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Genome-wide identification of microRNA targets in the neglected disease pathogens of the genus *Echinococcus*

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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 biological processes such as development. MiRNAs silence target mRNAs by binding to complementary sequences in the 3′untranslated regions (3′UTRs). The parasitic helminths of the genus *Echinococcus* are the causative agents of echinococcosis, a zoonotic neglected disease. In previous work, we performed a comprehensive identification and characterization of *Echinococcus* miRNAs. However, current knowledge about their targets is limited. Since target prediction algorithms rely on complementarity between 3′UTRs and miRNA sequences, a major limitation is the lack of accurate sequence information of 3′UTR for most species including parasitic helminths. We performed RNA-seq and developed a pipeline that integrates the transcriptomic data with available genomic data of this parasite in order to identify 3′UTRs of *Echinococcus canadensis*. The high confidence set of 3′UTRs obtained allowed the prediction of miRNA targets in *Echinococcus* through a bioinformatic approach. We performed for the first time a comparative analysis of miRNA targets in *Echinococcus* and *Taenia*. We found that many evolutionarily conserved target sites in *Echinococcus* and *Taenia* may be functional and under selective pressure. Signaling pathways such as MAPK and Wnt were among the most represented pathways indicating miRNA roles in parasite growth and development. Genome-wide identification and characterization of miRNA target genes in *Echinococcus* provide valuable information to guide experimental studies in order to understand miRNA functions in the parasites biology. miRNAs involved in essential functions, especially those being absent in the host or showing sequence divergence with respect to host orthologs, might be considered as novel therapeutic targets for echinococcosis control.

1. Introduction

The cestode parasites of the genus *Echinococcus* are the causative agents of the zoonotic neglected disease echinococcosis. The two main forms of the disease in humans are cystic echinococcosis (hydatid disease) caused by *Echinococcus granulosus sensu lato* and alveolar echinococcosis caused by *Echinococcus multilocularis*. Both are life-threatening diseases very difficult to control due to the lack of efficient drugs and vaccines. Differences in gene regulation mechanisms between parasites and their hosts could be exploited for drug development [1,2]. Among relevant molecules involved in gene regulation, microRNAs (miRNAs), a class of small non-coding RNAs, have been widely recognized as key regulators of gene expression being involved in many different biological processes such as development [3–5]. Furthermore, miRNAs have been proposed as potential therapeutic targets for the control of parasitic helminths [6].

In previous work, we performed a comprehensive analysis of miRNAs in the larval stages that develop in the intermediate host and human: metacestodes and protoscoleces from *Echinococcus canadensis* and *E. granulosus sensu stricto* (s. s.), two members of the *E. granulosus* complex [7], and metacestodes from *E. multilocularis* [8]. In these works, we found that miRNAs are the preponderant small RNA silencing molecules, suggesting that these small RNAs might be an essential mechanism of gene regulation in these species. In addition, we found differentially expressed miRNAs between life cycle stages and species, and identified both parasite specific and divergent miRNAs

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from host. Untangling miRNA roles in *Echinococcus* biology might help to find new strategies to control the disease by discovering essential molecules that may be considered novel targets for therapy.

miRNAs down regulate gene expression post-transcriptionally by binding to the mRNA of their target genes, mainly in the 3′untranslated region (UTR), promoting their cleavage or, more commonly in metazoans, their translational repression and/or destabilization. The main determinant of this interaction is the seed region, a conserved sequence situated in the 5′ end of the miRNA (nucleotides 2–8) [9,10]. Recently, it has been suggested that the identification of relevant miRNA target genes is a priority to advance in the characterization of miRNAs from parasitic helminths, and thus to significantly improve the understanding of the biology of these parasites [11]. Regarding parasitic platyhelminthes, miRNA target genes have been predicted through computational tools (*e.g.*, miRanda, RNAhybrid) in the cestode *E. granulosus* s. s. [12], the trematodes *Schistosoma mansoni* [13], *Schistosoma japonicum* [14,15], *Eurytrema pancreaticum* [16], *Orientobilharzia turkestanicum* [17], *Fasciola gigantica* and *Fasciola hepatica* [18]. Since these studies are mainly focused on miRNA identification, they lack of comprehensive functional and conservation analyses of miRNA targets. So far, neither 3′UTR annotation or miRNA target prediction have been addressed in *E. canadensis*, *E. multilocularis* or *Taenia solium*.

Experimental identification of miRNA targets is expensive, time consuming and difficult especially for non-model organisms. Parasites have complex life cycles and parasite material is often limited since appropriate *in vitro* or *in vivo* systems have not been developed for several parasites such as for *E. granulosus sensu lato*. In addition, transfection and silencing protocols are also lacking for some of them. In this context, bioinformatic prediction from integrated genomic and transcriptomic data offers an alternative approach to guide experimental validation of potential miRNA-target interactions. Algorithms for target prediction rely on: (i) complementarity between 3′UTRs and the miRNA seed region; (ii) miRNA-mRNA duplex thermodynamics; (iii) evolutionary conservation of target sites across orthologous 3′UTRs being in some algorithms a further refinement criteria [19]. Thus, to identify miRNA targets in a given species, it is important to obtain reliable 3′UTR and mature miRNA sequences since most algorithms for miRNA target prediction requires both of them as input datasets. Therefore, the main limitation to perform this strategy is the lack of accurate 3′UTR sequences of mRNAs for most species, including parasitic helminths. Here we generated transcriptomic data from protoscolex stage of *E. canadensis* that together with the recently generated whole genome sequences for this species [20] provided us basic information to perform a 3′UTR annotation in *E. canadensis* and a computational miRNA target prediction using experimentally identified *Echinococcus* miRNA sequences [7]. The aims of this study were to define a set of 3′UTRs in *E. canadensis* and to predict miRNA targets through a bioinformatic approach. The present study also provides information about miRNA targets in *E. multilocularis*, *E. granulosus* s. s. and *T. solium* since genomic sequences have been recently generated for these related flatworms [21]. Genome-wide identification and characterization of miRNA target genes in *Echinococcus* provides valuable information to guide experimental studies in order to understand miRNA functions in the biology of the parasite.

2. Material and methods

2.1. Parasite material

Three samples of protoscoleces were extracted under aseptic conditions from three different hydatid cysts from the liver of a naturally infected swine provided by local abattoirs from Buenos Aires, Argentina. One fraction of freshly isolated protoscoleces from each cyst was used to determine viability by eosine exclusion test. Samples showing more than 90% viability were stored at −20 °C in RNA later (Ambion) until RNA extraction. The species was determined by sequencing a fragment of the mitochondrial cytochrome c oxidase subunit 1 (CO1), as previously described in [22]. The resulting species was *E. canadensis*.

2.2. RNA isolation

Protoscoleces samples were mechanically homogenized in Trizol (Invitrogen) 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.

2.3. RNA library construction and sequencing

mRNA libraries were prepared using the Illumina mRNA-seq kit. Polyadenylated mRNA was purified from total RNA using oligo-dT dynabead selection followed by fragmentation by metal ion hydrolysis. First strand synthesis, primed using random oligonucleotides, was followed by 2nd strand synthesis with RNaseH and DNApolI to produce double-stranded cDNA. Template DNA fragments were end-repaired with T4 and Klenow DNA polymerases and blunt-ended with T4 polynucleotide kinase. A single 3′ adenosine was added to the repaired ends using Klenow exo- and dATP to reduce template concatemerization and adapter dimer formation, and to increase the efficiency of adapter ligation. After adaptor ligation, individual libraries made with the Illumina mRNA-seq kit were size selected using the caliper before PCR amplification. Kapa Illumina SYBR Fast qPCR kit was used to quantify the Illumina mRNA-seq libraries before pooling. Then, linearization, blocking and hybridization of the R1 sequencing primer were performed. The hybridized flow cells were loaded onto the Illumina sequencing platforms for sequencing-by-synthesis (100 cycles) using the V5 SBS sequencing kit. Then, *in situ* linearization, blocking and hybridization steps were repeated to regenerate clusters, release the second strand for sequencing and to hybridize the R2 sequencing primer followed by another 100 cycles of sequencing to produce paired end reads. Data were analyzed from the HiSeq sequencing machine using the RTA1.8 analysis pipelines. Samples were sequenced at the Wellcome Trust Sanger Institute, UK. The RNAseq data are available in ArrayExpress under accession code E-ERAD-236 (samples ERS242847, ERS242859 and ERS242867) (https://www.ebi.ac.uk/arrayexpress/experiments/E-ERAD-236/samples/?s_page=1&s_pagesize=25).

2.4. Source of genome assemblies and annotations

The high quality *E. canadensis* genome assembly and the gene annotations of 11,449 coding genes [20] were downloaded from the WormBase Parasite database (http://parasite.wormbase.org/). Also, the high quality *E. multilocularis* genome assembly, *E. granulosus* s. s. and *T. solium* draft genome assemblies [21] and the corresponding gene models were retrieved from the WormBase Parasite database (http://parasite.wormbase.org/).

2.5. Structural annotation of 3′untranslated regions (3′UTRs) in E. canadensis

A pipeline for structural annotation of 3′UTR was developed (Fig. 1). RNA sequencing (RNA-Seq) data from polyadenylated mRNA libraries from the *E. canadensis* protoscolex stage were mapped onto the *E. canadensis* genome assembly using TopHat2 [23] with the following parameters: –I 40,000, –i 20 –coverage search –microexon search, –mate inner dist 300, –mate std dev 100, –library type fr –unstranded. Previously, low quality reads were filtered out. To optimize RNA-Seq mapping the length distribution of the libraries was evaluated and

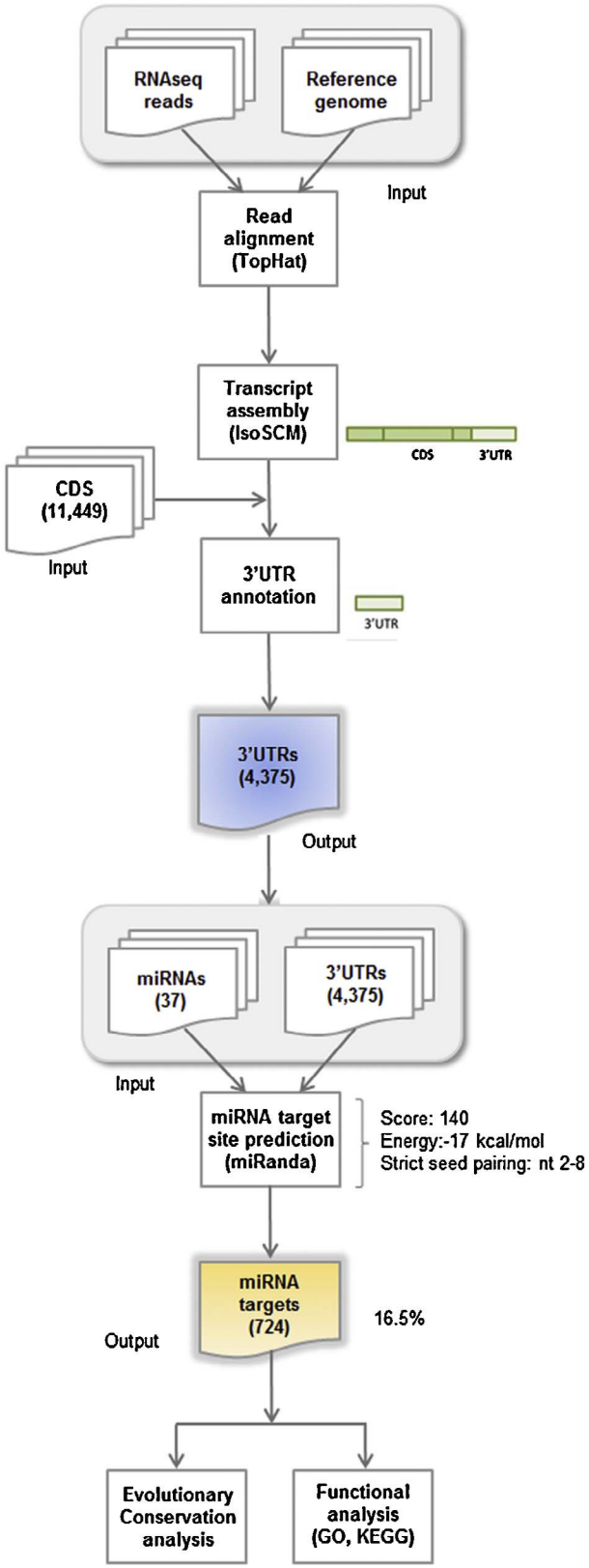

Fig. 1. Bioinformatic workflow developed for 3′UTR annotation, prediction of miRNA target sites and analysis in *Echinococcus canadensis* (reference species). The number of assembled transcripts, CDS and the number of 3′UTRs obtained are shown. The number of miRNAs used for prediction and the number of miRNA targets obtained are also indicated.

mapping statistics were calculated. Then, alignment files were used as input for transcript assembly with Isoform Structural Change Model (IsoSCM) [24], a method that incorporates change-point analysis to improve the 3′UTR annotation process, using default parameters. Since this method provides alternative last exons for the same transcript, only the last exons with the deepest coverage were chosen for further analyses. Finally, last exons from assembled transcripts by IsoSCM were integrated with *E. canadensis* coding DNA sequences (CDSs) using BEDTools (v2.19.1) [25]. The 3′UTR coordinates were extracted from the intersecting structural annotation using custom scripts. The 3′UTR sequences were extracted from *E. canadensis* genome and a length distribution analysis was performed using R v3.2.5 (https://www.r-project.org/).

2.6. Validation of 3′UTRs in E. canadensis by RT-PCR

RT-PCR was performed to validate both, the expression and the length of a random subset of 3′UTRs in *E. canadensis* protoscoleces. cDNA was synthesized from 1 μg of RNA from *E. canadensis* protoscoleces using SuperScript III Reverse Transcriptase (Invitrogen) and oligo-dT in a 20 μl reaction volume. Controls without reverse transcriptase were included. Reverse transcription was performed by using the following program: 60 min at 50 °C, 15 min at 70 °C. Prior to the reverse transcription reaction, the RNA was treated with DNase I (Invitrogen) according to the protocol of the manufacturer. For each PCR, 2 μl of cDNA (25 ng) or DNA (25 ng) were mixed with 2.5 μl of each primer (10 μM), 5 μl of buffer (10×), 1.5 μl of $MgCl_2$ (50 mM), 1 μl of dNTPs 10 mM (Invitrogen) 0.2 μl (1 U) of Taq DNA polymerase (Invitrogen) and 35.3 μl of sterile water in a final volume of 50 μl. Cycling conditions were: 95 °C for 5 min, followed by 35 cycles of 95 °C for 45 s, 57 °C for 50 s and 72 °C for 60 s, and a final incubation at 72 °C for 5 min. Amplification products were run in 1% agarose gels stained with GelRed for 45 min at 90 V to verify the presence or absence of the amplification product. The primer sequences and primer design used for validation are in Supplementary Table S1.

2.7. Prediction of miRNA target sites in Echinococcus

The *Echinococcus* mature miRNA sequences were obtained from previous studies [7,8]. The *E. canadensis* 3′UTR sequences were annotated in this study as described in Section 2.5. The orthologous 3′UTR sequences of *E. multilocularis*, *E. granulosus* s. s. and *T. solium* were predicted in this study as described in Section 2.8. The miRanda algorithm (v3.3a) [26] was used to perform an independent prediction of miRNA target sites in each species using each specific set of 3′UTRs. The parameters used were as follows: (i) strict seed pairing; (ii) score threshold: 140; (iii) energy threshold: −17 kcal/mol; (iv) gap open penalty: −9; (v) gap extend penalty: −4; (vi) scaling parameter: 4.

2.8. Evolutionary conservation analysis of miRNA target sites in Echinococcus and Taenia

Orthologous gene clusters were identified as described in [20]. Briefly, the protein complements of *E. canadensis*, *E. granulosus* s. s., *E. multilocularis* and *T. solium* were searched for reciprocal best hits using BLAST, and the outputs were subsequently used for identifying orthologous gene clusters by using the MCL algorithm. From each orthologous gene identified in *E. granulosus* s. s. and *T. solium* a sequence of the same length of the corresponding 3′UTR in *E. canadensis* was extracted downstream the gene model using custom scripts. For *E. multilocularis*, the 3′UTRs were determined using the pipeline described for *E. canadensis*, since Illumina RNAseq data were available. A target site was considered to be evolutionary conserved in *E. canadensis* if the position fell within ± 20 nucleotides in the 3′UTRs of a corresponding orthologous gene. To determine whether these target sites were significantly more conserved than other random sequences taken from

the 3′UTRs, the conservation between the predicted target sites and other random sequences on the 3′UTR was analyzed quantifying the levels of nucleotide substitution.

Pairs of orthologous 3′UTR sequences containing conserved target sites were aligned using MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). Then, we searched for nucleotide substitutions lying within the miRNA seed region and in the 50 bp up- and down-stream regions on the 3′UTR. The substitution positions were calculated relative to the miRNA seed, *i.e.* the 5′ end of the miRNA mature sequence. The nucleotide substitutions from all of the alignments were pooled for each position. Then, a histogram of number of nucleotide substitutions per position was built using R v3.2.5 (https://www.r-project.org/) for each pair of species compared. The average number of nucleotide substitution at the miRNA seed region was compared with that of five randomly selected regions taken from the 50 bp around the miRNA seed region on the 3′UTR. Statistical significance was assessed by performing an ANOVA test, with a confidence level of 95% using R.

2.9. Functional analysis of miRNA targets in E. canadensis

Functional annotations of *E. canadensis* CDSs were transferred to *E. canadensis* predicted miRNA targets using custom scripts. Gene ontology (GO) [27] and KEEG pathway analyses were performed. Gene ontology terms corresponding to Biological Process were assigned to *Echinococcus* miRNA targets using InterPro2GO databases (version 01-29-2015). Pathway mappings of miRNA targets were obtained from the KEGG database [28] using the KAAS tool [29]. Functional enrichment analysis of miRNA targets was performed using Hypergeometric Test in R v3.2.5 (https://www.r-project.org/) and using as reference the set of genes with an annotated 3′UTR. The significance of GO and KEEG pathway enrichment analyses was set at a p-value of FDR ≤ 0.05.

3. Results

3.1. Identification of 3′untranslated regions (3′UTRs) in E. canadensis

We developed a pipeline that integrates both available genomic data of *E. canadensis* and RNA-seq data from the protoscolex stage of *E. canadensis* obtained in this work. This pipeline allowed us to obtain a highly confident set of 4375 3′UTRs that corresponded to assembled transcripts matching gene models (CDSs) of *E. canadensis* (Fig. 1 and Supplementary File 1). In addition, the set of annotated 3′UTRs corresponded to genes with expression evidence at this stage. The number of gene models with expression evidence (at least 10 reads) in the protoscolex stage of *E. canadensis* was 8387. Therefore, the 3′UTR sequences represented 52% of the expressed gene models (4375/8387). Fig. 2A shows the length distribution of the annotated *E. canadensis* 3′UTR sequences. A major proportion of the 4375 3′UTRs (96%) were shorter than 1000 nucleotides and the mode was 73 nucleotides. The median length of the total set of 3′UTRs was 141 nucleotides and the average GC content was 40.39%. We validated both, the expression and the length of a random subset of *E. canadensis* 3′UTRs by RT-PCR (Fig. 2B). The primer sequences and primer design used for validation are in Supplementary Table S1.

3.2. Prediction of miRNA target sites in E. canadensis

We found 1049 miRNA-target interactions between the set of 4375 *E. canadensis* 3′UTRs and the set of 37 miRNAs used as input (Supplementary Tables S2 and S3). Since miRNAs that share the seed region binds the same miRNA target sites, the 1049 miRNA-target interactions corresponded to 941 potential miRNA target sites. These sites were distributed in 724 *E. canadensis* 3′UTRs, which represented 16.5% of the total set of *E. canadensis* 3′UTRs used as input (724/4375). We found that each *E. canadensis* miRNA family had a different number of predicted miRNA target genes (Fig. 3). MiR-71 and miR-2 (miR-2a/2b/2c) families, which are part of a cluster in *Echinococcus* [22], had the higher number of target genes, accounting for ~30% (211/724) of all target genes. These results are in agreement with those obtained for miR-71 and miR-2 families in *S. japonicum* where both miRNAs are clustered together and account for a major proportion of the predicted miRNAs targets in that parasite [13].

We classified the 724 predicted miRNA target genes according to the number of miRNA target sites present in each gene. We found that 556 3′UTRs (77%) had one miRNA target site, whereas 168 3′UTRs (23%) had multiple miRNA target sites (totaling 385). Among 3′UTRs with multiple sites, 129 3′UTRs had 2 sites, 32 3′UTRs had 3 sites, 4 3′UTRs had 4 sites and 3 3′UTRs had 5 sites (Supplementary Fig. S1). A total number of 17 3′UTRs had more than one miRNA target site for the same miRNA (Table 1).

3.3. Evolutionary conservation analysis of miRNA target sites in Echinococcus and Taenia

We identified a set of orthologous 3′UTRs to the 4375 3′UTRs of *E. canadensis* in *E. granulosus* s. s. and *T. solium*. We obtained a set of 3902 orthologous 3′UTRs in *E. granulosus* s. s. and 3663 orthologous 3′UTRs in *T. solium*. Also, we determined 6227 3′UTRs in *E. multilocularis* based on RNAseq data using the same pipeline used for *E. canadensis*. Then, we carried out a prediction of miRNA target sites in these species as performed for *E. canadensis*. We predicted 1053 miRNA-target interactions (928 target sites) distributed in 740 target genes in *E. granulosus*; 974 miRNA-target interactions (862 target sites) distributed in 686 target genes in *T. solium* and 1547 miRNA-target interactions (1382 target sites) distributed in 1105 miRNA target genes in *E. multilocularis*. The higher number of miRNA-target interactions and target genes obtained for *E. multilocularis* was due to the higher number of 3′UTRs used as input for miRNA target prediction.

We determined evolutionary conservation of each of the 941 predicted miRNA target sites and the 724 targets of *E. canadensis*. The number of conserved miRNA target sites and target genes between *E. canadensis* and (i) *E. granulosus*, (ii) *E. multilocularis* and (iii) *T. solium* are shown in Fig. 4A, C and E, respectively. In addition, we determined whether these conserved target sites were significantly more conserved than other random sequences taken from the 3′UTRs. The results showed a significant decrease in the average number of nucleotide substitutions at miRNA seed regions compared with random regions within the 3′UTRs (ANOVA test, Supplementary Fig. S2). This decrease was observed for conserved miRNA target sites between *E. canadensis* and (i) *E. granulosus*, (ii) *E. multilocularis*, and (iii) *T. solium* (Fig. 4B, D, F, respectively). Also, we performed a general analysis considering all predicted target sites. We found no significant differences between conservation of all sites compared to other random sequences taken from the 3′UTRs (ANOVA test, Supplementary Fig. S3). Evolutionarily conserved miRNA sites and targets in *E. canadensis* and their corresponding orthologs in *E. granulosus*, *E. multilocularis* and *T. solium* are shown in Supplementary Tables S4, S5 and S6, respectively.

3.4. Functional analysis of miRNA targets in E. canadensis

3.4.1. Functional annotation of miRNA targets

To analyze the possible roles of the miRNA repertoire in *E. canadensis*, we transferred the functional annotations of *E. canadensis* CDSs to the whole set of 4375 *E. canadensis* genes with an annotated 3′UTR. This set included those predicted as miRNA targets (724). A total number of 610 miRNA target genes had a functional annotation (84%). We performed a gene ontology (GO) analysis and 241 different miRNA target genes were assigned to 119 distinct GO terms (328 GO terms in total) corresponding to biological process category. The most represented GO terms were protein phosphorylation (GO: 0006468) (11%), transmembrane transport (GO: 0055085) (6%), regulation of transcription (GO: 0006355) (5%) with frequencies greater than 5%

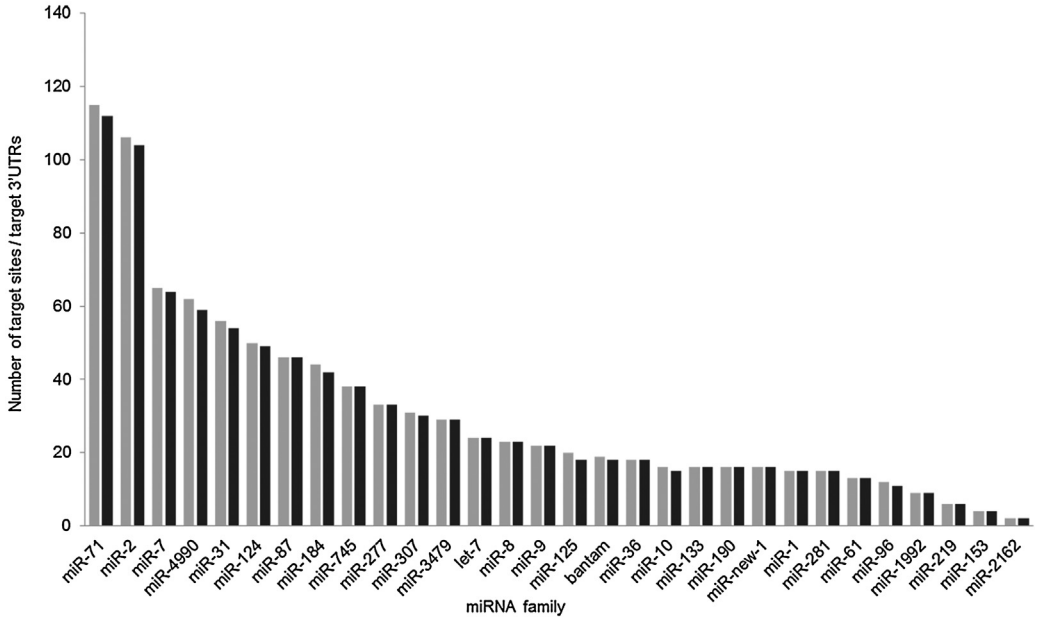

Fig. 3. Number of potential miRNA target sites (gray bars) and number of potential 3′UTR targets (black bars) in *Echinococcus canadensis* for each miRNA family.

Table 1
Potential miRNA targets with at least two sites for the same miRNA.

Gene_ID	miRNA sites	Gene description
ECANG7_00366	bantam-3p bantam-3p	Ribosomal protein S2
ECANG7_01292	miR-125-5p miR-125-5p miR-125-5p mir-87-3p	Zinc finger, C2H2
ECANG7_02182	miR-31-5p miR-31-5p miR-9-5p	Adenylate cyclase-associated CAP, C-terminal adenylate cyclase-associated CAP, N-terminal
ECANG7_02342	miR-184-3p miR-184-3p miR-71-5p miR-8-3p	Occludin/RNA polymerase II elongation factor, ELL domain RNA polymerase II elongation factor ELL
ECANG7_02601	miR-2c-3p miR-2c-3p	DNA-binding HORMA
ECANG7_03238	miR-7a-5p miR-7a-5p	Sister chromatid cohesion C-terminal domain HEAT repeat associated with sister chromatid cohesion protein
ECANG7_04012	miR-71-5p miR-71-5p	TFIIH subunit TTDA/Tfb5
ECANG7_04326	miR-4990-5p miR-4990-5p	Calponin homology domain
ECANG7_04917	miR-71-5p miR-71-5p	Protein kinase domain, protein kinase C-like, phorbol ester/diacylglycerol-binding domain
ECANG7_05326	miR-2c-3p miR-31-5p miR-4990-5p miR-4990-5p miR-745-3p	G2:M phase specific E3 ubiquitin protein ligase
ECANG7_06007	miR-71-5p miR-71-5p	Snf7
ECANG7_06901	mir-96-5p mir-96-5p	Conserved oligomeric Golgi complex, subunit 2, N-terminalCOG complex component, COG2, C-terminal
ECANG7_07082	miR-10-5p miR-10-5p	High mobility group box domain
ECANG7_07531	miR-184-3p miR-184-3p miR-1-3p	Nucleoside diphosphate kinase
ECANG7_07571	miR-31-5p miR-31-5p	Zinc finger, C2H2
ECANG7_10164	miR-124a-3p miR-124a-3p	Mob1/phocein
ECANG7_10172	miR-307-3p miR-307-3p miR-2b-3p	Ubiquitin-conjugating enzyme, E2

(Fig. S4). Also, the term protein phosphorylation (GO: 0006468) was significantly enriched (Hypergeometric test, FDR < 0.05). We also carried out a KEGG pathway analysis and assigned 181 different predicted miRNA target genes to 174 distinct KEGG pathways (622 KEGG mappings in total) (Supplementary Table S7). The most represented pathway was endocytosis (PATH: ko04144), followed by MAPK signaling pathway (PATH: ko04010) and Wnt signaling pathway (PATH: ko04310). Among other pathways, MAPK signaling pathway was significantly enriched (hypergeometric test, FDR < 0.05) (Supplementary Table S8).

3.4.2. MiRNA targets involved in developmental pathways

The MAPK signaling pathway might be regulated by miRNAs in *Echinococcus* (Fig. 5B, Supplementary Table S9). Members of this pathway included mitogen-activated protein kinases such as ECANG7_06033 (miR-71) and ECANG7_05589 (miR-10) and the gene son of sevenless (*sos*, ECANG7_04447) targeted by miR-10, miR-71 and miR-31. In addition, the gene basic fibroblast growth factor receptor 1 A (*fgfr1*, ECANG7_04102) had a conserved site for miR-124. In addition, two relevant members of the TGF-beta signaling pathway, Rho-associated protein kinase 1 (ECANG7_07875) and the growth factor TGF-β/activin (ECANG7_08823) were predicted to be conserved targets of miR-71 and miR-1, respectively. Also, FGFR1 (miR-124) and TGF-β (miR-1) mapped to Signaling pathways regulating pluripotency of stem cells (Supplementary Table S7). In *S. japonicum*, a member of the TGF-β pathway, the TGF-β receptor II that plays important roles in development and host-parasite interaction may be regulated by miRNAs [15].

The Wnt signaling pathway might be under miRNA regulation in *Echinococcus* (Fig. 5A, Supplementary Table S9). However, most of the possible targets in Wnt pathway are not only involved in this pathway since they also mapped to other pathways. Some components of the Wnt/Ca^{2+} pathway such as phosphatidylinositol phospholipase C gene (PLC, ECANG7_05735) and calcium/calmodulin-dependent protein kinase gene (CAMK2, ECANG7_00867), may be regulated by miR-184 in *Echinococcus*. In addition, casein kinase II (CK2, ECANG7_09002) from Canonical pathway may be a conserved target for miR-184. Another conserved target of the Canonical pathway in *Echinococcus* was the ortholog gene of lymphoid enhancer-binding factor 1 (LEF 1,

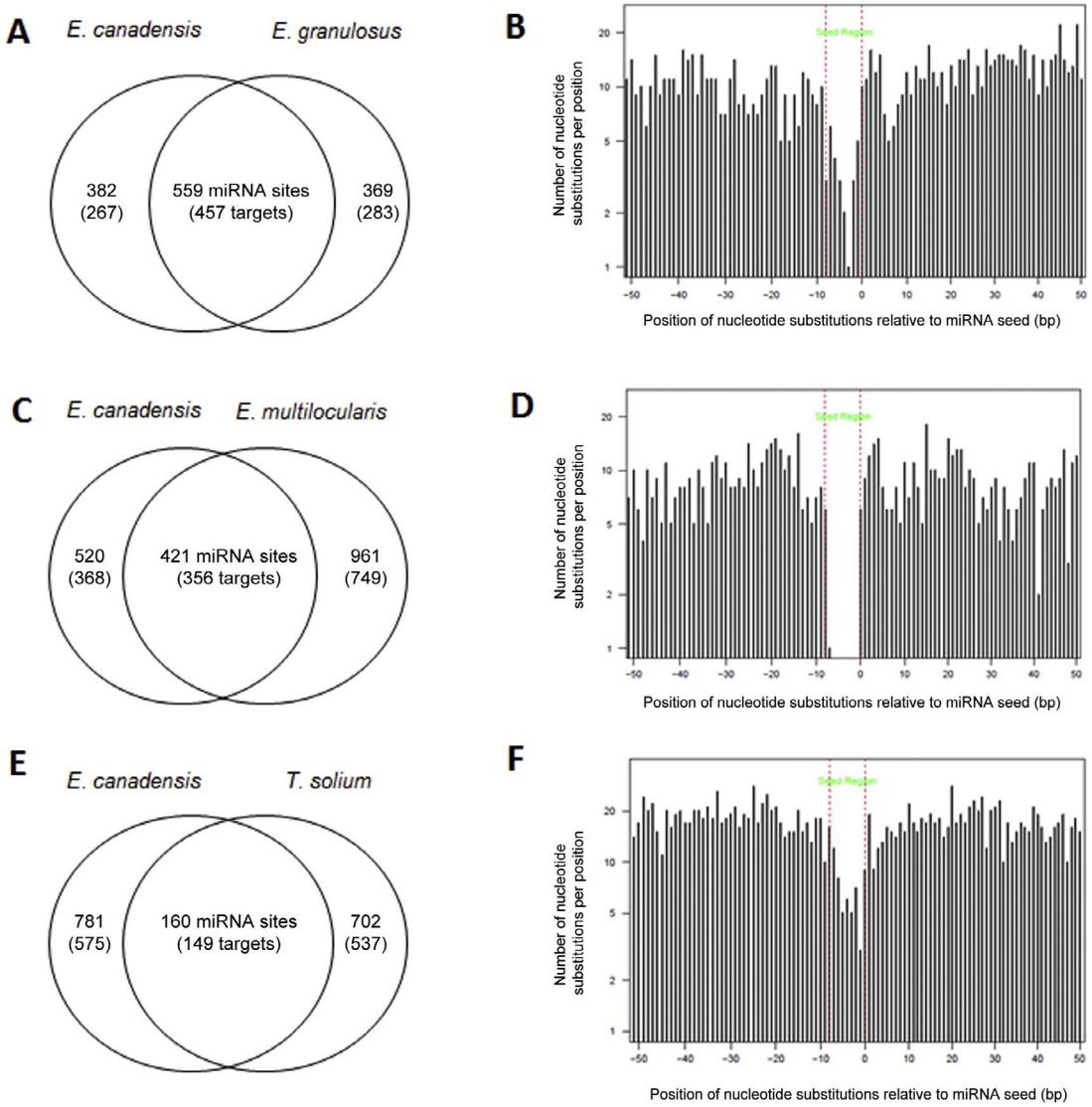

Fig. 4. Evolutionary conservation of miRNA target sites predicted in *Echinococcus canadensis* (reference species). Number of conserved and not conserved miRNA sites and targets between *Echinococcus canadensis* and *Echinococcus granulosus sensu stricto* (A), *Echinococcus canadensis* and *Echinococcus multilocularis* (C), *Echinococcus canadensis* and *T. solium* (E). Number of nucleotide substitutions per position (relative to miRNA seed) within the 3′UTRs between *Echinococcus canadensis* and *Echinococcus granulosus sensu stricto* (B), *Echinococcus canadensis* and *Echinococcus multilocularis* (D), *Echinococcus canadensis* and *T. solium* (F). Only pairs of orthologous 3′UTRs with conserved sites were considered in the analyses.

ECANG7_01705) that was predicted to be targeted by miR-10. Also, segment polarity protein disheveled (DVL, ECANG7_01278) and glycogen synthase kinase 3 beta (GSK3B, ECANG7_02522) may be regulated by miR-277. These two genes also mapped to Signaling pathways regulating pluripotency of stem cells (Supplementary Table S7).

Recently, it has been found that these pathways are complete in *Echinococcus* [20,30]. Several components of MAPK, TGF-β and Wnt signaling pathways have been identified in *Echinoccocus* [31,32]. Also, the existence of stem cells has been demonstrated in *E. multilocularis* [33].

3.4.3. Additional miRNA targets related to development

In addition to targets mapping to KEGG pathways, we predicted other target genes potentially involved in development. Some relevant examples found were a Bromodomain containing protein (ECANG7_05944) which had conserved target sites for miR-9, let-7 and miR-281. This target gene is an ortholog of *Caenorhabditis elegans* lin-49, which is involved in nematode larval development. In addition, four HMG-box containing proteins were predicted targets of miRNAs. Two of them were targets of miR-10, a deeply conserved miRNA known by its role in development and Hox regulation (Supplementary Fig. S5). Also, one homeobox containing protein (ECANG7_09658) from the Meis family [22] may be targeted by miR-10 in all *Echinococcus* species. We also predicted a homeodomain transcription factor (ECANG7_00514) which had conserved sites for miR-125 and miR-124a. Also, one TAFH/NHR1 Transcription initiation factor TFIID component TAF4 (ECANG7_02390) had conserved sites for miR-124, miR-2c and miR-184. In addition, we predicted two nuclear hormone receptors ECANG7_02627 and ECANG7_01524, which are potentially involved in steroid hormone mediated signaling pathway, as conserved targets of miR-71 and miR-125, respectively. This target gene (ECANG7_01524) is the ortholog of the nuclear receptor VDR of *E. granulosus* (EG_04794) [12]. The VDR gene is a putative target of let-7 and its expression is positively correlated with that of let-7 in *E. granulosus* [12]. Also, a relevant gene potentially involved in segmentation, Armadillo Importin-alpha (ECANG7_01054), which is the ortholog of EG_01111 [12] may be regulated by miR-124 and miR-71 in *Echinococcus*. Besides, two glypicans (ECANG7_04919, ECANG7_00818), which are potentially involved in developmental morphogenesis, had sites for miR-281 or miR-7a, among *Echinococcus* species (Supplementary Tables S4 and S5). Other relevant examples found were *nos-1*, a gene that is expressed in stem cells [2] and that was suggested to be involved in segmentation in

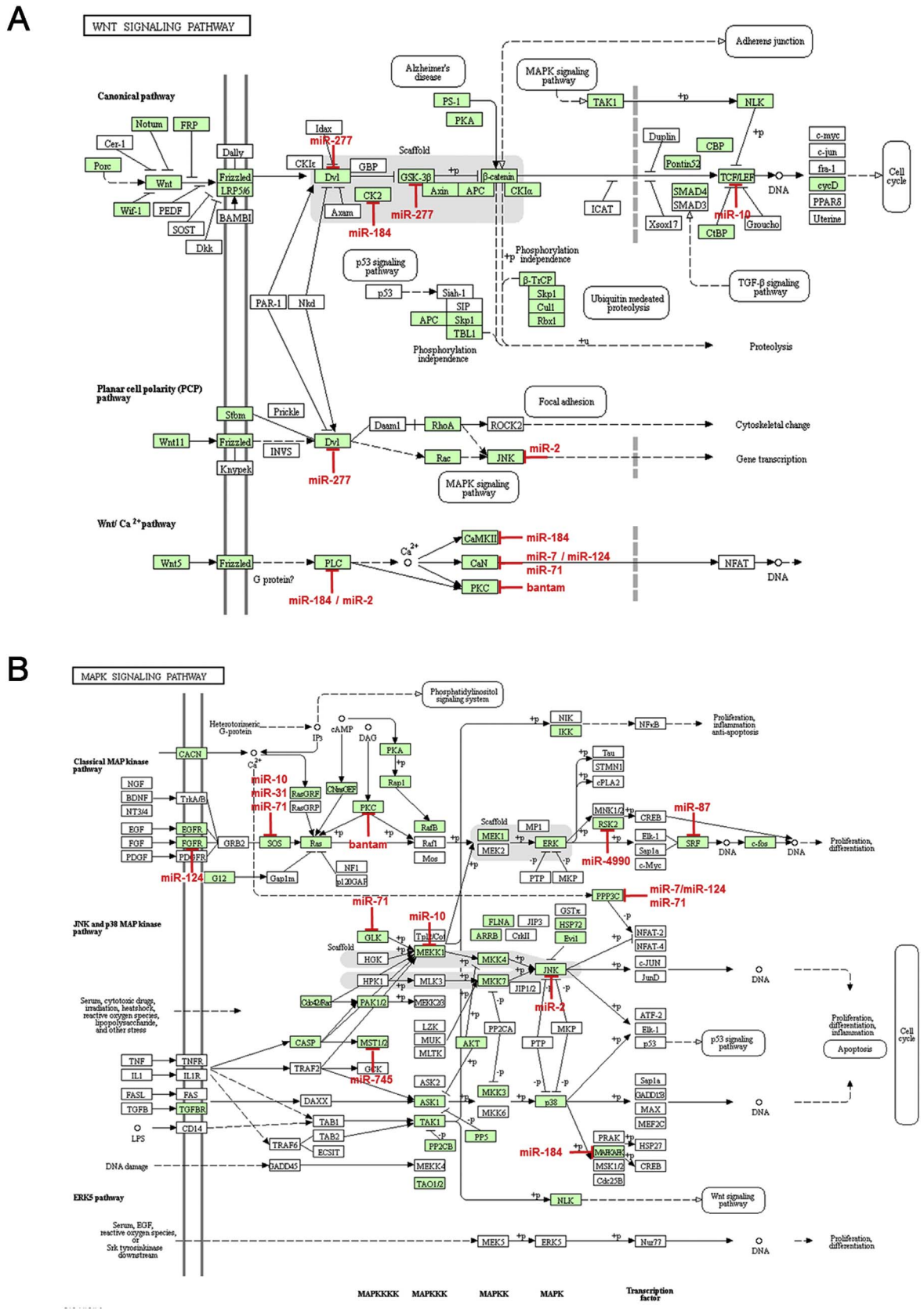

Fig. 5. Predicted miRNA targets within the context of selected signaling pathways in *Echinococcus*. (A) Wnt signaling pathway, (B) MAPK signaling pathway. Green boxes represent orthologous genes present in *Echinococcus canadensis*. miRNAs that target genes of these pathways are shown in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Echinococcus [30] was predicted to have conserved sites for miR-281 and miR-2c. The ortholog of human *ago2*, another gene expressed in stem cells, may be regulated by miR-184 suggesting a regulatory loop in miRNA biogenesis in *Echinococcus*. Interestingly, in human keratinocytes it was experimentally shown that miR-184, a miRNA conserved among bilaterians, down regulates Ago2 expression [34] giving further support to our findings.

3.4.4. MiRNAs as drug targets

MiR-71 is a bilaterian miRNA absent in the vertebrate hosts that is highly expressed in *Echinococcus* larval stages [7,8]. Also, this miRNA had the highest number of predicted targets in *Echinococcus* (Fig. 3). We found that additional conserved predicted targets of miR-71 were two basal transcription factors, which may be potentially involved in transcription process in *Echinococcus*: TFIIH subunit TTDA/Tfb5 (ECANG7_04012) that had two sites for miR-71 (Table 1), and TFIIE beta subunit, DNA-binding domain (ECANG7_07534) that had one site for miR-71. In addition to the basal transcription factors, we found a relevant conserved target of miR-71 in *Echinococcus*: the gene regulatory associated protein of mTOR (Raptor, ECANG7_04603) that is an ortholog of *C. elegans* daf-15. Interestingly, the gene raptor is a member of the Insulin-PI3K-mTor pathways and might be involved in the Longevity regulating pathway in *Echinococcus* (Supplementary Table S7). MiR-71 is known to be involved in *C. elegans* lifespan regulation [35] and stress responses [36]. We hypothesize that miR-71 may be essential for *Echinococcus* to survive in the intermediate or definitive host environment. Recently, miRNAs targeting basal transcription factors and signaling pathways were proposed as drug targets in the nematode parasite *Toxocara canis* [37].

4. Discussion

To address miRNA target prediction it is necessary to have a reliable set of 3′UTR sequences since most canonical sites are found in these regions [38]. A major challenge is the lack of accurate information of 3′UTR sequences within parasitic helminth species [11]. Here, we carried out the 3′UTR annotation in *E. canadensis* due to the generation of RNA-seq data from the protoscolex stage and the recent availability of the genome of *E. canadensis*. This process is challenging because there is no single tool for this purpose and due to the lack of genomic or RNA-seq data for many parasitic helminths [11,39]. For example, in some of the few species of helminth parasites where a bioinformatic prediction of miRNA targets has been performed, ESTs or mRNA are used [18] or a certain number of nucleotides downstream the stop codon of the annotated genes are taken [40]. Although these strategies are useful in the absence of a defined set of 3′UTR, they might produce false positives. The annotation of 4375 3′UTRs performed in this study for *E. canadensis* by integrating genomic and transcriptomic data allowed us an accurate identification of miRNA target genes. Since the annotation was carried out using RNA-seq data, the whole set of 3′UTRs has evidence of expression in the parasite. We validated both, the expression and the length of a random subset of *E. canadensis* 3′UTRs by RT-PCR adding confidence to the prediction pipeline. Also, they resulted from matching of two independent methods of gene annotation: MAKER (genes annotation) and IsoSCM (transcripts assembly). Our results suggest that the length distribution of 3′UTR sequences of *E. canadensis* is similar to that of the free living helminth *C. elegans* [41]. In that work, a median length of 130 nucleotides was determined using an experimental approach.

The number of target genes found in *E. canadensis* represents 16.5% of all genes with an annotated 3′UTR region (724/4375). According to the predictions made in *Drosophila* and in vertebrates, the number of coding genes that might be regulated by miRNAs is about 15% and 20–30% of all coding genes, respectively [5,42]. Therefore, the number of target genes obtained in this study is consistent with these results. This result may be due to the stringent parameters we used for miRNA target sites prediction such as strict seed base pairing. Binding of nucleotides 2–8 constitutes the minimum miRNA region required to exert its effect [9,10] being the most frequently type of binding site found in metazoans [43]. In addition, the predictions here obtained are reliable since they were generated using a set of miRNAs and 3′UTRs, both with expression evidence at the protoscolex stage of *E. canadensis*. Co-expression of miRNA and its predicted target gene is an important requirement for the interaction to be biologically possible [44]. It is known that a single miRNA might regulate different target genes and that the same target gene could be regulated cooperatively by distinct miRNAs [3]. Our results indicate that both possibilities may also occur in *Echinococcus*. Also, these results are in agreement with previous reports from other flatworm parasites [12,15,13]. The fact that the same target gene presents multiple target sites for the same or different miRNAs, increases the likelihood that it may be a true target [45,46] and therefore this kind of targets represent possible candidates to be validated.

Evolutionary conservation of potential miRNA target sites in phylogenetically related species is an additional criterion that gives greater confidence to the predictions obtained [26,47]. Although many target genes were predicted in parasitic helminths using a bioinformatic approach, the accuracy of these data was compromised since strict complementarity in the seed region, co-expression of both the miRNAs and their targets and evolutionary conservation were overlooked [11]. Our results indicate that evolutionarily conserved target sites identified in *E. canadensis* 3′UTRs are significantly more conserved than other random sequences taken from the 3′UTRs suggesting that many of these interactions may be functional sites and under selective pressure. The miRNA repertoire is conserved between the *Echinococcus* species [7,8] and displays a high degree of gene synteny (Supplementary Fig. S6). These genomic features together with the evolutionary conservation of miRNA targets suggest further functional conservation of the miRNA complement in *Echinococcus*. Identification of conserved miRNA targets in *Echinococcus* species might lead to the identification of relevant miRNAs that may be useful for anti-*Echinococcus* therapeutic control. Also, *E. canadensis* miRNA targets that are conserved in *E. multilocularis* may be relevant targets to be validated experimentally in this parasite since it has been recently proposed as an *in vitro* model for tapeworm research [31]. Furthermore, miRNA targets that are evolutionarily conserved in a more distant species such as *T. solium* may also be relevant targets to be validated experimentally since conservation at larger evolutionary distances would be stronger evidence of functionality. Our findings also show a proportion of miRNA target sites only present in *E. canadensis*. These sites might be valid targets [47] and may have implications for differential gene regulation, contributing to the existence of distinctive features in each species of the genus.

The functional analyses suggest that highly expressed miRNAs such as miR-71, miR-1, miR-184 and miR-10; and differentially expressed miRNAs such as miR-124 and miR-277 [7,8] may be involved in the regulation of MAPK and Wnt signaling pathways in *Echinococcus*. It is interesting to point out that several miRNAs that are up regulated in the protoscolex stage of *E. canadensis* such as miR-125, miR-124, miR-7 and miR-281 [7], might regulate developmental genes in *Echinococcus*. The protoscolex is the larval stage that is able to develop to an adult stage that is segmented (strobilated). In addition, miR-277, miR-1 and miR-124 may be involved in the regulation of Signaling pathways regulating pluripotency of stem cells in *Echinococcus*. Interestingly, it has been shown that miR-1 or miR-124 are able to change the whole cellular mRNA profile in HeLa cells thus defining cell fate [48]. Our findings indicate that other relevant miRNA target genes may be transcription factors, which are potentially involved in regulating developmental transitions and in the response to environmental changes and/or stress. Taken together, these results suggest that miRNAs might play a role in growth and development and suggest a key role of *Echinococcus* miRNAs as master regulators of gene expression. The fact that MAPK signaling pathway may be under miRNA regulation suggests the

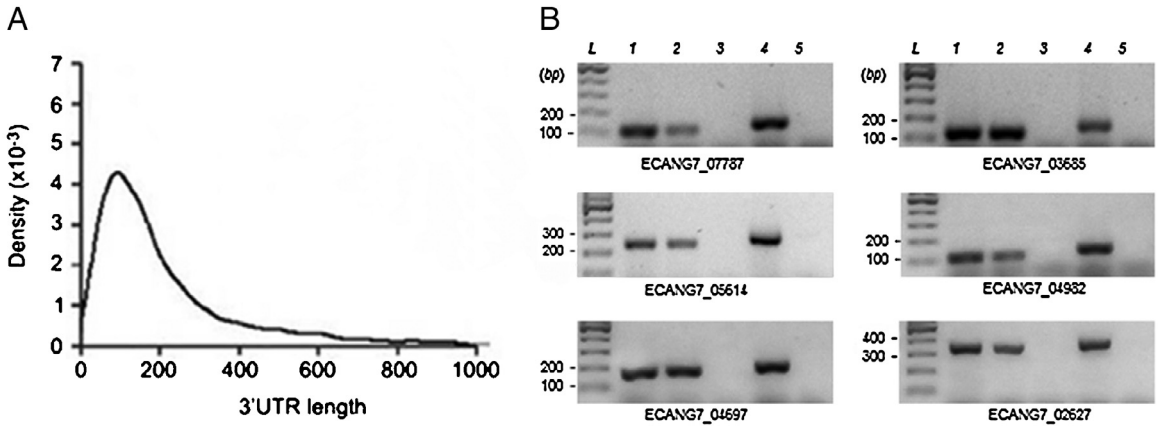

Fig. 2. Length distribution and validation of *Echinococcus canadensis* 3′UTR. (A) Length distribution analysis of *Echinococcus canadensis* 3′UTR. (B) Validation of a random subset of *Echinococcus canadensis* 3′UTRs by RT-PCR. L: ladder GeneRuler 100 bp. Lanes 1, 2, 3 correspond to gDNA, cDNA and RT-, respectively; amplified with primers F and R1. Lanes 4 and 5 correspond to gDNA and cDNA, respectively; amplified with primers F and R2. gDNA and cDNA samples were prepared from *Echinococcus canadensis* protoscoleces. Primer design: F within the 3′UTR, R1 at the end of the 3′UTR and R2 outside of the 3′UTR (up to 13 bp downstream).

potential of miRNAs as anti-*Echinococcus* intervention molecules. MAPKs were proposed as therapeutic targets for *Echinococcus* being *E. multilocularis* p38-like MAPK [49] and MEK1/2 and MKK3/6-like MAPKKs [50] experimentally validated as potential anti-*Echinococcus* therapeutic targets. MiRNAs associated to relevant functions that are highly expressed and absent in the host miRNome, such as miR-71, represent molecules for development of new therapeutic intervention strategies for echinococcosis control.

5. Conclusions

The recent availability of transcriptomic and genomic data from the zoonotic parasites of *Echinococcus* genus and the limited knowledge about gene regulation mechanisms highlight the importance of characterizing miRNAs, noncoding RNAs that have emerged as major components of the eukaryotic transcriptome and more recently as potential biomarkers and therapeutic targets. In this work, we applied an integrated pipeline to define 3′UTRs in *E. canadensis* and to identify and characterize miRNA target genes at the genome-wide level for the first time in *E. canadensis*, *E. multilocularis* and *T. solium*. Here, we perform for the first time a comparative analysis of miRNA targets in *Echinococcus* and *Taenia* providing new insights about miRNA function in *Echinococcus* biology. We found that miRNAs may regulate a large proportion of genes in all three species, which could be essential for development and survival of the parasites as well as for the host-parasite interaction, suggesting the importance of this mechanism of gene regulation in the biology of these parasites and their potential as targets for therapeutic intervention. This study provides a valuable resource to guide future experimental studies such as reporter assays or miRNA silencing and/or overexpression. Highly expressed miRNAs, absent in the host or divergent from host orthologs that are involved in essential functions such as cell signaling, regulation, development and host-parasite interaction represent novel therapeutic targets for further experimental research that may lead to new intervention strategies for echinococcosis control.

Conflict of interest

All authors declare no conflict of interest.

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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.2017.04.001.

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