Genome Biology and Evolution Advance Access published July 6, 2009 doi:10.1093/gbe/evp017

Genome Biology and Evolution

SMBE

High throughput genome-wide survey of small RNAs from the parasitic protists *Giardia intestinalis and Trichomonas vaginalis*

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Abstract

RNA interference (RNAi) is a set of mechanisms which regulate gene expression in eukaryotes. Key elements of RNAi are small sense and antisense RNAs from 19 to 26 nucleotides generated from double-stranded RNAs. miRNAs are a major type of RNAi-associated small RNAs and are found in most eukaryotes studied to date. To investigate whether small RNAs associated with RNAi appear to be present in all eukaryotic lineages, and therefore present in the ancestral eukaryote, we studied two deep-branching protozoan parasites, *Giardia intestinalis* and *Trichomonas vaginalis*. Little is known about endogenous small RNAs involved in RNAi of these organisms. Using Illumina Solexa sequencing and genome-wide analysis of small RNAs from these distantly related deep-branching eukaryotes, we identified 10 strong miRNA candidates from *Giardia* and 11 from *Trichomonas*. We also found evidence of *Giardia* siRNAs potentially involved in the expression of variant-specific-surface proteins. In addition, 8 new snoRNAs from *Trichomonas* are identified. Our results indicate that miRNAs are likely to be general in ancestral eukaryotes, and therefore are likely to be a universal feature of eukaryotes.

Key words: ancestral eukaryotes, miRNA, protists, RNA evolution

Introduction

Since its discovery in 1998 (Fire, et al. 1998), RNA interference (RNAi) has been found in animals (Collins and Cheng 2006), plants (Gazzani, et al. 2004) and some protists (Ullu, et al. 2004). It is implicated in a wide range of gene silencing mechanisms including downregulating mRNA levels (Sen and Roy 2007), heterochromatin assembly and maintenance (Grewal and Elgin 2007), DNA elimination (Collins and Cheng 2006), promoter silencing, developmental control (Morris, et al. 2004), up-regulation of transcription during the cell cycle (Vasudevan, et al. 2007), and transposon silencing (Hartig, et al. 2007). Key elements that guide all the above processes are small RNAs with size ranges of 19 to 26 nucleotides (nt).

Four major types of small RNAs associated with RNAi have been extensively studied: short interfering RNAs (siRNAs), repeat-associated short interfering RNAs (rasiRNA), microRNAs (miRNAs) (Meister and Tuschl 2004), and piwi RNAs (piRNAs) (Lau, et al. 2006). These RNAs are processed from complementary or near-complementary double-stranded RNAs (dsRNAs) precursors into 21 to 26 nt RNAs by Dicer or Drosha RNase III family endonucleases in the cytoplasm (Bernstein, et al. 2001, Lee, et al. 2003). Dicer homologues have been found in most eukaryotes including deep-branching unicellular parasites such as *Giardia intestinalis* and *Trichomonas vaginalis* (Finn, et al. 2006) (hereafter referred to as *Giardia* and *Trichomonas* repectively). After cleavage by Dicer the short dsRNAs are then incorporated into ribonucleoprotein particles which assemble the RNA-induced silencing complex (RISC) (Hammond, et al. 2001). The assembly of RISC also requires energy-driven unwinding of the siRNA or miRNA duplexes and conformational changes of pre-assembled RNPs. The single-stranded siRNA or miRNA guides the RISC complex to the target mRNA, and is strongly bound to the Argonaute (Ago) protein which then cleaves the target mRNA (Song, et al. 2004). In some organisms such as *Neurospora crassa* (Forrest, et al. 2004), *C. elegans* (Smardon, et al. 2000), *S. pombe (Martienssen, et al. 2005)* and plants (Gazzani, et al. 2004), an RNA-dependent-RNA-polymerase (RdRp) is also essential for dsRNA-triggered gene silencing. The RdRp is likely to use the siRNA as primers and convert the target RNAs into dsRNAs and a second wave of gene silencing is initiated.

Several protozoan parasites have been studied in searching for evidence of RNAi, including *Trypanosoma* (Ullu, et al. 2004), *Plasmodium* (Malhotra, et al. 2002) and *Giardia* (Ullu, et al. 2005, Macrae, et al. 2006, Prucca, et al. 2008, Saraiya and Wang 2008). The presence of RNAi has been suggested in the deep-branching eukaryote *Giardia* (Macrae, et al. 2006, Prucca, et al. 2008, Saraiya and Wang 2008). Detailed biochemical and structural studies have been carried out for the *Giardia* Dicer protein homologue, showing that recombinant *Giardia* Dicer could cleave dsRNAs into 25 to 26 nt short fragments *in vitro* (Macrae, et al. 2006, MacRae, et al. 2007). The *Giardia* genome contains protein homologues of Argonaute and RdRp (Morrison, et al. 2007). Recent studies also showed that Dicer, Argonaute and RdRp are all available for RNAi regulation of *Giardia* variant-specific surface protein (VSP) expression (Prucca, et al. 2008), as well as a miRNA derived from a snoRNA (Saraiya and Wang 2008). Results from these studies also support the idea that RNAi mechanism is likely to have occurred in the last common ancestor of eukaryotes (Collins and Penny 2009).

Giardia and *Trichomonas* are both single cellular anaerobic eukaryotes belonging to the group of Excavates (Keeling, et al. 2005). They both have gone through reductive evolution which resulted in either mitosomes in *Giardia* (Tovar, et al. 2003) or hydrogenosomes in *Trichomonas* (Dyall, et al. 2004). Mitosomes and hydrogenosomes appear to be two reduced forms of mitochondria (Embley, et al. 2003, Mentel and Martin 2008). Despite the similarly reduced cellular components, *Giardia* and *Trichomonas* are separated by long evolutionary distance within Excavates (Hampl, et al. 2009), making them comparable yet distant models for our study. Previous studies on *Giardia* ncRNAs (Collins, et al. 2004, Chen, et al. 2007, Chen, et al. 2008) showed that sequences of ncRNAs from deep-branching eukaryotes can be highly divergent from other well studied eukaryotes. Therefore using traditional methods it is hard to identify functional ncRNAs.

In this study we used high throughput Solexa-sequencing technology (Illumina) to search for previously unidentified small RNAs (including miRNAs and siRNAs) from two protozoan parasites *Giardia* and *Trichomonas*. Despite extensive biochemical studies on the RNAi mechanism of *Giardia*, little is known about the endogenous small RNA (20 to 60nt) population in either *Giardia* or *Trichomonas*. Previous studies on ncRNAs from these two organisms showed presence of eukaryotic specific RNAs such as snoRNAs (Yang, et al. 2005, Chen, et al. 2007), spliceosomal snRNAs (Chen, et al. 2008, Simoes-Barbosa, et al. 2008) and RNase P (Marquez, et al. 2005). A number of antisense RNAs were also found in *Giardia* (Ullu, et al. 2005). Therefore the presence of other basic small RNAs such miRNAs and siRNAs is expected. In addition, there have been many previously uncharacterised non-coding RNAs identified in *Giardia* (Chen, et al. 2007), indicating the likely presence of new classes of ncRNAs in deep-branching eukaryotes. Large scale RNA analysis has not previously been done for *Trichomonas*, another member of the Metamonada subclade of Excavates (Hampl, et al. 2009). Comparing the ncRNA contents of *Trichomonas* with those of *Giardia* could lead to a better understanding of the evolution of RNA processing in eukaryotes. Using Illumina Solexa sequencing on small RNAs from *Giardia* and *Trichomonas*, we identified 10 strong miRNA candidates from *Giardia* and 11 from *Trichomonas*, as well as a number of putative miRNA candidates from both organisms. We also found evidence supporting the presence of siRNA in *Giardia.* In addition, 8 new snoRNAs from *Trichomonas* are identified. Our results strongly support RNAi-related small RNAs as a general feature of eukaryotes.

Results

High throughput sequencing of *Giardia* and *Trichomonas* small RNAs

Cultured *Giardia intestinalis* and *Trichomonas vaginalis* were each harvested for total RNA extraction at exponential growth phase, and small RNAs (10 to 200 nt) were purified by size fractionation. cDNA was synthesized following Illumina's small RNA preparation protocol and were then sequenced using an Illumina Genome Analyzer (aka. Solexa sequencing). The initial output of 36 nt sequences was filtered for adaptor sequences, and the resulting output contained 2,761,362 sequences for *Giardia* and 2,789,242 sequences for *Trichomonas*. All sequences from the Solexa sequencing were uploaded onto a MySQL database (v. 5.0.45) and also visualised with GBrowse (v.1.69).

To evaluate the RNA coverage of the sequencing, the filtered sequences were mapped (see Experimental Procedures) to previously known ncRNAs from each organism. Allowing the maximum of 2 nt mismatches, 30 tRNAs (Morrison, et al. 2007), 3 rRNAs (Morrison, et al. 2007), 51 snoRNAs (Yang, et al. 2005, Chen, et al. 2007), RNase P (Marquez, et al. 2005), 4 spliceosomal snRNAs (Collins, et al. 2004, Chen, et al. 2008) and 21 previously found but uncharacterised non-coding RNAs (Chen, et al. 2007) were recovered in *Giardia*. In *Trichomonas* 165 tRNAs (Aurrecoechea, et al. 2008), 3 rRNAs (Aurrecoechea, et al. 2008), 5 spliceosomal snRNAs (Simoes-Barbosa, et al. 2008), RNase P and MRP (Piccinelli, et al. 2005), and 8 new snoRNAs were recovered. In total, 188,425 unique sequences from *Giardia* and 648,707 unique sequences from *Trichomonas* mapped to ncRNAs indicated above or to transcripts of

protein-coding genes. The remainder correspond to unknown transcripts. The coverage of various RNA species in both organisms is shown in Figure-1.

Identification of miRNA candidates

In order to effectively represent Solexa output, all filtered sequences which did not map to any known transcripts were assembled into contigs using the *de novo* short sequence assembler Velvet (Zerbino and Birney 2008). The resulting contigs were checked by Blasting them against the respective genomes. Two strategies were used for further analysis.

Stragety-1: Identifying new miRNAs by sequence similarity

The first way to identify miRNA candidates was based on sequence similarity where we compared Solexa output sequences with known mature miRNAs. Initially, sequences of all published miRNAs with annotation were downloaded from the miRBase (Release 12.0 http://microrna.sanger.ac.uk/), and Blasted (Altschul, et al. 1990) against *de novo* assembled Solexa sequences. From the Blast-checked Solexa *de novo* contigs, candidates of miRNAs were identified. Six Solexa *de novo* contigs from *Giardia* contained sequences with a high degree of similarity to previously known miRNAs. The candidate sequences and alignments with known miRNAs are shown in Figure-2A. Although the miRNA candidates align well with the known miRNAs, the corresponding pre-mRNA sequences do not seem to share distinct sequence similarities. These candidate sequences all exist as single copy in the genome. A structural study of dsRNA cleavage by *Giardia* Dicer has previously shown that *Giardia* Dicer protein tends to cleave dsRNAs into small RNAs of 25 or 26 nt (MacRae, et al. 2007). This is consistent with a recent study on wild-type *Giardia* RNA (Saraiya and Wang 2008). Therefore, based on alignments, putative length, and sequence similarities among candidates, we can predict mature miRNA candidates of *Giardia*, and we found Solexa *de novo* contigs that fit our prediction.

Two of the *Giardia* miRNA candidates Gim5 and Gim6 are located on the antisense strands of annotated genes: GL50803_11290 Kinase (CMGC CDK), and GL50803_11912 hypothetical protein, respectively. The other *Giardia* miRNA candidates have antisense matches to predicted ORFs that do not contain annotated genes. Potential miRNA targets were also searched in the 3′-UTR regions of annotated genes. The UTR regions of *Giardia* are typically short with < 20 nt at 5′ end and < 50 nt at 3′ end (Elmendorf, et al. 2001). Therefore sequences 50 nt 3′ to all annotated genes were extracted to represent all possible 3′-UTRs. Blast results showed partial complementary matching of all 6 *Giardia* miRNA candidates to 3′-UTRs, and at least two of them (Gim1 and Gim5) showed extensive matches. Examples of potential *Giardia* miRNA-target binding are shown in Figure-3A, together with the Solexa contigs where the examples of miRNAs were found.

To date, there have not been any studies on RNAi in *Trichomonas*. Seven *Trichomonas de novo* contigs from our Solexa sequences had a high degree of sequence similarity with mature miRNAs known from other species. The candidate sequences and alignments with known miRNAs are shown in Figure-2B. The studied 3′-UTRs in *Trichomonas* are also relatively short (Davis-Hayman, et al. 2000, Leon-Sicairos Cdel, et al. 2003, Leon-Sicairos, et al. 2004). Consequently 50nt 3′ to all annotated genes were extracted to represent possible 3′-UTRs in *Trichomonas* in this study. Four out of seven miRNA candidates of *Trichomonas* (Tvm2, Tvm3, Tvm6

and Tvm7) showed extensive matches to 3′-UTRs, therefore, they were predicted to potentially target these UTR sequences. In addition, 4 *Trichomonas* candidates (Tvm2, Tvm3, Tvm4 and Tvm7) also have full-length antisense matches to annotated genes. Examples of *Trichomonas* potential miRNA-target binding are shown in Figure-3B, as well as the *de novo* contigs where the examples of miRNAs were found.

As a negative control, two randomised databases were generated with the size equivalent to the *de novo* assembled Solexa contigs of *Giardia* or *Trichomonas*, using a Markov chain (Lowe and Eddy 1999) based on di-nucleotide frequencies. Known miRNA sequences were Blasted against the two databases. Results showed an average of 11.3 nt match to the *Giardia* database and 11.4 nt for the *Trichomonas* database, indicating the homology search results presented in Figure-2 are significantly positive.

Stragety-2: Searching for new miRNAs by definition

Another way to identify additional candidates was through extracting putative miRNA-containing genomic regions. Previous studies have shown that the sequences of non-coding RNAs in *Giardia* are highly diverged from other eukaryotic model organisms (Chen, et al. 2007, Chen, et al. 2008), but at least some non-coding RNAs (e.g. spliceosomal snRNAs and RNase P) in *Trichomonas* have a high degree of sequence similarity to non-coding RNAs from other organisms (Simoes-Barbosa, et al. 2008). Therefore we expect that the majority of miRNAs in *Giardia* and at least some miRNAs in *Trichomonas* do not share sequence similarity with currently published miRNAs, because of the large evolutionary distance (Keeling, et al. 2005). In order to look for other possible miRNA candidates, additional analyses based on structural and sequence criteria were carried out as the second strategy to isolate genomic regions possibly containing miRNA precursors. These regions would then be confirmed by Solexa short-reads coverage.

Both genomes were obtained from EuPathDB (http://eupathdb.org/eupathdb/). However, due to its large size (~180Mb) the *Trichomonas* genome was first masked to exclude protein-coding and repeat regions. In general, most miRNA precursors adopt a conserved single hairpin structure, which is recognized by Dicer and Dicer-like proteins in the cytoplasm (Lee, et al. 2002, Bartel 2004). A number of computational tools have been developed and used to conduct genome-wide miRNA predictions (Lim, et al. 2003, Doran and Strauss 2007, Huang, et al. 2007). Here we used the algorithm srnaloop (Grad, et al. 2003) to look for hairpins with a length of less than 95 bases. Resulting hairpins were then filtered as described in the Experimental Procedures. To determine the threshold of miRNA target prediction based on complementary binding, we used a simulated control database with the size of *Giardia* genome. The control test showed that the average length of a random complementary binding is about 11 bp. Therefore to effectively avoid false positives, only hairpins with extensive complementary bindings (over 15 nt) to 3′-UTRs were considered as strong candidates. To justify the results of this approach, all the candidates were also run through the existing miRNA-target prediction software miRanda (Enright, et al. 2003). To include a negative control, we used a shuffled UTR search with miRanda. The output of miRanda contains numerous false positive predictions with average total scores of 107 for *Giardia* and 126 for *Trichomonas*. All the positive hits from miRanda are justified with infinite z-scores. The strong candidates from our own approach are also found by miRanda, with the *Giardia*

candidates' scores above 140 and *Trichomonas* candidates' scores above 160, indicating positive results. Information for all miRNA candidates found is listed in Table-1. Examples of predicted precursor structures and miRNA targets binding from additional computational analysis are shown in Figure-4A (*Giardia*) and Figure-4B (*Trichomonas*).

Most of the miRNA candidates we identified in *Giardia* and *Trichomonas* have predicted targets within the 3′-UTR regions of annotated genes and some have potential antisense targets to mRNAs. The majority of *Trichomonas* miRNA candidates have many identical or nearly identical copies scattered in different genomic contigs. It is possible that some *Trichomonas* ncRNAs are highly duplicated in the same way as observed for *Trichomonas* tRNAs, but the possibility of pseudogenes cannot be excluded, because the genome is currently very fragmented (with 17,290 scaffolds and 38,210 repeated genes (Aurrecoechea, et al. 2008), and many genes have not yet been characterised. Hence we cannot determine at this stage how many copies of each ncRNA could be present.

It is necessary to mention that although we present only a small number of sequences as miRNA candidates from *Giardia* and *Trichomonas*, a larger number of other Solexa *de novo* contigs also have potential to be miRNA candidates (data not shown). These sequences are not presented here as candidates, either due to shorter complementary binding (between 9 to 12 bp) to predicted targets, or due to the atypical folding of predicted precursors (e.g. low folding energy for many candidates in *Trichomonas*). It is possible that novel miRNAs exist in the two parasites, and further work is needed to characterise these transcripts.

Candidates of siRNAs in *Giardia*

In our previous study (Chen, et al. 2007) we characterised from *Giardia* an unusual long tandem repeated RNA named Girep-1. Continuing studies have revealed four other similar RNAs (7 to 10 copies in tandem), named Girep-2 to 5. The expression of RNAs from Girep-1 to 5 on both sense and antisense strands were confirmed (Figure-5A). All the Girep RNAs are non-protein-coding, with an exception of the antisense strand of Girep-1 being a hypothetical mRNA transcript (GL50803_227577). The Gireps are all direct repeats located at different positions in the genome. Multiple alignments of all Gireps revealed considerable homology among the five sets of sequences, and also the presence of shared motifs. The shared motifs and tandem-repeated pattern suggests that these RNAs belong to one group. All five Gireps show a high degree of sequence similarity with a number of variant-specific-surface protein (VSP) genes. The patterns of sequence match are variable, but all involve the repeating units of Gireps being partially aligned to repeating units of VSP genes (Figure-5B).

VSP gene expression is crucial for the surface antigenic variation of *Giardia* trophozoites (Nash, et al. 1988). The sequences and structures of VSP proteins are highly similar, however in a single trophozoite only one VSP is expressed at a time out of a total of 150 to 200 genes (Nash, et al. 2001). Both RNAi (Ullu, et al. 2004) and epigenetic mechanisms (Kulakova, et al. 2006) have been suggested for the regulation of VSP gene expression. A recent study has identified a snoRNA-derived miRNA that has the potential to regulate VSP expression (Saraiya and Wang 2008), and another study has demonstrated that Dicer, Argonaut and RdRp could be involved

in RNAi regulation of VSP expression (Prucca, et al. 2008). Based on previous studies on *Giardia* RNAi (Ullu, et al. 2005, Macrae, et al. 2006, MacRae, et al. 2007, Prucca, et al. 2008, Saraiya and Wang 2008), it is likely that small RNA regulation is involved in VSP expression. However the overall mechanism is still uncertain. Thus our findings of potential siRNAs show that the Girep family of RNAs have strong potential to be involved in regulation of VSP expression.

Analysis of our Solexa sequencing results reveals unequal frequencies of matching reads on the sense and antisense strands of Gireps, as shown in Table-2. (i.e. the numbers of Solexa short-reads matching to the plus and minus strands of Gireps are uneven.) In addition, BLAST showed that all the sense and antisense transcripts of Gireps have sequence matches to *Giardia* mRNAs including many VSP genes. In total there are 18 mRNAs that have regions with high degree of sequence similarity to Gireps. Each Girep sequence is similar to more than one VSP gene (Table-S1 of Supplementary Data). Searching the *Giardia* genome revealed additional non-repeated sequences that are similar to the Gireps. Also comparing Gireps with the latest *Giardia* EST database (Morrison, et al. 2007) has revealed many matches. This observation suggests that a large portion of the total VSP genes are covered by expressed homologous non-coding sequences. A recent study on *Giardia* RNAi showed that similar mRNAs of different VSPs could be cleaved by Dicer to produce short RNAs (Prucca, et al. 2008). This leads to the possibility that Girep RNAs can act as matching RNAs to bind VSPs and result in the production of siRNAs, which in turn silence other homologous VSPs. It has been previously shown that both sense and antisense siRNAs can down-regulate gene expression in other organisms (Schwarz, et al. 2003, Lin, et al. 2005, Clark, et al. 2008). Therefore the sense and antisense transcripts of Gireps are likely to function in a similar way.

Solexa-sequencing enables detection of transcription of both sense and antisense strands. Mapping Solexa output sequences to VSP genes revealed bi-directional transcription, consistent with results from a previous study (Prucca, et al. 2008). However the numbers of hits for each strand are highly unequal (Table-2). While 1,633 Solexa sequences map to the plus strands of 147 VSPs, only 133 sequences map to the minus strands of 69 VSPs. Among all the VSPs, 44 have Solexa hits on both strands. This phenomenon is consistent with results from other species (e.g. human) that both strands can be transcribed (Werner, et al. 2009). With both strands indicating expression in our results, we cannot determine if sense, antisense, or regulation from both strands is involved in *Giardia* Girep-VSP regulation. We do however suggest that such bi-directional expression may be more common among eukaryotes than originally thought.

New non-coding RNAs identified from *Trichomonas*

We identified new non-coding RNAs from *Trichomonas*, including 8 C/D box snoRNAs, and we also confirmed the expression of *Trichomonas* MRP which has only been predicted computationally (Piccinelli, et al. 2005). In this study C/D box snoRNAs were initially identified by snoscan-0.9b (Schattner, et al. 2005) with rRNAs used as potential targets. Solexa sequencing confirmed expression of these snoRNAs. Analysis of these *Trichomonas* C/D box snoRNAs indicated that the identified C/D box snoRNAs can adopt either of the two common structures shown in Figure-6 depending on the length of the RNA. Longer snoRNAs appear to have a D′-box and the antisense recognition regions to rRNAs are more likely to locate towards

the 3′ ends of snoRNAs, whereas shorter snoRNAs tend to have the antisense recognition regions towards the 5′ ends of snoRNAs.

The conserved structures of *Trichomonas* C/D box snoRNAs are relatively reduced compared to model eukaryotes by lacking C′ boxes and terminal stems. This is similar to snoRNAs previously identified in *Giardi*a (Yang, et al. 2005, Chen, et al. 2007), but the functional sequence motif C box is slightly longer and more conserved than in *Giardia*. Together with the fact that non-coding RNAs in *Trichomonas*, such as snRNAs (Simoes-Barbosa, et al. 2008), are more similar to those of higher eukaryotes, it is possible that the general RNA-processing in *Trichomonas* represents an evolutionarily less reduced state than *Giardia*.

Discussion

Evolution of RNAi inferred from studies of parasitic protists

The evolutionary relationships of the deepest lineages among eukaryotes is yet uncertain (Keeling, et al. 2005, Hampl, et al. 2009). Our strategy has been to look for common features in all deep eukaryotic lineages in order to infer the features of the last common ancestor of all living eukaryotes, thereafter Ancestral eukaryote (Collins and Penny 2005). Results from various studies (Malhotra, et al. 2002, Ullu, et al. 2004, Ullu, et al. 2005, Macrae, et al. 2006, MacRae, et al. 2007, Prucca, et al. 2008, Saraiya and Wang 2008) have led to the idea that the RNAi mechanism is likely to have occurred in the ancestral eukaryote (Collins and Penny 2009). *Giardia* and *Trichomonas* have both gone through reductive evolution (Tovar, et al. 2003, Dyall, et al. 2004), yet they are distantly related, making them comparable models for inferring properties of ancestral eukaryote.

Recent studies have begun to explore the mechanism of *Giardia* RNAi. However despite the extensive biochemical studies on the protein components (Macrae, et al. 2006, MacRae, et al. 2007, Prucca, et al. 2008), little is known about the endogenous RNAs that may be involved in RNAi and other types of small-RNA regulated gene expression. To date only one report has characterised a single miRNA in *Giardia* (Saraiya and Wang 2008). Our approach has revealed the existence of many miRNA candidates in both *Giardia* and *Trichomonas*, indicating that, like other well-studied model eukaryotes, excavates also possess small RNAs functioning in RNAi and other regulatory pathways. Therefore despite some differences among individuals, it appears that all major lineages of eukaryotes share common general features of RNAi.

There are some differences between *Giardia* and *Trichomonas* miRNAs. *Giardia* miRNA candidates generally have less extensive base-pairing to 3'-UTR targets than *Trichomonas*. However shorter complementary binding can result in effective RNAi (Lin, et al. 2005), and therefore it is possible that *Giardia* miRNAs do not require full complementarity to their targets, and a single miRNA may target a number of different UTR regions. Compared to *Giardia, Trichomonas* miRNA candidates appear more typical in their target recognition. A previous study on *Trichomonas* spliceosomal snRNAs (Simoes-Barbosa, et al. 2008) revealed high degree of similarity to human snRNAs. This is consistent with our observation that

Trichomonas miRNAs found in this study have a more typical eukaryotic target-recognition feature.

Both *Giardia* and *Trichomonas* appear to have reduced protein components in their RNAi pathways. The *Giardia* Dicer protein lacks the dsRNA-binding domain and DEAD-box helicase domain (compared to the human Dicer), but still can fully function to cleave synthetic dsRNAs *in vitro* and *in vivo* (Macrae, et al. 2006, MacRae, et al. 2007, Prucca, et al. 2008). The Argonaute protein of *Giardia* has also been shown to be functional despite lacking a PAZ domain (Prucca, et al. 2008). Biochemical studies have yet to be done on *Trichomonas* RNAi proteins. The predicted Dicer protein homologue in *Trichomonas* lacks a PAZ domain compared to *Giardia*, but it has a typical Argonaute protein homologue. From the presence of protein homologues and miRNA candidates, we can suggest that *Trichomonas* is likely to have a typical RNAi pathway.

In addition to miRNA candidates, it is highly likely that the Girep RNAs identified from *Giardia* can act as sense or antisense matching RNAs to VSP mRNAs and produce siRNAs in a pathway involving Dicer. Based on the evidence of VSP gene regulation by homologous VSP mRNAs in a recent study (Prucca, et al. 2008), Girep RNAs may well be a class of *Giardia* endogenous RNAs that participate in VSP gene regulation. However, this suggestion requires further experimental verification. Together with evidence from another study that snoRNA-derived miRNAs may also function in VSP gene regulation (Saraiya and Wang 2008); we may conclude at this stage that VSP genes may be regulated by a combination of small RNAs derived from difference sources.

In this study we used high throughput sequencing technology (Solexa sequencing, Illumina) to look for previously unidentified small RNAs from the genomes of two distantly related Excavate parasites *Giardia* and *Trichomonas*. Comparing the identified small RNA contents in the two parasites has led to a better understanding of the evolution of RNA processing throughout the Excavates group, and in relation with other eukaryotic lineages. As well as confirming previously identified RNAs in both organisms, including many lowly expressed transcripts, and in addition to the new ncRNAs reported here, the remaining Solexa output sequences are yet to be characterised. Current work is underway to analyse Solexa sequences for additional characteristics based on genome location (e. g. 5′-UTRs etc.), sequence and structural similarities. It is certain that next-generation RNA sequencing to a high coverage can uncover novel ncRNAs that cannot be characterised with traditional methods, thus providing valuable information on the genome-wide picture of RNA-processing.

Materials and Methods

Total RNA preparation and sequencing

Giardia intestinalis (WB strain) trophozoites were collected from TY1-S-33 growth media at a concentration of 1.4×10^7 cells/ml by centrifugation (10 min, 2,500 rpm, 4ºC). Total RNA was prepared using Trizol (Invitrogen) according to the protocol provided by the manufacturer. The pure RNA was resuspended in distilled water.

Trichomonas vaginalis was grown in Trichomonas broth (Fort Richard) at 37°C for 3 to 4 days, and harvested by centrifugation (10,000 rpm, 15 min at room temperature). Growth media was removed and cells were resuspended in equal volumes of 2×LETS buffer (200 mM LiCl, 20 mM EDTA, 20 mM Tris pH 7.8, and 2% SDS). An equal volume of phenol:chloroform (5:1, pH 5) was added to the suspension, and the mixture was vortexed for 10 sec. Phases were separated by centrifugation at 14,000rpm for 5min at room temperature, and the upper phase was further extracted twice with phenol:chloroform, then once with chloroform. Finally total RNA was precipitated by adding LiCl to a final concentration of 0.2 M and 3 volumes of 100% EtOH, then incubated at -80°C for 1 hr.

For Solexa sequencing, 10 µg of total RNAs were separated on a 15% denaturing acrylamide 8 M urea gel and RNAs ranging from 10 to 200 nt were cut out from the gel and prepared according to Illumina's small RNA preparation protocol. 8 and 12 pmol (in each lane) of *Giardia* and *Trichomonas* cDNA were used for sequencing on an Illumina Genome Analyzer for 35 cycles.

Analysis of Solexa short-read sequences

Initial Solexa data required computational filtering and trimming to remove an expected portion of adaptor sequences (due to the short length of some RNAs). These filtered sequences were mapped to previously identified and computationally predicted non-coding RNAs from *Giardia* and *Trichomonas* using seqmap-1.0.8 (source code from: http://biogibbs.stanford.edu/~jiangh/SeqMap/). Sequence assembly was performed using Velvet version 0.7.20 (source code from: http://www.ebi.ac.uk/~zerbino/velvet/). Solexa short reads with lowest length of 17 nt were used for assembly.

Analysis of miRNA candidates and snoRNAs

Prediction of possible miRNA precursors were initiated by searching for hairpin loops in the genomes of *Giardia* and *Trichomonas* using srnaloop (source code from: http://arep.med.harvard.edu/miRNA/pgmlicense.html). The output sequences were filtered based on structural criteria using RNAfold from the Vienna package (http://www.tbi.univie.ac.at/~ivo/RNA/) and custom C programmes according to the following criteria: location in non-protein-coding regions; complementary to 3'-UTRs; having an RNAfold-determined minimum free energy ≤ -32.5 kcal/mol for *Giardia* and ≤ -20kcal/mol for *Trichomonas*, (because of the latter's high A/T content); and no multiloops. Subsequently the filtered hairpin sequences were mapped with Solexa output sequences using Seqmap (Jiang and Wong 2008). Finally putative candidates were evaluated by their complementary binding to 3'-UTRs, as a primary feature of many miRNAs (Pasquinelli, et al. 2005). The filtered sequences were checked for expression by comparing with our Solexa sequencing results using seqmap, and then checked against 3'-UTR sequences using Blast. The 3'-UTR sequences of 50 nt were extracted from the genomes by a custom C programme. Prediction of snoRNAs was done using snoscan-0.9 (source code from: http://lowelab.ucsc.edu/snoRNAdb/code/). The custom C programmes are available upon request from the corresponding author.

RT-PCR

All the RT-PCR reactions were done using Invitrogen Thermoscript 1st strand cDNA synthesis kit and subsequent PCR reactions were done using Roche Taq polymerase. The primers used are listed in Table-S2 of Supplementary Data.

Acknowledgements:
We thank Lorraine Berry and Maurice Collins of the AWCGS for sample preparation and sequencing. The *Giardia* culture was kindly provided by Errol Kwan, Protozoa Research Unit, Hopkirk Institute, Massey University; and the *Trichomonas* samples were collected by Lynn Rogers at Medlab Central, Palmerston North. Tim White helped with simulated databases. This work is funded by the Allan Wilson Centre of Molecular Ecology and Evolution and the Institute of Molecular Biosciences.

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Figure Legends

Figure-1 Coverage of RNA species by Solexa sequencing
The filtered reads were mapped against known RNA sequences in Giardia and Trichomonas, and the numbers of reads were counted for the corresponding transcripts. The majority of reads in both organisms are mapped to unknown transcripts, which potentially contain novel non-coding RNAs.
A Reads count for *Giardia*
B Reads count for *Trichomonas*

Figure-2 Predicted miRNA candidates and their alignments with published miRNAs:
A *Giardia* miRNA candidates and alignments
B. *Trichomonas* miRNA candidates and alignments
6 *Giardia* miRNA candidates and 7 *Trichomonas* miRNA candidates all show extensive sequence homology with known miRNAs from a number of organisms. The full names of known miRNAs are: gga-miR-1791 MIMAT0007705 *Gallus gallus* miR-1791; dan-miR-311a MIMAT0008471 *Drosophila ananassae* miR-311a; gga-miR-202 MIMAT0003355 *Gallus gallus* miR-202; oan-miR-1336 MIMAT0006829 *Ornithorhynchus anatinus* miR-1336; cel-miR-34 MIMAT0000005 *Caenorhabditis elegans* miR-34; gga-miR-1673 MIMAT0007557 *Gallus gallus* miR-1673; fru-miR-152 MIMAT0003103 *Fugu rubripes* miR-152; tni-miR-152 MIMAT0003104 *Tetraodon nigroviridis* miR-152; dre-miR-725 MIMAT0003753 *Danio rerio* miR-725; ath-miR862-5p MIMAT0004307 *Arabidopsis thaliana* miR862-5p; mmu-miR-743b-3p MIMAT0004840 *Mus musculus* miR-743b-3p; gma-miR1534 MIMAT0007397 *Glycine max* miR1534; mml-miR-891 MIMAT0006530 *Macaca mulatta* miR-891; osa-miR1852 MIMAT0007772 *Oryza sativa* miR1852.

Figure-3 Possible miRNA-3'-UTR target binding for *Giardia* and *Trichomonas*
miRNA candidates resulting from the homology search:
A. Examples of *Giardia* miRNA candidates
B. Examples of *Trichomonas* miRNA candidates
Examples of *Giardia* and *Trichomonas* miRNA candidates show extensive base pairing with predicted 3′-UTR targets. In general *Trichomonas c*andidates have longer base pairing compared with *Giardia* candidates, and all predicted target sites are within 50 nt 3′ to the stop codon. The candidate sequences are marked red on corresponding Solexa contigs.

Figure-4 Examples of additional miRNA candidates from genomic sequence analysis:
A. Examples of *Giardia* miRNA candidates and predicted target binding
B. Examples of *Trichomonas* miRNA candidates and predicted target binding
Regions on the predicted precursor sequences marked by red are covered by Solexa *de novo* contigs. The example of *Trichomonas* miRNA (B) shows a feature of many candidates that the mature miRNA may be in the loop region of the precursor hairpin. However more evidence is needed to show if this feature is general in *Trichomonas*. The *Giardia* example (A) shows a typical stem location of the mature miRNA.

Figure-5 Expression of Girep RNAs and alignment with VSP mRNA sequence

A. Expression of sense and antisense strands of Girep RNAs
From the figure, it is clear that at least one of each of the Girep sequences are transcribed at both sense and antisense strands, indicated by the RT-PCR. The products of RT-PCR and positive control PCR all have multiple bands, indicating the tandem repeating pattern of Girep sequences.
B. General pattern of matching between a Girep sequence and a VSP mRNA
This figure shows the sequence alignment between the 222 nt repeating unit of Girep-1 and the repeating unit of *Giardia* VSP gene (GL50803_137740). The two sequences are highly similar at sequence level, indicating a strong relation between them.

Figure-6 Common structures of *Trichomonas* C/D box snoRNAs
Trichomonas C/D box snoRNAs adopt either of the above common structures. Shorter snoRNAs tend to have the left-hand-side form and longer ones the right-hand-side form. Shorter sequences usually have ribosomal recognition sites close to their 5′ ends whereas longer sequences generally have less conserved D′ boxes and ribosomal recognition regions close to their 3′ ends.

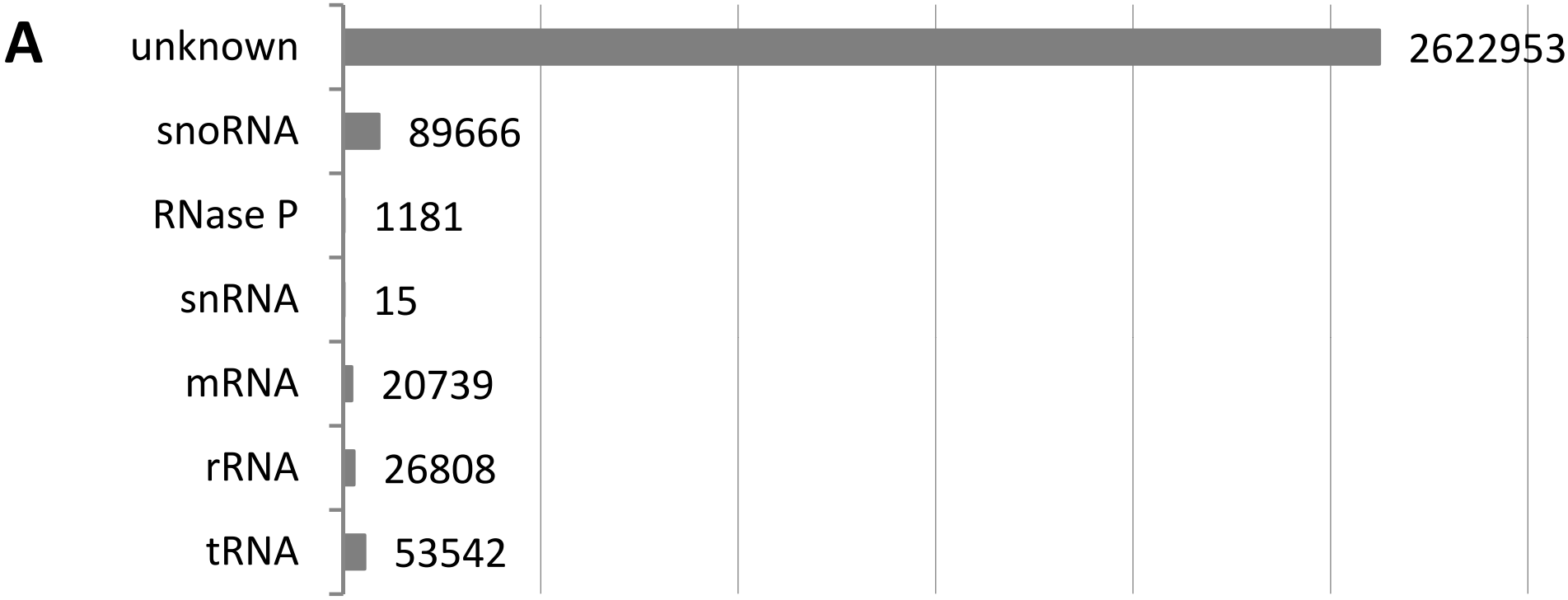
Table-1 miRNA candidates and predicted targets from *Giardia* and *Trichomonas*:

mir candidate	genomic coordinates	predicted mature sequence	possible target(s)
Gim1	CH991782: 610570-610593	AUCAACGUGACU GAUGCUGGCUCU	3'-UTR GL50803_8508 hypothetical protein
Gim2	CH991767: 1378309-1378332	AUCUCGCACAUA UACCGGCCUCCU	Not found
Gim3	CH991769: 132337-132314	GUGCAGAGGCAU GGAGCACGGGAA	3'-UTR GL50803_23634 hypothetical protein; 3'-UTR GL50803_9079 ERP2
Gim4	CH991779: 949975-949998	GUGGUCUGCAUC UGGACCUUCACU	3'-UTR GL50803_14602 hypothetical protein
Gim5	CH991779: 908521-908498	GGCCGUGUGGUU AGGUGGUUGUUG	3'-UTR GL50803_48432 hypothetical protein; 3'-UTR GL50803_10843 Thymus-specific serine protease precursor
Gim6	CH991782: 669487-669463	GUGGUGAGUAGA AGUCAGAUUAUA A	3'-UTR GL50803_15063 Long chain fatty acid CoA ligase 5; 3'-UTR GL50803_114815 Tenascin precursor
Gim7	CH991769: 687079-687104; 687267-687242	GCGGUCGCUUGG GUCCCAGCGGGU TC	3'-UTR GL50803_88901 hypothetical protein
Gim8	CH991813: 431-68 (5 copies in tandem)	GGUCGGUUAGCU CAGUCGGUAGAG CG	3'-UTR GL50803_20250 hypothetical protein
Gim9	CH991776: 417414-417439	GUAGGAUGCCCC AGAGACUGCCGA G	3'-UTR GL50803_13412 acidic ribosomal protein P0
Gim10	CH991782: 268174-268149	AAACUCUCCGCA CAGGGGCGCGCC UG	3'-UTR GL50803_94658 hypothetical protein
Tvm1	DS114515: 7733-7712	GUAAUAGGUCGA GCUUGUGAAU	Not found
Tvm2	DS177933: 152-132 (1st of 50 copies)	CAAUUUGGGUAA AUGGUCAAU	3'-UTR TVAG_416040 conserved hypothetical protein; mRNA TVAG_225980 conserved hypothetical protein
Tvm3	DS176142: 693-673 (1st of 7 copies)	CAAUUCAGUCAU UCUUUCUGU	3'-UTR TVAG_493570 conserved hypothetical protein; mRNA TVAG_450550 conserved hypothetical protein
Tvm4	DS160029: 395-416 (1st of 21 copies)	CAACAGACAUAA UGCUGAAUAG	mRNA TVAG_389700 conserved hypothetical protein
Tvm5	DS113666:	UAAUAUGGAAUC	Not found

	32359-32338	AGAAUGCAGU	
Tvm6	DS177803: 248-269 (1st of many copies)	UCAUCCUUACCU CAGUCAUUGA	3'-UTR TVAG_186870 conserved hypothetical protein
Tvm7	DS177310: 460-481(1st of 49 copies)	AUAUGGCAUAAU AGAACUUUGC	3''UTR TVAG_592550 conserved hypothetical protein; mRNA TVAG_089350 conserved hypothetical protein
Tvm8	DS174663: 730-750(1st of many copies)	UUGAAAAAUAAG AUGGUUCGC	3'-UTR TVAG_080530 conserved hypothetical protein; mRNA TVAG_567710 conserved hypothetical protein
Tvm9	DS162040: 804-784 (1st of 2 copies)	UUUCAAUUGGAC AAUUUGAAU	3'-UTR TVAG_518140 conserved hypothetical protein; mRNA TVAG_258870 conserved hypothetical protein
Tvm10	DS176757: 229-209 (1st of many copies)	CUGUUUGGAAGU UGUAUCCAU	3'-UTR TVAG_029730 conserved hypothetical protein
Tvm11	DS177474: 213-232 (1st of many copies)	UGCGACAAGCUU GCCCAUGG	3'-UTR TVAG_140140 conserved hypothetical protein; mRNA TVAG_123455 conserved hypothetical protein

Table-2 Gireps and Solexa short-read coverage

Name	No. of repeating units	Length of repeating unit	Location	Solexa +strand hits	Solexa -strand hits
Girep-1	9	222	CH991779:330469-332457	7	0
Girep-2	8	222	CH991779:395054-396828	14	1
Girep-3	7	228	CH991782:209411-210987	44	5
Girep-4	8	228	CH991804:1-1810	2	46
Girep-5	10	225	CH991763:274924-277375	13	2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snRNA
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mRNA
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rRNA
26808
tRNA
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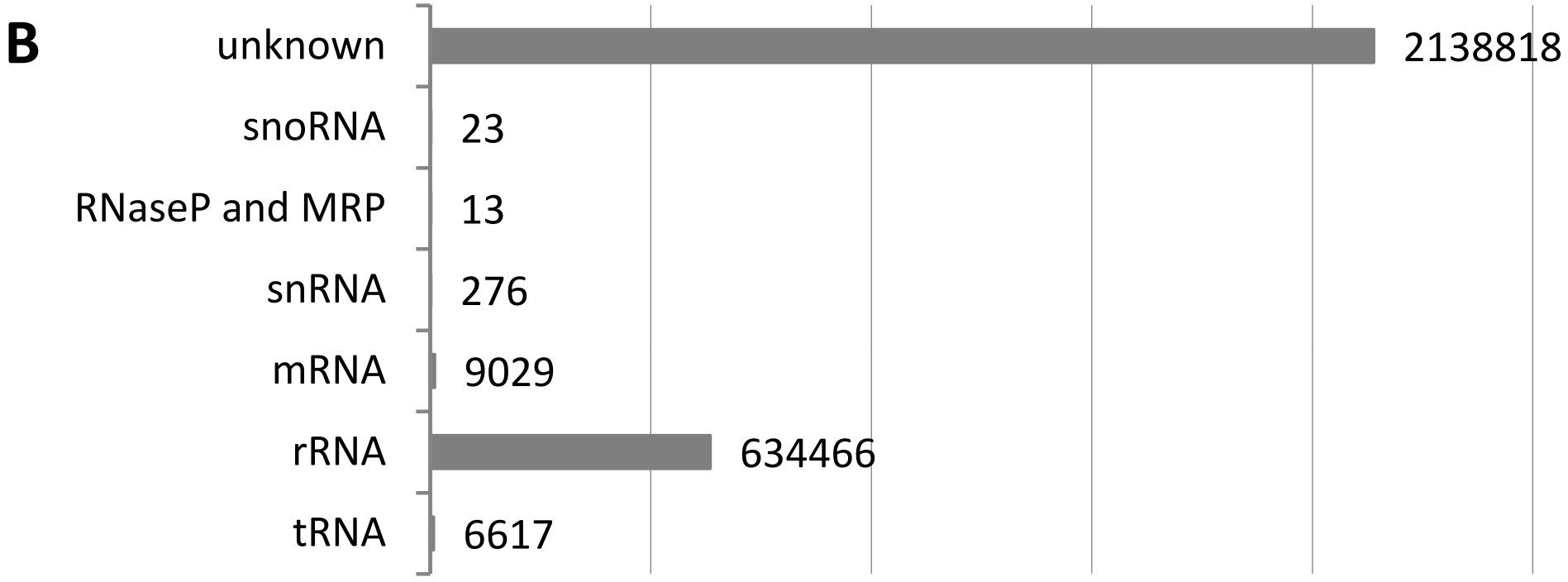
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snoRNA
23
RNaseP and MRP
13
snRNA
276
mRNA
9029
rRNA
634466
tRNA
6617

A

```
Gim1            AUCAACGUGACUGAUGCUGGCUCU
gga-miR-1791    -GCGAUGUGACUGAUGCAGGCUG-
                  * *  ***************

Gim2            AUCUCGCACAUAUACCGGCCUCCU
dan-miR-311a    -UAUUGCACUUAUACCGGCCUGA-
                 * * **** ***********

Gim3          GUGCAGAGGCAUGGAGCACGGGAA--
gga-miR-202   ----AGAGGCAUAGAGCAUGGGAAAA
                  ******** ***********

Gim4            GUGGUCUGCAUCUGGACCUUCACU
oan-miR-1336    UCUUUCUGCAUCUGAACCUUUUC-
                    ********** *****  *

Gim5             -GGCCGUGUGGUUAGGUGGUUGUUG
cel-miR-34       AGGCAGUGUGGUUAGCUGGUUG---
                  *** ********** ******

Gim6             GUGGUGAGUAGAAGUCAGAUUAUAA
gga-miR-1673     GGUGUGAGUGGAAGUCAGAGGU---
                 *  ****** *********
```

B

```
Tvm1              -GUAAUAGGUCGAGCUUGUGAAU
ath-miR862-5p     UCCAAUAGGUCGAGCAUGUGC--
                    ************ ****

Tvm2            CAAUUUGGGUAAAUGGUCAAU
gma-miR1534     UAUUUUGGGUAAAUAGUCAU-
                 * ********** ****

Tvm3            CAAUUCAGUCAUUCUUUCUGU----
dre-miR-725     ---UUCAGUCAUUGUUUCUAGUAGU
                   ********** *****

Tvm4              CAACAGACAUAAUGCUGAAUAG-
mmu-miR-743b-3p   -GAAAGACAUCAUGCUGAAUAGA
                   * ****** ***********

Tvm5            UAAUAUGGAAUCAGAAUGCAGU-
osa-miR1852     --AUAUGGAUUCAGAAUGCAGGU
                  ******* ***********

Tvm6            UCAUCCUUACCUCAGUCAUUGA
mml-miR-891     UGCAACUUACCUGAGUCAUUGA
                *    ******* *********

fru-miR-152     UCAGUGCAUAACAGAACUUUGU
tni-miR-152     UCAGUGCAUAACAGAACUUUGU
Tvm7            AUAUGGCAUAAUAGAACUUUGC
                  *  ****** *********
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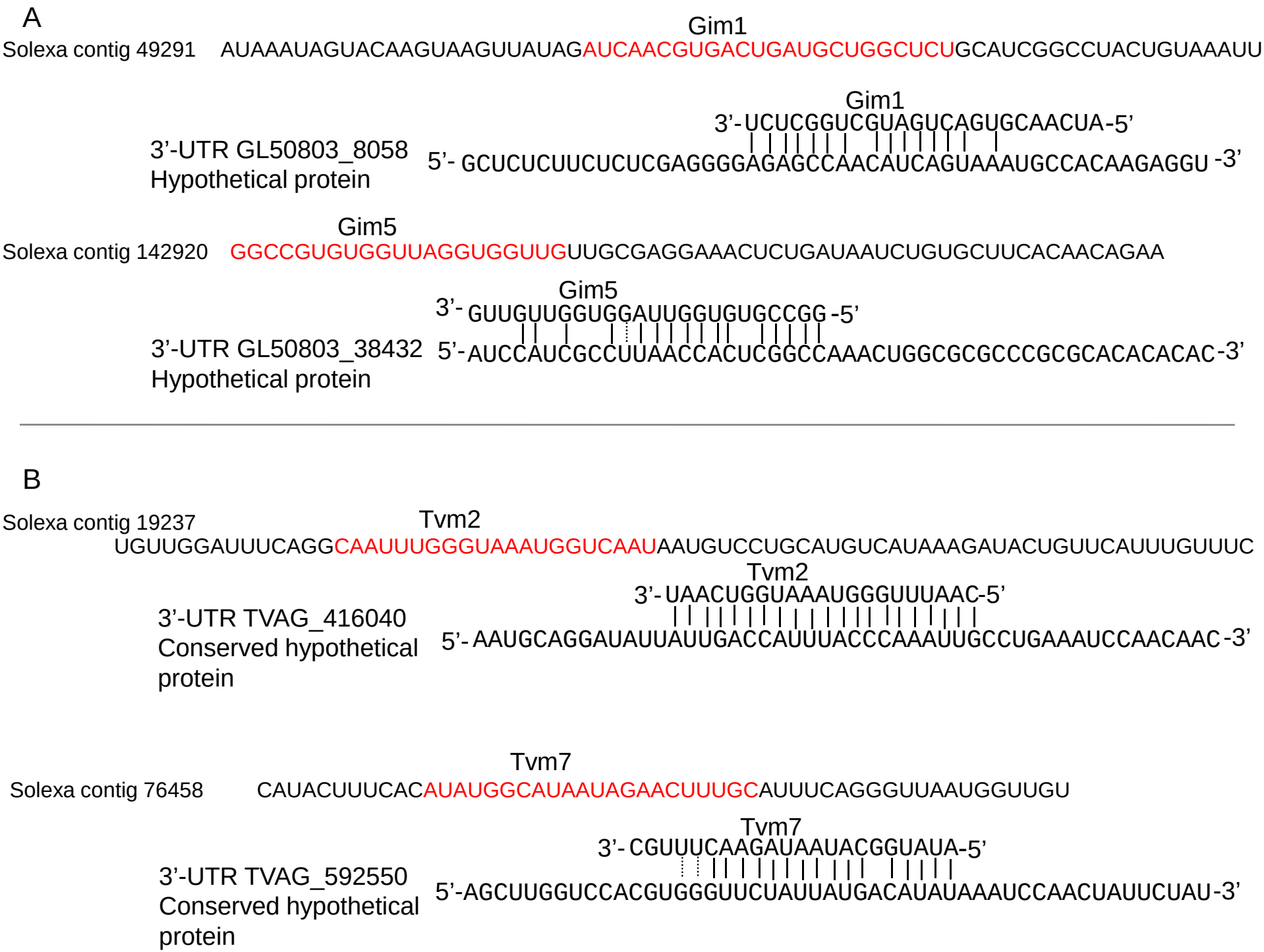
A
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Gim1
3'- UCUCGGUCGUAGUCAGUGCAACUA -5'
3'-UTR GL50803_8058
Hypothetical protein
5'- GCUCUCUUCUCUCGAGGGGAGAGCCAACAUCAGUAAAUGCCACAAGAGGU -3'
Gim5
Solexa contig 142920 GGCCGUGUGGUUAGGUGGUUGUUGCGAGGAAACUCUGAUAAUCUGUGCUUCACAACAGAA
Gim5
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Hypothetical protein
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B
Solexa contig 19237
Tvm2
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3'-UTR TVAG_416040
Conserved hypothetical protein
5'- AAUGCAGGAUAUUAUUGACCAUUUACCCAAAUUGCCUGAAAUCCAACAAC -3'
Tvm7
Solexa contig 76458 CAUACUUUCACAUAUGGCAUAAUAGAACUUUGCAUUUCAGGGUUAAUGGUUGU
Tvm7
3'- CGUUUCAAGAUAAUACGGUAUA-5'
3'-UTR TVAG_592550
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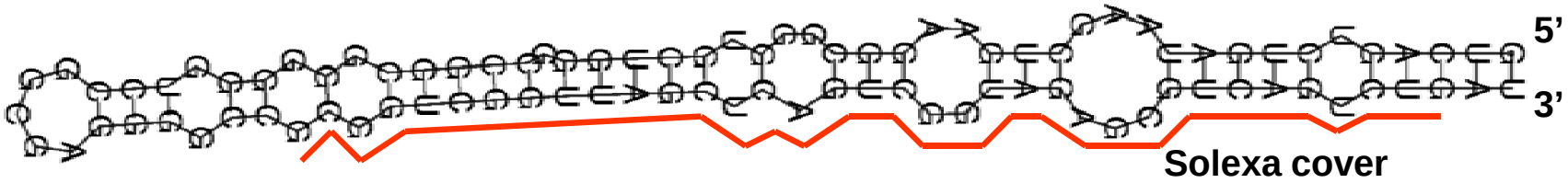
Precursor structure of Gim8



Gim8

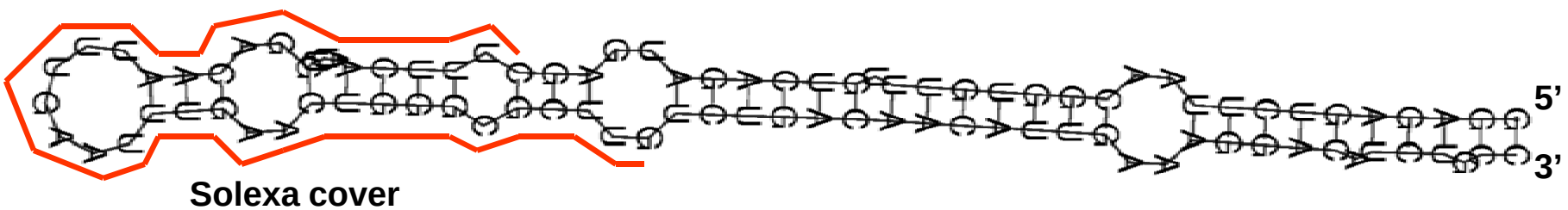
3’- GCGAGAUGGCUGACUCGAUUGGCUGG -5’

5’-GACUAUCGCUCUGCCGACUGAGCUACACAGACGCGCGGCGCGCCACUUAG -3’

3’-UTR GL50803_20250
Hypothetical protein

B

Precursor structure of Tvm9



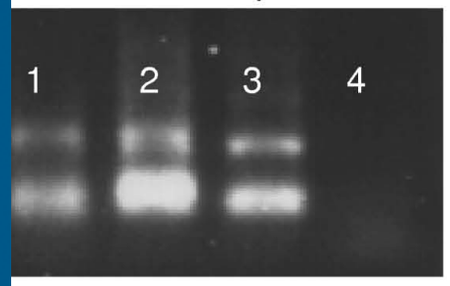
Tvm9

3’-UUUAAGUUUAACAGGUUAACUUU-5’

5’-AAUUCAAUCUCAAAAUUCAAAUUUUCCAAAUGAAAAUUUGGAAAAUUCCC -3’

3’-UTR TVAG_518140
Conserved hypothetical protein

T-PCR for Girep-1



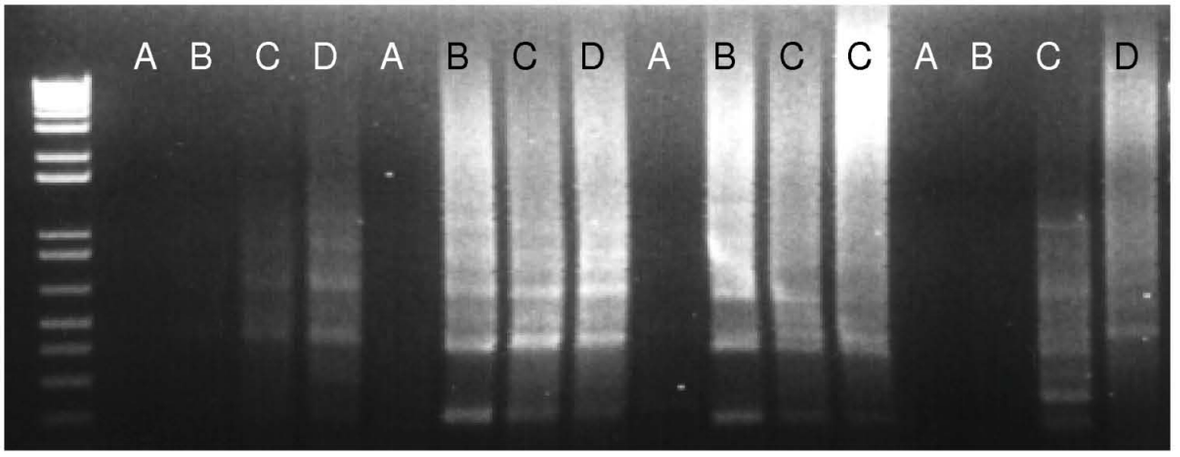
RT-PCR sense strand

RT-PCR antisense strand

+ control (genomic PCR)

- control

RT-PCR for Girep-2 to -5



A: - control

B: + control (genomic PCR)

C: RT-PCR sense strand

D: RT-PCR antisense strand

rep-1: 9 direct tandem repeats

222nt

Alignment of Girep1 repeating unit to VSP: GL50803_112207 mRNA repeating unit

```
 21 CCGGCTGTGCGACGTGCACAACGACTGGGAGCGAGC-AGACCTGCACAAGCTGTGCGACG 79
    | ||||| ||||| |||||  || | |||  | ||| |||| |||   | |||  | ||
806 CGGGCTGCGCGACCTGCACCCCGGC-GGGCTCCAGCCAGACGTGCCTCACCTGCACCACT 864

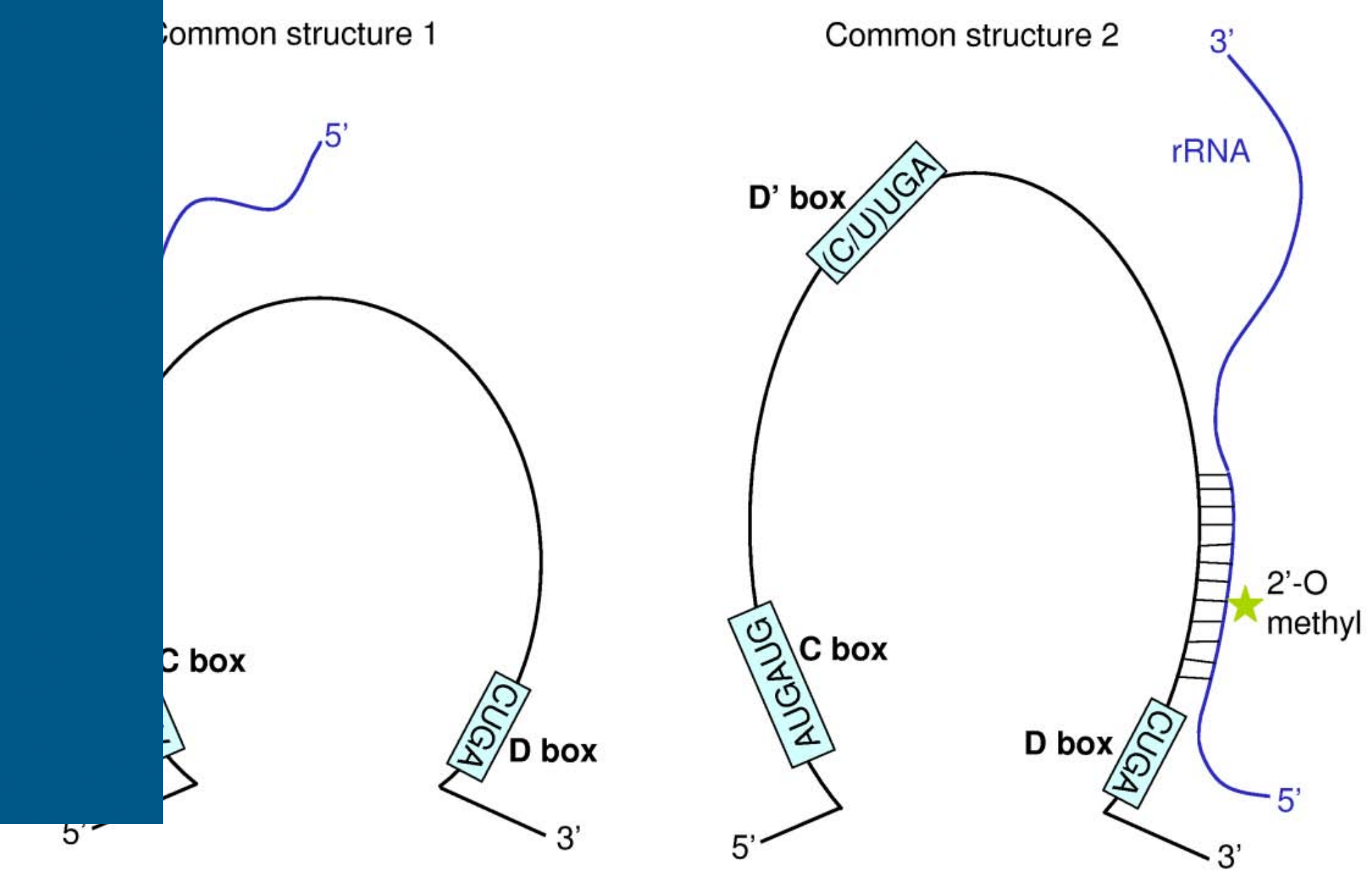
 80 GGCGGAG-AGAAGGTCAGGCCGGACAAGAAGGGCTGCATC-CCGCAGTGCCCTCCTGACG 137
      ||  | | ||| |||||||||| |||||||||||||| ||| |||||||  | ||||
865 T-CGTCGCATAAGATCAGGCCGGACGAGAAGGGCTGCATCTCCG-AGTGCCCCGCGGACG 922

138 TGAGCACAGAGAGCGGTGAGTTCTGCGAGTGCAAGAGCACGCACCAGCCCTCGCCGGACG 197
    ||||||||||   || ||  |||||| ||||||||||  | ||  ||| ||| || |||
923 TGAGCACAGACGTCGATGGATTCTGCAAGTGCAAGAGCGGGTACACGCCGTCGACGAACG 982

198 GGCAGACGTGTGTCCCGAAGACAGG 222
    |||||||||| |  | |||||| ||
983 GGCAGACGTGCGAGCAGAAGACGGG 1007
```

551 578 779 806 1007 1034 1226

VSP: GI50803_137740


Common structure 1
5'
C box
CUGA
D box
3'
5'
Common structure 2
3'
rRNA
D' box
(C/U)UGA
AUGAUG
C box
2'-O
methyl
D box
CUGA
5'
5'
3'