	CCCUGAG	1	1	1	1	3	1	3
mir-133	UGGUCCC	1	1	1	1	–	–	2
mir-153	UGCAUAG	1	1	1	1	–	1	1
mir-184	GGACGGA	1	1	1	1	–	3	2
mir-190	GAUAUGU	1	1	1	1	1	1	3
mir-210	UGUGCGU	1	–	1	2	–	–	1
mir-219	GAUUGUC	1	1	1	1	1	1	1
mir-277/4989	AAAUGCA	3	3	3	3	2	2	4
mir-281	GUCAUGG	1	1	1	1	1	1	1
mir-307/67	CACAACC	1	1	1	1	–	1	1
mir-745/22	GCUGCCU	1	1	1	1	1	–	1
mir-1992	CAGCAGU	–	1	1	–	–	1	1
mir-2162/1993	AUUAUGC	1	1	1	1	1	1	1
mir-3479/92	AUUGCAC	2	2	2	2	1	–	1
Number of conserved miRNAs (families)		37 (27)	36 (28)	36 (28)	36 (27)	33 (21)	36 (25)	75 (29)

[a] This study.
[b] Cucher et al. (2015); Macchiaroli et al. (2015).
[c] Perez et al. (2017).
[d] Basika et al. (2016).
[e] de Souza Gomes et al. (2011); Marco et al. (2013); Protasio et al. (2017).
[f] Fromm et al. (2013).
[g] Friedländer et al. (2009); Lu et al. (2009).

It would be interesting to analyze small RNAseq data of other stages in order to confirm whether these two miRNAs and other known cestode miRNA families are identified, especially miR-1992 which is the only lophotrochozoan-specific miRNA present in cestodes.

3.4. miRNA clusters in Hymenolepis

miRNAs can be grouped into clusters in the genome if they are less than 10 kb apart (miRBase v22), suggesting co-expression as a single transcriptional unit (Bartel, 2004). miRNA clusters have been found in the genomes of many species, including helminth parasites (miRBase 22).

To investigate the presence of miRNA clusters in *Hymenolepis*, the genomic arrangements of the 37 miRNAs identified in this study were assessed. We found three miRNA clusters conserved in both *Hymenolepis* species: mir-71/2c/2b, mir-277a/4989 and mir-36c/36d/36e. Each miRNA cluster comprised a genomic region of up to 320 bp, located in intergenic regions (Supplementary Table S4). The predicted secondary structure of the three clusters found in *Hymenolepis* is shown in Fig. 3. The miRNA clusters mir-71/2c/2b and mir-277a/4989 were previously reported in *H. microstoma* (Jin et al., 2013), whereas the miRNA cluster mir-36c/36d/36e was identified for the first time in the class Cestoda in this study. All three miRNA clusters were described for the first time in *H. nana.*

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It has been proposed that mir-1 and mir-133 form another cluster in *H. microstoma* (Jin et al., 2013). Although miR-1 and miR-133 clustering is highly conserved across metazoan species (Campo-Paysaa et al., 2011), we found that mir-1 is located 15 kb further from mir-133, suggesting that these miRNAs are not co-expressed as a single transcriptional unit in *Hymenolepis,* consistent with the situation reported in *Echinococcus* (Cucher et al., 2015; Macchiaroli et al., 2015), *M. corti* (Basika et al., 2016) and *T. solium* (Perez et al., 2017).

It is estimated that more than 40% of human miRNAs and more than 30% in worms and flies are found in clusters with other miRNAs (Griffiths-Jones et al., 2008). Here, we found a smaller percentage of miRNAs in clusters in both *Hymenolepis* genomes ~ 20% (8/37) and other cestodes analyzed. The fact that miRNA clusters are conserved across evolution suggests evolutionary and functional importance.

3.4.1. The miRNA cluster mir-71/mir-2c/mir-2b in Hymenolepis

The miRNA cluster mir-71/mir-2c/mir-2b is a protostomian-specific miRNA cluster highly conserved within platyhelminths. The mir-71 cluster with members of the mir-2 family is conserved in *Schistosoma* (Huang et al., 2009; de Souza Gomes et al., 2011) and *S. mediterranea* (Palakodeti et al., 2006), among other species within platyhelminths. Also, clustering of mir-71 and mir-2 has been found in nematodes indicating wider evolutionary conservation (Winter et al., 2012). Phylogenetic analyses have been done recently in other platyhelminths that showed multiple copies of a mir-71 cluster (Fromm et al., 2013; Jin et al., 2013). We did not find additional copies of this cluster in *Hymenolepis,* concurring with previous studies from *Echinococcus*, *M. corti* and *T. solium* (Cucher et al., 2015; Macchiaroli et al., 2015; Basika et al., 2016; Perez et al., 2017). Regarding the evolutionary origin of the members of this cluster, miR-71 is a bilaterian-specific miRNA family absent in vertebrata, whereas miR-2 is a protostomian-specific miRNA family. Whether the conserved genomic arrangement among platyhelminths means functional conservation of this cluster within this lineage remains to be investigated.

Regarding expression of *H. microstoma* miRNAs encoded in clusters, we found different levels of expression between members of the cluster mir-71/2c/2b. This result agrees with previous observations in *Echinococcus* (Cucher et al., 2015), *M. corti* (Basika et al., 2016) and *T. crassiceps* (Perez et al., 2017). This could be explained by the fact that miRNA themselves are subject to sophisticated regulation through control of miRNA processing, RNA editing or miRNA decay (Krol et al., 2010).

3.4.2. The miRNA cluster mir-277a/4989 in Hymenolepis

The miRNA cluster mir-277a/4989 is a lophotrochozoan-specific cluster highly conserved across Platyhelminthes. The alignment of the *Hymenolepis* cluster mir-277/4989 and its orthologs across Platyhelminthes is shown in Fig. 4A. The mature miRNA sequences miR-4989 and miR-277a are located in the 3′arm of their corresponding precursor sequences. The alignment showed a high level of nucleotide conservation in the mature miRNA sequences of all platyhelminths analyzed, especially in the seed regions, and the whole sequences are highly conserved only in

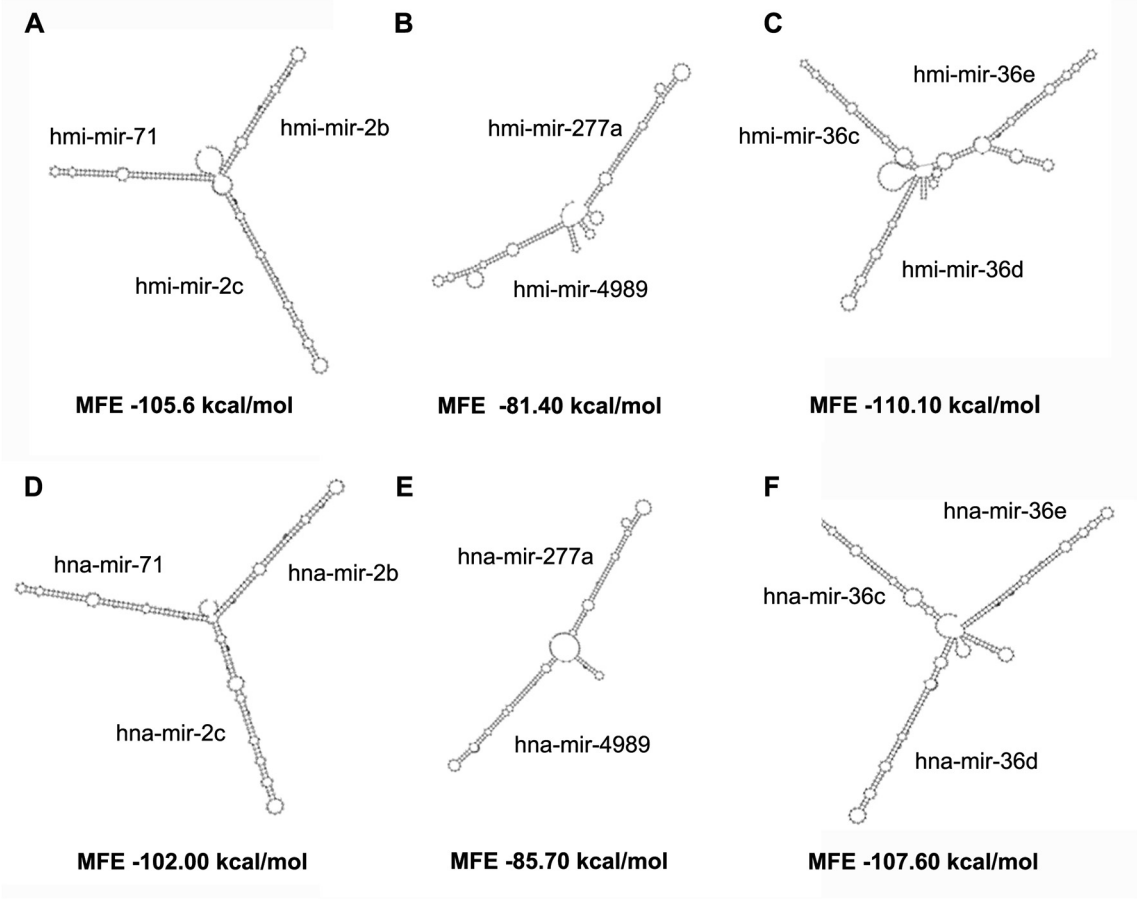

Fig. 3. Secondary structures of microRNA (miRNA) clusters identified in *Hymenolepis microstoma* (hmi) and *Hymenolepis nana* (hna) predicted with RNAfold. hmi-mir-71/2c/2b (A), hmi-mir-277a/4989 (B), hmi-mir-36c/36d/36e (C), hna-mir-71/2c/2b (D), hna-mir-277a/4989 (E), hna-mir-36c/36d/36e (F). Precursor miRNA sequences (mir) are indicated and the minimum free energy (MFE) of each cluster is shown.

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the Cestoda lineage. Although the two members of the cluster mir-277a/4989 belong to the protostomian miRNA family mir-277, their genomic organization in a cluster was only found in lophotrochozoan species (miRBase v22). One additional copy of a cluster that contains two members of the mir-277 family was described in the free living *S. mediterranea* (Palakodeti et al., 2006). Recently,

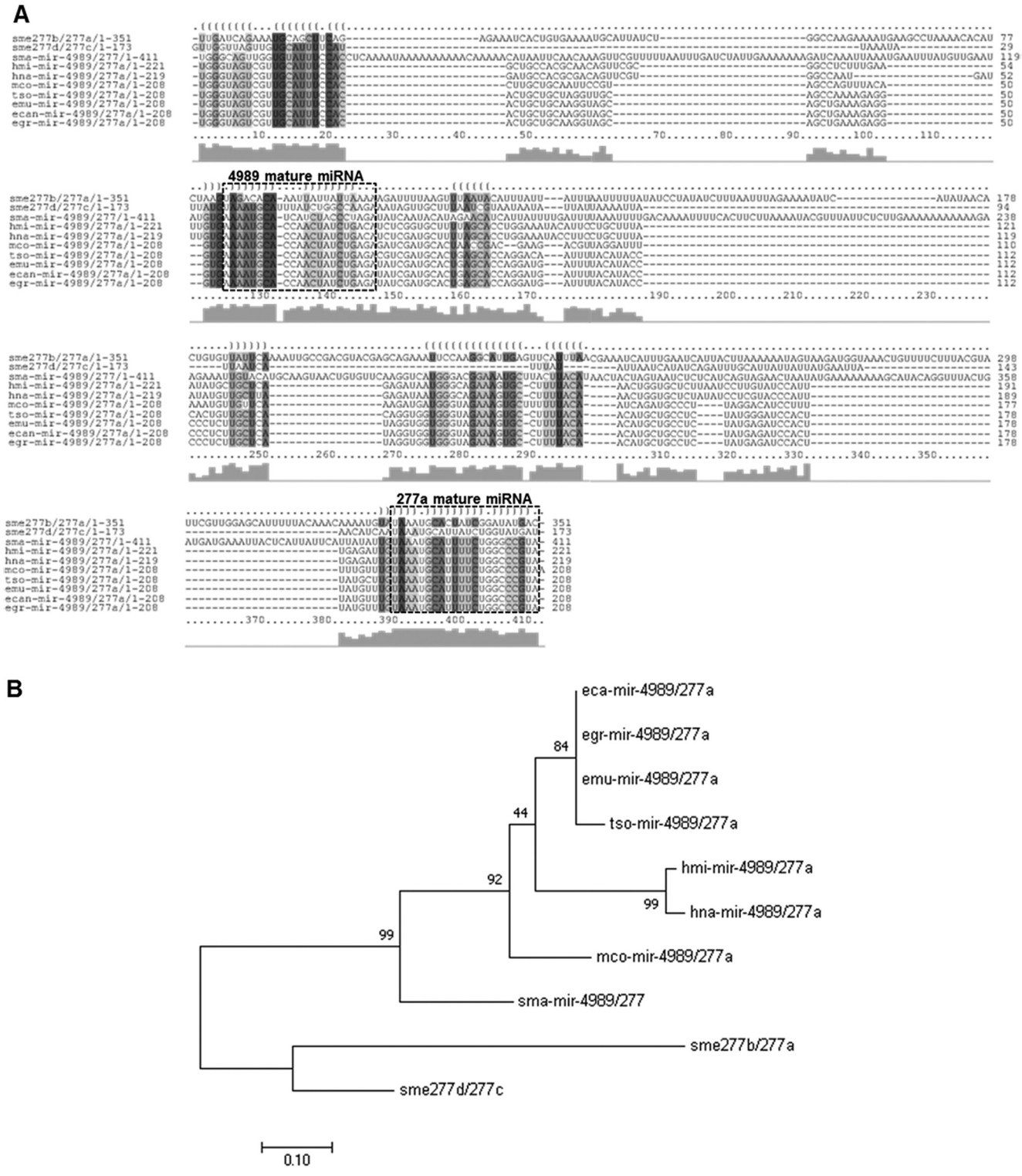

Fig. 4. Alignment and phylogenetic analysis of the *Hymenolepis* microRNA (miRNA) cluster mir-4989/277a with its orthologs across Plathyhelminthes: *Schmidtea mediterranea* (sme), *Schistosoma mansoni* (sma), *Hymenolepis microstoma* (hmi), *Hymenolepis nana* (hna), *Mesocestoides corti* (mco), *Taenia solim* (tso), *Echinococcus multilocularis* (emu), *Echinococcus canadensis* (eca), *Echinococcus granulosus* (egr). Alignment of precursor miRNA sequences (mir) was performed using MUSCLE followed by RNAalifold. The conserved nucleotides are highlighted in grey scale and the mature miRNAs are indicated with a box. The level of nucleotide identity is indicated with grey color below the alignment (A). A phylogenetic tree was inferred using the Maximum Likelihood method based on the Tamura three-parameter model in MEGA7. The topology of the tree with the highest log-likelihood value is shown. The sequence alignment used as input is shown above. The percentages of trees in which the sequences clustered together are shown next to the branches. The support for the node was assessed using 2000 bootstrap replicates (B).

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cluster mir-277/4989 was found in the trematode *S. mansoni* (Protasio et al., 2017). Interestingly, the results of that study suggest that the cluster mir-277/4989 might play a dominant role in post-transcriptional regulation during development of juvenile worms in *S. mansoni*. Whether this cluster plays a developmental role in *Hymenolepis* remains to be investigated. We also performed for the first known time a phylogenetic analysis of the cluster miR-277a/4989 that confirmed the relationship of the parasitic platyhelminths, highlighting the potential of miRNAs as phylogenetic markers (Fig. 4B).

3.4.3. The miRNA cluster mir-36c/36d/36e in Hymenolepis

The miRNA cluster mir-36c/36d/36e was identified for the first time in the class Cestoda in this study. Although this cluster is not conserved in Platyhelminthes (Lophotrochozoa), members of the mir-36 family were found to be in a cluster with members of the same or other miRNA family in *S. mediterranea* (sme-mir-36c/36a and sme-mir-36b/let7b) and *S. mansoni* (sma-mir-36b/8406). In addition, the miRNA family miR-36 has a protostomian origin, and their arrangement is in a cluster with members of the same family. Interestingly, this cluster is conserved in Nematoda (Ecdysozoa), where this miRNA family has multiple members organized in clusters. In the free-living *C. elegans* the miR-36 cluster is composed of seven members of this family (mir-35 to mir-41). In this model organism, deletion of the miR-36 cluster produces embryonic and larval lethality, suggesting an essential role in early development (Alvarez-Saavedra and Horvitz, 2010). In parasitic nematodes such as *Brugia malayi,* four members of the miR-36 family have been identified (Poole et al., 2014) with three of them (miR-36c/36d/36c) organized in a cluster with five members of other families. However, the whole cluster is contained in a genomic region of ~12,000 bp (miRBase v.22). Recently, miR-36 from *S. mansoni*, among others, was found to be secreted in vitro (Samoil et al., 2018).

3.5. Genomic arrangement of Hymenolepis miRNAs

The genomic arrangement of the 37 miRNAs identified in both *Hymenolepis* spp. was analyzed. This analysis showed that most precursor miRNAs (92% (34/37)) were located in intergenic regions distant from annotated genes, and 8% (3/37) were located in introns of protein coding genes in *Hymenolepis*. The bias in the genomic location found in this study was also observed in other platyhelminths such as *E. multilocularis* (Cucher et al., 2015) and *Schistosoma japonicum* (Cai et al., 2011) where 81% and 90% of the miRNA complement, respectively, was located in intergenic regions. The three intronic miRNAs found in this study in *Hymenolepis* were mir-190, mir-96 and mir-3479b (Supplementary Tables S2, S3).

3.5.1. The intronic miRNAs in Hymenolepis

We also performed a comparative analysis of the genomic location of the three intronic miRNAs identified in each *Hymenolepis* spp. across selected platyhelminths with available genomes: *S. mediterranea*, *S. mansoni*, *E. multilocularis*, *E. granulosus*, *E. canadensis*, *T. solium* and *M. corti*. The results of this analysis are shown in Table 3.

We found that the bilaterian miRNA mir-190 was located within the intron of the protein coding gene *talin* in both *Hymenolepis* spp. Also, we report for the first known time that the genomic location of miR-190 was conserved in *M. corti*, *T. solium, E. granulosus* and *E. canadensis.* These findings are consistent with previous studies, where mir-190 has been found in the intronic region of the *talin* gene in the platyhelminths *H. microstoma*, *E. multilocularis* and *S. mansoni* (Table 3), and in higher metazoans including *Homo sapiens* (Campo-Paysaa et al., 2011). Recently, mir-190 was also found to be located in an intron of the gene encoding *talin* protein in three Opisthorchiids (Ovchinnikov et al., 2015). Several functions have been proposed for miR-190 in mammals, for example it regulates *neurogenic differentiation 1* (NeuroD) activity and can also interact with other transcription factors that regulate neurogenesis, such as Pax6 (Zheng et al., 2012). *Talin* is an adhesion plaque protein that links the integrin-mediated cell–matrix contacts to the actin cytoskeleton. These interactions play an important role in regulating synapse morphology and number, neuron-neuron and neuromuscular synaptic transmission, and neuroplasticity that modulates neuronal cell proliferation, migration, and differentiation (Venstrom and Reichardt, 1993).

In addition, we found that the bilaterian miRNA mir-96 was located within the intron of the protein coding gene *Fras1-related extracellular matrix protein (Frem1)* in *H. microstoma,* consistent with Jin et al. (2013). Also, in this study we found that this arrangement was conserved in *E. granulosus,* concurring with previous reports from *E. multilocularis* and *S. mediterranea* (Table 3). Also, in this study we found that this arrangement was conserved in *E. granulosus,* concurring with previous reports from *E. multilocularis* and *S. mediterranea* (Table 3). Unlike miR-190, the intronic location of miR-96 is not conserved in mammals. Recently, it was shown that miR-96 is a sensory organ-specific miRNA expressed in the mammalian cochlea that regulates the progression of differentiation of inner and outer hair cells during development (Kuhn et al., 2011). The extracellular matrix protein *Frem1* plays a role in epidermal differentiation and is essential for epidermal adhesion during embryonic development in mice (Smyth et al., 2004).

Here we found for the first known time that the bilaterian miRNA mir-3479b (family miR-92/25, seed AUUGCA) was located within the intron of the protein coding gene *minichromosome maintenance complex component 2* (*mcm2*) in *H. microstoma*. Also, in this study, we found that this arrangement was conserved in *E. granulosus,* consistent with previous reports in *E. multilocularis* (Cucher et al., 2015). Interestingly, the human miRNA mir-25 (family miR-92/25, seed AUUGCA) is a member of the miRNA cluster mir-25/93/106b that is located in the thirteenth intron of the gene *mcm-7* in *Homo sapiens* (Rodriguez et al., 2004). Recently, the miR-25-93-106b cluster was shown to regulate tumor metastasis and immune evasion (Cioffi et al., 2017). Also, miR-92 was shown to be part of a cancer miRNA signature composed of a large portion of overexpressed miRNAs (Volinia et al., 2006). The protein encoded by *mcm7* belongs to the highly conserved *mcm* protein family of DNA helicases that are essential for the initiation of genome replication in eukaryotes. High expression levels of this protein promote cancer progression (Qu et al., 2017).

Interestingly, the functions of both the intronic miRNAs and their host genes suggest that they may be functionally related in higher organisms. It remains to be determined whether the intronic miRNAs are functionally related with their host gene in *Hymenolepis* and in other flatworms. The three intronic miRNAs found here are bilaterian-specific and are located in the same orientation of their corresponding host genes, suggesting that they may depend on their host gene promoter for transcription, resulting in a coordinated expression. Also, the small length of mir-190 and mir-3479b harboring introns in all species supports this hypothesis. The only exception was the mir-190 harboring intron in *S. mansoni* (Table 3). In addition, we found that all intronic miRNAs were located in the last introns of their corresponding host genes and this structure is conserved among the platyhelminths analyzed (Table 3). It would be interesting to determine whether this location has implications for the regulation of their expression. Interestingly, the three intronic miRNAs and their host genes are expressed at very low levels; less than 1% (Table 1) and less than 100 fragments per kilobase per million mapped reads (FPKM), respectively. Finally, the lack of conservation of the genomic loca-

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Table 3
Intronic microRNAs (miRNAs) identified in *Hymenolepis*, the structure of their host genes and their conservation across selected platyhelminths.

miRNA	Species	Gene ID[a]	Gene length (bp)	Number of exons	Intron[b]	Intron length (bp)[b]	Gene description
mir-190	*Hymenolepis microstoma*	HmN_000220000	44,564	40	36–37	356	tln-1[c]
	Hymenolepis nana	HNAJ_0000550001	3248	11	7–8	350	Putative talin 2
	Mesocestoides corti	MCOS_0000787901	23,883	44	40–41	236	Putative talin 2
	Taenia solium	TsM_000902500	27,323	44	40–41	225	Putative talin 2
	Echinococcus multilocularis	EmuJ_000736000	26,742	43	39–40	224	Talin
	Echinococcus granulosus	EgrG_000736000	26,854	43	39–40	225	Talin
	Echinococcus canadensis	ECANG7_01300	36,662	47	42–43	223	tln-1[c]
	Schistosoma mansoni	Smp_037860	12,433	10	7–8	1237	Putative talin
mir-96	*H. microstoma*	HmN_000594600	28,507	11	10–11	22,109	Fras1 related extracellular matrix protein
	Schmidtea mediterranea	mk4.010175.01	11,452	5	4–5	293	Fras1 related extracellular matrix protein[d]
	E. multilocularis	EmuJ_000086300	28,529	36	35–36	7203	Fras1 related extracellular matrix protein
	E. granulosus	EgrG_000086300	28,654	36	35–36	7301	Fras1 related extracellular matrix protein
mir-3479b	*H. microstoma*	HmN_000372000	8996	11	10–11	219	mcm-2[e]
	E. multilocularis	EmuJ_000212600	4678	11	10–11	184	mcm-2[e]
	E. granulosus	EgrG_000212600	4680	11	10–11	184	DNA replication licensing factor MCM2[e]

[a] Overlapping gene in sense orientation.
[b] Intron harboring miRNA.
[c] tln-1: talin 1 (*Caenorhabditis elegans* ortholog).
[d] *E. multilocularis* ortholog.
[e] mcm-2: minichromosome maintenance complex component 2 (*C. elegans* ortholog).

tion of mir-96 and mir-3479b in the *H. nana* genome could be due to the draft nature of the genome assembly in this species. The *H. nana* genome is highly fragmented (16,212 scaffolds in the WBPS9 release available from Wormbase Parasite compared with 3643 scaffolds for *H. microstoma*), making the genome localization of miRNAs and the analysis of their genomic context more difficult. The same interpretation may be valid for *E. canadensis*.

3.5.2. The mir-10 genomic organization in Hymenolepis

Mir-10 is one of the most ancient miRNAs that is present in all species of metazoans. In most bilateral animal species, mir-10 is encoded within Hox clusters (Campo-Paysaa et al., 2011). In this work, we found that one of the neighboring genes of mir-10 in *H. microstoma* was a Hox gene (**HmN_000772500**) and was located 41 kb apart from mir-10. We also found that this genome arrangement was conserved in *E. granulosus* and *E. canadensis* (Supplementary Table S5). These findings are consistent with our previous results in the cestodes *E. multilocularis* (Cucher et al., 2015) and *T. solium* (Perez et al., 2017) where the neighboring genes in these species were the Homeobox protein Hox B4a (**EmuJ_000813900**) and the Homeobox protein mab 5 (**TsM_000864600**), respectively. These genes were found to be located 25 kb apart from mir-10 in both species (Supplementary Table S5). In addition, we found that one of the neighboring genes of mir-10 in the trematode *S. mansoni* was a Gsx family Homeobox protein (**Smp_081620**) and was located 84 kb far from mir-10, consistent with previous results (de Souza Gomes et al., 2011). Recently, miR-10 was predicted to target Hox genes and transcription factors in *T. solium* (Perez et al., 2017). Also, miR-10 was predicted to target one homeobox containing protein from the Meis family in all *Echinococcus* spp. (Macchiaroli et al., 2017). In mammals, mir-10a resides upstream from Hoxb4 and mir-10b upstream from Hoxd4 (Lund, 2010). We found that the above mentioned flatworm Homeobox proteins are all orthologs of Hox4 in vertebrates (Tsai et al., 2013). Here, we showed a strong conservation of the genomic organization of mir-10 and a Hox4 gene in flatworms. However, the distance between mir-10 and the corresponding Hox4 gene is greater than in vertebrates (i.e. whole HoxA cluster ~100 kb) (Santini et al., 2003). Whether the genomic organization of mir-10 in *Hymenolepis* and related flatworms is functionally linked with the Hox genes remains to be investigated.

In many species, miR-10 is co-expressed with Hox genes (Lund, 2010; Tehler et al., 2011). Interestingly, we found that miR-10 was one of the most expressed miRNAs in *H. microstoma* larvae. It would be interesting to determine whether the neighboring Hox gene is expressed in a similar pattern in this parasite stage.

Parasitic flatworms, including *Hymenolepis*, have the smallest complement of Homeobox genes of any studied bilaterian animal (Tsai et al., 2013). In addition, Hox genes are at least partially dispersed within the genome and flatworms, and may not exhibit temporal colinearity in the expression patterns characteristic of Hox genes of many other animal groups (Olson, 2008). As mentioned above, parasitic flatworms have only one copy of miR-10 in the genome, whereas mammals and zebrafish have two and five copies, respectively (Tehler et al., 2011).

The other neighboring gene of mir-10 in *H. microstoma* was **HmN_002012500**, a protein coding gene that is the ortholog of the nuclear hormone receptor protein nhr-25 of *C. elegans*. Thus, mir-10 is flanked by two transcription factors in *H. microstoma*, and this genomic organization is conserved in *E. multilocularis* and *E. granulosus*, but not in *S. mansoni*.

3.6. Hymenolepis miRNAs as potential biomarkers

In this work, we identified some mature miRNAs in *Hymenolepis* that could represent potential biomarkers or therapeutic targets. Some parasite miRNAs are protostome-specific, such as miR-277 and bantam, or bilaterian-specific but absent in the vertebrate host, such as miR-71. Others are bilateria-specific but divergent at the sequence level from their host orthologs, such as miR-3479 (miR-92 family). In addition, other miRNAs are highly conserved across metazoans, such as miR-10. Interestingly, several recent works have shown that members of these families are secreted in parasitic helminths and can be detected in the serum of the host.

In recent work from our group, miR-71 and miR-277 from the cestode *T. crassiceps* were found to be secreted in vitro (Ancarola et al., 2017). Recently, miR-71, bantam and miR-3479 from the trematode *S. mansoni* were found to be secreted in vitro, and miR-71 and bantam were detected in sera of infected mice (Samoil et al., 2018). In a previous study, miR-277, miR-3479 and bantam from *S. mansoni* were detected in the serum of infected mice and human patients and could distinguish infected individu-

als with high specificity and sensitivity (Hoy et al., 2014). In addition, miR-10 was only found in serum of mice infected with *S. mansoni* (Hoy et al., 2014). Also, miR-100 (miR-10 family), bantam and miR-71 from the filarial nematode *Litomosoides sigmodontis* were among the most abundant miRNAs detected in sera of infected mice, thus confirming in vivo secretion of parasite miRNAs (Buck et al., 2014). Also, miR-10 and miR-71 from the filarial nematode *Dirofilaria immitis* were detected in plasma of infected dogs (Tritten et al., 2014). Among others, miR-71, miR-100 and bantam from the filarial nematode *Onchocerca volvulus* were detected in sera of infected humans (Quintana et al., 2015).

Taken together, these results suggest that parasite miRNAs might be evaluated as novel biomarkers for detecting helminth infection. It will be important to determine whether these miRNAs can also be secreted by *Hymenolepis* and to assess whether they may be detected in sera of infected humans. Since miRNAs are the main components of the eukaryotic transcriptome they require further investigation in *Hymenolepis*. Parasite miRNAs could complement existing diagnostic techniques to improve diagnosis and may provide a platform for further research in the area of therapeutic targets of neglected parasites.

The recent availability of the genome assemblies of *Hymenolepis* and the limited knowledge about miRNAs in these neglected zoonotic parasites encouraged us to identify and characterize these small, non-coding RNAs that have recently emerged as potential biomarkers and therapeutic targets of infections. In this work we described for the first known time the expression profile of the miRNA complement in *H. microstoma,* and discovered miRNA genes at the genome-wide level in *H. nana* using two different approaches. The high expression of a few miRNAs in the larval stage of *H. microstoma* is conserved in other cestodes, suggesting that these miRNAs may have important roles in development, survival and for host-parasite interplay. We found a reduced complement of evolutionarily conserved miRNAs in both *Hymenolepis* spp., putatively reflecting their low morphological complexity and parasitic lifestyle. We performed a comparative analysis of the identified miRNAs and examined their genomic arrangement across the Cestoda, providing new insights about their post-transcriptional mechanisms. Our results showed that most of the miRNAs in *Hymenolepis* are located in intergenic regions, implying that they are independently transcribed. Interestingly, we found a *Hymenolepis*-specific cluster composed of three members of the mir-36 family. Also, we found that one of the neighboring genes of mir-10 in *H. microstoma* was a Hox gene as in most bilaterial species. In addition, some *Hymenolepis* miRNAs are protostome-specific or bilaterian-specific, but divergent from host orthologs and therefore could represent novel biomarkers of *Hymenolepis* infection. This study provides a valuable resource for further experimental research in cestode biology that might lead to improved detection and control of these neglected parasites.

Note

Nucleotide sequence data reported in this paper have been submitted to the miRBase database.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2018.07.005.

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