

Identification and characterisation of microRNAs in young adults of *Angiostrongylus cantonensis* via a deep-sequencing approach

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Angiostrongylus cantonensis is an important causative agent of eosinophilic meningitis and eosinophilic meningoencephalitis in humans. MicroRNAs (miRNAs) are small non-coding RNAs that participate in a wide range of biological processes. This study employed a deep-sequencing approach to study miRNAs from young adults of *A. cantonensis*. Based on 16,880,456 high-quality reads, 252 conserved mature miRNAs including 10 antisense miRNAs that belonging to 90 families, together with 10 antisense miRNAs were identified and characterised. Among these sequences, 53 miRNAs from 25 families displayed 50 or more reads. The conserved miRNA families were divided into four groups according to their phylogenetic distribution and a total of nine families without any members showing homology to other nematodes or adult worms were identified. Stem-loop real-time polymerase chain reaction analysis of *aca-miR-1-1* and *aca-miR-71-1* demonstrated that their level of expression increased dramatically from infective larvae to young adults and then decreased in adult worms, with the male worms exhibiting significantly higher levels of expression than female worms. These findings provide information related to the regulation of gene expression during the growth, development and pathogenesis of young adults of *A. cantonensis*.

Key words: *Angiostrongylus cantonensis* - deep-sequencing approach - microRNA - stem-loop real-time polymerase chain reaction - young adults

Angiostrongylus cantonensis, the rat lungworm, is the primary causative agent of eosinophilic meningitis and eosinophilic meningoencephalitis in humans (Ramirez-Avila et al. 2009). This species was first discovered in rats from China in 1935 (Chen 1935) and the first human case was reported in Taiwan in 1945 (Nomura & Lin 1945). Although this infection is mainly prevalent in Southeast Asia and the Pacific Basin (Asato et al. 2004, Hochberg et al. 2011, Qu et al. 2011, Tsai et al. 2011), many new cases have been reported among travellers returning from endemic areas (Bärtschi et al. 2004, Ali et al. 2008, Malvy et al. 2008, Luessi et al. 2009) and, by 2008, more than 2,800 cases had been recorded worldwide (Wang et al. 2008). The major clinical manifestations of this disease include headache, neck stiffness, muscle weakness, nausea, vomiting and paraesthesia. Although the clinical course of infection is usually benign and self-limited, severe infections may lead to irreversible outcomes and even death (Punyangupta et al. 1975).

The life cycle of *A. cantonensis* is complex, requiring both an intermediate host and a final definitive host. The

giant African snail *Achatina fulica* and the channelled apple snail *Pomacea canaliculata* are the two most common intermediate hosts for *A. cantonensis* worldwide (Kliks & Palumbo 1992, Lv et al. 2009). Following entry into the intermediate host through the oral or percutaneous route, the first-stage larvae undergo two moults and develop into the infective larvae within two-three weeks. Humans are an accidental host for this parasite, becoming infected with the parasite by ingesting raw or uncooked individuals of intermediate host species (*A. fulica*, *P. canaliculata*, *Hyla aurea*), paratenic host species (*Macrobrachium* lar, *Ocypode ceratophthalma*, *Hoplobatrachus rugulosus*, *Rana plancyi*, *Tilapia mossambica*, *Vaginulus plebeius*, *Laevicaulis alte*) (freshwater crustaceans, frogs, fish and planarians) or contaminated vegetables carrying the third-stage larvae (Wallace & Rosen 1966, 1967, Ash 1968, Tsai et al. 2004). These larvae then penetrate the intestinal tract and migrate to the central nervous system, where they moult twice and develop into young adults, approximately one week after infection (Alicata 1965).

The available genomic information for *A. cantonensis* is limited to data from small-scale transcriptomic investigations and cloning studies. Following the submission of 1,226 expressed sequence tags (ESTs) from young adult *A. cantonensis* (5th-stage larva) to the National Center for Biotechnology Information dbEST by our laboratory in 2005, these and some additional sequences were analysed and were found to encode proteins participating in metabolism, cellular development, immune evasion and host-parasite interactions (Xu et al. 2009) and were subsequently grouped into 13 categories (Fang et al. 2010). Among proteins encoded by cDNA

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clusters found in the fourth-stage larvae, an enrichment of binding and catalytic activity was also revealed (He et al. 2009). The transcripts found in pepsin-activated infective larvae (3rd-stage) encode proteins that participate in a wide range of biological processes (Chang et al. 2011). Although cloning of galectin (Hao et al. 2007), cystatin (Liu et al. 2010), Acl6 (Li et al. 2011), cathepsin B (Han et al. 2011), a cathepsin B-like cysteine proteinase (Cheng et al. 2012) and a protein disulphide isomerase (Liu et al. 2012) has been accomplished in *A. cantonensis*, the characterisation of these proteins is far from complete. Moreover, the genome of this parasite has not yet been sequenced.

MicroRNAs (miRNAs) are a type of small non-coding RNAs, approximately 21-23 nucleotides (nt) in length, that have been discovered in diverse organisms, including animals, plants, viruses and fungi (Ambros 2004, Lee et al. 2010, Khan et al. 2011, Plaisance-Bonstaff & Renne 2011). Because the first two miRNAs, *lin-4* and *let-7*, were discovered and demonstrated to be involved in the regulation of developmental timing in *Caenorhabditis elegans* (Lee et al. 1993, Reinhart et al. 2000), more than 15,000 microRNA gene *loci* in over 140 species have been reported in miRBase v16 (Kozomara & Griffiths-Jones 2011). MiRNAs regulate a wide range of biological processes, including development, metabolism, cell proliferation and differentiation (Bartel 2009, Krol et al. 2010). In animals, miRNAs usually silence their target mRNAs through degradation or translational repression (Nilsen 2007). With respect to experimental methods, computational approaches are considered a useful strategy for identifying miRNAs, even in species whose genomes have not been fully sequenced (Berezikov et al. 2006). Computational approaches can also be utilised to quantify miRNA expression (Luo 2012).

As parasitic helminths usually exhibit a complex life cycle, investigations involving miRNAs enable us to not only understand the roles of these riboregulators in the physiology, development and evolution of these organisms, but also the mechanisms of host invasion and pathogenesis (Xue et al. 2008, Hao et al. 2010, Poole et al. 2010, Chen et al. 2011a, Winter et al. 2012). MiRNA expression in adult worms of *A. cantonensis* was recently identified and compared between the sexes (Chen et al. 2011b). The adult worms may only cause severe obstruction of the pulmonary arteries and respiratory failure (Wang et al. 2012). In contrast, because the young adults of *A. cantonensis* are at a pathogenic developmental stage associated with the central nervous system, miRNA expression profiling may provide further information on the pathogenesis of eosinophilic meningitis and eosinophilic meningoencephalitis. In the present study, we employed a deep-sequencing approach to identify conserved miRNAs among young adults of *A. cantonensis*. Following a phylogenetic distribution analysis, the expression profiles of selected miRNAs at different developmental stages were analysed via stem-loop real-time polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Parasites - A strain of *A. cantonensis* has been maintained in our laboratory in *Biomphalaria glabrata* snails and Sprague-Dawley rats since 1980 (Wang et al. 1989). Young adults and adult worms were collected on days 21 and 50 post-infection, respectively, from the cerebral tissues and pulmonary arteries of rats. The sex of these worms was determined based on their morphological characteristics: male worms are usually shorter and exhibit copulatory bursa and long spicules. Infective larvae were collected from the tissues of infected snails through digestion with 0.6% (w/v) pepsin-HCl (pH 2-3) for 1 h. These worms were washed with normal saline, phosphate buffered saline and distilled water and then stored at -80°C for further analyses (Hwang et al. 2010). This experiments performed in this study followed the recommendations of the Institutional Animal Care and Use Committee of Chang Gung University.

Small RNA library construction and high-throughput sequencing - Total RNA was isolated from young adults of *A. cantonensis* (500 worms of each sex) using the TRI Reagent, according to the instructions of the manufacturer (Molecular Research Center, Cincinnati, OH, USA). RNA integrity was assessed via 1% (w/v) agarose gel electrophoresis, while RNA purity was determined based on the absorbance recorded at 260/280 nm using an SMA1000 UV Spectrophotometer (Merinton Technology, Beijing, China). The RNA preparations were then stored at -80°C for further experiments.

RNA fragments of 18-30 nt in length were separated from total RNA using the Small RNA Sample Prep Kit (Illumina, San Diego, CA, USA). Following 15% Novex TBE-urea polyacrylamide gel electrophoresis (PAGE), the purified fragments were ligated to 5' and 3' RNA adaptors, reverse transcribed to produce single-stranded cDNAs and amplified via PCR. RNA fragments (approximately 92 bp) were isolated from the PCR products and sequenced using the Illumina HiSeq 2000 system (Illumina, San Diego, CA, USA).

Bioinformatic analysis - The small RNA library was analysed through a deep-sequencing small RNA analysis pipeline (DSAP) (dsap.cgu.edu.tw) (Huang et al. 2010). Following the removal of adaptors and poly-A/T/C/G/N nt, sequences longer than 16 nt were considered clean sequence tags. Using the clustering module of DSAP, redundant clean reads were merged into unique tags. The reads for each unique tag were counted as its expression abundance.

To identify transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs) and other annotated non-coding RNAs (ncRNAs), the unique tags were subjected to searches against the transcribed sequence library of the ncRNA (Rfam) database (version 10.0). The remaining sequences were compared with mature miRNA sequences in miRBase (version 16) using BLASTN.

Stem-loop RT-PCR - Total RNAs were isolated from infective larvae (20,000 worms), young adults (500 worms of each sex) and adult worms (50 worms of each

sex). The expression levels of aca-miR-1-1 and aca-miR-71-1 were determined via modified stem-loop RT-PCR (Chen et al. 2005). The stem-loop reverse transcription primer and forward primer for aca-miR-1-1 were 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTC-GCACTGGATACGACTACATAC-3' and 5'-CGCGGC-TGGAATGTAAAGAAGT-3', respectively, and those for aca-miR-71-1 were 5'-GTCGTATCCAGTGCAGGGT-CCGAG-GTATTCGCACTGGATACGACCGTCTCA-3' and 5'-GCGCGGTGAAAGACATGG-GTAGTG-3', respectively. The reverse primer employed in these assays was 5'-GTGCAGGGTCCGAGGT-3'.

Small RNAs from *A. cantonensis* at different developmental stages were extracted using an miRNA isolation kit (Geneaid, Sijhih City, Taipei County, Taiwan) according to the instructions of the manufacturer. First-strand cDNAs were synthesised using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Briefly, reaction mixtures (20 μ L) containing the purified small RNAs (160 ng), 5X First-Strand buffer (4 μ L), individual specific stem-loop RT primers (0.5 μ M), dNTP mix (0.5 mM), DTT (5 mM), SuperScript III Reverse Transcriptase (10 units) and RNaseOUT (2 units) were incubated at 55°C for 50 min, then inactivated by heating at 70°C for 15 min and subsequently incubated with two units of RNase H at 37°C for 20 min.

Quantitative RT-PCR was performed using RealQ PCR Master Mix (Ampliqon A/S, Skovlunde, Denmark) in the Stratagene Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA, USA). The PCR mixtures (20 μ L) included the reverse transcription product for each miRNA (3 μ L), RealQ-PCR 2X Master Mix (10 μ L), a specific forward primer (0.5 μ M) and the reverse primer (0.5 μ M). The RT-PCR assays were carried out with an initial step at 95°C for 10 min, followed by 40 cycles of amplification, with denaturation at 95°C for 30 s, primer annealing at 58°C for 1 min and elongation at 72°C for 30 s. The specificity of this assay was confirmed via 15% PAGE. Finally, the expression levels of the targeted miRNAs were determined through three rounds of stem-loop RT-PCR amplification and analysed according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001).

Statistical analysis - The expression levels of the miRNAs were expressed as the mean \pm standard deviation. Means were compared via one-way ANOVA and the least significance difference test was used for post-hoc multiple range comparisons. $p < 0.05$ was considered to be statistically significant.

RESULTS

Analysis of short RNAs - A total of 22,484,156 raw sequence reads were obtained through high-throughput sequencing from the small RNA library generated for young adults of *A. cantonensis*. The removal of adapters, contaminated nt and low-quality sequences resulted in 16,880,456 (75.1%) high-quality, clean reads of 16-31 nt in length, averaging 22.8 nt. A majority of the sequences were 23 nt in length (46.1%), followed by lengths of 22 nt (26.3%) and 24 nt (12.1%) sequences. Among the clean reads, 10,766,590 (63.8%) sequences were found to match

entries in the Rfam database. They included 7,736,727 tRNAs (45.8%), 1,191,869 rRNAs (7.1%), 228,262 snoRNAs (1.4%), 174,633 snRNAs (1%) and 1,435,099 ncRNAs (8.5%) (Fig. 1).

Identification of conserved miRNAs - From the remaining 7,548,965 clean reads, 252 conserved mature miRNAs including 10 antisense miRNAs were identified based on comparison with entities in miRbase. All of the identified miRNAs were homologous to miRNAs from Metazoa and their lengths ranged from 20-24 nt, with most being 22 nt in length (117), followed by lengths of 21 nt (54), 23 nt (49), 20 nt (19) and 24 nt (13). These miRNAs belonged to 90 families, in addition to which 10 antisense miRNAs were discovered. The number of reads obtained was 50 or more for 53 of these miRNAs, 21-49 for 28 miRNAs, 11-20 for 14 miRNAs, two-10 for 82 miRNAs and only a single read was observed in the remaining 75 miRNAs.

Supplementary data lists the 53 miRNAs with 50 or more reads, which belonged to 25 families. More than one miRNA was identified from the following families: let-7, mir-1, mir-34, mir-87, mir-71, mir-99, mir-2, mir-9, mir-31, mir-50 and mir-103. Only one miRNA was found in the remaining 14 families. Nine miRNAs displayed more than 1,000 reads. Aca-miR-1-1 exhibited the highest number of reads, at 184,946, followed by aca-miR-71-1 (92,433). These two highly expressed miRNAs constituted 50.7% and 25.3% of the total reads (365,112), respectively, among this group of 53 miRNAs.

Phylogenic distribution - To analyse the evolutionary features of *A. cantonensis* miRNAs, the identified miRNAs showing 50 or more reads, from 25 families, were compared with those from other nematodes in miR-Base. The families let-7, mir-1, mir-2, mir-9, mir-31, mir-34, mir-44, mir-50, mir-67, mir-71, mir-81, mir-87 and mir-124 contained members that were homologous to parasitic nematodes (*Ascaris suum* and *Brugia malayi*) and free-living nematodes (*C. elegans*, *Caenorhabditis*

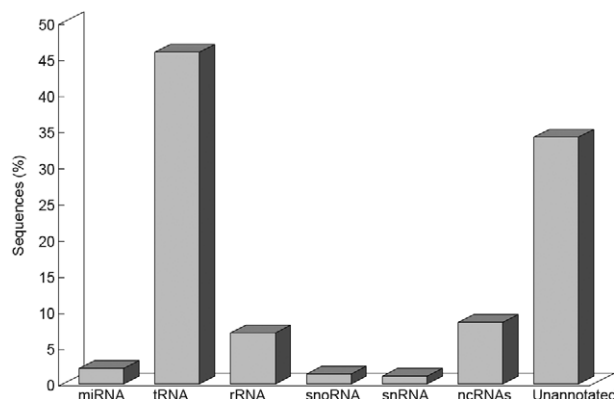


Fig. 1: classification of small RNAs of *Angiostrongylus cantonensis* young adults identified by a deep-sequencing approach. miRNA: microRNAs; ncRNA: non-coding RNAs; rRNA: ribosomal RNAs; snoRNA: small nucleolar RNAs; snRNA: small nuclear RNAs; tRNA: transfer RNAs.

briggsae, *Caenorhabditis remanei* and *Pristionchus pacificus*). Members of the mir-60 and mir-235 families were homologous to sequences from free-living nematodes, but not parasitic nematodes. The family mir-99 was homologous only to *A. suum*, while members of the mir-21, mir-29, mir-30, mir-103, mir-140, mir-146, mir-185, mir-191 and mir-320 families did not exhibit homology with sequences from other nematodes (Table).

Expression of miRNAs at different developmental stages - To confirm the expression of the miRNAs from *A. cantonensis* young adults that were identified through the applied high-throughput approach and to determine the expression levels of these miRNAs at different developmental stages, the levels of aca-miR-1-1 and aca-miR-71-1 transcripts were validated in a modified stem-loop quantitative RT-PCR analysis. Significant differences in the expression levels of these miRNAs were observed among different developmental stages (aca-miR-1-1: $F = 521.55$, $p < 0.001$; aca-miR-71-1: $F = 1585.86$, $p < 0.001$). In male worms, the expression of the two miRNAs was significantly higher in young adults than in infective larvae or adult worms ($p < 0.05$). Additionally, the expression in adult worms was significantly higher than in infective larvae ($p < 0.05$). In female worms, significantly higher expression was also observed in young adults ($p < 0.05$). However, there was no significant difference in aca-miR-1-1 expression observed between infective larvae and adult worms ($p > 0.05$) and the infective larvae exhibited significantly higher aca-miR-71-1 expression than adult worms ($p < 0.05$). Moreover, male worms displayed significantly higher expression than female worms in both the young adult and adult stages ($p < 0.05$) (Fig. 2).

DISCUSSION

Using a deep-sequencing approach, based on 7,548,965 reads, we identified and characterised 252 conserved mature miRNAs including 10 antisense miRNAs that belonging to 90 families from young adults of *A. cantonensis*. Previous authors identified miRNAs in adult male and female worms from 592,899 and 458,447 reads, respectively (Chen et al. 2011b). However, no novel miRNAs were discovered in either the present or previous studies. The sequences obtained from the adult male and female worms were matched to the *C. elegans* genome, although the percentage of perfect matches was quite low (18.94% in females and 22.58% in males). Although *C. elegans* and *A. cantonensis* are both nematodes, the former species is free-living in soil or water, whereas the latter is parasitic, being found in the pulmonary arteries of its definitive host and requiring a mollusc as an intermediate host (Chen et al. 2011b). In the present study, a search for sequences in miRBase discovered only conserved mature miRNAs. We hypothesise that novel miRNAs will be identified only when a reference genome for *A. cantonensis* becomes available.

In the present study, we identified nine miRNAs in young adults of *A. cantonensis* displaying more than a 1,000 reads: two in the let-7 family, four in the mir-1 family, one in the mir-44 family, one in the mir-71 family and one in the mir-99 family. In male adult worms, seven miRNAs exhibited more than 1,000 reads: miR-1,

miR-228, miR-44, miR-45, miR-71, miR-72 and miR-81. In the female worms, 10 miRNAs showed more than a 1,000 reads: miR-1, miR-2, miR-228, miR-44, miR-45, miR-60, miR-71, miR-72, miR-81 and miR-87 (Chen et al. 2011b). Mir-1, mir-71 and miR-44 show high expression in both the young adult and in adult stages. In contrast, let-7 and miR-99 are highly expressed only in young adults, whereas miR-45 and miR-81 are expressed only in adult worms. These findings indicate that a stage-specific expression of miRNAs occurs in *A. cantonensis*. Moreover, the regulatory functions of miR-99 require further investigation.

Let-7 and lin-4 are two important miRNA families associated with the lifespan of *C. elegans*. These miRNAs have been characterised as playing an essential role in the developmental timing of the worm by downregulating specific targets, such as the TRIM protein lin-41 and the transcription factor lin-14 (Ibáñez-Ventoso et al. 2006, Ambros 2011). Let-7 is an miRNA families that was discovered in ancient animals (Christodoulou et al. 2010). The let-7 family is expressed in a wide range of animals and the sequences of its members are highly conserved. However, significant variations in the size of the let-7 family occur among organisms: of the 55 organisms known to express members of the let-7 family, 13 express only one let-7 miRNA, including the nematodes *P. pacificus*, *C. remanei*, *C. elegans*, *C. briggsae* and *B. malayi*, whereas 19 mature let-7 sequences have been identified in the zebrafish (*Danio rerio*) (Pasquinelli et al. 2003). In the present study, we identified 19 members of the let-7 family in young adults of *A. cantonensis*, 11 of which exhibited 50 or more reads. In adult worms, the copy number of this miRNA was determined to be less than 50 in both sexes. These finding suggests the importance of let-7 in gene regulation in young adults of *A. cantonensis*. However, understanding the role of let-7 in the development of this parasite will require further studies.

Among the miRNA families found to be expressed in young adults of *A. cantonensis*, mir-1 displayed the highest number of total reads. Members of this family have been found in a wide range of organisms, including worms, flies, fishes, mice and humans (Bentwich et al. 2005, Sokol & Ambros 2005, Wienholds et al. 2005, Zhao et al. 2005). They are evolutionarily conserved and have been characterised as playing essential roles in regulating proliferation and the differentiation of muscle development via the regulation of synaptic transmission (Simon et al. 2008, Jones et al. 2011). In adult worms, the mir-71 family presented the highest number of reads in both sexes (Chen et al. 2011b). This family has been reported to promote longevity and stress resistance in worms (Pincus et al. 2011) and is involved in the sexual maturation of female worms (Gomes et al. 2011). These expression patterns suggest the different roles of miRNAs at different developmental stages.

Although the phylogenetic distribution has been reported for *Clonorchis sinensis* (Xu et al. 2010), there is no such information available for parasitic nematodes. The 25 conserved miRNA families found in *A. cantonensis* young adults showed a distribution bias. These conserved families can be divided into four groups: 13

families were homologous to sequences of other parasitic nematodes, two to sequences of free-living nematodes, but not parasitic nematodes, and one to *A. suum* sequences. Nine families did not exhibited any member that was homologous to either a free-living or parasitic nematode. Moreover, these miRNAs observed in young adults of *A. cantonensis* were not found in adult worms (Chen et al. 2011b). It is possible that the first two groups regulate general biological or physiological functions in nematodes. The miRNAs showing homology to *A. suum* may be specific to parasitic nematodes. As adult worms of *A. cantonensis* live within the central nervous system, the last group may regulate adaptive functions of worms related to this special environment, which may also cause pathological changes in the central nervous system. Further studies are required to confirm these hypotheses.

Analysis of the levels of aca-miR-1-1 and aca-miR-71-1 expression via the stem-loop RT-PCR revealed different expression patterns based on developmental stages and sex. These two miRNAs were selected because they are highly expressed not only in young adults, but also in adult worms of both sexes. In both male and female worms, the level of expression of these miRNAs

increased dramatically from the levels observed in infective larvae and peaked in young adults, subsequently declining to a low level in adult worms. Overall, the expressions levels of these miRNAs were found to be highest in male adult males. Similar expression patterns have been reported for 18 miRNAs in adult worms (Chen et al. 2011b). These miRNAs may be important in regulating sex differentiation, rather than developmental stages. The lower expression levels of these miRNAs observed in female worms indicate that females may require a lower degree of post-transcriptional regulation than male worms. Moreover, the higher expression levels of the miRNAs detected in young adults suggest that more significant changes may occur during the young adult stage than in the adult stage.

Replicate analyses were difficult in the present study because of the technical difficulties involved in obtaining sufficient sample sizes from infected animals. This limitation may restrict the quantitation of our findings. However, we identified 53 miRNAs, belonging to 25 families, that displayed 50 or more reads. These findings provide reliable information about the global miRNA expression profiles found in *A. cantonensis*. Al-

TABLE

Phylogenetic distributions of conserved microRNAs families of *Angiostrongylus cantonensis* young adults with 50 or more reads

Family	<i>A. cantonensis</i>	<i>Ascaris suum</i>	<i>Brugia malayi</i>	<i>Caenorhabditis elegans</i>	<i>Caenorhabditis briggsae</i>	<i>Caenorhabditis remanei</i>	<i>Pristionchus pacificus</i>
let-7	+	+	+	+	+	+	+
mir-1	+	+	-	+	-	+	+
mir-2	+	+	+	+	-	+	-
mir-9	+	+	+	+	+	+	+
mir-21	+	-	-	-	-	-	-
mir-29	+	-	-	-	-	-	-
mir-30	+	-	-	-	-	-	-
mir-31	+	-	+	-	-	+	+
mir-34	+	+	+	+	+	+	-
mir-44	+	+	-	+	+	+	+
mir-50	+	+	+	+	+	+	-
mir-60	+	-	-	+	+	+	-
mir-67	+	+	-	+	+	+	-
mir-71	+	+	+	+	+	+	-
mir-81	+	+	-	+	+	+	-
mir-87	+	+	+	+	+	+	+
mir-99	+	+	-	-	-	-	-
mir-103	+	-	-	-	-	-	-
mir-124	+	+	+	+	+	+	+
mir-140	+	-	-	-	-	-	-
mir-146	+	-	-	-	-	-	-
mir-185	+	-	-	-	-	-	-
mir-191	+	-	-	-	-	-	-
mir-235	+	-	-	+	-	+	-
mir-320	+	-	-	-	-	-	-

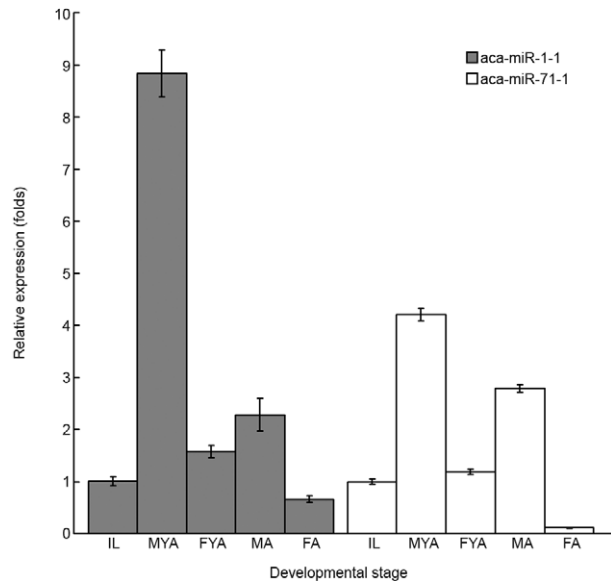


Fig. 2: expression profiles of selected microRNAs of *Angiostrongylus cantonensis* young adults in different developmental stages. FA: female adults; FYA: female young adults; IL: infective larvae; MA: male adults; MYA: male young adults.

though Northern blotting is considered a gold-standard approach for detecting miRNAs, this method is limited by its low sensitivity and difficulties in distinguishing homologous miRNAs from highly similar sequences (van Rooij 2011, Pritchard et al. 2012). Moreover, we succeeded in confirming the expression of two miRNAs initially identified via the high-throughput approach in *A. cantonensis* young adults through the more sensitive and specific technique of stem-loop quantitative RT-PCR. These findings demonstrated the reliability of the results obtained herein.

Based on the results of the present study, there are significant differences in the expression of miRNAs between young adults and adult worms of *A. cantonensis*. These differences are not only qualitative, but also quantitative. In the present study, we identified nine miRNA families without homologous members in the available sequences of other nematodes in the adult stage. Moreover, the expression levels of miR-1 and miR-71 increase from a low expression level in infective larvae to a peak in young adults and subsequently decrease in adult worms. These results suggest that miRNAs play a more important role in the regulation of biological functions in young adults than in adult worms of *A. cantonensis*.

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Identification of conserved microRNAs (miRNAs) of *Angiostrongylus cantonensis* young adults with 50 or more reads

Family	MiRNAs	Sequence [nucleotides (n)]	Reference miRNAs (accession)	Reads (n)
let-7	aca-let-7-1	UGAGGUAGUAGAUUGUAUAGUU (22)	ppy-let-7f (MIMAT0015726)	2,674
	aca-let-7-2	AACUAUACAACCUACUACCUCA (22)	gga-let-7a (MIMAT0001101)	1,858
	aca-let-7-3	UGAGGUAGUAGUUUGUGCUGUU (22)	ppy-let-7i (MIMAT0015728)	832
	aca-let-7-4	AACCACACAACCUACUACCUCA (22)	gga-let-7b (MIMAT0001102)	819
	aca-let-7-5	UGAGGUAGUAGUUUGUACAGUU (22)	ppy-let-7g (MIMAT0015727)	606
	aca-let-7-6	CUAUACA AUCUACUACCUCA (20)	hsa-let-7f-5p (MIMAT0000067)	276
	aca-let-7-7	UGAGGUAGUAGGUUGUAUGGUU (22)	ppy-let-7c (MIMAT0015723)	245
	aca-let-7-8	UGAGGUAGGAGGUUGUAUAGUU (22)	ppy-let-7e (MIMAT0015725)	217
	aca-let-7-9	UGAGGUAGUAGGUUGUAUAGU (21)	ngi-let-7 (MIMAT0018413)	201
	aca-let-7-10	UGAGGUAGUAGUUUGUGCUGU (21)	gga-let-7i (MIMAT0001098)	188
	aca-let-7-11	AGAGGUAGUAGGUUGCAUAGUU (22)	ppy-let-7d (MIMAT0015724)	79
mir-1	aca-miR-1-1	UGGAAUGUAAAGAAGUAUGUA (21)	ssc-miR-1 (MIMAT0010187)	184,946
	aca-miR-1-2	UGGAAUGUAAAGAAGUAUGUAU (22)	ppy-miR-1 (MIMAT0015720)	40,263
	aca-miR-1-3	UGGAAUGUAAAGAAGUAUGU (20)	ppc-miR-1 (MIMAT0011639)	6,652
	aca-miR-1-4	UGGAAUGUAAAGAAGUAUGUAG (22)	cte-miR-1 (MIMAT0009502)	2,196
mir-2	aca-miR-2-1	UAUCACAGCCAGCUUUGAUGU (21)	bma-miR-2a (MIMAT0014096)	413
	aca-miR-2-2	UAUCACAGCCAGCUUUGAUGUGC (23)	nvi-miR-2b (MIMAT0015667)	247
mir-9	aca-miR-9-1	UCUUUGGUUAUCUAGCUGUAUGA (23)	nlo-miR-9a (MIMAT0018442)	87
	aca-miR-9-2	UCUUUGGUUAUCUAGCUGUAUG (22)	ppc-miR-9 (MIMAT0011737)	60
mir-21	aca-miR-21-1	UAGCUUAUCAGACUGAUGUUGA (22)	tgu-miR-21 (MIMAT0014527)	149
mir-29	aca-miR-29-1	UAGCACCACUGAAAUCGGUUA (22)	eca-miR-29a (MIMAT0012940)	167
mir-30	aca-mir-30-1	UGUAAACAUCCCCGACUGGAAGCU (24)	bta-mir-30d (MIMAT0003533)	71
mir-31	aca-miR-31-1	AGGCAAGAUGUUGGCAUAGCU (21)	crm-miR-72 (MIMAT0011567)	96
	aca-miR-31-2	AGGCAAGAUGUUGGCAUAGCUGA (23)	bma-miR-72 (MIMAT0014107)	72
mir-34	aca-miR-34-1	UGGCAGUGUGGUUAGCUGGUUG (22)	nvi-miR-34 (MIMAT0015682)	598
	aca-miR-34-2	GGCAGUGUGGUUAGCUGGUUG (21)	nlo-miR-34 (MIMAT0018435)	77
	aca-miR-34-3	UGGCAGUGUGGUUAGCUGGU (20)	aga-miR-34 (MIMAT0005526)	66
	aca-miR-34-4	AGGCAGUGUAGUUAGCUGAUUG (22)	bta-miR-34c (MIMAT0003854)	65
mir-44	aca-miR-44	UGACUAGAGACACAUUCAGCU (21)	ppc-miR-45 (MIMAT0011640)	22,803
mir-50	aca-miR-50-1	UGAUUAGUCUGGUUUCUUGGGUU (24)	crm-miR-50 (MIMAT0011589)	702
	aca-miR-50-2	UGAUUAGUCUGGUUUCUUGGG (22)	asu-miR-50 (MIMAT0021461)	81
mir-60	aca-miR-60-1	UAUUUAGCACAUUUUCUAGUUCA (23)	cel-miR-60-3p (MIMAT0000032)	111
mir-67	aca-miR-307	UCACAACCUCCUUGAGUGAG (20)	isc-miR-307 (MIMAT0012701)	177
mir-71	aca-miR-71-1	UGAAAGACAUGGGUAGUGAGACG (23)	crm-miR-71 (MIMAT0011618)	92,433
	aca-miR-71-2	UGAAAGACAUGGGUAGUGAGAU (22)	bfl-miR-71 (MIMAT0009476)	129
	aca-miR-71-3	UGAAAGACAUGGGUAGUGAGAUG (23)	api-miR-71 (MIMAT0014735)	50
mir-81	aca-miR-81	UGAGAUCAUCGUGAAAGCUAGU (22)	crm-miR-81 (MIMAT0011570)	282
mir-87	aca-miR-87-1	GUGAGCAAAGUUUCAGGUGUG (21)	isc-miR-87 (MIMAT0012707)	314
	aca-miR-87-2	GUGAGCAAAGUUUCAGGUGUGC (22)	api-miR-87a (MIMAT0014739)	141
	aca-miR-87-3	GUGAGCAAAGUUUCAGGUGU (20)	ame-miR-87 (MIMAT0004434)	139
	aca-miR-87-4	GUGAGCAAAGUCUCAGGUGU (20)	bma-miR-87b (MIMAT0014110)	126
mir-99	aca-miR-99-1	AACCCGUAGAUCCGAACUUGUGU (23)	isc-miR-100 (MIMAT0012683)	1,783
	aca-miR-99-2	AACCCGUAGAUCCGAACUUGUG (22)	nvi-miR-100 (MIMAT0015677)	60
	aca-miR-99-3	AACCCGUAGAUCCGAACUUGU (21)	tgu-miR-100 (MIMAT0014511)	50
mir-103	aca-miR-103-1	AGCAGCAUUGUACAGGGCUAUGA (23)	tgu-miR-103 (MIMAT0014523)	482
	aca-miR-103-2	AGCAGCAUUGUACAGGGCUAU (21)	gga-miR-103 (MIMAT0001145)	220
mir-124	aca-miR-124-1	UAAGGCACGCGGUGAAUGCCAA (22)	bma-miR-124 (MIMAT0014116)	129
mir-140	aca-miR-140-1	ACCACAGGGUAGAACCACGGA (21)	cfa-miR-140 (MIMAT0006689)	60
mir-146	aca-miR-146-1	UGAGAACUGAAUUCUAGGGUU (22)	ppy-miR-146a (MIMAT0015767)	270
mir-185	aca-miR-185	UGGAGAGAAAGGCAGUCCUGA (22)	ppy-miR-185 (MIMAT0015781)	79



Family	MiRNAs	Sequence [nucleotides (n)]	Reference miRNAs (accession)	Reads (n)
mir-191	aca-miR-191-1	CAACGGAAUCCCAAAGCAGCUG (23)	ppy-miR-191 (MIMAT0015785)	50
mir-235	aca-miR-235-1	UAUUGCACUCGCCCCGGCCUG (21)	crm-miR-235 (MIMAT0011558)	143
mir-320	aca-miR-320-1	AAAAGCUGGGUUGAGAGGGCGA (22)	ppy-miR-320a (MIMAT0015821)	78

aga: *Anopheles gambiae*; ame: *Apis mellifera*; api: *Acyrtosiphon pisum*; asu: *Ascaris suum*; bfl: *Branchiostoma floridae*; bma: *Brugia malayi*; bta: *Bos taurus*; cel: *Caenorhabditis elegans*; cfa: *Canis familiaris*; crm: *Caenorhabditis remanei*; cte: *Capitella teleta*; eca: *Equus caballus*; gga: *Gallus gallus*; hsa: *Homo sapiens*; isc: *Ixodes scapularis*; ngi: *Nasonia giraulti*; nlo: *Nasonia longicornis*; nvi: *Nasonia vitripennis*; ppc: *Pristionchus pacificus*; ppy: *Pongo pygmaeus*; ssc: *Sus scrofa*; tgu: *Taeniopygia guttata*.