

Prediction of Structured Non-Coding RNAs in the Genomes of the Nematodes *Caenorhabditis elegans* and *Caenorhabditis briggsae*

KRISTIN MISSAL^{1*}, XIAOPENG ZHU², DOMINIC ROSE¹, WEI DENG², GEIR SKOGERBØ², RUNSHENG CHEN², AND PETER F. STADLER^{1,3-4*}

¹Bioinformatics Group, Department of Computer Science, and Interdisciplinary Center for Bioinformatics, University of Leipzig, Härtelstraße 16-18, D-04107 Leipzig, Germany

²Bioinformatics Laboratory, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China

³Department of Theoretical Chemistry, University of Vienna, A-1090 Wien, Austria

⁴The Santa Fe Institute, Santa Fe NM 87501

ABSTRACT We present a survey for non-coding RNAs and other structured RNA motifs in the genomes of *Caenorhabditis elegans* and *Caenorhabditis briggsae* using the RNAz program. This approach explicitly evaluates comparative sequence information to detect stabilizing selection acting on RNA secondary structure.

We detect 3,672 structured RNA motifs, of which only 678 are known non-translated RNAs (ncRNAs) or clear homologs of known *C. elegans* ncRNAs. Most of these signals are located in introns or at a distance from known protein-coding genes. With an estimated false positive rate of about 50% and a sensitivity on the order of 50%, we estimate that the nematode genomes contain between 3,000 and 4,000 RNAs with evolutionary conserved secondary structures. Only a small fraction of these belongs to the known RNA classes, including tRNAs, snoRNAs, snRNAs, or microRNAs. A relatively small class of ncRNA candidates is associated with previously observed RNA-specific upstream elements. *J. Exp. Zool. (Mol. Dev. Evol.)* 306B:379–392, 2006. © 2006 Wiley-Liss, Inc.

Within the last few years, non-coding RNAs have moved from a fringe existence to a central topic in molecular genetics. Starting with the discovery that microRNAs form a generic family of regulators of gene expression, small, non-translated RNAs (ncRNAs) have become a topic of utmost interest in molecular genetics (Mattick, 2003, 2004; Szymański et al., 2003; Bartel and Chen, 2004; Hobert, 2004; Storz et al., 2005). Unlike protein-coding genes, ncRNA gene sequences do not exhibit a strong *common* statistical signal that separates them from their genomic context. Individual families of ncRNAs, on the other hand, exhibit evolutionarily very-well-conserved secondary structures. Among these are the rRNAs and tRNAs (which are also very-well-conserved at the sequence level), as well as both classes of snoRNAs (C/D-box and H/ACA-box snoRNAs), microRNA precursors, the RNA components of RNase P, RNase MRP, SRP, and the

five spliceosomal snRNAs (U1, U2, U4, U5, and U6). Structure-based search algorithms such as ERPIN (Gautheret and Lambert, 2001), RNAMotif (Macke et al., 2001), Rsearch (Klein and Eddy, 2003), or FastR (Bafna and Zhang, 2004), can thus be used to identify members of these classes in genomic sequences even in the absence of significant sequence homology. These approaches cannot be employed, however, to identify novel RNA families.

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*Correspondence to: K. Missal, Bioinformatics Group, Department of Computer Science, and Interdisciplinary Center for Bioinformatics, University of Leipzig, Härtelstraße 16-18, D-04107 Leipzig, Germany. E-mail: studla@bioinf.uni-leipzig.de, kristin@bioinf.uni-leipzig.de

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The structural conservation of ncRNAs can be understood as a consequence of stabilizing selection acting (predominantly) on the secondary structure. Their sequences, on the other hand, are often highly variable. This results in a substitution pattern that can be utilized to design a general-purpose RNA genefinder based on comparative genomics: The first tool of this type, qrna (Rivas and Eddy, 2001), is based upon a stochastic context free grammar method to assess the probability that a pair of aligned sequences evolves under a constraint for preserving a secondary structure. RNAs that are under long-time selection for secondary structure can be expected to have sequences that are more resilient against mutations (Nimwegen et al., '99; Wagner and Stadler, '99), which in turn correlates with increased thermodynamic stability of the fold. Indeed, it has been observed that functional RNAs are more stable than the structures formed by randomized sequences (Bonnet et al., 2004; Washietl and Hofacker, 2004; Clote et al., 2005). The program RNAz (Washietl et al., 2005a) combines both approaches. It uses a *z*-score measuring thermodynamic stability of individual sequences and a *structure conservation index* obtained by comparing the folding energies of the individual sequences and the energy of the predicted consensus folding. Both quantities measure different aspects of stabilizing selection acting to preserve RNA structure.

In bacterial genomes, searches for ncRNAs based on the detection of promoter sequences without subsequent ORF were quite successful (Hershberg et al., 2003). In eukaryotes, such a procedure is limited by the diversity and complexity of promoter sequences, the highly variable organization of the genes themselves, and the sheer size of the genomes. The analysis of the flanking sequences of more than 100 experimentally determined ncRNAs in *Caenorhabditis elegans*, however, revealed three distinct upstream motifs (UMs) common to a number of ncRNA loci both in *C. elegans* and *Caenorhabditis briggsae* (Deng et al., 2006). One coincides with the RNA polymerase-III promoter motif of tRNAs, the second is characteristic for snRNAs, while the third one appears to be specific for a small number of nematode-specific ncRNA transcripts.

A computational survey (Washietl et al., 2005b) for non-coding RNAs with conserved secondary structure in vertebrate, and in particular mammalian, genomes, identified more than 30,000 putative ncRNAs. A similar analysis of the

genomes of urochordates (Missal et al., 2005), on the other hand, identified only a few thousand putative structured RNAs, consistent with the hypothesis that ncRNAs form the basis of a complex cellular regulation system that has been vastly expanded in vertebrates (Bartel and Chen, 2004; Mattick, 2004). Here we extend the phylogenetic range of systematic surveys for ncRNAs to nematodes.

METHODS

Data sources

The genomic sequence of *C. elegans* was retrieved from the website of the Sanger Institute, i.e., version WS120 of March 2004,¹ for which a gene and repeat annotation exists at UCSC genome browser. For the *C. briggsae* genome (Stein et al., 2004) we used the version cb25.agp8 of July 2002.² The WormBase gene annotation and the repeat annotation from the UCSC genome browser were taken to define non-coding DNA in the *C. elegans* genome.

Genome-wide alignments of non-coding DNA

We started with the collection of all contiguous regions of the *C. elegans* genome that are not annotated as either "protein coding in known genes" or as "repetitive elements" in WS120. Putative coding regions predicted by genscan or other gene prediction tools were not excluded from this initial dataset, which amounts to 61,067,263 bp of the 100,291,769 bp genomic DNA.

For each DNA interval, we determined potentially homologous regions in the *C. briggsae* genome by pairwise blast (Altschul et al., '90) searches with $E < 10^{-3}$. Regions separated by only short distances (≤ 30 nt) were combined provided the alignments passed the consistency checks outlined below. Global alignments of the resulting regions were then computed using clustalw (Thompson et al., '94). We obtained pairwise alignments for 13,567,851 bp (13.5%) of the *C. elegans* genome.

Structured RNAs are less conserved in regions without basepair interactions, which might prevent blast from extending the sequence alignment into such regions. In order to ensure that a global alignment constitutes a complete ncRNA gene,

¹ftp://ftp.sanger.ac.uk/pub/wormbase/FROZEN_RELEASES/WS120/CHROMOSOMES/

²ftp://hgdownload.cse.ucsc.edu/goldenPath/currentGenomes/Caenorhabditis_briggsae/sanger

blast hits with short distances between them were combined. But due to rearrangement, deletion, and duplication events during evolution, not all local alignments lead to a consistent global alignment. We therefore employed the following algorithm:

A global alignment is inconsistent if at least one region of sequence *A* is conserved with at least two regions of sequence *B* (duplication or deletion) or if at least two distinct regions of sequence *A* are conserved in different order in sequence *B* (rearrangement), see Figure 1. It is useful to construct a graph G_s in the following way: Local alignments are the vertices, and there is an edge between two local alignments if they have a distance less than a threshold value ℓ ; in our case $\ell = 30$ nt. The connected components of G_s thus comprise sets of alignments with pairwise short distance; within these, all combinations of consistent, global alignments have to be determined. To this end, one first checks whether each pair x and y of local alignments are consistent, in the sense that they can be derived from the same global alignment. Two further auxiliary graphs G_C and G_I store this consistency information. If x and y are consistent, an edge in G_C is introduced, otherwise an edge in G_I is added between x and y . Finally, the graph G_F is constructed by inserting edges between the two nodes x and y if at least one path between x and y exists in G_C which does not contain pairs of nodes that are inconsistent, i.e., connected by an edge in G_I . Complete subgraphs of G_F correspond to local alignments which can be combined to a consistent global alignment. Only maximal local alignments, i.e., the maximal cliques of G_F , are of interest for our purposes. These can be computed efficiently, e.g., by the program cliquer (Östergård, 2002). We remark that this approach is similar in spirit to the consistency checking algorithm implemented in

the tracker algorithm for phylogenetic footprinting (Prohaska et al., 2004).

For some regions, in particular tRNA genes, snRNA genes, and a few other loci we obtained more than one alignment for the same *C. elegans* sequence. This does not constitute a problem for the ncRNA detection, since we obtained essentially identical alignments with different paralogs. Two different alignments of the same reading direction were merged onto the same genomic loci if they overlap to at least 90% in the *C. elegans* genome. All such genomic regions were combined again if they overlap to at least 90% independent of the reading direction of their alignments.

Putative ncRNA clusters in close proximity might still cover a genomic region more than once. Of all merged regions which overlapped more than 20%, we discarded all except one leaving us with a unique genomic locus for each ncRNA gene. For each locus we choose the alignment with the maximal RNAz classification probability as the representative. Hence, for all statistics reported below, each genomic location is represented in at most one structured RNA candidate.

We used a database system to handle the huge amount of data. We set up a MySQL 4.1 database server providing sequence information on the *C. elegans* and *C. briggsæ* genome including various annotation data. The complete output of the major processing tools blast and RNAz is stored at the system to allow fast assaying. Currently, 18 tables containing up to 1,270,000 records provide a putative annotation of non-coding RNAs in *C. elegans* and *C. briggsæ*.

Detection of structured RNA motifs

The pairwise clustalw alignments described above were screened with RNAz (Washietl et al., 2005a) to detect regions that are also conserved on the level of RNA secondary structure. Due to computational limitations and restrictions in the training set of the support vector machine (SVM) underlying the RNAz program, alignments were scanned by moving a window of length 120 in steps of 50 nt. We only scanned alignments of at least 40 nt length, because most known ncRNA families are not shorter than this. The RNAz algorithm evaluates the thermodynamic stability of RNA secondary structures (relative to an ensemble of shuffled sequences) and quantifies the evidence for stabilizing selection by comparing the energy of a consensus structure with the

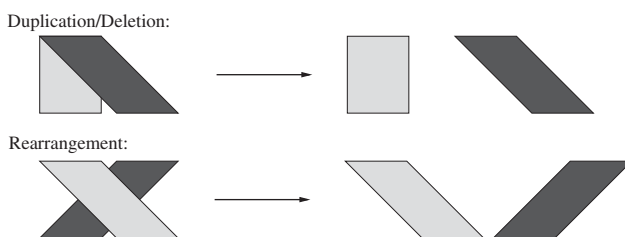


Fig. 1. Local pairwise alignments will lead to an inconsistent global alignment in case of duplication, deletion or rearrangement events. They are combined to a global alignment only if they are consistent.

ground-state energies of the individual structures. RNAz performs the classification by means of a support vector machine that takes into account (1) the length and sequence divergence of the alignment, (2) the number of aligned sequences, (3) the folding energy z -score, and (4) the structure conservation index. A probability estimate $p > p_c$ for the SVM decision value gives a convenient measure to interpret the RNAz classification. A value of $p_c = 0.5$ classifies the alignment as non-coding RNA with low significance, whereas $p_c = 0.9$ indicates a high significance for structured RNA. For details we refer to Washietl et al. (2005a). For each global alignment, both possible reading directions are considered, because the classification of RNAz is based on the thermodynamic stability of the potentially transcribed RNA, which is inherently direction dependent.

Specificity

In order to estimate the specificity of RNAz on the pairwise alignments of non-coding DNA, we repeated the entire screen with shuffled input alignments. The specificity in terms of individual RNAz scanning windows is defined as

Specificity

$$:= \frac{\text{number of shuffled scanning windows with } p \leq p_c}{\text{number of shuffled scanning windows}}$$

We found that RNAz has a specificity of more than 0.96 ($p_c = 0.5$) and 0.98 ($p_c = 0.9$). However, we observe “raw overall false positive rates” of the entire screen of 56% ($p_c = 0.5$) and 41% ($p_c = 0.9$) by comparing the number of genomic regions classified as structured RNA in the true data with the shuffled dataset. We define the raw overall false positive rate as

$$\text{raw overall false positive rate} := \frac{\sum_{\{i \in \text{shuffled screen}\}} l_i}{\sum_{\{j \in \text{original screen}\}} l_j},$$

where l_i and l_j are the length of the i th and j th unique genomic loci classified as ncRNA in the shuffled and original screen, respectively. These raw false positive rates are, however, dramatic overestimates since we shuffled each alignment independently. Thus, if there are $M > 1$ alignments for a given locus (which is the case for all ncRNA genes that appear in multiple copies in the genome), there are M independently shuffled alignments (Fig. 2). Our procedure, however, counts a locus as a false positive as soon as one of them is misclassified by RNAz. In order to correct for this effect, we counted each alignment with a weight $1/M$:

corrected overall false positive rate

$$:= \frac{\sum_{\{i \in \text{shuffled screen}\}} \frac{l_i}{M_i}}{\sum_{\{j \in \text{original screen}\}} \frac{l_j}{M_j}},$$

and obtained corrected false positive rates of 49% ($p_c = 0.5$) and 33% ($p_c = 0.9$), respectively.

Alternatively, we defined an individual false positive rate as

individual false positive rate :=

$$\frac{\text{number of shuffled scanning windows with } p > p_c}{\text{number of original scanning windows with } p > p_c}.$$

Based on this definition, we obtained the much smaller false positive rates of 10.9% ($p_c = 0.5$) and 5.5% ($p_c = 0.9$). The reason for this difference is that RNAz hits overlap due to the windowing technique. While overlapping windows typically agree on their classification in the true dataset, RNAz hits only sparsely cover a misclassified genomic locus in the shuffled dataset. This effect suggests the possibility for further methodological improvements that could increase the specificity of RNAz.

Estimating the sensitivity of RNAz

In order to estimate the sensitivity of our screen, we compared our data to a recent annotation of

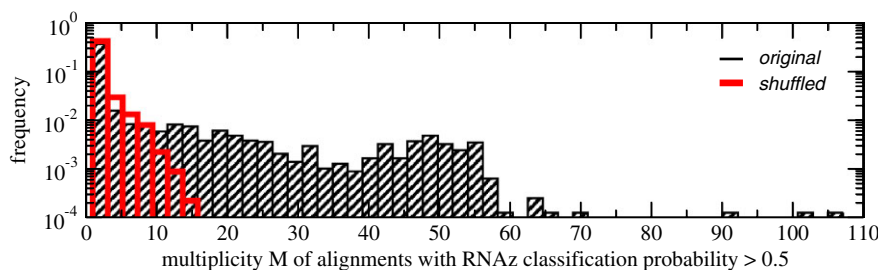


Fig. 2. Distribution of the number M of alignments, classified as structured RNA, mapping to a given genomic locus.

non-coding RNAs in *C. elegans* (Stricklin et al., 2005), Table 3, and (Deng et al., 2006), Table 4. We annotate a putative ncRNA candidate of our screen as known if its genomic locus overlaps to at least 70% with a ncRNA annotated in *C. elegans*, leading to the following definition of sensitivity:

$$s_g := \frac{N}{N_g}.$$

Here, N is the number of unique genomic loci, identified by RNAz, which overlap to at least 70% with a known member of a specific ncRNA family found in Stricklin et al. (2005) or Deng et al. (2006) and N_g is the entire number of ncRNAs of this family in the genome. The sensitivity of our screen largely depends on the number of ncRNAs which have a conserved primary structure between *C. elegans* and *C. briggsæ*. In order to assess how many known ncRNAs can in principle be detected by our screen, we also report the sensitivity of our alignment procedure defined as

$$s_a := \frac{N}{N_a},$$

where N_a is the number of known ncRNAs overlapping to at least 70% with our pairwise alignments scanned with RNAz.

While in Stricklin et al. (2005) the WS130 assembly of *C. elegans* was used, we based our screen on the WS120 assembly, because for WS120 a protein-coding gene and a repeat annotation track are provided by UCSC. This allowed us to summarize the results of our survey conveniently as RNAz custom track that can be readily viewed in the UCSC genome browser. All RNAz hits with classification probability $p_c = 0.5$ were mapped to the WS130 in order to facilitate comparison with the ‘‘Wormbook annotation’’ (Stricklin et al., 2005).

Upstream patterns

The putative regulatory motifs considered here were derived from the experimentally determined ncRNAs reported by Deng et al. (2006). The 100 bp upstream of these 198 genomic loci were extracted from the genomic DNA sequence and analyzed with the pattern discovery software MEME (Bailey and Elkan, '94) with the parameter `-dna-motif 10`. Three UMs were statistically highly significant and each of them belongs to more than three different RNAs or RNA families;

see Deng et al. (2006) for further details. Most probably, therefore, these elements constitute regulatory (promoter) elements.

The complete *C. elegans* genome was scanned for occurrences of these three UMs using the program MotifLocator from the software INCLUSIVE (Thijs et al., 2001). This program uses an adapted position-weight matrix scoring scheme based upon a higher-order background model. The score is computed as the normalized ratio of the motif score and the background score. The threshold value for the score is determined by counting the number of hits of the very abundant UM1 motif with different thresholds. In order to ensure that the results do not depend strongly on the software, we compared MotifLocator with PatSearch (Grillo et al., 2003). The threshold score value of 0.8 was chosen since the number of PatSearch hits increases sharply below this value. The results were similar for both softwares, and only the MotifLocator data were used for further analysis.

The motifs identified by the genome-wide MotifLocator scan were compared to the RNAz predictions. However, a comprehensive investigation of the upstream regions of the RNAz predictions, unfortunately, is complicated by both the large set of predictions and the fact that RNAz cannot reliably determine the direction and the ends of the putative ncRNAs.

RESULTS

Novel ncRNAs

We detected 3,672 structured RNA candidates ($p_c = 0.5$) of which 678 correspond to 665 known ncRNAs or clear homologs of known *C. elegans* ncRNAs (Table 1, Table 3 and Table 4). The complete dataset can be accessed as a gff file that is included in the electronic supplement.³ A few examples are shown in Figure 3.

Approximately a quarter of the RNAz hits are located in introns, and a comparable number is ‘‘intergenic’’ in the sense that it is located more than 1 kb away from any known protein-coding gene. Putative RNA structures in untranslated regions (UTRs) of protein-coding genes are identified using the GeneBounds track provided at the UCSC Genome Browser. Interestingly, ncRNA candidates have approximately equal densities in

³URL: <http://www.bioinf.uni-leipzig.de/Publications/SUPPLEMENTS/05-023/>

TABLE 1. Statistics of RNAz ncRNA screens for two different classification probability levels p_c

Genomic context	Blast alignments length	Number of ncRNA candidates	
		$p_c = 0.5$	$p_c = 0.9$
Intronic	597,128	1,235	891
5'UTR	116,193	119	65
3'UTR	128,766	130	69
Intergenic	810,989	1,221	726
Total		3,672	2,366
Length (nt)	13,567,851	432,536	291,499

A comparison of the number of initial blast alignments with the number of ncRNA candidates predicted by RNAz shows that ncRNAs are slightly enriched in introns, while UTR elements are rare.

A ncRNA is classified as “intergenic” if it is at least 1 kb away from the closest known protein coding gene in *Caenorhabditis elegans*; a ncRNA is classified as “UTR” if it is located within an interval of GeneBounds track either before the first or after the last coding exon of the gene in question. 54 ncRNAs are annotated as 5'UTR as well as 3'UTR, which might be regulatory elements for polycistronic transcripts (Blumenthal, 2004). All numbers refer to the *C. elegans* genome.

TABLE 2. Specificity and false positive rates of the RNAz ncRNA screens for two different classification probability levels p

	False positive rates	
	$p_c = 0.5$	$p_c = 0.9$
Individual RNAz hits	10.9%	5.5%
Genomic loci (raw)	55.9%	40.9%
Genomic loci (corrected)	48.8%	33.2%
Specificity per test	0.96	0.98

False positive rates can be estimated in different ways for our screen (see text for details). The estimate for the individual windows that are screened with RNAz appears optimistic, while the estimates for the entire screen are by construction pessimistic.

intron and intergenic regions, while they are under-represented by a factor of 10 in UTRs.

Specificity and sensitivity of the RNAz screen

Specificity and false positive rates can be estimated by different methods, as outlined in the Methods section. Using the individual alignment windows that are scored by RNAz, we observe a false positive rate of less than 11% ($p_c = 0.5$) in a comparison between real and randomly shuffled data. This is probably an optimistic estimate. On the other hand, the false positive rates of the entire screen, corrected for multiple alignments mapping to the same genomic

position are about 50% ($p_c = 0.5$) and 33% ($p_c = 0.9$), Table 2. As argued above, these are pessimistic estimates. The 3,672 ($p_c = 0.5$) and 2,366 ($p_c = 0.9$) predicted ncRNAs imply lower bounds between 1,600 and 1,900 structured RNAs, of which roughly one third (see Table 3) are annotated. It follows that we can expect at least roughly 1,000 bona fide novel ncRNAs and structured RNA elements in our dataset.

An accurate estimate of the overall sensitivity of an RNA gene finding approach is hard to derive since comprehensive annotations are available only for a few “classical” families of ncRNAs. In the following, we briefly outline our results for the major ncRNA families; see also Table 3.

To annotate the RNA classes below, we mapped the annotation in Stricklin et al. (2005), which is given in WS130 coordinates, to the *C. elegans* assembly WS120, from which we derived our sequences. An overlap of at least 70% is required for an ncRNA candidate to be annotated.

tRNAs: Of about 591 known tRNAs in *C. elegans*, we identified 509 ($p_c = 0.5$) and 465 ($p_c = 0.9$) in our screen. Only 70 of 1,072 tRNA pseudogenes are found in our global pairwise alignments of which 50 at $p_c = 0.5$ and 44 at $p_c = 0.9$ were detected by RNAz.

rRNAs: About three 18S, one 23S, one 26S, 15 5S and two 5.8S are known in *C. elegans*. We recovered all 18S rRNAs in both chromosome I and in the mitochondrial DNA. The mitochondrial 23S rRNA appears as two separated RNAz hits. The repeat unit of 26S rRNAs on chromosome I is also detected as a series of 10 separated RNAz hits. One single copy of the 5S rRNA in chromosome V (with an overlapping constraint of at least 60%) was detected by our screen, but none of the 5.8S rRNAs. Both 5.8S rRNAs are not conserved in *C. briggsae* (blast cutoff of $E < 10^{-3}$) and hence are not identifiable by our approach. Whereas the 15 known 5S rRNA loci are well conserved in *C. briggsae* (blast cutoff of $E < 10^{-3}$), their sequence similarities (96–100%) are beyond the favorable values for RNAz. In such alignments, no covariance information to predict a reliable consensus secondary structure is given and the high degree of structure conservation, resulting from almost identical sequences, is not significant.

miRNAs: In *C. elegans*, 117 miRNAs are annotated in Stricklin et al. (2005). Of these, 54 are conserved with *C. briggsae* at a blast cutoff of $E < 10^{-3}$. While the mature miRNA sequence is easily detected by blast, we failed to detect the precursor stem loop of some miRNAs in our

TABLE 3. Sensitivity of RNAz-detected ncRNAs based on known ncRNA annotations from the Wormbook (Stricklin et al., 2005)

		Known in genome N_g	In <i>C.el./C.br</i> alignment N_a s_g		RNAz					
					$p_c = 0.5$			$p_c = 0.9$		
					N	s_g	s_a	N	s_g	s_a
tRNA	Functional	591	584	0.98	509	0.86	[0.87]	465	0.78	[0.79]
	Pseudogene	1,072	70		50			44		
miRNA		117	40	0.34	34	0.29	[0.85]	34	0.29	[0.85]
snoRNA		31	26	0.84	13	0.41	[0.50]	9	0.29	[0.35]
snRNA	Spliceosomal	72	72	1.00	54	0.75	[0.75]	47	0.65	[0.65]
	Spliced leader	30	26	0.87	26	0.87	[1.00]	26	0.87	[1.00]
rRNA		22	20	0.9	5	0.22	[0.25]	4	0.18	[0.2]

We compare the numbers N_g of genes known in the genome (second column) with those contained in our input alignments (N_a), and those classified as structured RNAs by RNAz (N) at two different classification probability levels. In addition, sensitivities are listed as fraction s_g of known genomic sequences, and as fraction s_a of known sequences contained in the input alignments (given in brackets). The sensitivity of the miRNA genes refers to the 54 miRNA loci conserved in *C. briggsæ*. For all other ncRNA classes, the sensitivity values refer to the number of the known genomic loci in *C. elegans*. Known ncRNA genes are counted to be in our alignments if they overlap to at least 70% with a global alignment. Sensitivities are also reported relative to the *C. elegans* genome.

TABLE 4. Comparison of the RNAz results with the experimental small RNA screen (Deng et al., 2006)

		Deng et al., 2006 ncRNA loci N_g	<i>C.el/C.br.</i> alignments N_a s_g		RNAz					
					$p_c = 0.5$			$p_c = 0.9$		
Type					N	s_g	s_a	N	s_g	s_a
In wormbook		97	90	0.93	63	0.64	[0.70]	55	0.56	[0.61]
H/ACA		41	31	0.76	11	0.26	[0.35]	9	0.21	[0.29]
snoRNA										
CD snoRNA		28	19	0.68	3	0.10	[0.15]	2	0.07	[0.10]
sb RNA		9	3	0.33	2	0.22	[0.66]	2	0.22	[0.66]
snl RNA		8	3	0.38	3	0.37	[1.00]	2	0.25	[0.66]
Unknown		14	14	1.00	4	0.28	[0.28]	2	0.14	[0.14]
All novel		101	70	0.69	23	0.23	[0.33]	17	0.17	[0.24]
Total		198	160	0.81	86	0.43	[0.53]	72	0.36	[0.45]

All numbers refer to genomic locations in the *C. elegans* genome. Columns have the same meaning as in Table 3. The coordinates given by Stricklin et al. (2005) and Deng et al. (2006) are mapped to WS120, the coordinates of our ncRNA candidates. Annotations overlapping at least 70% with RNAz hits are counted as the same ncRNA gene. Sensitivities are given relative to both the genomic loci, and relative to the loci that are contained in our alignments (in square brackets).

pairwise alignments. Indeed, only 40 of the 54 conserved miRNAs overlap to at least 70% with pairwise alignments longer than 40 nt. Only those were scanned by RNAz and were therefore in principle identifiable by our screen. We detected 34 of these 40 miRNA precursor genes at both $p_c = 0.5$ and 0.9.

snoRNAs: Of the 31 known small nucleolar RNA genes, we found 13 at the $p_c = 0.5$ level and only nine at $p_c = 0.9$. Fifteen of the annotated snoRNAs are experimentally verified. We detected 10 of these at $p_c = 0.5$ and nine at $p_c = 0.9$. This amounts to a sensitivity of 0.66 ($p_c = 0.5$) and

0.60 ($p_c = 0.9$), respectively. Of the 16 annotated snoRNAs which are not experimentally verified, we could only identify three at $p_c = 0.5$ and none at $p_c = 0.9$.

RNaseP RNA: The one known copy of RNase P RNA was detected by our screen with a classification probability $p_c = 0.99$. In contrast, we do not find an RNase MRP RNA. If *Caenorhabditis* species have an RNase MRP RNA, it appears to be highly divergent from other species. A recent specific search for this ncRNA did not detect candidates in either *C. elegans* or *C. briggsæ* (Piccinelli et al., 2005).

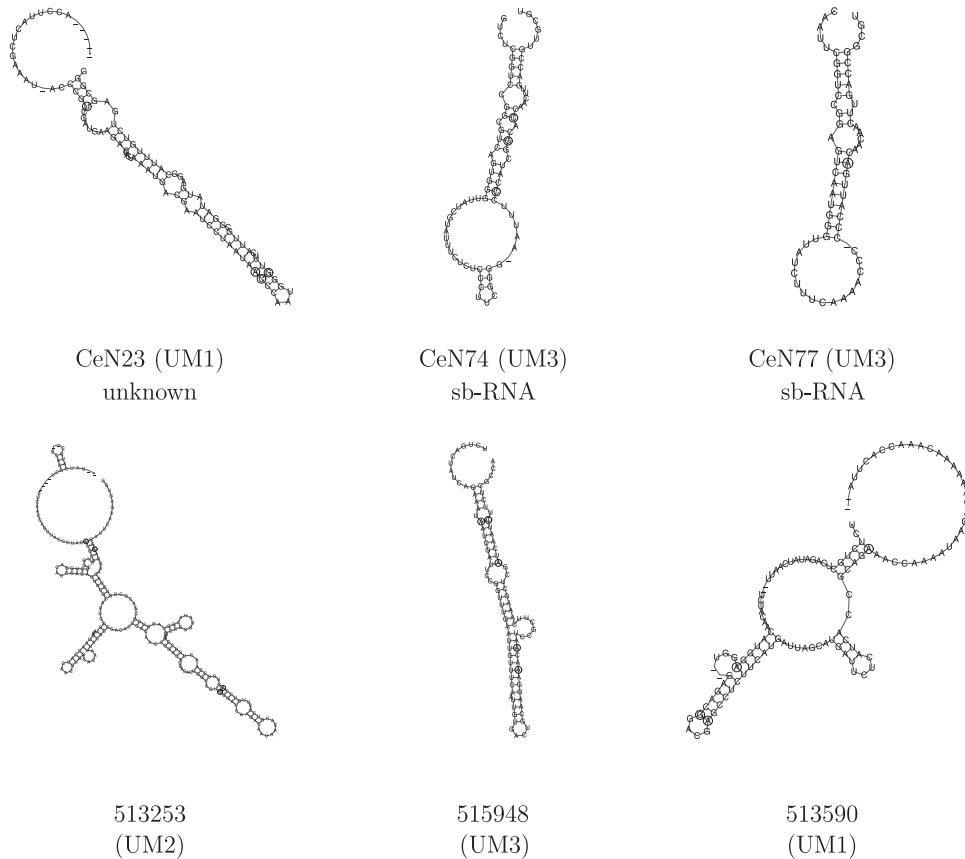


Fig. 3. Examples of ncRNAs in *C. elegans*. The top row shows predicted consensus structures for three ncRNAs experimentally verified by Deng et al. (2006), the associated UM is listed in parentheses. SbRNAs (stem-bulge RNAs) are a set of conserved nematode ncRNAs showing two conserved motifs located at the 5' and 3' end of the transcript, which together form an imperfect stem with a characteristic bulge. The second row shows three RNAz predictions that are associated with one of the UMs reported by Deng et al. (2006). Small circles indicate consistent and compensatory mutations, respectively.

Spliced Leader RNAs: The first form, SL1 RNA, occurs in 10 copies in a tandem repeat in chromosome V, whereas the second form, SL2 RNA, is found in 20 variants. At both $p_c = 0.5$ and 0.9 we found 10 regions in chromosome V which overlap with the 10 known SL1 RNA genes and 16 variants of the SL2 gene.

Spliceosomal RNAs: 12 U1, 19 U2, 5 U4, 13 U5, and 23 U6 spliceosomal RNA genes are known in *C. elegans*. At $p_c = 0.5$, we could identify all the known U1, U2, U4, and U5 genes and five of the U6 loci. At $p_c = 0.9$ we missed two U4, one U5 and four U6 RNA genes.

A recent experimental screen for ncRNAs in *C. elegans* (Deng et al., 2006) described 161 ncRNA transcripts mapping to 198 genomic loci, of which 100 transcripts at 101 loci were unknown before this study. A subset of 69 distinct sequences are putative snoRNA-like transcripts and 31 are functionally unassigned. This set of 100 “novel” ncRNAs provides us at least with a rough estimate

on how our comparative genomics approach performed beyond the realm of the “classical ncRNAs”. Since tRNAs and rRNAs (which form a substantial fraction of the known ncRNAs) are among the evolutionarily best conserved genes, it is to be expected that they are easier to find and recognize as structured RNAs than most other ncRNAs. Indeed, the sensitivity of RNAz on this dataset is significantly lower: we recovered only 23 of the 101 non-Wormbook loci, Table 4.

Tables 3 and 4 show that the sensitivity of our screen can be understood in terms of two effects: the classification accuracy of RNAz, and the probability that the corresponding genomic region is sufficiently conserved to yield a blast-based alignments. With the exception of the annotated microRNAs, which contain a large number of species-specific sequences annotated as “tiny non-coding RNAs” by Ambros et al. (2003), more than 80% of the well-conserved classical ncRNAs (tRNAs, rRNAs, RNase P and MRP, pre-miRNAs,

snRNAs) are contained in alignments, while the fraction is smaller for snoRNAs. In the case of snoRNAs, the Wormbook annotation seems to have a bias towards the few snoRNAs with rather well-conserved sequences.

The sensitivity of RNAz strongly depends on the RNA class. It is typically on the order of 80%, with the notable exception of snoRNAs, which are notoriously hard to recognize based on sequence alignments (Washietl et al., 2005b). For this class we have a sensitivity of one-third to one-half. The low sensitivity for rRNAs is due to the high degree of conservation of the 5S rRNAs between *C. elegans* and *C. briggsæ*, which makes it impossible for RNAz to make a significant decision, because the global alignments lack any covariance information. All other classes of rRNAs, with the exception of 5.8S rRNA which is not conserved in *C. briggsæ*, were successfully identified as structured ncRNAs. We estimate that the sensitivities observed on the dataset from Deng et al. (2006) are probably a plausible order of magnitude of the overall sensitivity of our screen, that is approximately 25–50%.

The support vector machine underlying the RNAz program classified the overwhelming majority of known ncRNAs as “structured RNA” with classification probabilities $p_c = 0.9$, Figure 4. Nevertheless, a significant number of true positives is identified with small values of p_c , indicating that a cutoff at a much higher value of p_c , than 0.5, would significantly decrease the sensitivity.

The distribution of classification probabilities p also provides us with an independent way of estimating the false positive rate, yielding a value of about 11%, in agreement with the observed false positive rate for individual RNAz hits. The much less favorable false positive rate of 49% for the entire screen has its roots in the overlapping RNAz hits and the fact that our procedure by construction systematically overestimates the false positive rate.

RNA-specific promoters

Deng et al. (2006) have identified three putative RNA-specific promoter sequences, denoted by UM1, UM2, and UM3.

UM1 was found at the loci of both snRNAs and a number of other known and novel *C. elegans* ncRNAs, and includes the *C. elegans* proximal sequence element characteristic for spliceosomal snRNAs (Thomas et al., '90; Hernandez, 2001).

UM2 was mainly found upstream of snoRNA genes. However, the motif also bears a strong resemblance to the internal tRNA promoter, and indeed 1,135 UM2 elements overlap 391 of 591 tRNA and 745 of 1,072 tRNA-pseudo-genes according to the annotation by Stricklin et al. (2005).

The third motif, UM3, was only found at the loci of nine transcripts, all belonging to the new class stem-bulge RNAs (sbrRNAs), see Figure 3.

A hand-curated list of ncRNA candidates from the RNAz screen that are associated with one or more of the three UMs was produced in the following way: We combined the positions from the annotation in Stricklin et al. (2005), of the predicted UMs from Deng et al. (2006), from our RNAz screen, and the transcripts reported in Deng et al. (2006). The positions were sorted numerically and combined into clusters if the distance of consecutive annotations was at most 500 nt. Table 5 summarizes the annotation of the putative ncRNAs that are associated with one of the three promoter motifs. Of the 536 initial hits we retained 506, in the remaining cases the promoter was directed away from the RNAz hit. As expected, the majority of the overlaps are tRNA/UM2 combinations.

Intronic ncRNAs

A large fraction of our ncRNA candidates are located in introns. Interesting examples are RNAz-515115 and RNAz-515227, which are located in introns of the putative protein-coding genes *C14A6.5* and *W04E12.5*, respectively. Both genes

TABLE 5. Three upstream motifs discovered by Deng et al. (2006) are associated with RNAz-predicted ncRNAs ($p_c = 0.5$)

Hit type	UM1	UM2	UM3	# Unique hits
tRNAs	0	391	0	391
Deng et al. (2006)	55	6	4	63
Other known	18	2	0	20
Unknown overlapping	1	3	1	4
unknown < 500	16	11	2	28
Total	90	413	7	506
Predicted (MotifLocator)	2,182	2,390	92	4,664

We separately show the association with tRNAs (mostly UM2), the experimentally verified ncRNAs described by Deng et al. (2006), other known ncRNAs according to the Wormbook annotation (Stricklin et al., 2005), and novel candidates. In the latter case we distinguish between motifs that overlap RNAz hits and those in a close distance upstream of the RNAz signal. The number of unique hits can be less than the sum of columns 2–4 if an RNAz-predicted ncRNA is associated to more than one putative promoter sequence.

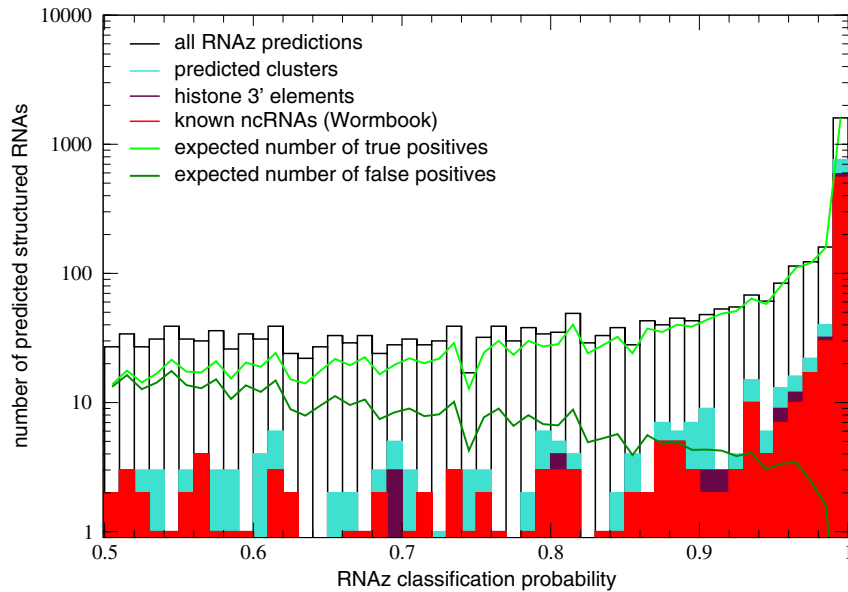
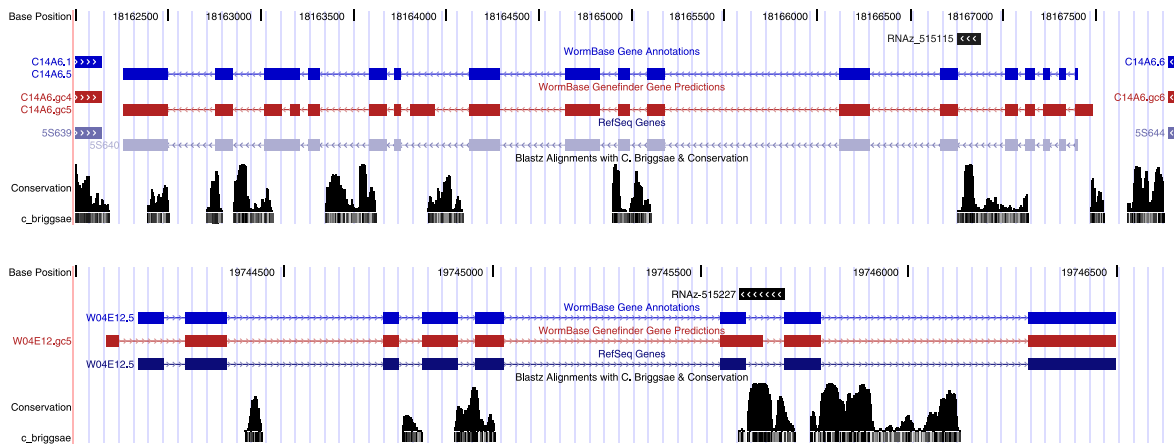
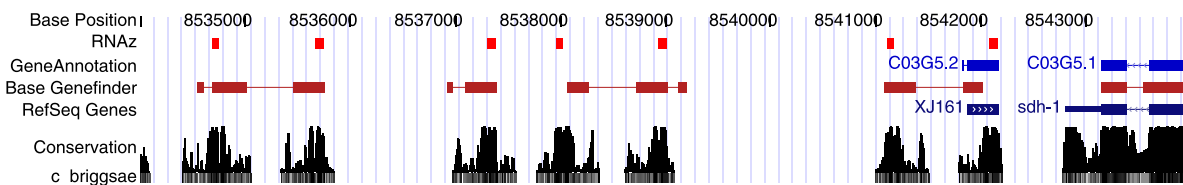


Fig. 4. Distribution of classification probabilities p among RNAz predictions. Colors indicate the fractions of known ncRNAs, predicted histone elements, and predicted families with two or more homologous in each histogram bar.



(a)



(b)

Fig. 5. (a) Location of *RNAz-515115* in the gene *C14A6.5* and *RNAz-515227* in the gene *W04E12.5*. The exonic sequences of those genes are not conserved in *C. briggsae*, while most of the intronic sequence is rather well conserved. (b) Genomic context of seven of the eight members of a cluster of related RNAz hits containing *RNAz-515800*. This cluster is localized at the *C. elegans* X-chromosome. Some of the cluster members overlap with a predicted protein-coding gene.

do not have annotated homologs in *C. briggsae* but their intronic sequences are fairly well-conserved in *C. briggsae*, Figure 5a. Such a structure is reminiscent of “host genes” whose only purpose is

to carry snoRNAs in their introns (Tycowski et al., '96; Tycowski and Steitz, 2001; Bachellerie et al., 2002). However, using snoscan (Lowe and Eddy, '99) to test for C/D box snoRNAs and checking the

secondary consensus structure for two hairpins typical for H/ACA box snoRNAs, failed to produce evidence that *RNAz-515115* and *RNAz-515227* may be snoRNAs. The consensus structures, predicted by RNAz, are well conserved and stable hairpins. We therefore presume that both ncRNA candidates may be miRNAs, which is supported by Rodriguez et al. (2004) where it is shown that also miRNAs occur in “host genes”.

Multi-copy structured RNAs

Clustering the RNAz hits using blastclust with a minimum overlap of 50% and a minimum sequence identity of 50% yields 148 clusters containing a total of 916 RNAz signals and 2,756 individual sequences. Most of the sequences in these clusters are known tRNAs, snRNAs, and other ncRNAs for which an unambiguous annotation is available (725 sequences in 134 clusters).

The largest remaining group is associated with histone genes. An initial analysis of the blastclust results of this group gave six clusters containing a total of 36 sequences that mapped to various annotated histone genes in *C. elegans*. The consensus of these sequences was then compared with the complete *C. elegans* genome, yielding a total of 47 RNAz hits. The motif appears in a region that is annotated as “a consensus sequence thought to contain a putative U7 snRNA” in two GenBank entries of *C. elegans* histone genes X15633 and X15634 (Roberts et al., '89). The U7 snRNA is part of the machinery for processing histone mRNAs (see Dominski and Marzluff, '99) for a review) but so far has not been verified directly in nematodes. We checked for Sm protein binding site, HDE binding site and the snRNA-like promoter element UM1, however with negative result. We conclude that the histone-3'-motif corresponds to the hairpin motif found in histone mRNAs of other species.

Seventeen additional blastclust clusters contain more than two genomic loci. One of these clusters appears to be associated with the multigene family of major sperm proteins, while the 16 other clusters are not related to annotated protein-coding genes. We extracted well-conserved consensus sequences for those elements and then performed a blast search against the database of RNAz hits with $E < 10^{-10}$. In total, we find 216 sequences in 127 blastclust clusters that match one of these consensus sequences. Of the remaining hits, 53 appear twice and 2,577 are single sequence motifs.

A few of these blastclust clusters are localized in one or a few narrow genomic regions, an example is shown in Figure 5b. Consensus sequences of these multi-copy sequences are given in the electronic supplement. In many cases, there is evidence for some form of concerted evolution since the *C. elegans* loci are more similar among themselves than compared to the homologous *C. briggsae* sequences. One of these families forms very stable hairpins and hence might be microRNA precursors.

To date, no telomerase RNA has been reported for *C. elegans* (Jones et al., 2001; Stein et al., 2004; Stricklin et al., 2005), although—in contrast to *Drosophila* (Melnikova, 2005)—this species has a “normal” telomeric repeat sequence. A putative telomerase reverse transcriptase was identified by Malik et al. (2000), which shows several atypical features, suggesting that a unique mechanism of telomere extension may have developed in the *Caenorhabditis* lineage. We therefore further investigated the RNAz hits containing the one-and-a-half repeat of the telomeric template that is characteristic for telomerase RNA (Jones et al., 2001), here CCTAAGCCTTAA. The set of 16 candidates (excluding intronic and UTR elements) does not contain the two putative telomerase RNA transcripts *tts-1* and *tts-2* discussed by Jones et al. (2001): The first is not conserved at sequence level, the second is not classified as structured RNA by RNAz. We checked, using pknotsRG (Reeder and Giegerich, 2004), for a locally stable pseudoknot domain immediately downstream of the template sequence, that is typically observed in vertebrate, ciliates and yeast telomerase RNAs (Chen et al., 2000; Lin et al., 2001; Chen and Greider, 2005). Inspection of the resulting five candidates showed, however, that the *C. briggsae* sequences in these alignments contain longer repetitive stretches of the (reverse complement of the) telomeric repeat sequence, suggesting that they may be false positives arising from aligning the *C. elegans* sequence with repetitive sequences from *C. briggsae*. Consequently, our survey did not detect a plausible candidate for telomerase RNA with a conserved secondary structure.

Novel microRNA candidates

Possible novel microRNA precursors can be identified by a rather crude filtering procedure from the set of all RNAz hits. All RNAz hits are realigned with their homologs in *C. briggsae* and those without a conserved hairpin structure are

discarded. The conserved hairpin structure is extracted and the restricted alignment is scanned with RNAz. It is accepted as a pre-miRNA candidate provided: (1) it forms a stem-loop structure with a total length between 40 and 130, and (2) its z -score is below $z = -3.0$. This threshold value was identified by assessing pairwise alignments of random chosen homologous sequences from the Rfam database (Missal et al., 2005). Figure 6 summarizes the comparison of the filtered RNAz hits with the candidate set proposed by Grad et al. (2003) and the set of known miRNAs of *C. elegans* (Stricklin et al., 2005).

We expect that this simple filter has a rather large false positive rate. In addition, its sensitivity is rather limited, recovering only 22 of the 34 known microRNA precursors detected by our screen (Table 3). A more sophisticated post-processing using, e.g., miRscan (Lim et al., 2003) should provide better results; this program, however, is only available as a web-service and hence not suitable to screen the entire set of thousands of RNAz predictions.

DISCUSSION

The systematic comparison of the genomic DNA of *C. elegans* and *C. briggsae* reveals evidence for a large number of structured RNA motifs. Most are located either within introns or relatively far away from known protein-coding regions. This strongly suggests that the majority of these signals are bona fide non-coding RNAs. The comparable density of signals in introns and intergenic regions, and the

very sparse occurrence of signals in UTRs also tally well with a recent experimental study of *C. elegans* ncRNAs, in which 56% of 198 loci were found overlapping an intron vs. 42% in intergenic regions, and only very few loci found in UTRs (Deng et al., 2006). The argument for RNAz signals representing actual ncRNA loci is further supported by the fact that some subclasses of both intronic and intergenic ncRNAs are associated with UMs that appear to be characteristic for *C. elegans* ncRNAs.

With an estimated sensitivity of around 50% we therefore predict the total number of structured RNA motifs at 3,000–4,000, comprising about 1 Mb of the genome. We emphasize that our survey is based on the RNAz program (Washietl et al., 2005a), which is based on both primary sequence conservation and secondary structure conservation. Both are factors which may reduce the sensitivity of our screen, because much of the recently detected non-coding transcription is poorly conserved between relatively close species (Hyashizaki, 2004; Wang et al., 2004) and RNAs which might perform their function without the need for a well-defined structure, for example, anti-sense transcripts (FANTOM Consortium et al., 2005), are not detectable by our method. This could also explain the small fraction of RNAz hits that are associated with UMs. Nevertheless, estimates based on intron conservation and conserved UMs have arrived at figures in the range 1,600–4,100 different ncRNAs *C. elegans* (Deng et al., 2006), thus lending support to our estimate for structured RNA motifs.

These numbers have to be compared with estimates for the ncRNA content in other genomes. An RNAz survey based on the most conserved parts of the vertebrate genomes estimates that the ncRNA content of mammalian genomes is comparable to their protein-coding genes (Washietl et al., 2005b), and hence at least an order of magnitude larger than in nematodes. In contrast, the predicted number of structured RNAs in the urochordate *Ciona intestinalis* is comparable to our results for the nematodes (Missal et al., 2005). This indicates that higher vertebrates have dramatically expanded their ncRNA inventory relative to their complement of protein-coding genes. This is consistent with the assumption that the function of the ncRNAs is primarily regulatory (Mattick, 2003, 2004).

The partial analysis of the predicted *C. elegans* ncRNAs highlights important open problems in computational RNomics. With the exception of rRNAs and tRNAs, efficient and reliable tools for

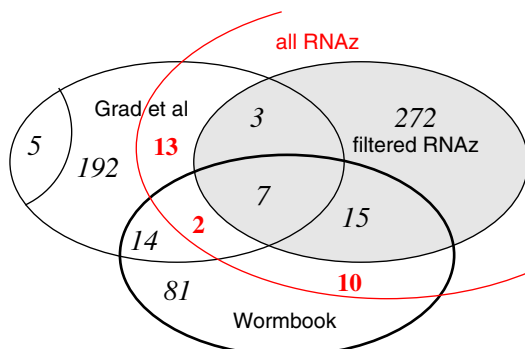


Fig. 6. Comparison of microRNA candidates derived from the RNAz screen with the 222 microRNA candidates from (Grad et al., 2003) (four of these could not be mapped to the WS120 genome assembly and one is apparently a repetitive element) and the 117 known miRNAs listed by Stricklin et al. (2005). Numbers in red refer to RNAz hits that did not pass the simple filter described in the text.

classifying ncRNAs are not available. Recent advances in snoRNA detection (Schattner et al., 2005) still require explicit knowledge of the modification targets and hence cannot correctly classify snoRNAs with non-canonical targets such as mRNAs. Even for microRNAs, reliable classification tools that could be used for genome-wide studies are not available. Indeed, for the majority of predicted structured RNAs we have no annotation at all, and the overwhelming majority of them have no homologs outside the nematodes that could be detected unambiguously by means of sequence comparison.

In the near future, several additional nematode genomes will become available, including both distant species with a parasitic lifestyle such as *Brugia malayi* (Ghedini et al., 2004) and close relatives such as *C. remanei*.⁴ These additions will bring the total number of sequenced nematode genomes to a total of ten. A denser taxon coverage of nematodes will undoubtedly also increase the specificity of the non-coding RNA annotation in this phylum. Of particular interest are the close relatives within the *Caenorhabditis* taxon, because for these the sequence similarity is sufficient to obtain reasonable-quality genomic alignments. For example, 3,066 of the 3,672 RNAz predicts show significant sequence homology (blast with $E < 10^{-5}$) in the current assembly⁵ of the *C. remanei* genome. Of these, 1,872 are classified as structured RNAs using RNAz on multiple alignments composed of the sequences from all three species.

In contrast, only 694 hits are found in a comparison with the *B. malayi* genome.⁶ More than 90% of these can be identified as tRNAs and other well-known ncRNAs. Since both sensitivity and specificity of comparative genomics approaches such as RNAz increase with the amount of available data, a reliable annotation of nematode structured RNAs is at least within reach.

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⁴<http://www.genome.wustl.edu/projects/cremanei/>
⁵<ftp://genome.wustl.edu/pub/seqmgr/remanei/pcap/remanei-041227/>

⁶<http://www.tigr.org/>. "Preliminary sequence data for *B. malayi* are deposited regularly into the GSS division of GenBank. The sequencing effort is part of the International *Brugia* Genome Sequencing Project and is supported by an award from the National Institute of Allergy and Infectious Diseases, National Institutes of Health."

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