An Abundant Class of Tiny RNAs with Probable Regulatory Roles in Caenorhabditis elegans.

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Supplementary Materials

Predicted Fold-backs

Fold-back secondary structures were predicted with the computer program RNAfold, using the Zuker algorithm (6, 7). The cloned RNA (or a predicted homolog) was folded with flanking sequence in two contexts, either 70 nt of upstream sequence with 15 nt of downstream sequence, or 15 nt of upstream sequence with 70 nt of downstream sequence. Optimal folds, as predicted by RNAfold, can be viewed using the links of Supplemental Table 1. To determine the chance occurrence of predicted fold-back structures within the *C. elegans* genome, 1000 genomic segments 22 nt in length were randomly selected and folded as for the cloned RNAs and their homologs. Less than 5% of these randomly generated segments resided within a sequence context that might have been scored as a plausible miRNA fold-back structure.

C. elegans	C. briggsae	D. melanogaster	H. sapiens
<u>let-7</u>	let-7	<u>let-7</u>	<u>let-7</u>
<u>lin-4</u>	lin-4		
miR-1	miR-1	<u>miR-1</u>	miR-1
miR-2	miR-2	miR-2	
<u>miR-34</u>	<u>miR-34</u>	<u>miR-34</u>	<u>miR-34</u>
<u>mir-35</u>	miR-35:miR-41		
miR-36	"-		
miR-37	" -		
miR-38	" -		
miR-39	" -		
miR-40	" -		
miR-41	" -		
miR-42	miR-42		
miR-43	miR-43		
miR-44	miR-44		
miR-45	miR-45		
miR-46	miR-46		
miR-47	miR-47		

miR-49 miR-50 miR-50 miR-50 miR-51 miR-51 miR-52 miR-52 miR-53 miR-52 miR-53 """ miR-55 """ miR-56 """ miR-57 miR-57 miR-58 miR-58 miR-59 miR-59 miR-60 miR-60 miR-61 miR-61 miR-62 miR-62 miR-63 miR-62 miR-64 miR-65 miR-65 miR-65 miR-66 miR-67 miR-68 miR-70 miR-71 miR-71 miR-72 miR-73 miR-74 miR-74	<u>miR-48</u>	<u>miR-48</u>		
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<u>miR-72</u> <u>miR-73</u> <u>miR-73</u>	miR-70	<u>miR-70</u>		
<u>miR-73</u> <u>miR-73</u>	miR-71			
		<u>miR-71</u>		
miR-74 miR-74		miR-71		
	<u>miR-72</u>			

<u>miR-75</u>	<u>miR-75</u>		
<u>miR-76</u>	<u>miR-76</u>		
<u>miR-77</u>	miR-77		
<u>miR-78</u>			
<u>miR-79</u>	<u>miR-79</u>	<u>miR-79</u>	
<u>miR-80</u>	<u>miR-80</u>		
<u>miR-81</u>	<u>miR-81</u>		
<u>miR-82</u>	<u>miR-82</u>		
<u>miR-83</u>	<u>miR-83</u>		
<u>miR-84</u>	<u>miR-84</u>	<u>miR-84</u>	<u>miR-84</u>
<u>miR-85</u>	<u>miR-85</u>		
<u>miR-86</u>	<u>miR-86</u>		

Methods for Cloning miRNAs.

Total RNA was extracted from a flash-frozen pellet of mixed-stage wild-type N2-strain nematodes by using the Tri-Reagent protocol (Molecular Research Center, Inc.). Extracted RNA (500 μg) was combined with trace amounts of 5´-32P-labeled RNA standards, AGCGUGUAGGGAUCCAAA and GGCCAACGUUCUCAACAAUAGUG++, 18 and 24-26 nt in length, respectively (where the length heterogeneity of the second standard was due to 0, 1, or 2 nontemplated nucleotides added during in vitro transcription, indicated by ++). RNA was then electrophoresed through a single 1.5 mm x 23 mm lane on a 15% polyacrylamide, 8 M urea gel. A gel fragment spanning both the 18 nt and 24-26 nt internal standards was excised, and RNA was eluted and ethanol-precipitated in siliconized tubes, with 20 µg of glycogen as carrier. Gel-purified 18-26 nt RNA (less than 1 µg) was incubated with 40 mM pre-adenylylated 3'-adaptor oligonucleotide, 50 mM HEPES pH 8.3, 10 mM MgCl₂, 3.3 mM dithiothreitol, 10 mg/mL BSA, 8.3% glycerol and 1 U/mL T4 RNA ligase (Amersham Pharmacia Biotech) in a 20 µl reaction at 22°C for 2 hours. The body of the 3'-adaptor oligonucleotide, a DNA oligo, pCTGTAGGCACCATCAATx (p, phosphate; x, O-C3 linker, which blocks the 3'-terminus, preventing it from participating in ligation) was synthesized by standard phosphoramidite chemistry, then activated by adenylylation with adenosine 5'-phosphorimidazolide (2) and gel purified prior to use. The T4 RNA ligase reaction was purified on a 10% sequencing gel, by using the ligated forms of the standards as a guide, then added to a second T4 RNA ligase reaction, like the first except ATP (0.4 mM) was included, and a 5'-adaptor oligonucleotide, ATCGTaggcaccugaaa (Dharmacon Research; uppercase, DNA; lowercase RNA) replaced the 3'-adaptor oligonucleotide. The ligated products from the second ligation reaction were gel-purified, excising the gel fragment spanning the doubly ligated standards. Based on the ligation efficiencies of the internal standards, the overall ligation efficiencies of C. elegans RNAs are estimated to have ranged from 30-90%, depending upon how well the C. elegans sequences matched the preferences of T4 RNA ligase (3). One quarter of the gel-purified doubly ligated RNA was used in a standard 50 µl reverse transcription (RT) reaction (SuperScript II, Gibco BRL) with the RT primer, ATTGATGGTGCCTACAG. The cDNA was amplified by PCR, using the RT primer and a completely DNA version of the 5'-adaptor oligo. PCR product was phenol-extracted, ethanol-precipitated, and then digested with Ban I (NEB), a restriction enzyme that cuts the non-palindromic sites present in the 3' and 5' adaptor oligos. After further phenol extraction and ethanol precipitation, the digested products were concatemerized with T4 DNA ligase (NEB). Concatemers ranging from 200-600 bp were isolated from a low-melt agarose gel, processed with Taq polymerase, and cloned into the pCR2.1-TOPO vector using the TOPO TA cloning kit (Invitrogen), as similarly described (1). PCR products spanning the

Sequences Not Matching C. elegans or E. coli

insert were directly submitted for sequencing (Research Genetics, Inc.).

Fourty-four clones that did not match *C. elegans* or *E. coli* genomic sequences. Eleven matched the two oligoribonucleotides used as internal standards to follow the progress of the cloning. The remaining clones might represent RNAs of fungal contaminants, *C. elegans* RNAs processed or edited beyond recognition, or portions of the *C. elegans* genome that have not been sequenced. None matched *C. elegans* mitochondrial sequence. A few *C. elegans* clones were not perfect matches to the *C. elegans* genomic sequence. These single-nucleotide mismatches were attributed to errors introduced by reverse transcription or PCR, and were ignored if the other clones for the same miRNA matched the genomic sequence. When there were no other clones, the location of the mismatch is indicated by a lower-case nucleotide in Table 1. Two clones for miR-47 and one clone for miR-58 had an untemplated U at their 3´ terminus, and one clone for miR-57 had an untemplated A at its 5´ terminus.

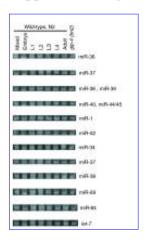
Methods for Developmental Northerns

Eggs were harvested from gravid wild-type N2-strain nematodes by the standard bleaching method, and were hatched at 20°C without food for 20 hours, leading to arrest in early L1. Upon feeding, worms were allowed to grow at 20°C and harvested at specific time intervals based on inspection of P cell and vulval development to obtain synchronized cultures of L1, L2, L3, L4 and young adult worms. Gravid adult *glp-4 (bn2ts)* worms were first cultured at the permissive temperature (15°C), and were then bleached to harvest eggs. These eggs were then cultured at the non-permissive temperature (25°C) to adults. Total RNA was extracted as described for the cloning protocol and separated on a denaturing 15% polyacrylamide gel, with about 30 μg of RNA loaded per lane. RNA was electrophorectically transferred to Zeta-Probe GT membranes (BioRad) or GeneScreen Plus membranes (NEN) as indicated in Supplemental Table 2. A marker lane containing radiolabeled RNAs was also electrophoresed and transferred to the membranes. Membranes were baked and crosslinked as described (4), and hybridization and washing conditions were as prescribed for oligonucleotide probes by the ZetaProbe membrane manual. The oligonucleotides used as probes are listed in Supplemental Table 2. Probes were designed based on their predicted melting temperatures (5), and all probes were hybridized and washed at 50°C. Blots were analyzed by phosphorimaging (BAS2000, FujiMed). The miR-35—41 consensus probes were used as specificity controls. They did not match any miRNAs of the miR-35—41 cluster but instead corresponded to the miR-35—41 consensus sequence. The absence of signal from these control probes indicated that there was little if any cross-hybridization among probes matching members of the cluster.

miRNA	Membrane	Probe
miR-1	Zeta-Probe GT	TACATACTTCTTTACATTCCA
miR-34	GeneScreen Plus	CAACCAGCTAACCACACTGC
mir-35	Zeta-Probe GT	ACTGCTAGTTTCCACCC
miR-36	Zeta-Probe GT	CATGCGAATTTTCACCCGG
miR-37	Zeta-Probe GT	ACTGCAAGTGTTCACCCG
miR-38	Zeta-Probe GT	ACTCCAGTTTTTCTCCC
miR-39	Zeta-Probe GT	CAAGCTGATTTACACCCGGT
miR-40	Zeta-Probe GT	TTAGCTGATGTACACCCGG
miR-41	Zeta-Probe GT	TAGGTGATTTTCACCCGG
miR-42	Zeta-Probe GT	CTGTAGATGTTAACCCGGTG
miR-43	GeneScreen Plus	GCGACAGCAAGTAAACTGTGATA
miR-44/45	Zeta-Probe GT	AGCTGAATGTGTCTCTAGT
miR-46/47	GeneScreen Plus	TGAAGAGAGCGACTCCATGA

miR-52	Zeta-Probe GT	AGCACGGAAACATATGTACGGGTG
miR-56	GeneScreen Plus	CTCAGCGGAAACATTACGGGTA
miR-56*	GeneScreen Plus	TACAACCCAAAATGGATCCGCCA
miR-61	GeneScreen Plus	GATGAGTAACGGTTCTAGTCA
miR-62	GeneScreen Plus	CTGTAAGCTAGATTACATATCA
miR-68	GeneScreen Plus	TCTACACTTTTGAGTCTTCGA
miR-69	GeneScreen Plus	TCTACACTTTTAATTTTCGA
miR-80	GeneScreen Plus	TCGGCTTTCAACTAATGATCTCA
miR-84	GeneScreen Plus	TACAATATTACATACTACCTCA
miR-35—41 consensus1	Zeta-Probe GT	AATGCTAATTTTCACCCGGT
miR-35—41 consensus2	Zeta-Probe GT	ATTGCTAATTTTCACCCGGT
let-7	GeneScreen Plus	ACTATACAACCTACTACCTCA

Supplemental Figure 1. 5S rRNA bands, used as loading controls for Northern blots.

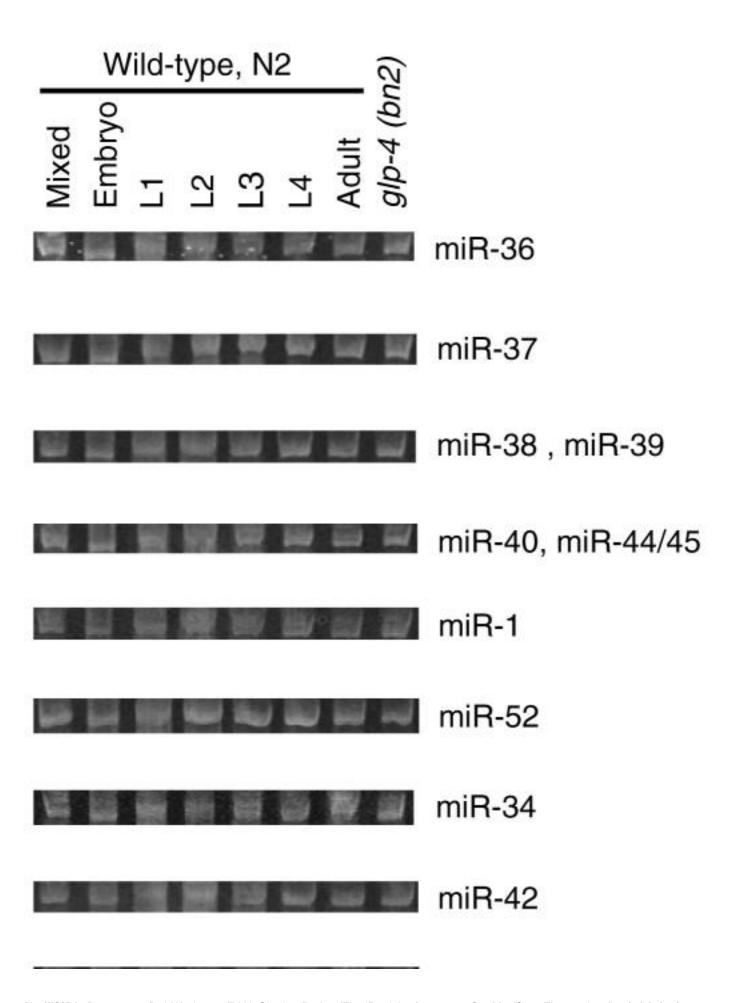


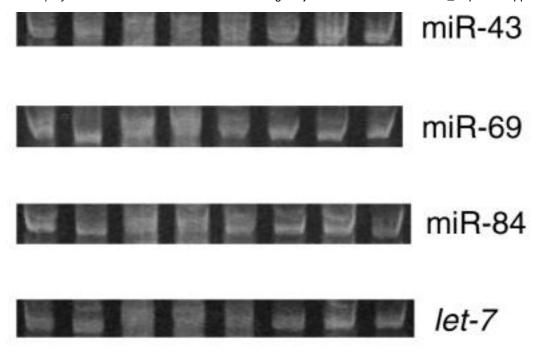
Full Size Version

References

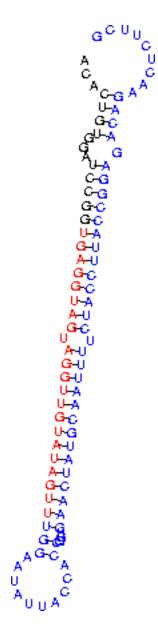
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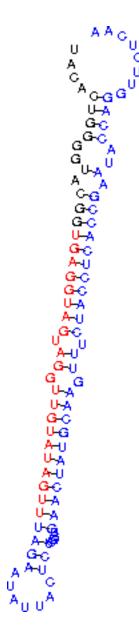




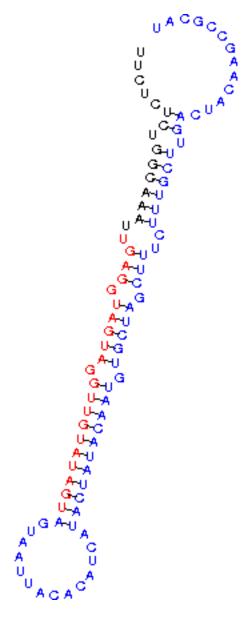
let-7



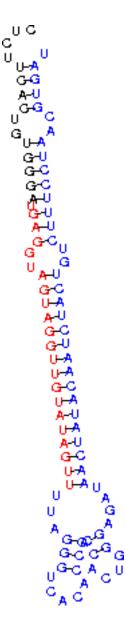
potential ortholog of let-7 in C. briggsae



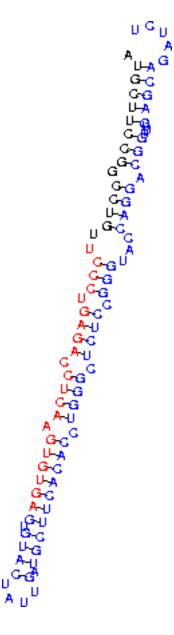
potential ortholog of let-7 in *D. melanogaster*



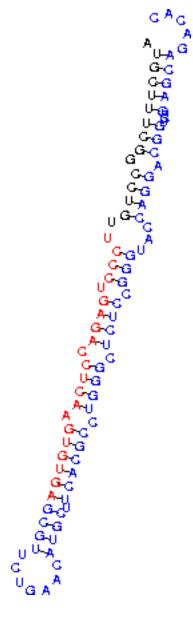
potential ortholog of let-7 in *H. sapiens*



lin-4

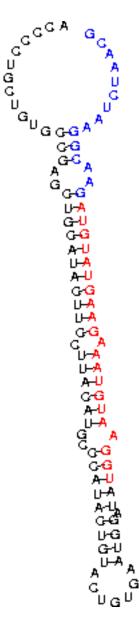


potential ortholog of lin-4 in C. briggsae

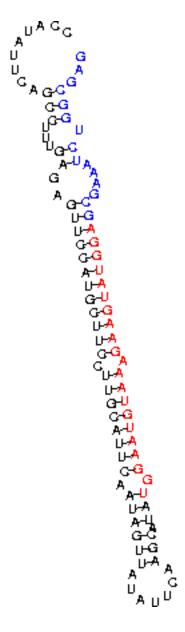




potential ortholog of miR-1 in C. briggsae



potential ortholog of miR-1 in *D. melanogaster*



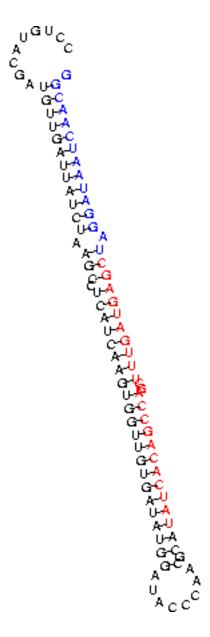
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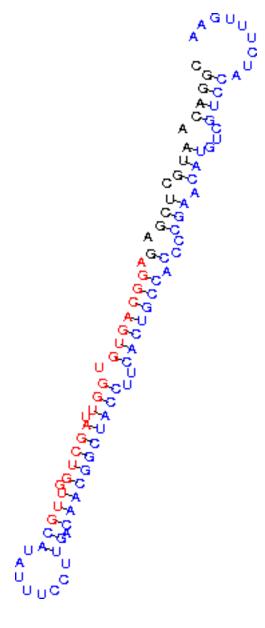


potential ortholog of miR-2 in C. briggsae

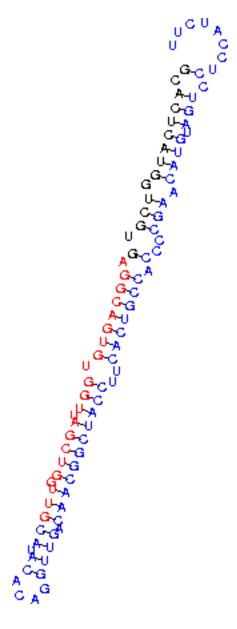


potential ortholog of miR-2 in *D. melanogaster*

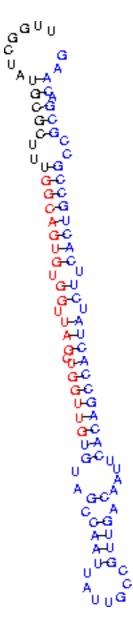




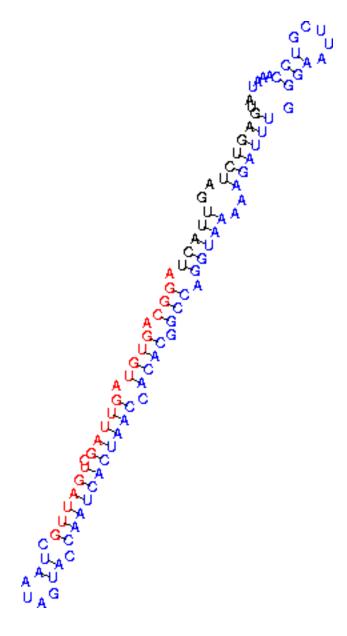
potential ortholog of miR-34 in C. briggsae



potential ortholog of miR-34 in *D. melanogaster*

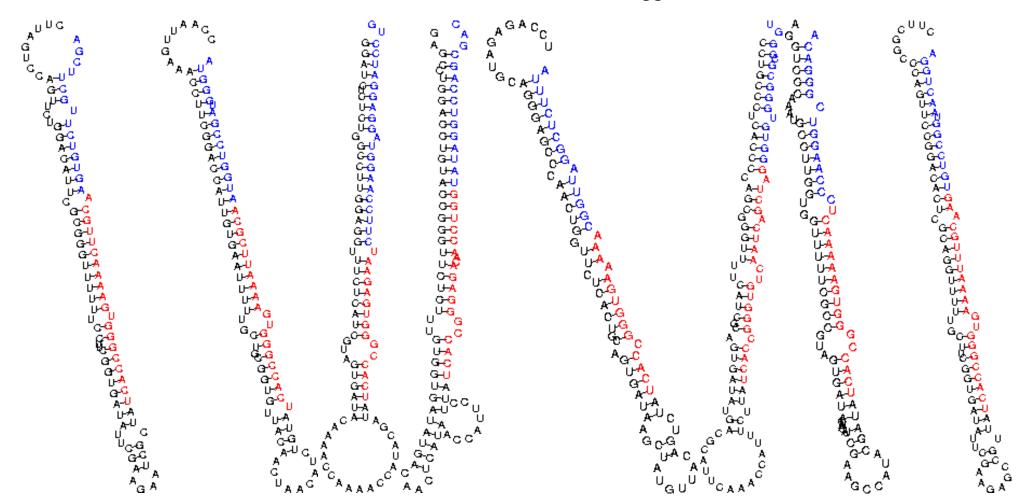


potential ortholog of miR-34 in *H. sapiens*

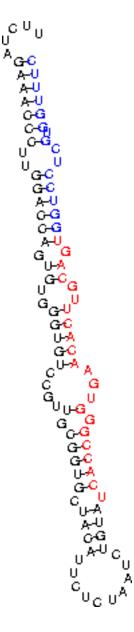


mir-35

miR-35 to miR-41 cluster in *C. briggsae*

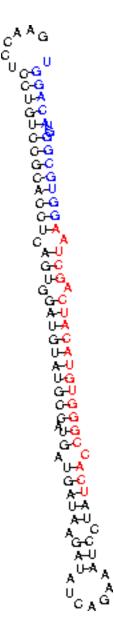










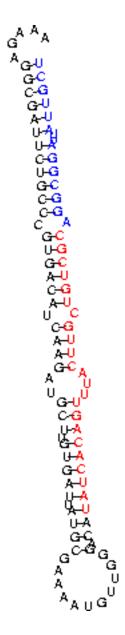






potential ortholog of miR-42 in C. briggsae

potential ortholog of miR-43 in C. briggsae





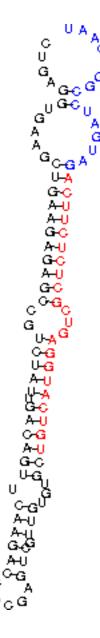
potential ortholog of miR-44 in C. briggsae



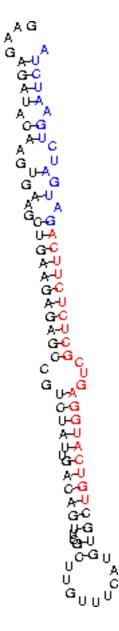


potential ortholog of miR-45 in *C. briggsae*



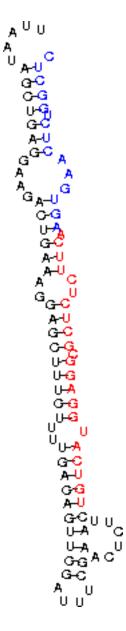


potential ortholog of miR-46 in *C. briggsae*

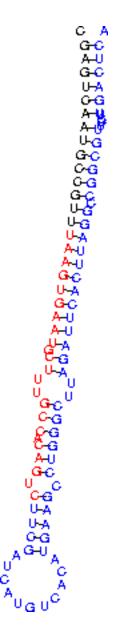


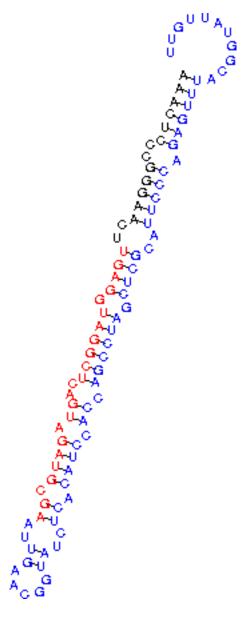


potential ortholog of miR-47 in *C. briggsae*

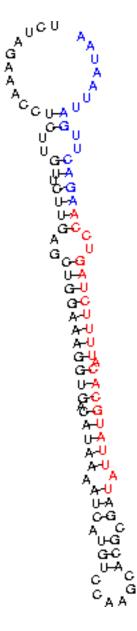


potential ortholog of miR-86 in C. briggsae

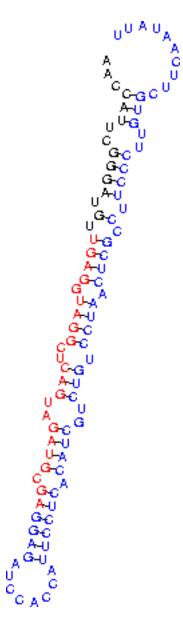


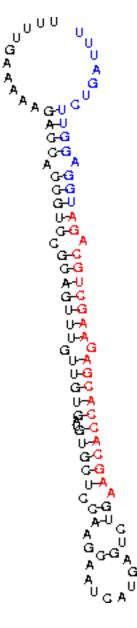


potential ortholog of miR-60 in C. briggsae



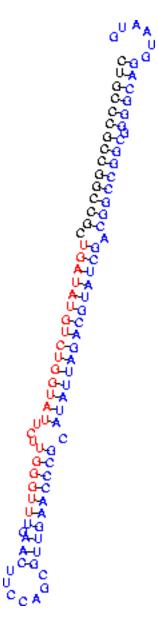
potential ortholog of miR-48 in C. briggsae





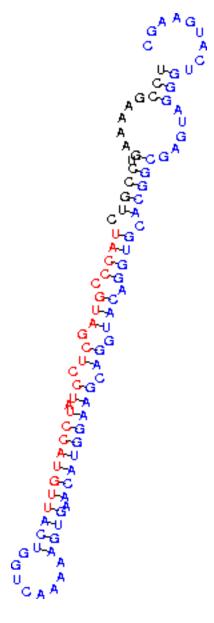
potential ortholog of miR-49 in C. briggsae



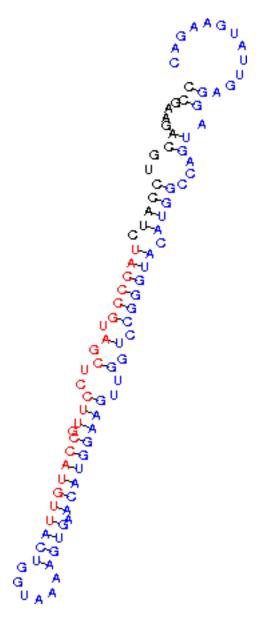


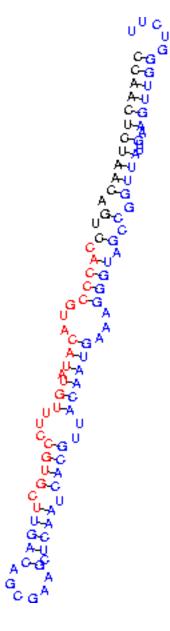
potential ortholog of miR-50 in C. briggsae



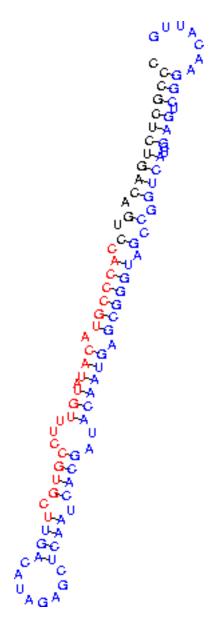


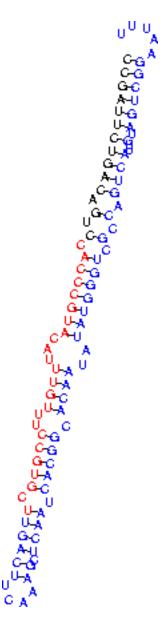
potential ortholog of miR-51 in C. briggsae





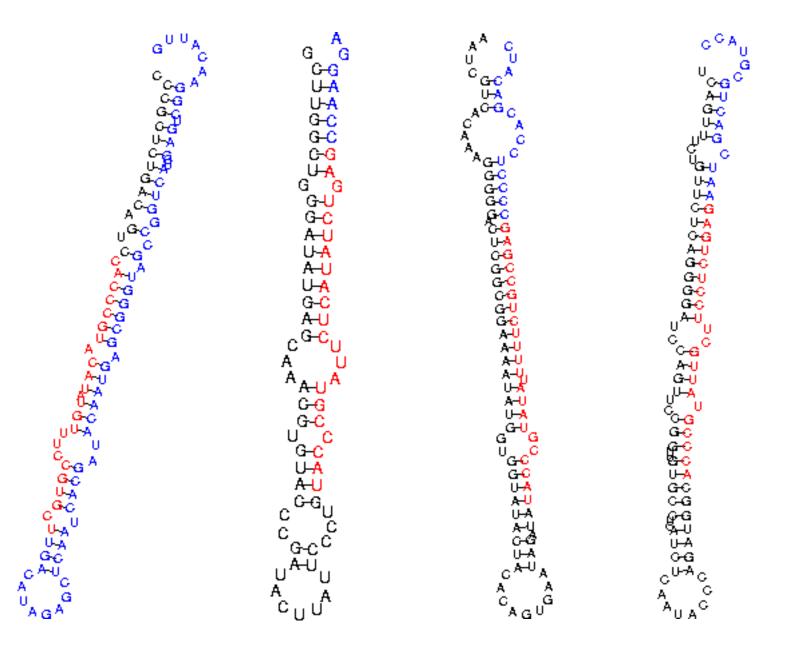
potential ortholog of miR-52 in C. briggsae

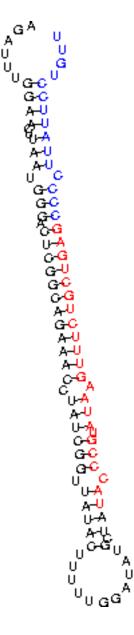




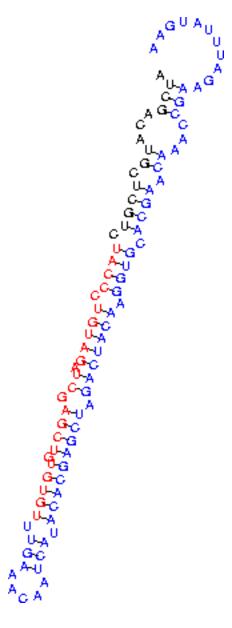


miR-54 to miR-56 cluster in *C. briggsae*

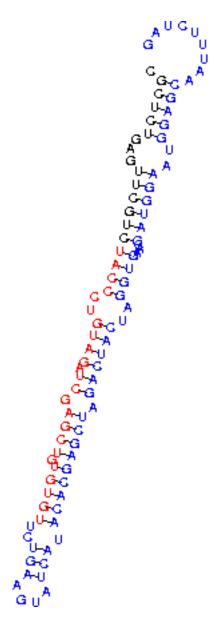


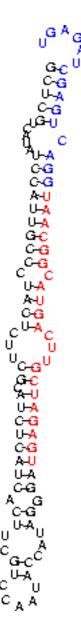




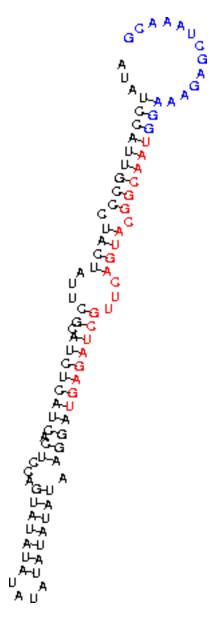


potential ortholog of miR-57 in C. briggsae





potential ortholog of miR-58 in C. briggsae



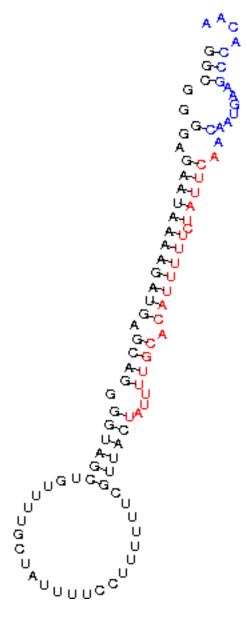


potential ortholog of miR-59 in *C. briggsae*

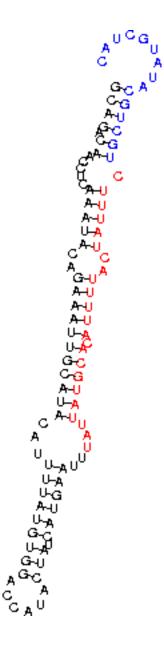




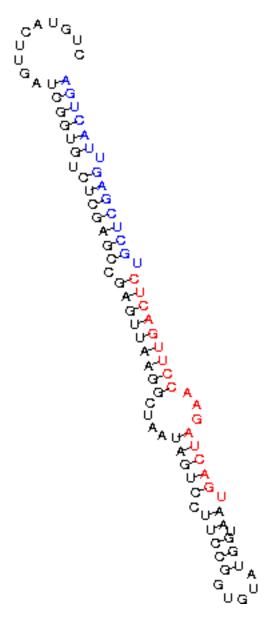
potential ortholog of miR-60 in *D. melanogaster*

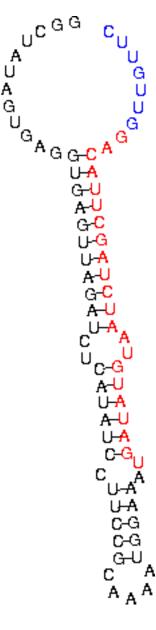


potential ortholog of miR-60 in *H. sapiens*

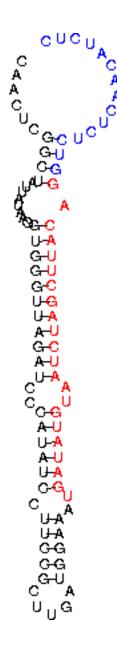


potential ortholog of miR-61 in C. briggsae

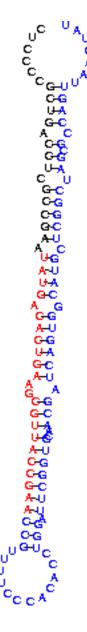


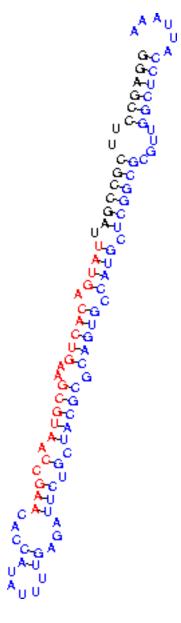


potential ortholog of miR-62 in *C. briggsae*

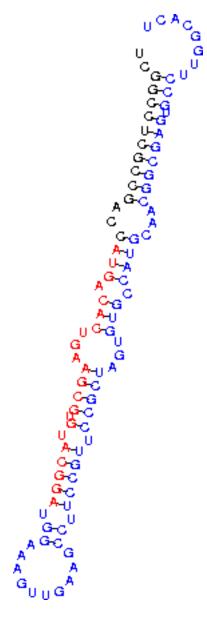


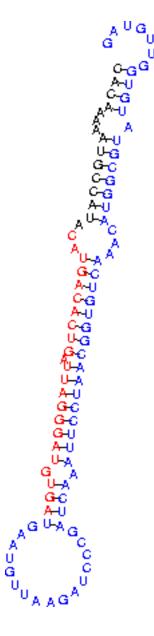


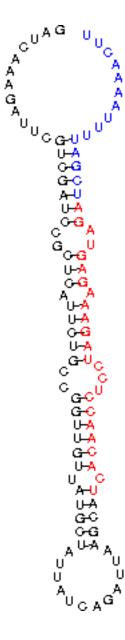




potential ortholog of miR-65 in C. briggsae

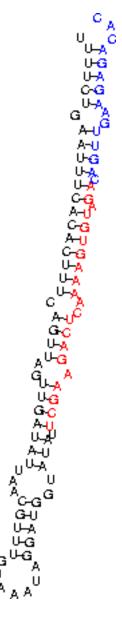




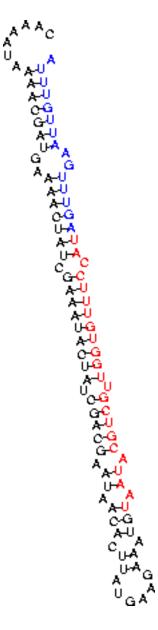


potential ortholog of miR-67 in *C. briggsae*

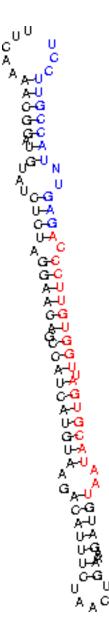


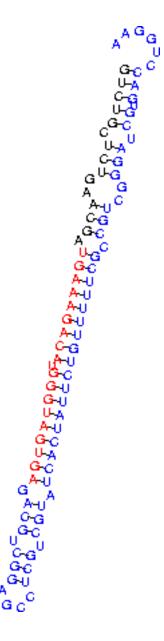




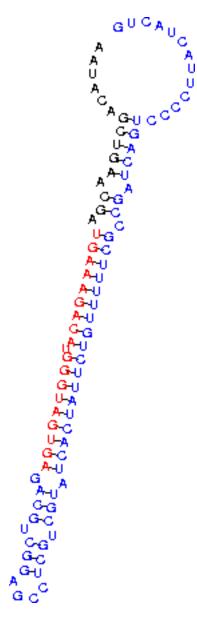


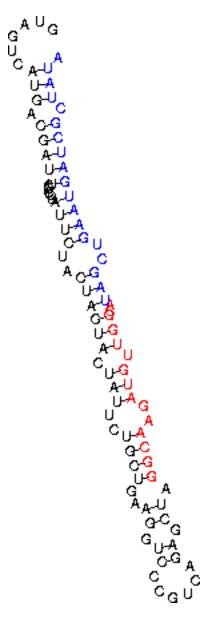
potential ortholog of miR-70 in C. briggsae

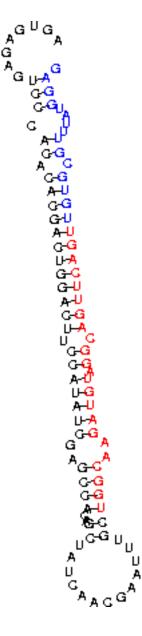




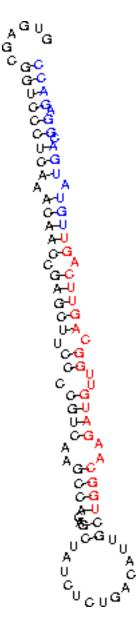
potential ortholog of miR-71 in C. briggsae







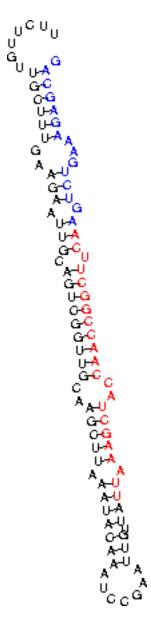
potential ortholog of miR-73 in C. briggsae





potential ortholog of miR-74 in C. briggsae





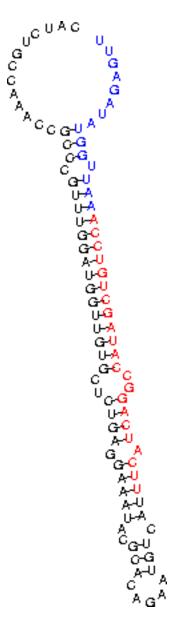
potential ortholog of miR-75 in C. briggsae



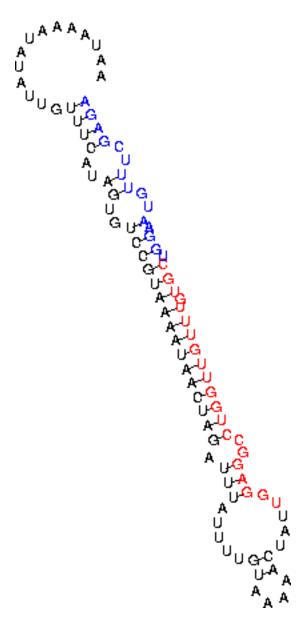


potential ortholog of miR-76 in C. briggsae





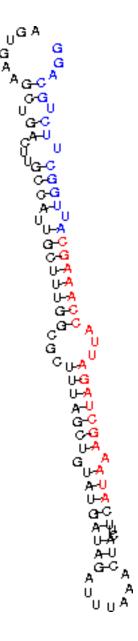
potential ortholog of miR-77 in C. briggsae





potential ortholog of miR-79 in *C. briggsae*

potential ortholog of miR-79 in *D. melanogaster*





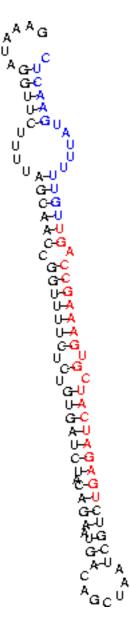
potential ortholog of miR-80 in *C. briggsae*





potential ortholog of miR-81 in C. briggsae





potential ortholog of miR-82 in C. briggsae



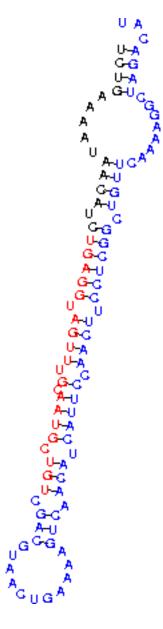


potential ortholog of miR-83 in C. briggsae

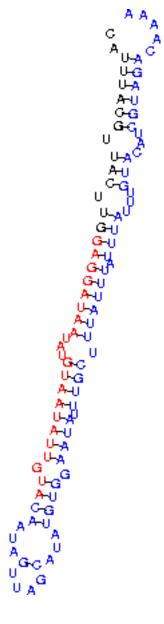




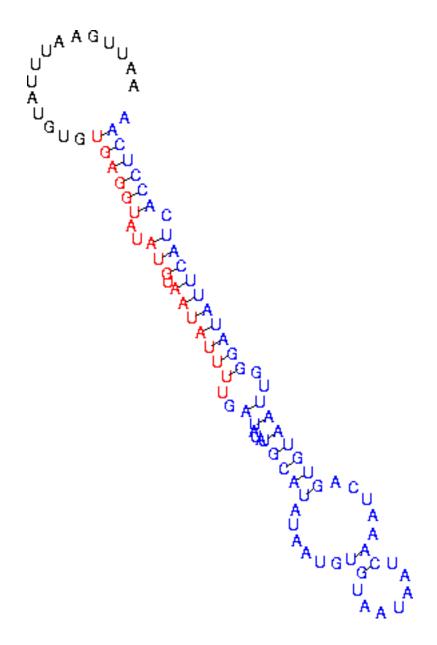
potential ortholog of miR-84 in *C. briggsae*



potential ortholog of miR-84 in *D. melanogaster*



potential ortholog of miR-84 in H. sapiens





potential ortholog of miR-85 in C. briggsae

