Preliminary analysis of miRNA pathway in *Schistosoma mansoni*

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ARTICLE INFO

ABSTRACT

RNA silencing refers to a series of nuclear and cytoplasmatic processes involved in the post-transcriptional regulation of gene expression or post-transcriptional gene silencing (PTGS), either by sequence-specific mRNA degradation or by translational arrest. The best characterized small RNAs are microRNAs (miRNAs), which predominantly perform gene silencing through post-transcriptional mechanisms. In this work we used bioinformatic approaches to identify the parasitic trematode *Schistosoma mansoni* sequences that are similar to enzymes involved in the post-transcriptional gene silencing mediated by miRNA pathway. We used amino acid sequences of well-known proteins involved in the miRNA pathway against *S. mansoni* genome and transcriptome databases identifying a total of 13 putative proteins in the parasite. In addition, the transcript levels of *SmDicer1* and *SmAgo2/3/4* were identified by qRT-PCR using cercariae, adult worms, eggs and *in vitro* cultivated schistosomula. Our results showed that the *SmDicer1* and *SmAgo2/3/4* are differentially expressed during schistosomula development, suggesting that the miRNA pathway is regulated at the transcript level and therefore may control gene expression during the life cycle of *S. mansoni*.

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1. Introduction

Post-transcriptional gene silencing (PTGS) or RNA silencing is a complex mechanism involving conserved proteins and at least two distinct pathways. These are known as the siRNAs (shorts interfering RNAs) and miRNAs (microRNAs) pathways involved in the silencing of endogenous or exogenous mRNAs each with differing specificity but with several overlapping components [1]. These RNAs were initially identified as complex structures of dsRNAs, processed in mature small RNAs of 17 to 25 nt in size, which play a role on cleavage or inhibition of specific translation of mRNAs. siRNAs are the cleavage products of longer dsRNAs that are generated by RNA-dependent RNA polymerases, or from bidirectional transcription of genes or transposable elements. miRNAs mediate translational repression, although, in some cases, they can also direct mRNA degradation. The isolation and characterization of diverse genes that codify components of the microRNAs pathway in *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Homo sapiens* support the hypothesis

that the silencing of RNA constitutes a well conserved system found in different organisms [2–5].

The machinery of RNA silencing mediated by miRNA is dependent on several proteins [6]. In the initiation stage, the primary miRNA (pri-miRNA) is transcribed by a type II RNA polymerase. Inside the nucleus the pri-miRNA is cleaved into pre-miRNA that is an ATP-dependent microprocessor complex coordinated by RNase III Drosha [7]. The pre-miRNA, which exhibits a cramp-like structure and contains approximately 60–70 nt, is transported from the nucleus to the cytoplasm by the complex Ran-GTP/Exportina-5 [8,9]. Dicer and Argonaute consist of two core proteins involved in the silencing of RNA [6]. In the cytoplasm the pre-miRNA is cleaved by Dicer generating the mature miRNA. The latter is presented to the RNA induced silencing complex (RISC) which in *D. melanogaster* is composed of the main constituents: Argonaute, Tudor-SN, Fmr1 and Vig [10]. Different Argonaute paralogs were identified in various organisms where they give rise to different forms of RISC, each playing a distinct role [11–14].

Dicer is a ribonuclease in the RNase III family that contains two RNase III domains and one PAZ domain. A main feature of the Argonaute proteins is the presence of PAZ and PIWI domains in their structure [15]. The PIWI domain functions by directing the dsRNA to hydrolysis and it is structurally similar to a domain found in the RNAse H. The PAZ domain (*Piwi*/*Argonaute*/*Zwile*) identified in proteins Argonaute and Dicer, consists of 130 amino acids and it coordinates the interaction between the two proteins forming a heterodimer [16,17].

Table 1
Putative miRNA pathway sequences in *Schistosoma mansoni*

miRNA pathway	*S. mansoni* putative orthologs	Function	Domains and Pfam number	Identity with *D. melanogaster* (%)	*D. melanogaster* orthologs (FlyBase)	*C. elegans* orthologs (WormBase)	*S. japonicum* putative orthologs
Endoribonuclease							
Dicer	Smp_169750 (SmDicer1)	Processing of miRNA precursor	dsRNA_bind (PF03368); PAZ (PF02170); Ribonuclease_3 (PF00636)	47	FBgn0039016	WBGene00000939	SJCHGC08817
Drosha	Smp_142510.2 (SmDrosha2)	Processing of primary miRNA transcripts	Ribonuclease_3 (PF00636); dsrm (PF00035)	46	FBgn0026722	WBGene00009163	SJCHGC08309
	Smp_142510.1 (SmDrosha1)			48			
Component of RISC							
Argonaute	Smp_140010 (SmAgo1)	Short RNA binding	DUF1785 (PF08699); PAZ (PF02170); Piwi (PF02171)	68	FBgn0026611	WBGene00000105	SJCHGC07755
	Smp_179320 (SmAgo2)			33	FBgn0046812	WBGene00000106	SJCHGC07884
	Smp_102690.2 (SmAgo3)			29			SJCHGC01111
	Smp_102690.3 (SmAgo4)			33			SJCHGC05069
							SJCHGC07014
							SJCHGC02502
Tudor-SN	Sm01663	Nuclease	SNase (PF00565); TUDOR (PF00567)	36	FBgn0035121	WBGene00006626	SJCHGC09149
							SJCHGC04700
							SJCHGC05245
Fmr1	Smp_099630	RNA binding	KH_1 (PF00013)	25	FBgn0028734	–	SJCHGC09283
Other factors							
Partner Drosha	Smp_087220	RNA binding	dsrm (PF00035)	29	FBgn0039861	WBGene00011908	SJCHGC09172
Partner Dicer	Smp_023670	RNA binding	dsrm (PF00035)	33	FBgn0032515	WBGene00017025	–
Exportin-5	Smp_152800.1	Nuclear export of miRNA precursors	Xpo1 (PF08389);	21	FBgn0031051	–	SJCHGC09381
	Smp_152800.2			21			SJCHGC05562

In *Schistosoma mansoni* the silencing mechanism mediated by post-transcriptional modification was suggested by Blanton and Licate [18] as part of the hypothesis that during the cercariae to schistosomula transformation there is a block in translation. Based on this statement we used bioinformatic approaches to identify miRNA pathway components in this parasite. In addition, we performed quantitative Real-Time PCR of two key proteins of the pathway, Dicer and Argonaute, to determine the relative levels of mRNA in adult worms, cercariae, eggs and mechanically transformed schistosomula cultivated from 3.5 h to 3 days. Even though our study has evidenced the presence of these transcripts in all investigated stages we highlight its importance for post-transcriptional control during the cercariae to schistosomula transition.

2. Materials and methods

2.1. Sequence retrieving of the miRNA components pathway

Sequences involved in the miRNA pathway were searched in *S. mansoni* genome database version 4.0 from GeneDB (http://www.genedb.org/genedb/smansoni/) and *S. mansoni* transcriptome project (http://verjo18.iq.usp.br/schisto/) using amino acid sequences of *D. melanogaster* and *C. elegans* orthologs as queries. The BLASTp algorithm, underpinned by the Pfam (v22.0) and CDD databases was used for searches of conserved protein domains or motifs from *S. mansoni* sequences.
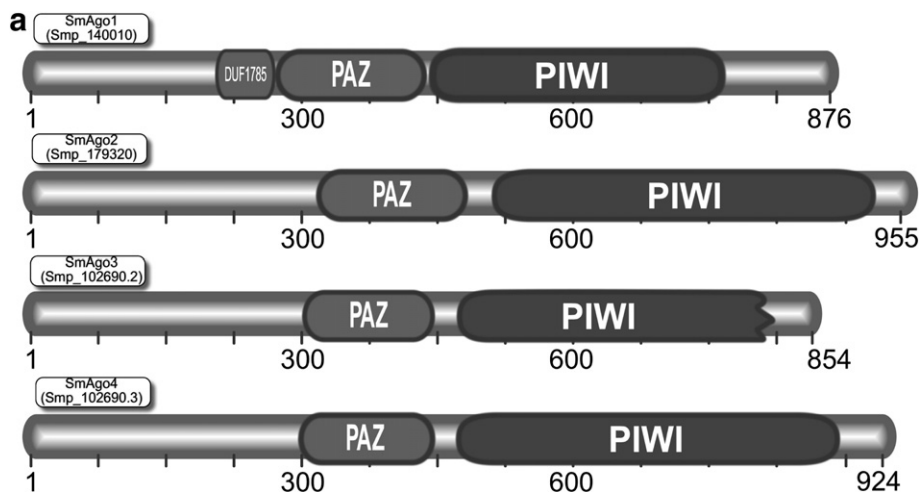

Fig. 1. Argonaute family in *S. mansoni* and AGOs lacking the key catalytic residues. (a) Representatives of Argonaute proteins of *S. mansoni*: SmAgo1/2/3/4. Domain boundaries are indicated with boxed ranges of amino acid sequence. (b) Alignment of PIWI (PF02171) and PAZ (PF02170) domains of SmAgo1/2/4 and their orthologs. Within the PIWI domain two key aspartic acid and histidine residues coordinate a magnesium ion at the catalytic center of the RNase H enzyme marked with arrow. Accession numbers: SmAgo2 (Smp_179320), Sj.1 (AAW26476.1), SmAgo4 (Smp_102690.3), Ce.ALG1 (NP_510322.2), CeALG2 (NP_493837.1), Hs.AGO1 (Q9UL18), MmAGO1 (Q8CJG1), HsAGO2 (Q9UKV8), MmAGO2 (Q8CJG0), DmAgo1 (NP_725342), SmAgo1 (Smp_140010), At.AGO1 (AAB91987.1), and AtAGO9 (CAD66636.1).

b

PAZ (PF02170)

Hs. AGO1	CEVLDI RNI D- EQPKPLTDSQRVRFTKEI KGLKVEVTHCGQMRKKYRVCNVTRRPASHQTFPLQLESGQ- - - TVECTVAQYFKQKYNLQLKYPHLPCLQVGQEQKHTYLPLEVCNI VAGQRCI KKLTDNQTSTM KATAR
Mm. AGO1	CEVLDI RNI D- EQPKPLTDSQRVRFTKEI KGLKVEVTHCGQMRKKYRVCNVTRRPASHQTFPLQLESGQ- - - TVECTVAQHFKQKYNLQLKYPHLPCLQVGQEQKHTYLPLEVCNI VAGQRCI KKLTDNQTSTM KATAR
Hs. AGO2	CEVLDFKSI E- EQQKPLTDSQRVKFTKEI THCGQMRKRYRVCNVTRRPASHQTFPLQQESGQ- - - TVECTVAQYFKDRHKLVLRYPHLPCLQVGQEQKHTYLPLEVCNI VAGQRCI KKLTDNQTSTM RATAR
Mm. AGO2	CEVLDFKSI E- EQQKPLTDSQRVKFTKEI THCGQMRKRYRVCNVTRRPASHQTFPLQQESGQ- - - TVECTVAQYFKDRHKLVLRYPHLPCLQVGQEQKHTYLPLEVCNI VAGQRCI KKLTDNQTSTM RATAR
Dm. Ago1	CEVLDI RDI N- EQRKPLTDSQRVKFTKEI KGLKI EI THCGQMRKRYRVCNVTRRPAQMQSFPLQLENGQ- - - TVECTVAKYFLDKYRMKLRYPHLPCLQVGQEHKHTYLPLEVCNI VAGQRCI KKLTDMQTSTM KATAR
Sm. Ago1	CEVLDI SDKN- EQRRPLTDSQRVKFTKEI KGLKVEI THCGTMRRKYRVCNVTRRPSQTQSFPLQLDTGA- - - TVECTVAKYFQERYNI RLDYPHLPCLQVGQEQKHTYLPLEVCNMVAGQRCI KKLTDMQTSTM KATAR
Ce. ALG1	AEVLELPVQALAERRALSDAQRVKFTKEI RGLKI EI THCGQMRKRYRVCNVTRRPAQTQTFPLQLETGQ- - - TI ECTVAKYFYDKYRI QLKYPHLPCLQVGQEQKHTYLPPEVCDI VPGQRCLKKLTDVQTSTM KATAR
Ce. ALG2	AEVLELPVQALAERRALSDAQRVKFTKEI RGLKI EI THCGAVRRKYRVCNVTRRPAQTQTFPLQLETGQ- - - TI ECTVAKYFFDKYRI QLKYPHLPCLQVGQEQKHTYLPPEVCDI VPGQRCLKKLTDVQTSTM KATAR
At. AGO1	KANQSV- - - - - - ETPRQI DWI KVAAKMLKHMRVKATHR- - - NMEFKI I GLSSKPCNQQLFSMKI KDGEREVPI REI TVYDYFKQTYTEPI SSAYFPCLQVGQEQKHTYLPLPLEFCNLVSLQRYTKPLSGRQRVLLVESSRQ
At. AGO9	LANQNK- - - - - - - KDPYGMDWNK- ARRVLKNLRVQI TLS- - - NREYKI SGLSEHSCKDQLFTWRKPNDKGEFEEVEI TVLNYYKERNI EVRYSGDFPCI NVGKPKRPTYFPI EFCNLVSLQRYTKSLTNFQRAALVEKSRQ
Sj. 1	- QHHHI KLKYPELPCVKVDQ- KREVYMPLELLNI LPYQAPNASKAEVAS- EVVRCAAV
Sm. Ago2	MYEKYGDNI AR- - - - CSSQMAHDLRRI RVETDKFYKNENGDAYSRRFTVHGI SSVSADKLM EER- - - - - - - - - - KQSVAAYFDEHHHI KLKYPDLPCVKVDQ- KREVYMPLELLNI LPFQAPNASKADVAS- EVI RCAAV
Sm. Ago4	MYEKYGDNI AR- - - - CSSQMAHDLRRI RVETDKFYKNENGDVYSRRFTVHGI SSVPADKLM EER- - - - - - - - - - KQSI AAYFDEHHHI KLKYPDLPCVKVDQ- KREVYMPEQLNI LPFQAPNASKADVAS- EVI RCAAV

REGION BETWEEN THE PAZ AND PIWI DOMAIN	PIWI (PF02171)
~ 140-160 aa	

Hs. AGO1	LI I VI LPG- - - KTPVYAEVKRVGDTLLGMATQCVQVKNVVKTSPQTLSNLCLKI NVKLGGI NNI LVPHQR- - - SAVFQQPVI FLGADVTHPPAG- DGKKPSI TAVVGS- MDAHPSR
Mm. AGO1	LI I VI LPG- - - KTPVYAEVKRVGDTLLGMATQCVQVKNAVKTSPQTLSNLCLKI NVKLGGI NNI LVPHQR- - - SAVFQQPVI FLGADVTHPPAG- DGKKPSI TAVVGS- MDAHPSR
Hs. AGO2	LVVVI LPG- - - KTPVYAEVKRVGDTVLGMATQCVQMKNVQRTTPQTLSNLCLKI NVKLGGVNNI LLPQGR- - - PPVFQQPVI FLGADVTHPPAG- DGKKPSI AAVVGS- MDAHPNR
Mm. AGO2	LVVVI LPG- - - KTPVYAEVKRVGDTVLGMATQCVQMKNVQRTTPQTLSNLCLKI NVKLGGVNNI LLPQGR- - - PPVFQQPVI FLGADVTHPPAG- DGKKPSI AAVVGS- MDAHPNR
Dm. Ago1	LVVVVLPG- - - KTPVYAEVKRVGDTVLGMATQCVQAKNVNKTSPQTLSNLCLKI NVKLGGI NSI LVPSI R- - - PKVFNEPVI FLGADVTHPPAG- DNKKPSI AAVVGS- MDAHPSR
Sm. Ago1	LI VVLPG- - - KTPVYAEVKRVGDI MFGLATQCVQKHKXXX- XXLGADVTHPPAG- DKTKPSI AAVVAS- MDAHPSR
Ce. ALG1	LVVVVLPG- - - KTPVYAEVKRVGDTVLGI ATQCVQAKNAI RTTPQTLSNLCLKMNVKLGGVNSI LLPNVR- - - PRI FNEPVI FFGCDI THPPAG- DSRKPSI AAVVGS- MDAHPSR
Ce. ALG2	LI VVLPG- - - KTPI VAEVKRVGDTVLGI ATQCVQAKNAI RTTPQTLSNLCLKMNVKLGGVNSI LLPNVR- - - PRI FNEPVI FLGCDI THPAAG- DTRKPSI AAVVGS- MDAHPSR
At. AGO1	HFI LCI LPERKTSDI YGPWKKI CLTEEGI HTQCI CP- - - I KI SDQYLTNVLLKI NSKLGGI NSLLGI EYSYNI PLI NKI PTLI LGMDVSHGPPG- RADVPSVAAVVGSKCWPLI SR
At. AGO9	LFLLCI LAERKNSDVYGPWKKKDLVDLGI VTQCI AP- - - TRLNDQYLTNVLLKI NAKLGGLNSLLAVERSPAMPKVTQVPTI I VGMDVSHGPG- QSDI PSI AAVVSSRQWPLI SK
Sj. 1	FLLLI LYD- - - EYSYPTI KRLSDLQMGI RTQCVRGR- - TLDKPNVFPNLLLKI NGKLGGVNWQI PDLI K- - - - NGNELI MVFGADVTHPAPTQNQQI RKSVAAI GS- VSPDLMR
Sm. Ago2	FLLLI LYD- - - EHAYPAI KRLSDLQI GI RTQCVRSR- - TLDKPNVFPNLLLKI NGKLGGVNWQI PDLI K- - - - - NSDELI MVFGADVTHPAQT- - QQVRKSVAAVLGS- VSPDLMR
Sm. Ago4	FI M.I LYD- - - DLAYGTI KRLSDLKMGI RTQCVRGS- - TLRKPNVFPNLLLKLNGKLGGVNWI VPDLTE- - - - - YSKDLI MVFGADVTHPAPTQTHQVLKSVAAVVGS- VSPELMR

PIWI (PF02171)

Hs. AGO1	RQ- - - - - - EI I EDLSY- - - - - - - - - - - MVRELLI QFYKSTR- FKPTRI I FYRDGVPEGQLPQI LHYELLAI RDACI KLEKDYQPGI TYI VVQKRHHTRLFCADKNERI GKSGNI PAGTTVDTNI THPFEFDFYLCSHAG
Mm. AGO1	RQ- - - - - - EI I EDLSY- - - - - - - - - - - MVRELLI QFYKSTR- FKPTRI I FYRDGVPEGQLPQI LHYELLAI RDACI KLEKDYQPGI TYI VVQKRHHTRLFCADKNERI GKSGNI PAGTTVDTNI THPFEFDFYLCSHAG
Hs. AGO2	RQ- - - - - - EI I QDLAA- - - - - - - - - - - MVRELLI QFYKSTR- FKPTRI I FYRDGVSEGQFQQVLHHELLAI REACI KLEKDYQPGI TFI VVQKRHHTRLFCTDKNERVGKSGNI PAGTTVDTKI THPTEFDFYLCSHAG
Mm. AGO2	RQ- - - - - - EI I QDLAA- - - - - - - - - - - MVRELLI QFYKSTR- FKPTRI I FYRDGVSEGQFQQVLHHELLAI REACI KLEKDYQPGI TFI VVQKRHHTRLFCTDKNERVGKSGNI PAGTTVDTKI THPTEFDFYLCSHAG
Dm. Ago1	RQ- - - - - - EI I QELSS- - - - - - - - - - - MVRELLI MFYKSTGGYKPHRI LYRDGVSEGQFPHVLQHELTAI REACI KLEPEYRPGI TFI VVQKRHHTRLFCAEKKEQSGKSGNI PAGTTVDVGI THPTEFDFYLCHQG
Sm. Ago1	RQ- - - - - - EI I HDLYP- - - - - - - - - - - MVRDLLLQFYRATR- FKPTRI I YYRDGVGEQFLNVLNHELRAI REACVKLELGYQPGI TYI I VQKRHHTRLFCADKKDQMGKSGNI PAGTTVDQVI THPTEFDFYLCSHAG
Ce. ALG1	RQ- - - - - - EI I SDLTY- - - - - - - - - - - MVRELLVQFYNRTR- FKPARI VVYRDGVSEGQFNVLQVELKA I REACMLERGYQPGI TFI AVQKRHHTRLFAVDKKDQVGKAYNI PPGTTVDVGI THPTEFDFYLCSHAG
Ce. ALG2	RQ- - - - - - EI I TDLTY- - - - - - - - - - - MVRELLVQFYNTR- FKPARI VVYRDGVSEGQFNVLQVELKA I REACMLESGYQPGI TFI AVQKRHHTRLFAADKADQVGKAFNI PPGTTVDVGI THPFDFFLCSHAG
At. AGO1	RL- - - - - - EM DSLFQPI ENTEKGDNGI MNELFVEFYRTSRARKPKQI I I FRDGVSESQFEQVLKI EVDQI I KAYQRLGESDVPKFTVI VAQKNHHTKLFQAKGPE- - - - - NVPAGTVVDTKI VHPTNYDFYMCAHAG
At. AGO9	KM - - - - - - EM DNLFKPVNGK- - - DEGMFRELLLDFYYSSENRKPEHI I I FRDGVSEGQFNQVLNI ELDQMQACKFLDDTWHPKFTVI VAQKNHHTKKFFQSRGPD- - - - - NVPPGTI I DSQI CHPRNFDFYLCAHAG
Sj. 1	TEKGNKTAREI I DDVRL- - - - - - - - - - - I VKELLQLYLRNTNGRFPNRM FYRDGVSEGQFENVLVEELAAI QRACADVRPGEEPAI TYI VVQKRHHI RFKPSDPRAR- - - - - NVEPGTI I VDTEI THPREFDFYLCSQDG
Sm. Ago2	TEKGNKAAREI I DDVRL- - - - - - - - - - - I VKELLQLYLRNTNGRFPTRM FYRDGVSEGQFENVLVEELAAI QRACSDVRPGEEPAI TYI VVQKRHHI RFKPSDPRAR- - - - - NVEPGTI VVDTEI THPREFDFYLCSQDG
Sm. Ago4	TERGNKTTREI I DNLHL- - - - - - - - - - - SVGELLTLYLRNTKGRFPKRI I FYRDGVSEGQFENVLVEELSAI OKACSDI RPGEEPAI TFI VVQKRHHI RLKPQDPRAR- - - - - NVEPGTVVDTDI THRREFDFYI CSHEG

PIWI (PF02171)

Hs. AGO1	I QGTSRPSHYYVLWDDNRFTADELQI LTYQLCHTYVRCTRSVS- - - - - - - - I PAPAYYARLVAFRARYHLV
Mm. AGO1	I QGTSRPSHYYVLWDDNRFTADELQI LTYQLCHTYVRCTRSVS- - - - - - - - I PAPAYYARLVAFRARYHLV
Hs. AGO2	I QGTSRPSHYHVLWDDNRFSSDELQI LTYQLCHTYVRCTRSVS- - - - - - - - I PAPAYYAHLVAFRARYHLV
Mm. AGO2	I QGTSRPSHYHVLWDDNRFSSDELQI LTYQLCHTYVRCTRSVS- - - - - - - - I PAPAYYAHLVAFRARYHLV
Dm. Ago1	I QGTSRPSHYHVLWDDNHFDSDLQCLTYQLCHTYVRCTRSVS- - - - - - - - I PAPAYYAHLVAFRARYHLV
Sm. Ago1	I QGTSRPSHYHVLWDDNRFSADDI QNLTYQLCHTYVRCTRSVS- - - - - - - I PAPAYYAHLVAFRARYHLV
Ce. ALG1	I QGTSRPSHYHVLWDDNNLTADELQQLTYQMCHTYVRCTRS- - - - - - - - I PAPAYYAHLVAFRARYHLV
Ce. ALG2	I QGTSRPSHYHVLWDDNDLTADELQQLTYQMCHTYVRCTRS- - - - - - - - I PAPAYYAHLVAFRARYHLV
At. AGO1	KI GTSRPAHYVLLDEI GFSPDDLQNLI HSLSYKLLNSI FNVSSLLCVFVLSVAPVRYAHLAAAQVAQFTK
At. AGO9	M GTTRPTHYHVLYDEI GFATDDLQELVHSLSHVYQRSTTAI S- - - - - - - VVAPVVAQMGTVWK
Sj. 1	I QGTSKPAHYHVLYDDSNWTSDALQMFTYYLCYAYMRCSRSVS- - - - - - - YPAPTYYSHLAAFRARDWLS
Sm. Ago2	I QGTSKPAHYHVLYDDSNWSSDALQMFTYHLCYTYMRCSRSVS- - - - - - - YPAPTYYSHLAAFRARDWLS
Sm. Ago4	I QGTSKPSHYHVLYDDSNFTSDSLQMFTYHLCYAYMRCSRSVS- - - - - - - YPAPVYYSHLAAFRARDWLS

Fig. 1 (continued).

2.2. Alignment and phylogenetic analyses

Multiple alignment of *SmDicer1* and *SmAgo1/2/3/4* were performed by ClustalX 2.0 and phylogenetic analyses were conducted in MEGA 4 [19]. Phylogenetic tree of these sequences were inferred using the Neighbor-Joining method [20]. The bootstrap consensus tree inferred from 1000 replicates was used to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [21]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset.

2.3. Parasites

S. mansoni LE strain was maintained by routine passage through *Biomphalaria glabrata* snails and BALB/c mice. The infected snails were induced to shed cercariae under light exposure for 2 h and the cercariae were recovered by sedimentation on ice. Adult worm parasites were obtained by liver perfusion of mice after 50 days of infection. Mechanically transformed schistosomula (MTS) were prepared as described by Harrop and Wilson [22]. Briefly, cercariae were recovered, and washed in RPMI 1640 medium (Invitrogen), before vortexing at maximum speed for 90 s and immediately cultivated during 3.5 h at 37 °C, 5% CO_2. Then the recovered schistosomula were washed with RPMI 1640 until no tails were detected. For subsequent incubations, the parasites were maintained in M169 medium supplemented with 10% FBS, penicillin and streptomycin (100 µg/mL) and 5% of Schneider's medium [23] at 37 °C on 5% of CO_2 during 8.5; 18.5; 24; 48 and 72 h.

2.4. RNA preparation and quantitative Real Time PCR

Total RNA was obtained using a combination of the Trizol reagent (GIBCO-BRL) and chloroform for extraction, and then purified on column using the "Purelink Micro-to-Midi Total RNA purification system" (Invitrogen). The preparation was treated with RNase-free DNase I in 3 different rounds by decreasing enzyme concentration (RQ1 DNase; Promega, Belo Horizonte, Brazil). RNA was quantified using a spectrophotometer and 5 µg reverse transcribed using an oligodT primer from the Thermoscript RT-PCR System (Invitrogen São Paulo, Brazil) as described by the manufacturer. The cDNAs encoding *SmAgo2/3/4* and *SmDicer1* were obtained by PCR amplification using the forward primer 5′-TCACGCACGGTAGATCAGG-3′ and reverse primer 5′-AGGTCCGCGTCAATTTGG-3′ for *SmAgo2/3/4* and the forward 5′-TCTTCCGTCCACCATTCG-3′ and reverse 5′-TGCCAACAACACAATTCC-3′ for *SmDicer1*. The efficiency of DNAse I treatment was evaluated by

PCR amplification of the cDNA reaction mix without the addition of the Thermoscript enzyme. All the primers were designed using the program GeneRunner®. All PCR products were sequenced in the ABI 3100 automated sequencer (Applied Biosystems) using the Dye terminator kit. Reverse-transcribed cDNA samples were used as templates for PCR amplification using SYBR Green Master Mix UDG-ROX® (Invitrogen) and 7500 Real Time PCR System (Applied Biosystems). Specific primers for *S. mansoni* α-tubulin were used as an endogenous control (GenBank access no. M80214) [24] (forward 5′-CGTATTCG-CAAGTTGGCTGACCA-3′, reverse 5′-CCATCGAAGCGCAGTGATGCA-3′). The efficiency for each pair of primers was evaluated according to the protocol developed by the Applied Biosystems application (cDNA dilutions were 1:10, 1:100 and 1:1000). For both investigated transcripts three biological replicates were performed and their gene expression normalized against the α-tubulin transcript according to the $2^{-\Delta\Delta Ct}$ method [25] using the Applied Biosystems 7500 software.

3. Results

3.1. Data mining of miRNA pathway related sequences in S. mansoni databases and analyses of their putative domains

The primary goal of our study was to use bioinformatic tools for an *in silico* reconstitution of the miRNA pathway in *S. mansoni*. Our analyses revealed that the members of this pathway are well-conserved at the amino acid level when compared to their orthologs found in diverse organisms such as *D. melanogaster*, *C. elegans*, *H. sapiens*, *Mus musculus* and *A. thaliana*. Due to the high representation of members of this pathway in *D. melanogaster* we decided to evaluate their degree of similarity with the related *S. mansoni* sequences (Table 1). When comparing between the *S. mansoni* predicted genes and their related ESTs from *S. japonicum* up to 70% sequence identity could be found.

Based on our database mining we suggest that *S. mansoni* contains four members of Argonaute proteins termed SmAgo1, SmAgo2, SmAgo3 and SmAgo4 containing 876, 955, 854 and 924 amino acid residues, respectively (Fig. 1a). Alignments between SmAgo2/3/4 proteins revealed 58% identity and 17% between SmAgo1/2/3/4. When comparing the degree of identity of SmAgo1/2/3/4 proteins with their orthologs in *D. melanogaster* we observed 68, 33, 29 and 33%, respectively (Table 1). Moreover, the alignment of AGO-like members revealed that most of their components, including SmAgo1/2/4 exhibited the conservation of key amino acid (D/D/H) residues that coordinate the Mg^{2+} ion at the PIWI domain (Fig. 1b).

We also identified three putative RNase III proteins, including SmDicer1, SmDrosha1 and SmDrosha2 containing 2174, 1531 and 1577 amino acid residues, respectively (Fig. 2a). Furthermore, we can clearly observe the four catalytic residues ED and DE of endoND 1/2 domains of SmDicer1 and SmDrosha1/2 (Fig. 2b).

3.2. Phylogenetic relationship among Argonautes and RNAse III family protein members

The phylogenetic tree created with the Neighbor-Joining method was used to separate the putative SmAgo1/2/3/4, SmDicer1 and
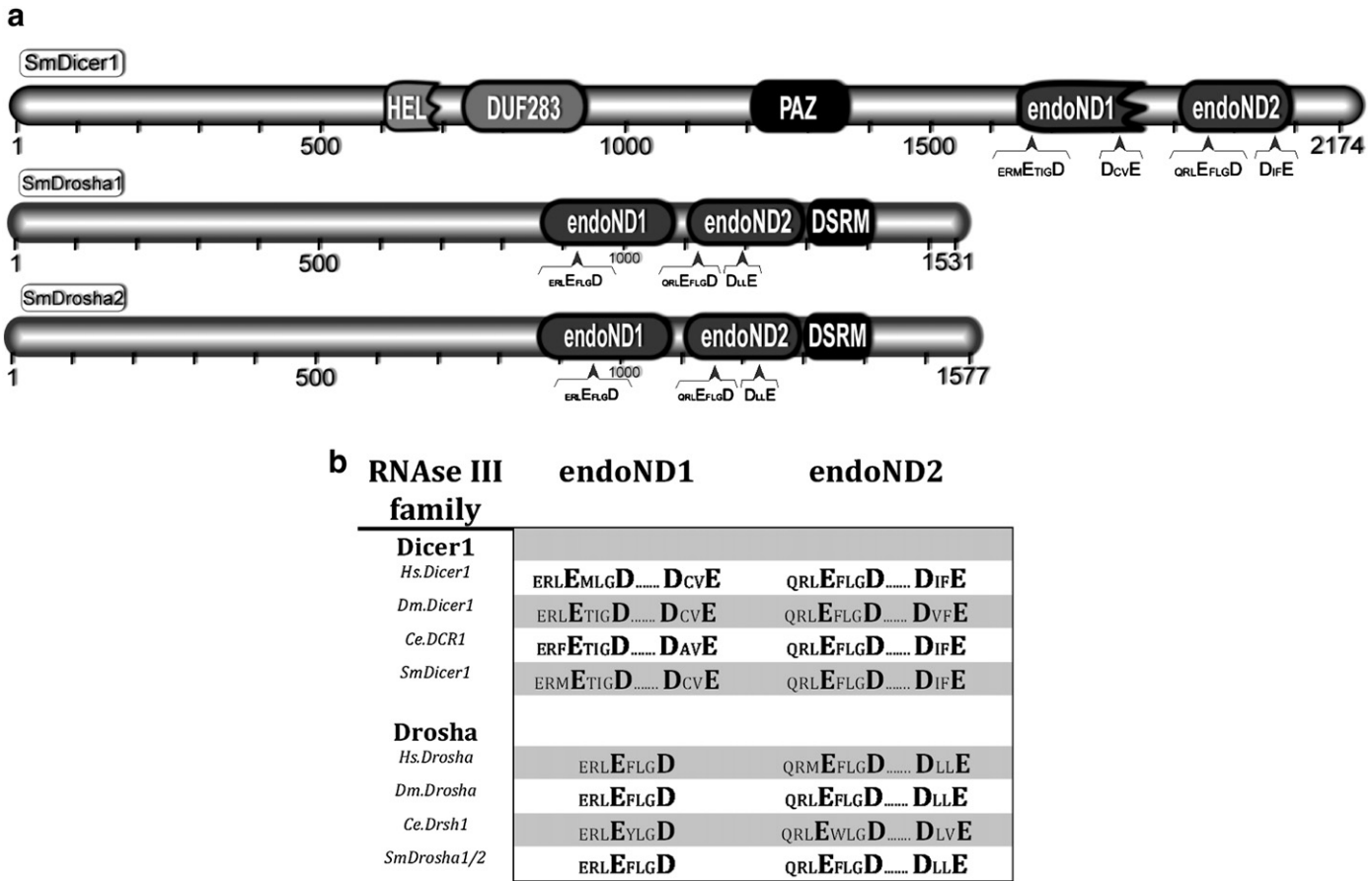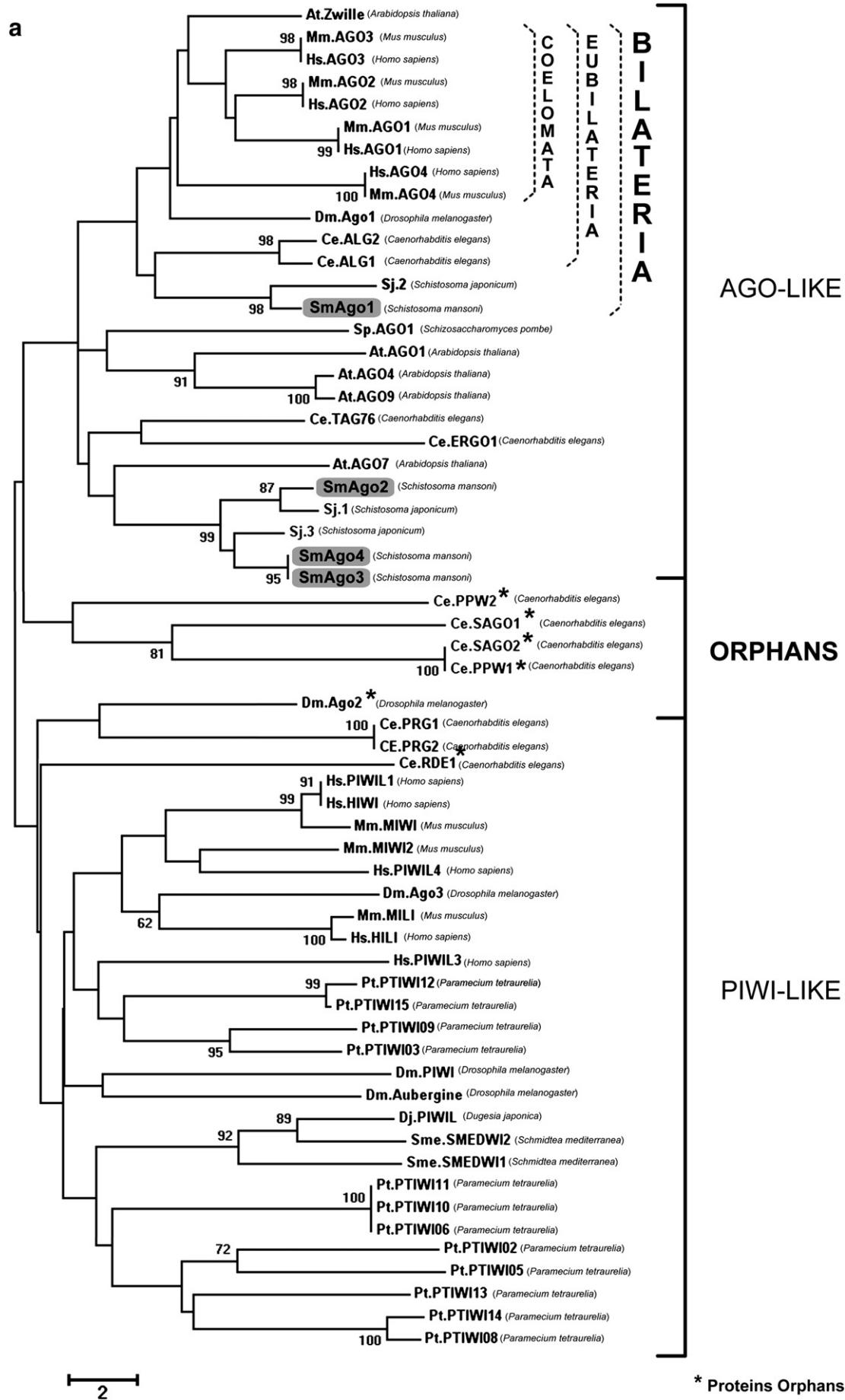

Fig. 2. The RNase III family in *S. mansoni* and their catalytic residues. (a) Representatives of RNase III-like proteins of *S. mansoni*: SmDicer1 and SmDrosha1/2. Domain boundaries are indicated with boxed ranges of amino acid sequence. The gray arrow represents the positions of signature motif in the endoND1 (PF00636) and endoND2 (PF00636). (b) Signature motif in the endoND and four catalytic residues of *H. sapiens*, *D. melanogaster*, *C. elegans*, and *S. mansoni*. Accession numbers: Hs.Dicer1 (NP_085124.2), Dm.Dicer1 (NP_524453.1), Ce. DCR1 (NP_498761.1), SmDicer1 (Smp_169750), Hs.Drosha (NP_037367.3), Dm.Drosha (NP_477436.1), Ce.Drsh1 (NP_492599.1), and SmDrosha1/2 (Smp_142510.1 and Smp_142510.2).

a

At.Zwille (*Arabidopsis thaliana*)
98 ⌐ Mm.AGO3 (*Mus musculus*)
└ Hs.AGO3 (*Homo sapiens*)
98 ⌐ Mm.AGO2 (*Mus musculus*)
└ Hs.AGO2 (*Homo sapiens*)
Mm.AGO1 (*Mus musculus*)
99 └ Hs.AGO1 (*Homo sapiens*)
Hs.AGO4 (*Homo sapiens*)
100 └ Mm.AGO4 (*Mus musculus*)
Dm.Ago1 (*Drosophila melanogaster*)
98 ⌐ Ce.ALG2 (*Caenorhabditis elegans*)
└ Ce.ALG1 (*Caenorhabditis elegans*)
Sj.2 (*Schistosoma japonicum*)
98 └ SmAgo1 (*Schistosoma mansoni*)
Sp.AGO1 (*Schizosaccharomyces pombe*)
At.AGO1 (*Arabidopsis thaliana*)
91 ⌐ At.AGO4 (*Arabidopsis thaliana*)
100 └ At.AGO9 (*Arabidopsis thaliana*)
Ce.TAG76 (*Caenorhabditis elegans*)
Ce.ERGO1 (*Caenorhabditis elegans*)
At.AGO7 (*Arabidopsis thaliana*)
87 ⌐ SmAgo2 (*Schistosoma mansoni*)
└ Sj.1 (*Schistosoma japonicum*)
Sj.3 (*Schistosoma japonicum*)
99 SmAgo4 (*Schistosoma mansoni*)
95 └ SmAgo3 (*Schistosoma mansoni*)

C O E L O M A T A / E U B I L A T E R I A / **B I L A T E R I A**

AGO-LIKE

Ce.PPW2 * (*Caenorhabditis elegans*)
Ce.SAGO1 * (*Caenorhabditis elegans*)
Ce.SAGO2 * (*Caenorhabditis elegans*)
81 100 └ Ce.PPW1 * (*Caenorhabditis elegans*)

ORPHANS

Dm.Ago2 * (*Drosophila melanogaster*)
100 ⌐ Ce.PRG1 (*Caenorhabditis elegans*)
└ CE.PRG2 (*Caenorhabditis elegans*)
Ce.RDE1 * (*Caenorhabditis elegans*)
91 ⌐ Hs.PIWIL1 (*Homo sapiens*)
99 └ Hs.HIWI (*Homo sapiens*)
Mm.MIWI (*Mus musculus*)
Mm.MIWI2 (*Mus musculus*)
Hs.PIWIL4 (*Homo sapiens*)
Dm.Ago3 (*Drosophila melanogaster*)
Mm.MILI (*Mus musculus*)
62 100 └ Hs.HILI (*Homo sapiens*)
Hs.PIWIL3 (*Homo sapiens*)
99 ⌐ Pt.PTIWI12 (*Paramecium tetraurelia*)
└ Pt.PTIWI15 (*Paramecium tetraurelia*)
Pt.PTIWI09 (*Paramecium tetraurelia*)
95 Pt.PTIWI03 (*Paramecium tetraurelia*)
Dm.PIWI (*Drosophila melanogaster*)
Dm.Aubergine (*Drosophila melanogaster*)
89 ⌐ Dj.PIWIL (*Dugesia japonica*)
92 └ Sme.SMEDWI2 (*Schmidtea mediterranea*)
Sme.SMEDWI1 (*Schmidtea mediterranea*)
Pt.PTIWI11 (*Paramecium tetraurelia*)
100 Pt.PTIWI10 (*Paramecium tetraurelia*)
Pt.PTIWI06 (*Paramecium tetraurelia*)
72 Pt.PTIWI02 (*Paramecium tetraurelia*)
Pt.PTIWI05 (*Paramecium tetraurelia*)
Pt.PTIWI13 (*Paramecium tetraurelia*)
Pt.PTIWI14 (*Paramecium tetraurelia*)
100 └ Pt.PTIWI08 (*Paramecium tetraurelia*)
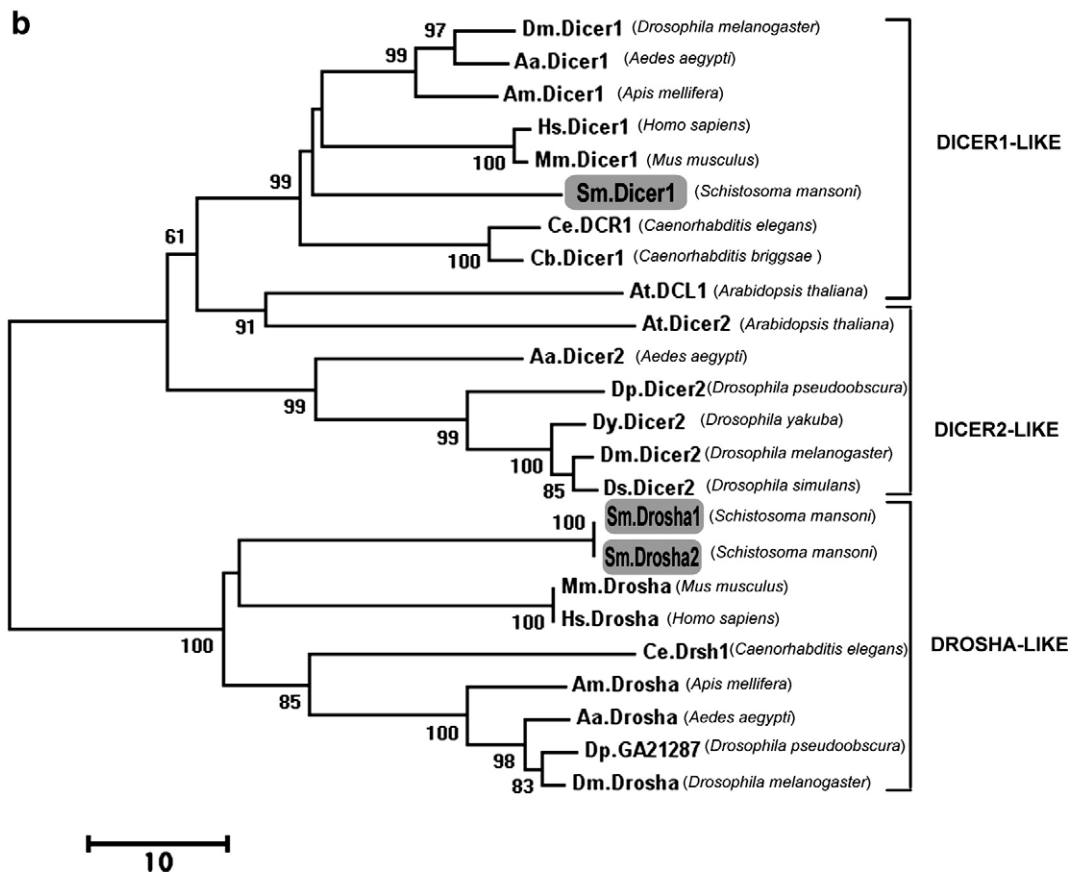
PIWI-LIKE

2

* Proteins Orphans

b

Fig. 3. Phylogenetic trees of Argonaute and RNAse III proteins. Multiple alignments were performed using ClustalX 2.0 and Mega 4.0 with bootstrap analysis. Bootstrap percentages are indicated at each branch. (a) Phylogenetic tree of Argonaute proteins from plants, animals (coelomata, bilateria and eubilateria) and fungi using the conserved domain PIWI. Accession numbers of sequences for Argonaute proteins: Dm.Ago1 (NP_725342), Dm.Ago2 (ABB54719.1), Dm.Ago3 (ABO26294), Dm.Aubergine (CAA64320), Dm.PIWI (AAD08705.1), Ce.ALG1 (NP_510322.2), Ce.ALG2 (NP_493837.1), Ce.RDE1 (NP_7411.1), Ce.ERGO1(NP_503362.2), Ce.SAGO2 (NP_871859.1), Ce.SAGO1 (NP_5040.1), Ce.PPW1 (NP_740835.1), Ce.PPW2 (NP_491535.1), Ce.PRG1(NP_492.1), At.AGO1(gbAAB91987.1), At.AGO4 (NP_565633.1), Ce.TAG76 (NP_499192.1), CE.PRG2(NP_500994.1), Sp.AGO1 (CAA19275.1), At.AGO7 (NP_177103.1), At.Zwille (CAA11429.1), At.AGO9 (CAD66636.1), Pt.PTIWI06 (CAI39071.1), Pt.PTIWI11 (CAI39069.1), Pt.PTIWI03 (CAI39076.1), Pt.PTIWI08 (CAI39073.1), Pt.PTIWI15 (CAI39065.1), Pt.PTIWI13 (CAI39067.1), Pt.PTIWI14 (CAI39066.1), Pt.PTIWI09 (CAI39072.1), Hs.HIWI (AAK92281.1), Pt.PTIWI02 (CAI44470.1), Pt.PTIWI12 (CAI39068.1), Pt.PTIWI05 (CAI44468.1), Hs.PIWIL1 (NP_004755.2), Hs.PIWIL4 (NP_689644.1), Hs.PIWIL3 (BAC81343.1), Hs.HILI (BAC81342.1), Hs.AGO1 (Q9UL18), Hs.AGO2 (Q9UKV8), Hs.AGO3 (Q9H9G7), Hs.AGO4 (Q9HCK5), Mm.AGO2 (Q8CJG0), Mm.AGO1 (Q8CJG1), Mm.AGO3 (Q8CJF9), Mm.AGO4 (Q8CJF8), Mm.MIWI2 (AAN75583.1), Mm.MIWI (EDL19532.1), Mm.MILI (AAK31965.1), Sme.SMEDWI1 (Q2Q5Y9), Sme.SMEDWI2 (Q2Q5Y8), Sj.3 (AAW25407.1), Sj.2 (AAX25645.2), Sj.1 (AAW26476.1), Dj.PIWIL (Q2PC95), and SmAgo3 (Smp_102690.2). (b) Phylogenetic tree of ribonuclease III proteins from animals and plants using the conserved domain endoND2. Accession numbers of sequences for RNAse III proteins: Dm.Dicer1 (NP_524453.1), Hs.Dicer1 (NP_085124.2), Mm.Dicer1 (NP_683750.2), Aa.Dicer1 (XP_001659747.1), At.DCL1 (NP_171612.1), Am.Dicer1 (XP_624510.2), Ce.DCR1 (NP_498761.1), Cb.Dicer1 (XP_001666666.1), Dm.Dicer2 (ABB54751.1), Ds.Dicer2 (ABB54760.1), Hs.Drosha (Q9NRR4), Dp.GA21287 (XP_001361471.1), Dy.Dicer2 (ABB54764.1), Aa.Drosha (XP_001653338.1), Dm.Drosha (NP_477436.1), Am.Drosha (XP_394444.2), Aa.Dicer2 (AAW48725.1), At.Dicer2 (NP_001078101.1), Dp.Dicer2 (XP_001360634.1), Mm.Drosha (XP_001473806.1), and Ce.Drsh1 (NP_492599.1).

SmDrosha1/2 proteins in their respective subclades. The phylogenetic relationships of amino acid sequences are shown in Fig. 3a/b. The sequences were constituted as an independent branch supported by high bootstrap values (1000 samples). It could be seen that SmAgo1/2/3/4 are orthologs of the AGO-like group and are related to *C. elegans* and *D. melanogaster* orthologs.

Phylogenetic analysis of the RNAse III family in *S. mansoni*, using the highly conserved endoND domain, revealed the clustering SmDicer1 and SmDrosha1/2 proteins into respective subclades: Dicer1-like and Drosha-like (Fig. 3b).

3.3. Analysis of the relative gene expression profile by quantitative Real Time PCR

We analyzed the expression of *SmDicer1* and *SmAgo2/3/4* transcripts by relative qRT-PCR in different developmental stages of *S. mansoni*: cercariae, MTS-3.5, MTS-8.5, MTS-18.5, MTS-S24, MTS-48, MTS-72, adult worms and eggs (Fig. 4a/b). Control reactions (where no reverse transcriptase was used) revealed no amplification. Data were normalized relative to an endogenous transcript (Sm α-tubulin) and were represented as the fold-change in expression relative to the levels found in 24 h schistosomula (MTS-24) stage. We observed that *SmDicer1* and *SmAgo2/3/4* transcripts were expressed in all investigated stages and their levels of expression differed significantly ($p < 0.001$) (Fig. 4a/b).

It is worth mentioning that the expression of *SmDicer1* increased significantly following the mechanical transformation from cercariae to schistosomula stage (Fig. 4a). For *SmAgo2/3/4* transcripts we found a significant decrease from cercariae to MTS-8.5 followed by an increase in MTS-18.5 peaking at MTS-48 stage and then declining in the MTS-72. High and comparable transcript levels for *SmAgo2/3/4* were found in the adult and cercariae stages. *SmDicer1* and *SmAgo2/3/4* transcripts reached their high expression levels in the egg stage.

4. Discussion

The miRNA pathway consists of a conserved core of proteins and enzymes found in human, *C. elegans*, *D. melanogaster* and *A. thaliana* and known to function in the recognition and processing of dsRNA. dsRNAs are involved in important mechanisms of gene expression and therefore regulate genes involved in the maintenance of the genome integrity [1]. The first evidence of the presence of constituents of the

miRNA pathway in *S. mansoni* came from analysis of its transcriptome using the ORESTES strategy [26]. One of the remarkable features of this pathway refers to the fact that miRNAs are small enough to move between cells and sufficiently long to ensure their specificity to the target mRNA. Although several groups have shown that the incorporation of exogenous RNA and its processing into RNA-antisense occur in various stages of *S. mansoni*, it is not yet known how the miRNAs contribute to the differential regulation of gene expression [4,27–30].

Through a combination of phylogenetic tree, similarity between domains and mining of the *S. mansoni* database we were able to show putative sequences with conserved domains of the miRNA pathway in this parasite. Our searches have identified thirteen putative sequences that showed significant homology with orthologs of *D. melanogaster* and *C. elegans*. In addition, there are a number of *S. japonicum* EST sequences displaying a high degree of similarity to components of the miRNA pathway, supporting the existence of this mechanism in *Schistosoma* spp. During this investigation we were able to verify some species-specific peculiarities, for example, *C. elegans* does not contain orthologs of Fmr1, Loquacious or Exportin-5. Considering the experimental data that emphasize the importance of the miRNA pathway in *C. elegans*, the absence of those proteins might be balanced by the existence of others engaged in the same function [31].

In order to understand how closely related the *S. mansoni* miRNA machinery is compared to orthologs found in model organisms, we selected two key components termed *SmAgos* and *SmDicer* for phylogenetic analyses. Using the PIWI domain in the Argonaute

Fig. 4. Developmental expression analysis of *S. mansoni* (a) *SmDicer1* and (b) *SmAgo2/3/4*. The mRNA expression levels were measured, based on three replicates, in the stages: cercariae, MTS-3.5; MTS-8.5; MTS-18.5; MTS-24; MTS-48, MTS-72 h, adult worms, and eggs using quantitative RT-PCR. Expression levels were calibrated according to the comparative $2^{-\Delta\Delta Ct}$ method, using the constitutively expressed Sm α-tubulin as an endogenous control and were normalized relative to the MTS-24 stage. In (a) a, b, c, d and e indicate statistically different expression levels for *SmDicer1*. In (b) a, b, c, d and e indicate statistically different expression levels for *SmAgo2/3/4* (one-way variance analysis followed by Tukey pairwise comparison $p < 0.001$).

proteins it could be seen that these proteins can be divided into two subclades: the AGO-like, based on *A. thaliana* Ago1 and the PIWI-like, based on *D. melanogaster* Piwi. The PIWI-like and AGO-like exhibit structural differences that may help explain their distinct biological activities. In this work, we have shown that four *S. mansoni* representatives of Argonautes are AGO-like proteins suggesting their role in the recognition of small RNAs, as a first step towards the silencing of its complementary transcript.

Different Ago proteins have been found in several organisms, ranging from a single member in *Schizosaccharomyces pombe*, to more than twenty in *C. elegans* [14,32]. Among the four *S. mansoni* Argonaute proteins, SmAgo3 and SmAgo4 are alternative spliced and the same mechanism for generating transcript diversity seems to occur for their orthologs DmAgo1 and CeAlg-2 in *Drosophila* and *C. elegans* (FlyBase — http://flybase.bio.indiana.edu/ and WormBase — http://www.wormbase.org/).

Alignment of members of the AGO-like revealed that all members of this family, including SmAgo1/2/4, exhibit conservation of the key metal coordinating residues in the PIWI domain known to be responsible for the Slicer activity of Ago proteins. Although the mechanism is far from clear, such activity is the one directly involved in the cleavage of the target mRNA [17,33]. The activity of SmAgos proteins in the regulation of gene expression in different stages of *S. mansoni* awaits to be demonstrated.

Blaszczyk et al. [43] provided evidence that RNase III family can be divided into four classes. In *D. melanogaster* the fourth class can be separated in 2 types of Dicer as exemplified by Dcr-1 and Dcr-2 [34]. Our phylogenetic analyses revealed that the RNAse III family representatives *SmDrosha1/2* and *SmDicer* could be grouped in their respective categories. The amino acid sequence of these three components showed the conserved endoND domain [35]. However, as shown for other organisms [36], these proteins might operate in different sub-cellular compartments in *S. mansoni*; SmDrosha1/2 in the nucleus and SmDicer1 in the cytoplasm.

A more detailed investigation of the transcriptome data revealed that ESTs coding for components of the miRNA pathway were sequenced from different stages of *S. mansoni* (http://verjo18.iq.usp.br/schisto/). Based on observations that the newly transformed schistosomula are characterized by a semi-quiescent state where no net protein synthesis occur [37,38] and the cell-cycle is under arrest [39], we hypothesize that the miRNA pathway may play a role in preventing protein translation, which could favor parasite remodeling at this stage. Blanton and Licate [18] anticipated that the very low protein synthesis at this stage is probably not due to the lack of mRNA, but to a post-transcriptional mechanism that blocks translation. In order to test this hypothesis, we cultivated schistosomula at various time points after transformation and analyzed by qRT-PCR two key transcripts of miRNA pathway, *SmAgo2/3/4* and *SmDicer1*.

Our results confirmed the expression of the *SmDicer1* gene in various life-cycle stages of *S. mansoni* as demonstrated by Krautz-Peterson and Skelly [40]. However, we are the first to investigate its expression in the newly transformed schistosomula. The significant increase in *SmDicer1* transcript occurring straight after transformation and its remarkable abundance in eggs suggest an important role in gene silencing at these stages. As a preliminary investigation we decided to perform a global analysis of the expression of *SmAgo2/3/4* transcripts in different stages of the *S. mansoni* life-cycle. This allowed us to infer in which stages the miRNA pathway is likely to contribute to gene silencing in the parasite. *SmAgo2/3/4* expression was similarly high in cercariae and adult worms and lower in the newly transformed schistosomula. In agreement, a recent proteomic investigation of the *S. japonicum* cercariae secretome revealed the presence of Argonaute-like proteins likely present in high abundance on the cercarial secretions [41]. It seems obvious that Argonaute proteins detected in the secretome were probably not derived from it but contributed as a cytosolic contaminant of the preparation. The high expression levels of *SmAgo2/3/4* and *SmDicer1*
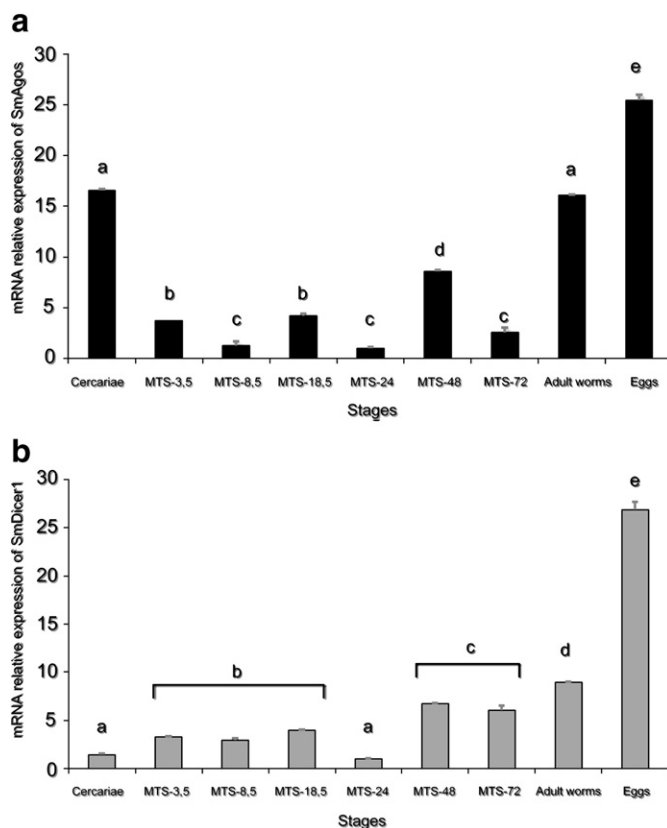
expression in eggs suggest a role in targeting maternal mRNAs to repress their translation and consequently accelerate their decay as reviewed by Schier [42]. Further experiments are ongoing to test this hypothesis.

Recently, various groups have used the RNAi technique to regulate gene expression in sporocysts, schistosomula, and adult worms [27–30] meaning that gene silencing mechanisms are naturally present on the parasite. Our experiments aimed to uncover a few aspects of the miRNA pathway in *S. mansoni*; however, several issues still need to be clarified. Of particular importance is to determine the pattern of expression of all components of this pathway, the stage-specific repertoire of miRNA genes and the elucidation of their target transcripts.

Acknowledgments

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