Mirtrons are a special type of pre-miRNA which originate from intronic regions and are spliced directly from the transcript instead of being processed by Drosha. The splicing mechanism is better understood for the processing of mRNA for which was established that there is a characteristic CG content around splice sites. Here we analyse the CG-content ratio of pre-miRNAs and mirtrons and compare them with their genomic neighbourhood in an attempt to establish key properties which are easy to evaluate and to understand their biogenesis. We propose a simple log-ratio of the CG-content comparing the precursor sequence and is flanking region. We discovered that *Caenorhabditis elegans* and *Drosophila melanogaster* mirtrons, so far without exception, have smaller CG-content than their genomic neighbourhood. This is markedly different from usual pre-miRNAs which mostly have larger CG-content when compared to their genomic neighbourhood. We also analysed some mammalian and primate mirtrons which, in contrast the invertebrate mirtrons, have higher CG-content ratio.

**INTRODUCTION**

During the last decade, a wealth of small RNAs were discovered and with them new classes of biological regulators emerged. Among those, microRNAs (or miRNAs) due to their crucial role in genomic regulation are perhaps the most intensively studied. miRNAs are involved in the regulation of numerous cellular processes including differentiation, development, apoptosis, proliferation, the stress response and they change the expression of genes during differentiation, development, apoptosis, proliferation, the stress response and they change the expression of genes in several human diseases such as diabetes, cancer and neuromuscular dystrophy [1–3].

miRNAs are non-coding RNAs first identified in 1993 in the nematode *Caenorhabditis elegans* [4]. Canonical miRNAs are derived from primary miRNA transcripts (pri-miRNA), usually long nucleotide sequences that form specific hairpin-shaped stem–loop secondary structures. Pri-miRNA may originate one or more hairpins typically with 55–70 nucleotide (nt) in length. In animals, pri-miRNAs are cleaved by the nuclear Drosha RNase III enzyme to release precursor miRNA (pre-miRNA) hairpins. These are then transported to the cytoplasm by Exportin-5 (Exp5) and cleaved by the Dicer RNase III enzyme to generate a very short mature miRNA (22–25nt), is incorporated into a RISC miRNA/miRNA* duplex [5]. One of the strands, called mature miRNA (22–25nt), is incorporated into a RISC complex (RNA induced silencing complex) and guides the complex to the target mRNA to regulate gene expression while the other strand seems to take on other biological functions [5–7]. In animals, most of the miRNA functions are related to down-regulation of genes.

Ruby et al. [8] showed the existence of intronic pre-miRNAs in *Drosophila melanogaster* and *C. elegans* that bypass Drosha processing providing an alternative pathway for miRNA biogenesis [8]. These pre-miRNAs were called ‘mirtrons’ and the main difference between them and canonical miRNAs is that intronic sequences form lariats and the mirtrons are originated by splicing [8–10]. Flynt et al. [11] reclassified a subset of mirtrons in *D. melanogaster* as “tailed mirtrons”, which have substantial 3′ overhangs and are targets of exosome-mediated 3′ – 5′ trimming, which allows functional pre-miRNA to be generated. The existence of mirtrons in mammalians (human, macaque, chimpanzee, rat and/or mouse) was reported by Berezikov et al. [10] where they identified, using computational and experimental strategies, 3 well conserved mirtrons expressed in diverse mammals, 16 primate specific mirtrons, and 46 candidate mirtrons in primates.

For mRNA, which is processed by splicing, Zhang et al. [12] determined that there is a characteristic CG content around splice sites. Also, it was shown that alternative splicing is promoted by the secondary RNA structure [13] which is strongly determined by CG content [14]. MicroRNAs are co-expressed with miRNAs [15] and, in particular, mirtrons are seemingly not processed by the Drosha microprocessor but by splicing only. With splicing being dependent on thermodynamic stability could there be some characteristic CG content which would set aside mirtrons from ordinary pre-miRNAs? Of special interest would be properties which would help to understand the splicing mechanism proposed for mirtrons [6].

Here we set out to characterise precursor sequences of miRNAs and mirtrons in terms of CG-content and also Gibbs free energies for *D. melanogaster* and *C. elegans*. We found that the CG-content shows marked differences for both types of small RNA. Also, we performed the same analysis for mammalian mirtrons reported by Berezikov et al. [10], again our results show important differences albeit opposite of those for the two invertebrates.
METHODS

To characterise the small RNAs we compare the CG-content of the precursor sequences which originate the pre-miRNAs and mirtrons to the CG-content of their neighbouring regions. The rationale for this approach is that if the neighbouring DNA sequence has an important difference in thermodynamic stability, when compared to the precursor sequence there should be tell tale signs of it in the CG-content fractions. We define the CG-content fraction as

\[ f = \frac{\text{number of } C \text{ and } G \text{ nucleotides}}{\text{total number of nucleotides}} \] (1)

Two types of CG-content fractions are used, one \( f_p \) is related to either the precursor miRNA or the precursor mirtron. The other \( f_N \) accounts for the total CG-contents of the 150 base pairs downstream and upstream of the precursor sequence which forms the neighbourhood of the precursor. The flanking sequence length was chosen to be of the same order of magnitude of the length of canonical pre-miRNAs. We performed the same analysis with longer flanking sequences (up to 250 nt, not presented), but found no difference from the results reported in this work. Both CG-contents are combined to form a log-ratio between the precursor and its neighbourhood

\[ R = \log_2 \left( \frac{f_p}{f_N} \right) \] (2)

A positive ratio means that the CG-content \( f_P \) of the precursor sequence is larger than that of its neighbours. Since CG-content is related to thermodynamic stability we may infer that \( R > 0 \) generally means that the flanking DNA region is less stable than the precursor region. To ease the notation we use

\[ R^+ \rightarrow R > 0 \quad \text{precursor region has larger CG-content, } f_P > f_N \]
\[ R^- \rightarrow R < 0 \quad \text{flanking region has larger CG-content, } f_N > f_P \]

To evaluate the statistical significance of our findings we use the Kolmogorov-Smirnov test [17]. Even though this statistical test is well established, given the question which is posed in this work it is perhaps more intuitive and simpler to quantify the significance by using simple combinatorial probabilities. Therefore, we also calculate the probability \( p^- \) of drawing \( k \) pre-miRNAs, all with \( R^- \), purely by chance

\[ p^- = \left( \frac{n^-}{n^- + n^+} \right)^k \] (3)

where \( n^- \) and \( n^+ \) are number of known pre-miRNAs with \( R^- \) and \( R^+ \), respectively.

The database used to obtain the precursor miRNA and mirtrons of \( D. \ melanogaster \) and \( C. \ elegans \) was from mirBASE version 16 [18, 19], which is one of the main on-line repositories for microRNA sequences. For extracting the flanking sequences we used the complete genome file of \( D. \ melanogaster \) version r5.34 [20] and version WS223 for \( C. \ elegans \) [21]. We retrieved the precursor miRNA/mirtron sequences by searching for an exact match within the complete genome files. For each sequence four types of matches were performed: the original sequence, the reversed sequence, the complementary sequence and the reversed-complementary sequence.

The mirtrons reported by Berezikov et al. [10] were collected from the supplemental data, the neighbouring sequences for each these mirtrons were obtained from Ensemble API and databases [22].

To complete our analysis we also calculated the average Gibbs free energies of mirtrons and ordinary pre-miRNA. In this work we use the RNAfold program from the Vienna package [23] with default parameters to obtain the Gibbs free energies, \( \Delta G \).

RESULTS AND DISCUSSION

![Figure 1](image-url) FIG. 1. CG-content ratio \( R \) distribution for a) 151 canonical miRNAs and b) 18 mirtrons of \( D. \ melanogaster \) and c) 170 canonical miRNAs and d) 5 mirtrons of \( C. \ elegans \).

In Fig. 1 we show the distribution of CG-content log-ratio \( R \) for canonical pre-miRNAs and mirtrons, defined in Methods, of \( D. \ melanogaster \) and \( C. \ elegans \). The content log-ratio \( R \) for canonical pre-miRNAs, Fig. 1a, is roughly gaussian with a peak around \( R = 0 \). This means that for this type pre-miRNA there appears to be no strong preferential ratio for CG-content within the precursor sequence and its neighbours, although a bias towards \( R^+ \) is clearly noticeable. In stark contrast, all 18 mirtrons of \( D. \ melanogaster \) have \( R < 0 \) (\( R^- \)) as shown in Fig. 1b. Even though the number of reported mirtrons is still small, the probability of picking 18 small RNAs with \( R^- \) by chance alone, considering the distribution for canonical pre-miRNA, is \( p^- = 9.1 \times 10^{-9} \), see
Eq. (3). The Kolmogorov-Smirnov distribution test yields a similar gaussian shaped distribution of the ratio $R$ with $p^- = 7.8 \times 10^{-9}$ which essentially confirms the simple combinatorial probability. Therefore, the occurrence of 18 $R^-$ pre-miRNA entirely by chance is very unlikely.

Some authors describe mirtrons as tightly packed between exons [3], but in our analysis we have found that this is not the case. Most mirtrons are surrounded by intronic sequences not exons. This seems consistent if one considers that intronic regions of D. melanogaster are about 750 to 1000 nt in length on average [24] and that mirtrons are typically 60 nt in length. Therefore, $R^-$ means that the immediate flanking region which is also intronic is more stable than the precursor region. One possible explanation for the predominance of $R^-$ would be if intronic regions were of highest CG-content. However, the intronic regions of D. melanogaster have one of the smallest CG-content in this genome: 0.44 as compared to 0.52 for coding regions. The fact that the surrounding region of mirtrons has a higher CG content, which is unusual for intronic regions, suggest a role in the processing of these special types of miRNAs. Therefore, we may speculate that $R^-$ may play a role in the mirtron splicing mechanism in a similar fashion to what happens for messenger RNA [12].

For canonical C. elegans pre-miRNAs we observe a similar gaussian shaped distribution of the ratio $R$ (Fig. 3) but with strong bias towards $R^+$. Tab. I shows that the $n^+ : n^-$ ratio of three $R^+$ pre-miRNAs for each $R^-$ pre-miRNA. To date there are only five mirtrons reported and they all show $R^-$ (Fig. 1), similar to the mirtrons of D. melanogaster. Even though this number is very small, it is still intriguing given the strong bias toward $R^+$ in canonical pre-miRNAs. Indeed, the probability of picking 5 pre-miRNAs all with $R^-$ is small, the combinatorial probability being $p^- = 6.3 \times 10^{-4}$. Again, the Kolmogorov-Smirnov test provides $5.5 \times 10^{-4}$ in agreement with the combinatorial probability.

To complete our comparative analysis of mirtrons and canonical pre-miRNAs, we also calculated the Gibbs free energies. Clearly, given the $R^-$ nature of the mirtrons, one would expect these to be generally less stable than the average pre-miRNAs. In Fig. 2 we show the distribution of free energy $\Delta G$ for both invertebrates, and detailed quantities are also given in Tab. II. Except for one notable exception, all mirtrons show $\Delta G$ larger than $-30$ kcal/mol, confirming their instability. In contrast, canonical pre-miRNAs are distributed over a much larger range of energies. Certainly, the fact that mirtrons are much shorter than canonical pre-miRNAs, 60 nt compared to 90 nt on average, largely accounts for this. But is a free energy larger than $-30$ kcal/mol sufficient to result in $R^-$? To answer this, we isolated all canonical pre-miRNAs with $R^-$ and recalculated the their $\Delta G$ distribution, which are shown as red bars in Figs. 2a and 2b: and summarised in Tab. III. Essentially, we find a considerable number of $R^+$ pre-miRNAs with $\Delta G > -30$ kcal/mol. In other words, a pre-miRNAs with $\Delta G > -30$ kcal/mol does not imply in $R^-$. Therefore, the free energy distribution alone does not explain why all mirtrons of D. melanogaster and C. elegans are $R^-$. The next question is whether other types of reported mirtrons, such as primate and mammalian mirtrons show the same $R$ distribution as D. melanogaster and C. elegans? As shown in Tab. III in terms of CG-content ration $R$ and average free energy $\Delta G$ these mirtrons appear not to be biased to any particular value. Berezikov et al. [10] found that the GC content of mammalian mirtrons was much higher than that of invertebrate miRNAs but, in comparison with their neighbours regions, we found that they tend generally to $R^+$ (precursor region has larger CG-content), see Fig. 3. We have not attempted to generate the distribution of mammalian pre-miRNAs due to the number of large genomes which would have to be processed.

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**Table I.** CG-content ration $R$ and free energy $\Delta G$ characteristics and of canonical pre-miRNAs and mirtrons of invertebrates. Also shown are number of sequences $n^+ \text{ with } R^+$, average CG-content ratio $\langle R \rangle$, average free energy $\langle \Delta G \rangle$ and average precursor length $\langle N \rangle$.

| Organism     | RNA type  | $n^+$ | $n^-$ | $R$ | $\langle \Delta G \rangle$ (kcal/mol) | $\langle N \rangle$ (nt) |
|--------------|-----------|-------|-------|-----|-------------------------------------|-------------------------|
| C. elegans   | mirtrons  | 5     | 0     | 5   | 0.81 ± 0.08                         | 20.28 ± 4.21            |
| D. melanogaster | mirtrons | 18    | 0     | 18  | 0.76 ± 0.10                         | -22.03 ± 6.55           |
| D. melanogaster | tailed mirtrons | 7     | 2     | 5   | 1.8 0.84 ± 0.24                     | -20.36 ± 10.19          |
| C. elegans   | canonical miRNAs | 170   | 131   | 39  | 3.3 1.18 ± 0.23                     | -35.10 ± 9.09           |
| D. melanogaster | canonical miRNAs | 151   | 97    | 54  | 1.8 ± 0.21                         | -33.89 ± 8.25           |

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**Table II.** CG-content ratio $R$ and free energy $\Delta G$ characteristics and of canonical miRNAs considering only those with $R^-$.}

| Organism     | RNA type  | $R$ | $\langle R^- \rangle$ | $\langle \Delta G \rangle$ (kcal/mol) | $\langle N \rangle$ (nt) |
|--------------|-----------|-----|-----------------------|-------------------------------------|-------------------------|
| C. elegans   | canonical miRNAs | 39  | 0.90 ± 0.08            | -30.17 ± 8.72                      | 90.95 ± 17.50           |
| D. melanogaster | canonical miRNAs | 54  | 0.87 ± 0.10            | -30.0 ± 5.40                       | 90.40 ± 11.78           |
TABLE III. CG-content ratio $R$ and free energy $\Delta G$ characteristics of specific vertebrate mirtrons.

| Organism      | RNA type        | total $n^+$ | $n^-$ | $n^+:n^-$ | $\langle R \rangle$ | $\Delta G$ (kcal/mol) | $\langle N \rangle$ (nt) |
|---------------|-----------------|-------------|-------|------------|----------------------|------------------------|------------------------|
| mammals       | putative mirtrons | 13          | 8     | 5          | 1.5 : 1              | $0.98 \pm 0.16$         | $-30.02 \pm 13.20$     | $87.92 \pm 9.52$       |
| primates      | specific mirtrons | 16          | 13    | 3          | 4.3 : 1              | $1.12 \pm 0.11$         | $-30.32 \pm 11.69$     | $83.62 \pm 24.36$      |
| primates      | candidate mirtrons | 45          | 40    | 5          | 8 : 1                | $1.11 \pm 0.09$         | $-41.18 \pm 12.39$     | $90.44 \pm 21.94$      |

Ref. 10 reports 46 candidates mirtrons for primates, yet supplementary tables only show 45 sequences.

FIG. 2. Average free energy $\Delta G$ distribution for a) 151 canonical pre-miRNAs and b) 18 mirtrons of D. melanogaster and c) 170 canonical miRNAs and d) 5 mirtrons of C. elegans. Red bars are for $R^-$ miRNAs.

FIG. 3. CG-content ratio $R$ distribution for a) 13 putative mammalian mirtrons, b) 16 specific primate mirtrons and c) 46 candidate primate mirtrons.

CONCLUSIONS

We have introduced the concept of CG-content log-ratio of precursor sequences and flanking regions and discovered that all D. melanogaster and C. elegans mirtrons are $R^-$. This cannot be explained by the CG-content of the intronic region and neither by the fact that mirtrons are generally shorter and less stable than pre-miRNAs. Usual pre-miRNAs of these organisms only show a moderate bias towards $R^+$. This finding appears to support the notion that mirtrons are spliced in a similar fashion to mRNA instead of being processed by Drosha. For mammalian mirtrons we have found no such bias, but we noticed that these also display several important differences when compared to the vertebrate mirtrons which were considered in this work, such as differences in length and free energy.

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