Letter - Discovery

Title:

Run or die in the evolution of new microRNAs - Testing the Red Queen hypothesis on *de novo* new genes

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Abstract:

The Red Queen hypothesis depicts evolution as the continual struggle to adapt. According to this hypothesis, new genes, especially those originating from non-genic sequences (i.e., *de novo* genes), are eliminated unless they evolve continually in adaptation to a changing environment. Here, we analyze two Drosophila *de novo* miRNAs that are expressed in a testis-specific manner with very high rates of evolution in their DNA sequence. We knocked out these miRNAs in two sibling species and investigated their contributions to different fitness components. We observed that the fitness contributions of miR-975 in *D. simulans* seem positive, in contrast to its neutral contributions in *D. melanogaster*, while miR-983 appears to have negative contributions in both species, as the fitness of the knockout mutant increases. As predicted by the Red Queen hypothesis, the fitness difference of these *de novo* miRNAs indicates their different fates.

Introduction:

Organisms evolve to adapt to changing environments via the acquisition of adaptive traits. Adaptations are generally transient because continual changes are needed to keep pace with a moving world, as the Red Queen advises Alice (Carroll 1893). Van Valen (1973) emphasized biotic factors as the driving selective force, which has subsequently been expanded to include the coevolution of host/parasite, prey/predator, male/female (sexual selection), as well as antagonistic evolution (Tobler and Schlupp 2005; King, et al. 2009; Brockhurst 2011; Liow, et al. 2011; Morran, et al. 2011; Brockhurst, et al. 2014; Nordbotten and Stenseth 2016; Greenspoon and Mideo 2017). Nevertheless, the essence of this metaphor is continual adaptation, which could be driven by biotic, abiotic or both factors. Van Valen (1973) first reported that genera and species, regardless of their age, often become extinct when they cannot sufficiently adapt. We proposed to test the Red Queen hypothesis by studying the evolution of new genes, which can be compared to those in younger taxa. If the

adaptive landscape shifts rapidly, as posited by the Red Queen hypothesis, this may suggest that newly emerging genes have been under strong pressure to evolve continually or face elimination.

New genes fall in two broad categories: those that originated from reshuffling of existing gene components and those emerging *de novo* from non-genic sequences (Long, et al. 2013; Andersson, et al. 2015; Schlotterer 2015; VanKuren and Long 2018). The majority of new genes belong to the former, comprising duplicated, (retro-)transposed, and chimeric genes (Chen, et al. 2013). In comparison, *de novo* genes are more appropriate for testing the Red Queen hypothesis because their products are truly new in the cellular environment. The most common source of *de novo* genes may be small non-coding loci represented by microRNAs (miRNAs) (Lu, Shen, et al. 2008; Lyu, et al. 2014). Given the ease with which miRNAs can be formed *de novo* (Meunier, et al. 2013; Lyu, et al. 2014), many could have emerged adaptively (Lu, Fu, et al. 2008; Mohammed, et al. 2013; Lyu, et al. 2014; Mohammed, et al. 2014; Mohammed, et al. 2018) although the majority may be functionless (Lu, Shen, et al. 2008; Berezikov, et al. 2010), or dead-on-arrival (Petrov, et al. 1996; Petrov and Hartl 1998). Following the molecular evolutionary analysis performed by Lyu et al. (2014), we use the term "*de novo* miRNAs" to specifically refer to the first group of adaptive miRNAs. We hypothesize that "*de novo* miRNAs" must continuously evolve and survive in different fitness landscapes or face elimination, as inferred by the Red Queen hypothesis.

In order to test this possibility, experiments must be conducted in close sibling species, as their divergence time is sufficiently short such that rapidly evolving genes remain identifiable. More importantly, they provide distinct fitness landscapes, and thus *de novo* genes might end up with different fates. Therefore, we performed functional and molecular analysis on two typical *de novo* miRNA genes (miR-983 and miR-975) in closely related *Drosophila* species that diverged about 5 – 50 Myrs ago (Kumar, et al. 2017).

61 **Result:**

62 **Sequence and expression divergence of miR-983 and miR-975 in *Drosophila***

63 We collected and sequenced 14 small RNA libraries from the testes of 5 *Drosophila* species to survey the
64 sequence and expression patterns of the *de novo* miRNAs (**Fig. 1A**). miR-975 is present in all *Drosophila*
65 species, while miR-983 is absent in *D. virilis*. Similar to most young miRNA genes, these two *de novo*
66 miRNAs have rapidly evolved across species, as divergence can be seen in both the seed and non-seed regions
67 compared to conserved miRNAs (bantam and miR-184) (**Fig. 1B**). The number of substitutions in these
68 miRNAs is far higher than can be sustained by the neutral process, suggesting adaptive evolution (Lyu, et al.
69 2014; Mohammed, et al. 2014). In contrast, bantam and miR-184 are highly conserved across *Drosophila*
70 species, even in non-seed regions, a common trend among older miRNAs.

71 We then examined the expression of these miRNAs in different species and multiple lines of *D.*
72 *melanogaster* and *D. virilis* as available (**Table S1**). **Figure 1A** shows the expression of bantam, miR-184,
73 miR-975 and miR-983 relative to all miRNAs detected in the testes. Bantam and miR-184 are highly expressed
74 and almost always ranked in the first quantile among the evaluated *Drosophila* species. In contrast, miR-975
75 shows substantial variation between and even within species. In *D. simluans*, *D. erecta* and *D. virillis*, its
76 expression is high and comparable with bantam and miR-184, while in *D. sechellia*, its expression drops below
77 the average level. In *D. melanogaster*, miR-975 expression is generally low, ranking in or close to the fourth
78 quantile in six of nine samples. Compared with miR-975, miR-983 shows less variation across species and
79 has higher expression, except in *D. simulans*.

80 Each miRNA locus, either new or old, can potentially generate two mature products, which are usually
81 referred as the -5p and -3p arm, respectively (Ameres and Zamore 2013). The starting position of each arm
82 may shift, resulting in distinct mature miRNAs. For example, miR-983-3p has four distinct seeds in four
83 sibling species, and miR-975-5p has three distinct seeds in five sibling species (**Fig. 1B**), which determines

84 the repertoire of target genes and, presumably, their functions. We also observed arm switching for young
85 miRNAs among different species (Berezikov 2011; Zhao, et al. 2018; Kim, et al. 2020). **Figure 1C** shows that
86 the conserved miRNAs (bantam and miR-184) predominantly express one product. In contrast, the expression
87 levels of miR-983 and miR-975 are divergent among *Drosophila* species. For example, expression of miR-
88 975-3p is strong in *D. virilis* but rather weak in *D. simluans, D. sechellia* and *D. erecta* (**Fig. 1C**). These three
89 sibling species show arm-switching vis-à-vis *D. virilis,* with the 5p miR being the major form. We should also
90 note that the divergence pattern can be observed even between different lines of the same *D. melanogaster*
91 species.

92 In short, the mature products of *de novo* miRNAs are extremely variable in sequence, expression and
93 biogenesis among species, which indicates their rapid evolution across different species.

94 **Rapid functional evolution of miR-983 and miR-975**

95 We then surveyed the functional evolution of miR-983 and miR-975 by constructing loss-of-function
96 mutants. For a direct comparison with our previous constructs in *D. melanogaster* (Lu, et al. 2018), we used
97 TALEN (Transcription activator-like effector nuclease) to construct miRNA mutants in *D. simulans*, a non-
98 model organism (**Fig. S1**). Mutants were confirmed by PCR followed by DNA sequencing (**Fig. 2A**), and the
99 absence of mature miRNA expression was further validated by RT-qPCR in testes (**Fig. S2**).

100 These KO lines were then subjected to a series of analyses focusing on the typical fitness components
101 associated with male reproductive functions, including male mating success, male ability to repress female re-
102 mating, sperm competition, and male fertility. Male mating success was evaluated by presenting virgin and
103 non-virgin females to the miRNA KO males and calculating the male mating success rate (Lu, et al. 2018).
104 We measured a male's ability to repress female re-mating according to the re-mating rejection rate of the
105 female (Lu, et al. 2018). Sperm competition was analyzed using by both offense and defense assays (Yeh, et
106 al. 2013; Civetta and Ranz 2019), and we measured male fertility by mating males to two batches of virgin

107 females in series, which aims to exhaust sperm reservoirs in order to obtain an accurate estimate of sperm
108 production (Liufu, et al. 2017).

109 We compared miR-975 KO in *D. simulans* vs. *D. melanogaster*, which shows no defects in all surveyed
110 components (**Fig. 2B;** also see Lu et al. (2018)). Surprisingly, miR-975 KO in *D. simulans* resulted in severe
111 defects in male mating success (**Fig. 2C**). More than one-third (16 of 43) of mutant *D. simulans* males failed
112 to mate with virgin female, compared with only 1 of 41 wild type males. The mating defect was even more
113 pronounced when miR-975 KO males were presented with non-virgin females, in which the mating success
114 rate drops to only 15% (8 of 53) compared with 73% (32 of 44) in wild type flies. This loss of mating success
115 would substantially decrease fitness due to fewer progeny produced in wild. We also found that miR-975 KO
116 males appeared to have reduced ability to repress female re-mating, but this did not reach statistical
117 significance, perhaps due to the limited numbers tested. According to these data, miR-975 KO seems to have
118 a neutral effect in *D. melanogaster* but was deleterious in *D. simulans*, which echoes its expression difference
119 between the two species (**Fig. 1A**). Compared with the distinct phenotypic effects of miR-975 KO, effects of
120 miR-983 KO were more similar between the two species (**Fig. 2D&E**). Intriguingly, we found that miR-983
121 KO males had higher offense scores, indicating that the sperm of miR-983 KO males are more competitive.
122 This enhanced performance is offense-specific because we did not observe an elevated score in the defense
123 assay (**Fig. S3**). In addition, we observed that miR-983 KO males likely have a stronger ability to repress
124 female re-mating (**Fig. 2D&E**), and miR-983 KO males produce slightly more progeny in *D. melanogaster*
125 (**Fig. S4**). This observation is surprising because the absence of a highly expressed miRNA might be expected
126 to reduce rather than improves fitness (See further discussion below).

127 Collectively, these functional assays demonstrate the diverse contributions of the studied miRNAs to male
128 reproduction functions in different species, suggesting the rapid functional evolution of these *de novo* miRNAs.

129 **Transcriptome divergence between species**

130 The phenotypic consequences of the *de novo* miRNA deletions (miR-983 KO and miR-975 KO) between
131 *D. melanogaster* and *D. simulans* should be a manifestation of the underlying transcriptomic dysregulation.
132 We next compared the number of dysregulated genes in the two species upon miRNA knockout. For miR-975,
133 we found only 396 genes that were significantly dysregulated in *D. melanogaster* (**Fig. 3A**) whereas 1369
134 genes were dysregulated in *D. simulans* (**Fig. 3B**), a 3-fold difference. This discrepancy, similar to the
135 differences observed in phenotypic assays, might be due to the weak expression of miR-975 in *D.*
136 *melanogaster* compared to a much higher expression level in *D. simulans* (**Fig. 1A**). Indeed, for miR-983,
137 which has a similar expression in the two species, the magnitude of transcriptomic change was much closer,
138 with 854 genes dysregulated in *D. melanogaster* (**Fig. 3C**) and 1151 in *D. simulans* (**Fig. 3D**). We found the
139 overlap among these mis-regulated genes to be minimal between the two species (**Fig. S5**). Therefore, it is not
140 surprising that dysregulated genes are enriched in distinct gene ontology terms (**Table S2**), indicating
141 significant divergence in target gene regulation between the two species, which results in downstream effects
142 on the entire transcriptome.

143 Discussion:

144 Van Valen suggested that lower taxa (e.g. genera) are subjected to more unpredictable selective pressure
145 compared to the higher taxa (e.g. classes or orders). Hence, these lower groups often go extinct, even following
146 a period of adaptive existence. In this study, we demonstrated this scenario from a gene-level perspective by
147 focusing on *de novo* miRNA genes.

148 A unique feature of this study is the choice of genes. The selected *de novo* genes must have been adaptive
149 in the past. Many earlier studies have proposed high turnover rates of new elements in the genome, for both
150 coding (Tautz and Domazet-Loso 2011; Palmieri, et al. 2014) and non-coding genes (Lu, Shen, et al. 2008;

151 Meunier, et al. 2013; Lyu, et al. 2014). As these new elements are mostly non-functional, they may belong to
152 a class often characterized as dead-on-arrival (DOA) elements. The high turnover of neutral DOA elements is
153 unsurprising, whereas new genes that arrive in an adaptive manner are expected to remain functional.

154 Indeed, our functional assays revealed that miR-975 is functional in *D. simulans*, as deleting the miRNA
155 substantially decreased male mating success, and hence fitness (**Fig. 2C**). However, when the same gene
156 continuously evolves in *D. melanogaster*, a distinct fitness landscape, it may end up in a completely different
157 sequence and functional space due to its neutral contributions to fitness components (**Fig. 2B**). These
158 observations are direct evidence for the Red Queen hypothesis from a gene-level perspective.

159 In contrast, our assays showed that knockout of miR-983 increased fitness in general for both species.
160 This negative contribution of miR-983 is unexpected given its abundant expression level in both species. In
161 our previous study, we noted that the total fitness effect of miRNAs may be "quasi-neutral", which means the
162 miRNA KO flies could perform better in some fitness components and worse in others (Lu, et al. 2018). We
163 only measured four components of male reproduction, which may not be sufficient to draw definitive
164 conclusions. However, *de novo* genes die regularly, not only due to complete loss of functionality, but also
165 because of potentially opposing positive and negative fitness effects. This observation indicates that miR-983
166 may be on an evolutionary death trajectory, as it shows modest negative effects on some fitness components.

167 The driving force underlying the continual evolution of new genes has been widely discussed (McLysaght
168 and Hurst 2016). Here we should mention that most new genes are specific to male reproduction (Kaessmann
169 2010; Schlotterer 2015). The arms race in sexual competition is a never-ending battle (Gage 2004; Brennan
170 and Prum 2015; Perry and Rowe 2015; Morimoto, et al. 2019), and selective advantages are often transient.
171 In this sense, sexual selection may very likely operate in a Red Queen landscape. Because new genes,
172 including all the *de novo* miRNAs analyzed here, are often testis-specific in expression, it is plausible that the
173 evolution of new genes may be subject to the Red Queen effect via sexual selection.

Methods and Materials:

Experimental details are included in the supplementary materials. The small RNA-seq libraries generated for this study are curated in GEO with the accession numbers GSM2977400 and GSM2977401. RNA-seq libraries are available in the National Genomics Data Center (https://bigd.big.ac.cn/) with the accession number PRJCA002747.

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257
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Figure legends：

Figure 1 Sequence and expression divergence of miR-975 and miR-983 in Drosophila species.

A) The phylogenetic tree of five Drosophila sibling species is shown on the left. Adopted from TimeTree (Kumar, et al. 2017), scale in million years (Myrs). Expression levels (Reads Per Million, RPM) of miR-975 and miR-983 are shown on the right. Boxplot represents the expression level of all detected miRNAs, and dots represent the expression of selected miRNAs annotated with different colors. miR-5p and miR-3p expression levels are summed together for comparison.

B) Sequences of two highly conserved miRNAs, bantam and miR-184, across five Drosophila species are shown on the left. Sequences of miR-975 and miR-983 in the same species are depicted on the right. Seed regions of major and minor miRs are highlighted in yellow and green, respectively.

C) Arm switching of miR-975 and miR-983 in different Drosophila species. Relative expression levels of miR-5p and miR-3p are shown across species (right panel), with an annotation sketch of the miRNA structure (left panel).

Figure 2 Functional divergence of miR-975 and miR-983 in *D. melanogaster* and *D. simulans*.

A) miR-975 and miR-983 mutant sequences in *D. melanogaster* and *D. simulans*. miR-983 has two sequence identical paralogs in *D. melanogaster*: *dme-mir-983-1* and *dme-mir-983-2*. A 186-bp deletion disrupts both copies. WT, wildtype. KO, knockout.

B) Phenotypic assays for miR-975 KO in *D. melanogaster*. Four fitness measurement are shown from left to right: male mating success with virgin and non-virgin females, Fisher's exact test was used for significance, *** represents $p \leq 0.001$; male ability to repress female re-mating; offense score for sperm competition, Mann-Whitney U test was used for significant test, * represents $p \leq 0.05$ and ** represents $p \leq 0.01$.

C) Phenotypic assays for miR-975 KO in *D. simulans*

D) Phenotypic assays for miR-983 KO in *D. melanogaster*.

E) Phenotypic assays for miR-983 KO in *D. simulans*

Phenotypic assays for miR-975 and miR-983 in *D. melanogaster* are adopted from Lu et al. (2018).

286

287 **Figure 3 Genome-wide dysregulation in miR-975 and miR-983 KO flies**

288 A) MA plot shows the expression difference between miR-975 KO vs. WT flies in *D. melanogaster*. The
289 significantly differential genes with FDR (false discovery rate) < 0.05 are shown in red dots.

290 B) MA plot shows the expression difference between miR-975 KO vs. WT flies in *D. simulans*

291 C) MA plot shows the expression difference between miR-983 KO vs. WT flies in *D. melanogaster*.

292 D) MA plot shows the expression difference between miR-983 KO vs. WT flies in *D. simulans*.

293

294

295

Figure 1

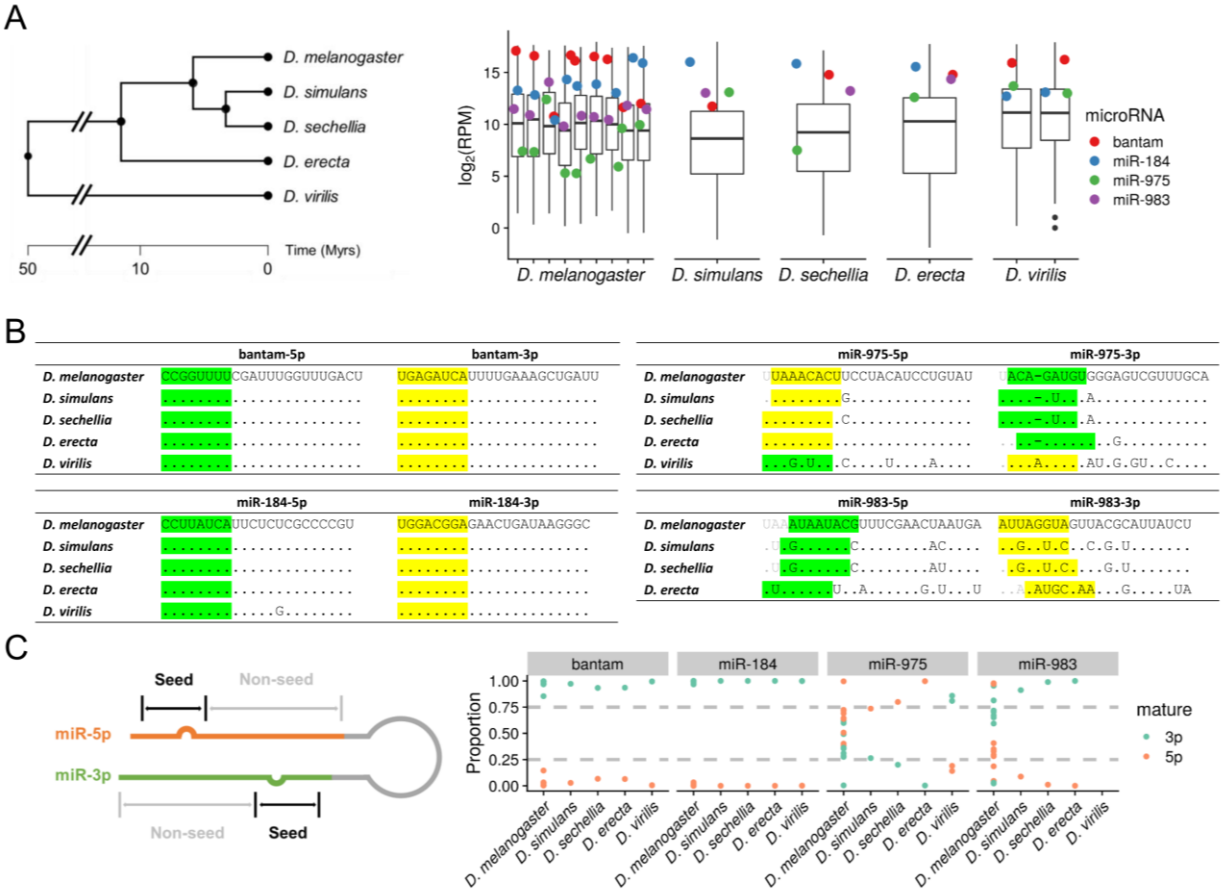

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Figure 2

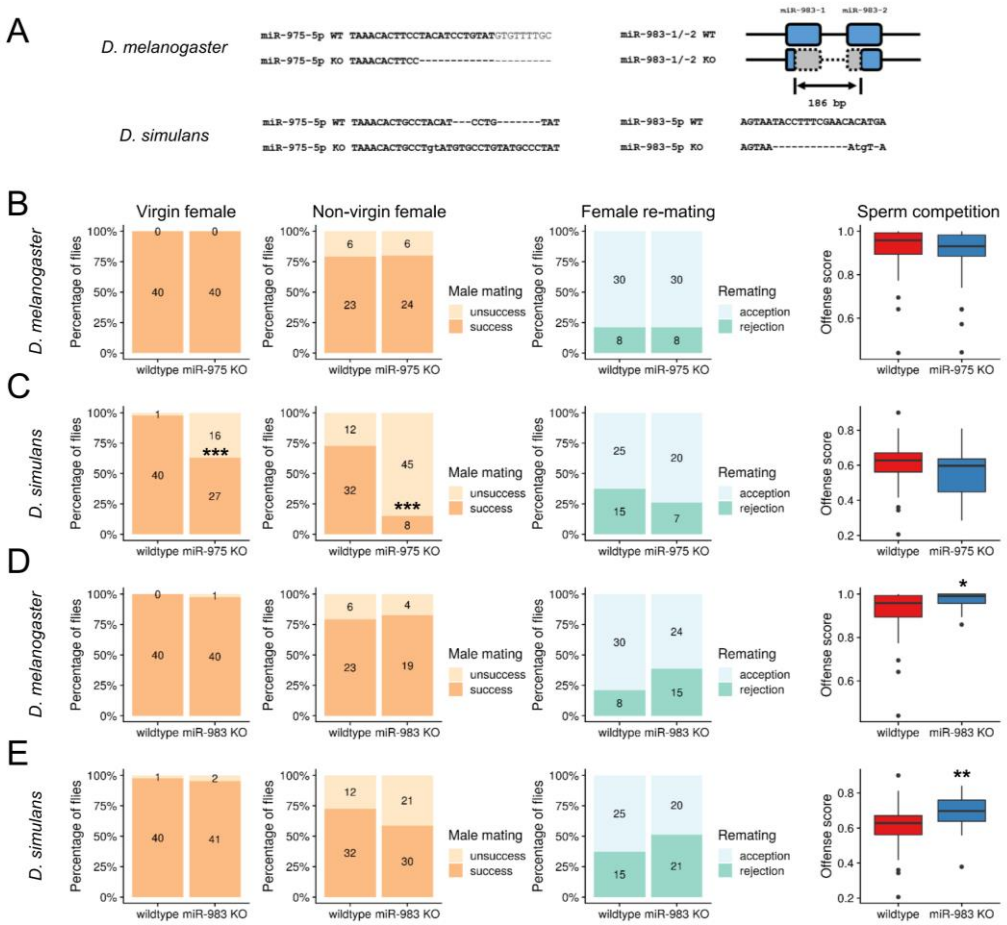
A
D. melanogaster
miR-975-5p WT TAAACACTTCCTACATCCTGTATGTGTTTTGC
miR-975-5p KO TAAACACTTCC----------
miR-983-1/-2 WT
miR-983-1/-2 KO
miR-983-1
miR-983-2
186 bp
D. simulans
miR-975-5p WT TAAACACTGCCTACAT---CCTG-------TAT
miR-975-5p KO TAAACACTGCCTgtATGTGCCTGTATGCCCTAT
miR-983-5p WT
AGTAATACCTTTCGAACACATGA
miR-983-5p KO
AGTAA----------AtgT-A
B
C
D
E
Virgin female
Non-virgin female
Female re-mating
Sperm competition
D. melanogaster
D. simulans
Percentage of flies
Offense score
Male mating
unsuccess
success
Remating
acception
rejection
wildtype
miR-975 KO
miR-983 KO
***
*
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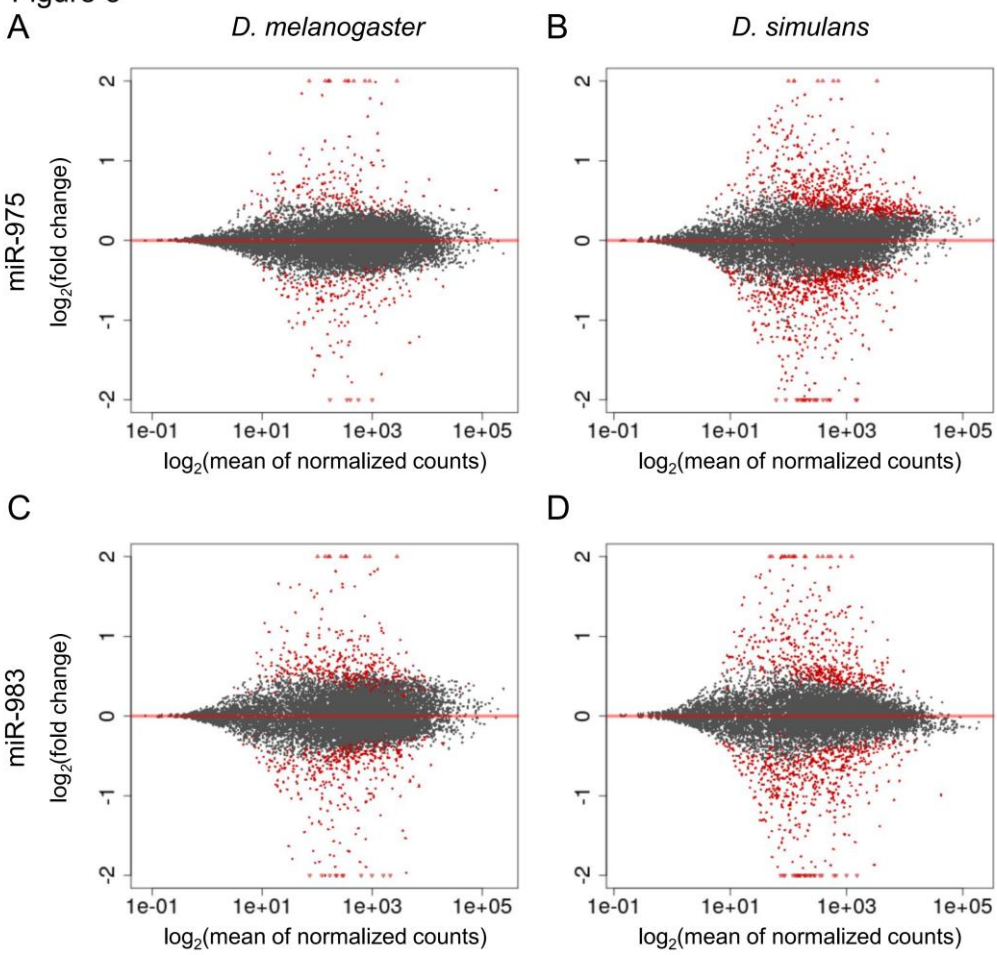
Figure 3



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