Title:

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

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Abstract

The Red Queen hypothesis depicts evolution as the continual struggle to adapt. The hypothesis, commonly invoked to explain organismal evolution, can also be applied to genic evolution. According to this hypothesis, new genes, especially those originating from non-genic sequences (i.e., *de novo* genes), would be eliminated unless they evolve continually to adapt to a changing world. Non-coding genes, represented by microRNAs (miRNAs), are the most common *de novo* genes. Here, we analyze six Drosophila *de novo* miRNAs that are testis-specific. These miRNAs exhibit a very high rate of evolution in their DNA sequence, transcript production, and expression pattern. We knock out two of the youngest miRNAs and observe three patterns. 1) The fitness advantage of miR-984 presence seems to have vanished in *D. melanogaster* even though its DNA sequence bears a signature of past adaptation. 2) Another gene, miR-983, appears to have become maladaptive in *D. melanogaster* as the fitness of the knockout mutant increases. 3) In *D. simulans*, the deletion of miR-983 is associated with extensive mis-regulation of male reproductive genes, resulting in a 70% loss in male fertility. The fitness contributions of these genes are, respectively, neutral, negative, and positive. As predicted by the Red Queen hypothesis, *de novo* genes either evolve rapidly or face elimination.

Introduction

Organisms evolve to adapt to changing environments. In response to these changes, a set of adaptive traits may evolve, for example, when terrestrial woody plants invade new habitats between land and sea (Xu, et al. 2017). These mangrove plants would then retain the evolved traits such as salt tolerance in the intertidal habitat. In the Red Queen metaphor of evolution (Van Valen 1973; Liow, et al. 2011), however, evolved traits are not necessarily long-lasting. Adaptations would generally be 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 force that has subsequently been expanded to include the coevolution of host/parasite, prey/predator and male/female (sexual selection) as well as antagonistic evolution (Tobler and Schlupp 2005; Otto and Gerstein 2006; Ebert 2008; King, et al. 2009; Brockhurst 2011; Liow, et al. 2011; Morran, et al. 2011; Brockhurst, et al. 2014). Nevertheless, the essence of this metaphor is continual adaptation, which could be driven by biotic, abiotic or both factors (see Supplementary text).

Van Valen (1973) first reported that genera and species, regardless of their age, would often go extinct when they cannot keep up (Van Valen 1973). In this study, we test the Red Queen hypothesis by studying the evolution of new genes, which may be compared to the younger taxa in organismal evolution. If the adaptive landscape shifts rapidly as posited by the Red Queen hypothesis, newly emerging genes may be 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). The majority of new genes belong to the first kind, comprising duplicated, (retro-)transposed, and chimeric genes (Chen, et al. 2013). In comparison, *de novo* genes would be more appropriate for testing the Red Queen hypothesis because their products are truly new in the cellular environment.

Despite the substantial interest in *de novo* genes, their prevalence is controversial if protein coding is involved (McLysaght and Hurst 2016; Moyers and Zhang 2016; Schmitz and Bornberg-Bauer 2017). Many have suggested that the stringent coding and length requirements preclude their frequent emergence (McLysaght and Hurst 2016). In this context, the most common source of *de novo* genes may be small non-coding loci represented by microRNAs (miRNAs) (Lu, et al. 2008; Lyu, et al. 2014). Biogenesis of miRNAs in animals requires a hairpin structure that is generally ~ 100 nucleotides (nt) in length, yielding mature products (miR) of ~ 22 nt (**Fig. 1A**). The number of potential miRNA loci in animal genomes is often in the

hundreds of thousands (Bentwich, et al. 2005; Lu, et al. 2008). Given the ease with which miRNAs can be formed *de novo* (Meunier, et al. 2013; Lyu, et al. 2014), many could have emerged adaptively (Berezikov, et al. 2010; Lu, Shen, et al. 2010; Mohammed, et al. 2013; Lyu, et al. 2014; Mohammed, et al. 2014) although the majority may be functionless, 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" specifically for the former, adaptive kind.

Lyu et al. identified nine *de novo* miRNAs in two X-linked clusters in *D. melanogaster* (Lyu, et al. 2014). The miR-972 cluster harbors both young (< 30 Myrs) and old (60-250 Myrs) members, while the miR-982 cluster contains only young ones (Lyu, et al. 2014). Being testis-specific and X-linked, these *de novo* miRNAs share the common characteristics of new genes (Lyu, et al. 2014; Mohammed, et al. 2014; Schlotterer 2015). We chose six highly expressed loci from the two clusters to investigate their evolutionary dynamics between Drosophila species (**Fig. S1**). In a companion study (Lu et al. 2018), the performance of these genes in *D. melanogaster* is analyzed in detail.

Results

I. Sequence and expression divergence of *de novo* miRNAs in Drosophila

We start by examining sequence and expression evolution of the six chosen *de novo* miRNAs. Given their testis-specific expression, we collected and sequenced, in total, 11 small RNA libraries from the testes of Drosophila species (Methods and Materials). Among them, *D. simulans* and *D. sechellia* are sibling species of *D. melanogaster*, which have diverged for ~ 4 Myrs ago, while *D. erecta* and *D. virilis* are more distantly related with the divergence time of ~10 and ~ 60 Myrs. We used multiple lines of *D. melanogaster* and *D.*

virilis. This comprehensive dataset permits us to survey miRNA expression within and between Drosophila species. The second and third section of this report will address adaptive evolution in detail by knocking out two of the youngest miRNAs.

Sequence divergence

The biogenesis of miRNAs is sketched in **Figure 1A**, where seed regions are depicted on a hairpin. Each seed, usually the first 2 – 7 nucleotides of the mature miRNA, contributes the most to target recognition (Friedman, et al. 2009; Agarwal, et al. 2015). DNA sequences of the mature products of 6 *de novo* miRNAs as well as two conserved molecules (bantam and miR-184) are shown in **Figure 1B** (see **Fig. 2A** for the phylogeny of species). *De novo* miRNAs are evolving rapidly between species as can be seen in the nucleotide substitutions in both the seed and non-seed regions (**Fig. 1B**). The number of substitutions is far higher than can be sustained by the neutral process, suggesting adaptive evolution (Lyu, et al. 2014). In contrast, bantam and miR-184 are highly conserved across Drosophila species, even in non-seed regions. The patterns of the two conserved miRNAs are representative of the common trend among older miRNAs.

Processing and expression divergence

Each miRNA locus, new or old, can potentially generate multiple mature products (Ameres and Zamore 2013), which, unlike the differential splice forms (or isomers) of coding genes, are functionally unrelated. These multiple miRs can be generated from either arm of the precursor hairpin, referred to as the -5p and -3p form (**Fig. 1A**). In **Figure 1B**, the more abundantly expressed form is labeled yellow and the other is labeled green. Seed shifting may also happen in either arm, depending on the starting position of the mature product (**Fig. 1B**). For example, miR-983-3p and miR-978-5p have four distinct seeds in four sibling species. Each product has its own unique seed, which determines the repertoire of target genes and, hence, its function.

For a comparison of the expression evolution among miRNAs, we chose the most abundant product from each of the two arms for analysis. They are designated "major and minor" miRs, according to the abundance in *D. melanogaster* and labeled accordingly in the heat map of **Figure 1C**. Obviously, the major form is more abundant than the minor form of the same miRNA. However, the minor miRs of the highly expressed miRNAs can sometimes be more abundant than the major miRs of the less highly expressed ones.

Figure 1C shows that the established miRNAs (bantam and miR-184) express predominantly one product. This major miR is consistently highly expressed across Drosophila species while the minor miRs fluctuate moderately at a much lower level. In sharp contrast, expression levels of the *de novo* miRNAs are divergent among Drosophila species. The divergence is so large that major and minor forms actually switch dominance in some species. For example, miR-973 expresses the 5p miR strongly in *D. melanogaster*, but rather weakly in *D. simulans* and *D. sechellia* (**Fig. 1C**). In these two latter species, there is arm-switching vis-à-vis *D. melanogaster* with the 3p miR being the dominant form. Another example of large divergence is miR-977-3p which is highly expressed in *D. melanogaster* but undetectable in *D. erecta* (**Fig. 1C**). We should also note that the divergent pattern can be observed even between lines of the same species. In *D. melanogaster*, *miR-983-3p* is the dominant product in only four of the six lines examined.

In short, many types of mature products are produced from each *de novo* miRNA gene. These mature products are extremely variable in sequence, biogenesis, and expression among species.

II. Rapid functional evolution, or death, of *de novo* genes

We chose the two youngest genes that are adjacent in the miR-982 cluster for further functional analyses (**Fig. 2A**). We used the TALEN (Transcription activator-like effector nuclease; see Materials and Methods)

method to construct miRNA KO lines in both *D. melanogaster* and *D. simulans*. Insertions or deletions are introduced to disrupt the miR-984 or miR-983 genes (**Fig. 2B & Fig. S2A-C**). We did not detect mature miRNAs in the testes of KO lines using RT-qPCR assays (**Fig. 2C-D**). These KO lines were then subjected to a series of analyses. Such direct experimentation would resolve many controversies about *de novo* genes in previous publications (McLysaght and Hurst 2016), which often address the issue *in silico*.

No contribution by miR-984 to the fitness of D. melanogaster

The youngest gene in this survey is miR-984, which can be found only in *D. melanogaster*. Given its high-level testis-specific expression, miR-984 KO line is expected to be defective in male reproduction. The sequential cell types in the testes, however, appear normal when examined under a phase-contrast microscope (**Fig. S3A-B**). Neither is there any obvious abnormality in mitotic cells or sperm visualized by DAPI staining (**Fig. S3C-D**). Most importantly, when male fertility is measured by counting hatched larvae or emerged adults, respectively (Methods & Materials), the difference between miR-984 KO and control males is undetectable (Two tailed Student's t test, $P > 0.05$) and scattered around zero (**Fig. 3A)**. While the fitness effect of miR-984 is weak and condition-dependent, the question is how weak it is. Lu et al. (2018) carried out a direct test of the total fitness in long-term laboratory populations whereby most fitness components (fertility, mating success, viability etc.) can be measured in aggregate and various experimental conditions can also be incorporated. They report that the fitness contribution of miR-984 centers on zero with a precision of 0.005.

The absence of any measurable fitness effect of miR-984 deficiency seems to contradict the molecular evolutionary history of the gene. Since its emergence, miR-984 has accumulated far more mutations in the critical portion of the locus than would have been expected (Lyu, et al. 2014), suggesting past adaptive evolution. The contrast between evolutionary and functional analyses thus raises an interesting possibility: that miR-984, after a brief adaptive phase, may have become fitness neutral and dispensable. However, our

analyses are somewhat limited by the absence of miR-984 in other taxa. We therefore turned to examining miR-983 which, unlike the *D. melanogaster*-specific miR-984, can be studied in a number of species.

Rapid fitness gain vs. loss of miR-983 between sibling species

The miR-983 gene, present in melanogaster subgroup (**Fig. 2A**), has been evolving rapidly. First, there has been seed-shifting, accompanied by many nucleotide substitutions (**Fig. 1B**). Second, the expression levels of both arms are substantially different between, as well as within, species (**Fig. 1C**). Third, there is one extra miR-983 copy in *D. melanogaster* (**Fig. 2A**). We therefore analyzed its fitness contributions in the two sibling species, *D. melanogaster* and *D. simulans*.

Male fertility of the miR-983 KO lines vs. their controls was measured in two *D. melanogaster* genetic backgrounds to avoid strain-specific biases. Progeny count was taken after each of two consecutive rounds of mating (see Materials and Methods). As partial sperm depletion is more likely to happen in the second round, the latter measurement may be a more sensitive detector of male fertility differences (Liufu, et al. 2017). To our surprise, the miR-983 KO males are more fertile than (or at least as fertile as) the control under several conditions (**Fig. 3B**). To verify this unexpected result, we used anti-miRNA oligonucleotides (AMO) to transiently knock down *dme-miR-983-5p in vivo* (**Fig. S3E**). Again, the knockdown flies appear to be more fertile than the control (**Fig. 3C**), even when only one mature product is incompletely removed.

While the deletion of miR-983 in *D. melanogaster* leads to the paradoxical increase in male fertility, the knockout line in *D. simulans* shows fertility reduction by more than 70% in the first round of mating (**Fig. 3D**). The fertility reduction in the second round is also highly significant (one-way ANOVA with Bonferroni correction, all $P < 0.0001$, **Fig. 3D**). Indeed, when we examined the sequential cell types in testes, many

mature sperm are abnormal and immotile in miR-983 KO flies. Since no difference was found in the sex ratio between the control and *dsi-mir-983a* KO flies (Two-sided Chi-square test, all $P > 0.05$, **Table S1**), the fertility reduction is not due to any unusual mechanism such as sex-linked meiotic drive.

These results show the rapid divergence of miR-983 function in the last 4 Myrs. miR-983 appears to face elimination in *D. melanogaster* because of its harmful effects, whereas it has evolved into an important gene for male fertility in *D. simulans*.

III. Transcriptome divergence between species

Differences in phenotypic consequences of miR-983 deletion between *D. melanogaster* and *D. simulans* should be a manifestation of the underlying divergence in transcriptomes that are influenced by this miRNA. We therefore compared repression strength of direct miR-983 targets (**Fig. 4A-B**) and the entire transcriptome (**Fig. 4C-D**) in each species.

From the cumulative plots depicted in **Figure 4A-4B**, it is clear that miR-983 target genes are expressed at a higher level in miR-983 KO lines than in the control, confirming the effect of target repression. It is also apparent that repression is stronger in *D. simulans* (**Fig. 4B**) than in *D. melanogaster* (**Fig. 4A**) , corroborating the fertility assay (**Fig. 3)**. Furthermore, in the analysis that separates the effects of 5p and 3p miRs, target repression strength follows this order: *dsi-miR-983a-3p* > *dsi-miR-983a-5p* > *dme-miR-983-5p* > *dme-miR-983-3p*. In particular, *dme-miR-983-5p* is a somewhat weaker repressor than *dsi-miR-983a-5p* (**Fig. S4A**), even though they are comparable in expression in their respective species (**Fig. 1C**). Target prediction from an independent method (PITA) gives concordant results (**Fig S4B-C** & **Table S2**).

The stronger repression by miR-983 in *D. simulans* than in *D. melanogaster* should be observable when analyzing the whole transcriptome, not just among direct targets. Indeed, only 121 genes are significantly mis-regulated in *D. melanogaster* whereas 1206 genes are mis-regulated in *D. simulans* (**Fig. 4C-D**), a 10-fold difference. The overlap among these mis-regulated genes is minimal between the two species (**Fig. S4D**). Because the overall expression patterns are quite similar and highly correlated in the testes of the two species (Methods & Materials, **Fig. S4E-F**), miR-983 likely represses direct targets with different strengths between the two species, which then cascade down the entire transcriptome downstream.

Our results suggest that the miR-983 gene has diverged in multiple aspects of male reproduction between these sibling species. Its presence has a large and positive influence on fitness of *D. simulans* but a small and negative impact in *D. melanogaster*.

Characteristics of genes mis-regulated in D. simulans

Gene expression changes associated with naturally-occurring male sterility (such as in inter-specific crosses) exhibit several patterns (Wu, et al. 1996; Wu and Ting 2004). We wanted to know whether male fertility reduction in our *D. simulans* miR-983 KO lines follows the same trend. From the results presented in **Table 1A**, it is apparent that mis-regulated genes tend to be on the autosomes rather than on the X chromosome (Two-sided Chi-square test, $P < 0.01$). Given that miR-983 is X-linked, it is interesting to note that X-linked male sterility genes in interspecific crosses tend to affect autosomal genes as well (Lu, Shapiro, et al. 2010). Such X-autosome interactions are often the genetic basis of hybrid male sterility (Wu, et al. 1996; Ting, et al. 1998; Sun, et al. 2004).

Not unexpectedly, the mis-regulated genes are highly enriched in male-biased transcripts (**Table S3**).

Interestingly, male-biased genes are prone to down-regulation rather than up-regulation, while the trend for female-biased genes is reversed in the KO lines (**Table 1B**). Furthermore, two related patterns are observed. 1) The mis-regulated genes are more likely to be involved in spermatogenesis (**Table 1C**). 2) They are also more testis-specific (**Table 1D**). Therefore, aberrant gene expression associated with male reproductive functions may indeed account for the large fertility reduction in *D. simulans* deficient in miR-983 (**Fig. 3D**)

The overall patterns of gene expression changes associated with miR-983 KO in *D. simulans* resemble those in interspecific crosses. In both cases, the fertility function has diverged only recently in the sibling species. As will be discussed below, newly emerged traits and genes are hypothesized to be evolving on a Red Queen landscape.

Discussion:

Over 40 years ago, Van Valen proposed an evolutionary landscape where continual adaptation is required (Van Valen 1973). This landscape is in contrast with the more conventional view of higher taxa, such as mammals with mammalian characters that are not continually and rapidly evolving. Other conventional examples may include characters associated with colonization of new habitats like the intertidal zone by woody plants (Xu, et al. 2017) or high altitude environments by vertebrates (Peng, et al. 2011). In these cases, once adaptive traits have evolved they remain the defining characters of the taxa. Van Valen found that lower taxa (e.g. genera), as opposed to the higher ones (e.g. classes or orders), would be under more unpredictable selective pressures. They hence often go extinct even after a period of adaptive existence. He borrowed the term “Red Queen” from *Through the Looking Glass* (Carroll 1893) to depict this ever-changing adaptive landscape.

Biotic forces are often believed to operate on a Red Queen landscape. In particular, sexual selection would lead to sundry sexual characters (Brockhurst, et al. 2014). Interestingly, most new genes are indeed male reproduction specific (Kaessmann 2010; Schlotterer 2015). Although Van Valen reported that newly evolved taxa go extinct at a constant rate, the Red Queen hypothesis (RQH) in fact merely suggests non-zero probability of extinction of previously adapted taxa or characters. After all, the probability of extinction should eventually decrease in higher taxa such as mammals or diptera. Similarly, older genes, like higher taxa, are not expected to become extinct at an appreciable rate.

As predicted by RQH, *de novo* miRNA genes that survive have indeed been rapidly evolving. They are highly variable in DNA sequence, miRNA production, and expression pattern within Drosophila (**Fig. 1B-C**), suggesting that each species may impose different adaptive pressures. Further functional tests of two miRNAs detect all three possible patterns – neutral, adaptive, and maladaptive evolution.

A distinction of this study is the choice of genes: the chosen *de novo* genes must have been adaptive in the past. Many earlier studies have proposed high turnovers of new elements in the genome for both coding genes (Tautz and Domazet-Loso 2011; Palmieri, et al. 2014) and non-coding RNAs (Lu, et al. 2008; Meunier, et al. 2013; Lyu, et al. 2014). Since these new elements are mostly non-functional, they may belong to the class often characterized as DOA (dead on arrival) elements. The high turnover of neutral DOA elements is obviously unsurprising. In contrast, new genes that arrive adaptively are expected to remain functional. Hence, the impending demise of previously adaptive *de novo* genes, chosen for this study, is intriguing. We should also note that, in the two clusters surveyed, miRNA death may have already occurred in some species. For example, while miR-977 is highly expressed in *D. melanogaster*, it does not form a functional hairpin in *D. erecta* (Mohammed, et al. 2014) and its expression is undetectable in the testes (**Fig. 1C**). Another case is miR-2582, which has several substitutions in *D. melanogaster* that disrupt its hairpin. Its death has been

suggested to be adaptive (Lyu, et al. 2014).

Three lines of evidence suggest previous adaptive evolution of miR-983 and miR-984. First, a gene that is not needed has a low probability of retaining the genic structure for longer than a few Myrs in Drosophila (Palmieri, et al. 2014). A maladaptive miRNA should have been even more quickly eliminated. Thus, the negative fitness contribution of miR-983 in *D. melanogaster* must be a recent phenomenon since the gene has existed for > 10 Myrs. Second, both genes have shown an adaptive signature in their DNA sequences from the time of their emergence (Lyu, et al. 2014). Third, a companion study (Lu et al. 2018) found that even when a *de novo* gene is evolving neutrally, it has both positive and negative contributions to different fitness components (fertility vs. viability, for example). In other words, such a "quasi-neutral" gene, unlike pseudogenes that have no function, appears to have been shedding fitness contributions incrementally.

Finally, the arms race in sexual competition is a never-ending battle (Gage 2004; Brennan and Prum 2015; Perry and Rowe 2015), and the selective advantages are often transient. In this sense, sexual selection may very likely operate in a Red Queen landscape. Because new genes, including all *de novo* miRNAs analyzed here, are often testis-specific in expression, it is plausible that the evolution of new genes may be connected to the Red Queen effect via sexual selection.

Methods and Materials:

Small RNA analyses

Testes were dissected and collected from three to five-day *D. erecta* (UCSD stock: 14021-0224.01) and *D. sechellia* (stock: 14021-0248.20) adults. Total RNA was extracted using the TRIzol® Reagent. Small RNA libraries were generated using Illumina Small RNA Sample Preparation kit, and subsequently sequenced using the Illumina HiSeq 2000 machine at the Beijing Genomics Institute (Shenzhen).

To capture expression patterns of *de novo* miRNAs, we also collected and surveyed six testes libraries in *D. melanogaster*, one in *D. simulans,* and two in *D. virilis*. Sequencing data were retrieved from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) and accession numbers are shown in **Table S4** (Edgar et al. 2002). Fly genomes were retrieved from FlyBase (http://flybase.org/) (Attrill et al. 2016). The genome versions used were: *D. melanogaster*: r6.04, *D. simulans*: r2.01, *D. sechellia*: r1.3, *D. erecta*: r1.04, and *D. virilis*: r1.03. miRNA precursor orthologous sequences were adopted from Mohammed et al. (Mohammed, et al. 2013). Mature miRNA sequences were retrieved from miRBase (http://www.mirbase.org; release 21) (Kozomara and Griffiths-Jones 2014). miR-982 and miR-972 cluster members without mature annotations were implemented by miRDeep2 prediction (Friedlander et al. 2012) or annotations in Mohammed et al. (Mohammed, et al. 2014). miRNA expression was measured using Mapper and Quantifier modules in miRDeep2 (version: 2.0.0.7). Short reads were mapped to genomes using mapper.pl with default parameters, allowing no mismatches in the first 18 nt of reads, and no more than two mismatches in the rest of the reads. Reads matching miRNAs in each library were normalized using all reads mappable to mature sequences and scaled as Reads Per Million (RPM).

miRNA mutant fly construction and stocks

miRNA mutant flies were generated using a TALEN (Transcription Activator-Like Effector Nuclease) method (Katsuyama, et al. 2013). TALEN plasmids were designed and constructed by ViewSolid Biotech Co., Ltd (http://www.v-solid.com/). TALEN binding sites are shown in supplementary **Table S5**. Plasmids were transcribed to mRNA *in vitro* by using mMESSAGE mMACHINE® T7 ULTRA Transcription Kit. w^{1118} and w^{501} embryos were used for microinjection in *D. melanogaster* and *D. simulans*, respectively. After microinjection, embryos were kept at 25℃ and emerging adult flies were crossed to flies bearing the FM7c balancer in *D. melanogaster* or Sim6 strain in *D. simulans* (see **Fig. S2D-G** for a detailed cross scheme). DNA was extracted from F_0 adult flies after five days of crossing. PCR was performed to amplify the regions containing miR-983 and miR-984 in *D. melanogaster* or miR-983 in *D. simulans* for mutation detection using the Surveyor® Mutation Detection Kit. Once a mutation was detected, single-pair cross was performed with F_1 flies. Sanger sequencing of mutants was performed to determine indel sequences. Primers used for PCR are shown in **Table S6**. Flies without miRNA mutations were handled using the same cross scheme and used for controls in further assays. All procedures using commercial kits were performed following manufactures' protocols. Fly stocks used in this study are listed in **Table S7**.

Quantitative miRNA analyses by qRT-PCR

We measured relative expression levels of miRNAs in miRNA knock out and control strains. Total RNA was extracted from testes of three to five-day-old flies using the TRIzol® Reagent (Thermo Fisher Scientific Inc., catalog #. 15596026). We used stem-loop primers for reverse transcription (Chen, et al. 2005), then Taqman PCR analysis was performed using the miRNA UPL (Roche Diagnostics) probe assay protocol (Varkonyi-Gasic, et al. 2007; He, et al. 2016). Primers are listed in **Table S8**. Three biological replicates were

conducted for each genotype and *2s* RNA was used as an endogenous control.

Phenotypic assays

Testes of three- to five-day-old male miR-984 KO flies were dissected for cell type examination using phase contrast and DAPI staining following protocols in White-Cooper (2004) (White-Cooper 2004). To measure male fertility, two assays were performed. 1) Each virgin female was exposed to a miR-984 KO or control male for two hours. Over 15 single-pair replicates for each genotype were performed. The number of hatched 1st instar larvae produced by the females was counted for two days. 2) Each three- to five-day-old male was mated with five virgin control females for two days, then all flies were removed. Emerged adult offspring were counted. Over 20 miR-984 KO and control males were examined.

Two rounds of mating were performed with miR-983 KO flies. In *D. melanogaster*, each three- to five-day-old male was mated with five three- to five-day-old w^{1118} virgin females for two days, and then transferred to a new vial to mate with another five virgin females for two days. To evaluate male fertility in other genetic backgrounds, we also introduced autosomes of wild type flies (Canton-S) into the miR-983 KO background. The detailed cross scheme is depicted in **Figure S3F-G**. F_2 males with or without miR-983 KO were used for fertility assays same as above. In *D. simulans*, the two rounds of mating procedure were the same except that *D. simulans* control virgin females were used for mating. All offspring were counted in each of the two consecutive rounds of mating. Over 15 males were examined for each genotype in *D. melanogaster* and *D. simulans.*

We injected 200μM AMO or mock into adult male (w^{1118}) abdomen to generate miR-983 knocked down flies, then let the flies recover for one day. 2'O methylation modifications were synthesized by Shanghai

GenePharma Co, Ltd. AMO sequence is perfectly complementary to *dme-miR-983-5p*, and the sequence of the mock oligonucleotide is agaggcaggaaguucggaucaa, which is not complementary to any known miRNAs in *D. melanogaster*. Each injected male was mated to four three- to five-day-old w^{1118} virgin females for five days. More than 10 males were examined for each chemical, and all emerging adults were counted. To evaluate the efficiency of chemicals, male abdomens were dissected and miR-983 expression was measured using the method described in the above section. Three replicates were used for both mock and AMO treatments. Each replicate contained three male abdomens.

Male fertility was measured as the average number of offspring per male. Two-tailed t-test or one-way ANOVA with Bonferroni correction was used to determine the statistical significance of phenotypic differences between genotypes.

Target and transcriptome analyses

To detect the effects of miR-983 on predicted targets, we performed an RNA-seq analysis. Total RNA was extracted from testes of flies crossed to five virgin females for 48h using the TRIzol® Reagent. RNA libraries were constructed and sequenced on Illumina HiSeq 2000 at Beijing Genomics Institute (Shenzhen). *D. melanogaster* (r6.04) and *D. simulans* (r2.01) genomes were used for mapping. Raw data from RNA-seq were mapped with default parameters using Tophat (Trapnell, et al. 2009), then normalized with SeqMonk version 1.36 (http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). The mapping statistics are listed in **Table S9**. Expression level of each gene was normalized as reads pre kilobase per million (RPKM) as previously described (Mortazavi, et al. 2008). All sequencing data for this study were deposited on GEO database under the accession number GSE110086.

3'UTR sequences were retrieved from FlyBase (Attrill, et al. 2016). For direct comparison between two species, we chose longest UTR for each gene, and retained genes with one to one ortholog in both species for downstream analysis. Target genes of miR-983-5p and 3p were predicted using the TargetScan algorithm with default settings (Ruby, et al. 2007). All predicted targets with 8mer, 7mer-m8 and 7mer-1A sites were used to examine the repression effect of miR-983-5p and 3p. In addition, PITA was also used to predict miR-983-5p targets for the regulation strength comparison (Kertesz, et al. 2007). Genes with $\log_2$(RPKM) > 1 were used to calculate expression fold change between miR-983 knock out and control flies. DEseq2 was used to detect significantly mis-regulated genes with $P < 0.05$ (Love, et al. 2014).

Sex-biased genes were retrieved from SEBIDA (sex bias database) (Gnad and Parsch 2006). Genes in *D. melanogaster* under the GO term "spermatogenesis (GO:0007283)" and all its child items were retrieved from FlyBase to represent spermatogenesis-related genes (Attrill, et al. 2016). Testes-specific genes were also retrieved from FlyBase (Attrill, et al. 2016) using the modENCODE dataset (Graveley, et al. 2010; Gelbart and Emmert 2013), with default requirements that expression level be not less than moderately high expression (26 – 50 RPKM) in testes and accessory glands in addition to no more than low expression (4 – 10 RPKM) in other tissues. Since all the gene features above are generated in *D. melanogaster*, and data in *D. simulans* are much less abundant than in *D. melanogaster*, we mapped genes in *D. simulans* to their 1 to 1 orthologs in *D. melanogaster* for all enrichment analyses. The Kolmogorov-Smirnov test and cumulative distributions were performed in R, the Chi-square test and Fisher's exact test were performed in GraphPad Prism.

Author contributions

Conceived and designed experiments: Yixin Zhao, Tian Tang, Chung-I Wu; Performed experiments: Yixin Zhao, Hao Yang, Zhongqi Liufu, Guang-An Lu; Analyzed transcriptome data: Yixin Zhao, Pei Lin; Interpreted

results and wrote the paper: Yixin Zhao, Jin Xu, Haijun Wen, Chung-I Wu.

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Figure Legends:

Figure 1 Sequence and expression divergence of *de novo* miRNAs in Drosophila

(A) Biogenesis of mature miRNAs. The miRNA hairpin structure has the potential to produce mature products from both arms, denoted miR-5p (in red) and miR-3p (in blue). The first 2-7nt from the 5' end is defined as the seed region, which is important for target recognition. The seed and non-seed regions are indicated.

(B) Alignment of miRNA mature sequences in five species. The first 8nt of the major and minor miRs are highlighted in yellow and green, respectively. 5' heterogeneous products expressed at more than 10% of the major miR are shown and labeled as 5p.2 or 3p.2.

(C) miRNA expression in testes across six lines of *D. melanogaster* and four other Drosophila species. Expressions of the most abundant products from either arm are shown. Major and minor miR in *D. melanogaster* are highlighted in yellow and green on the left. miRNAs without orthologs in sibling species are shaded in grey. RPM, reads per million.

Figure 2 Construction of miRNA KO lines

(A) The schematic diagram of the miR-982 cluster. This cluster comprises five miRNAs (miR-982, miR-303, miR-983-1/-2 and miR-984) in *D. melanogaster*, indicated by green, yellow, blue, and orange boxes, respectively. Orthologous miRNAs are represented by boxes of corresponding color. Bold bars indicate *CG3626* exons. Alignment gaps are illustrated with dashed lines. The genomic region is not drawn to scale. Species abbreviations: D. mel: *D. melanogaster*, D. sim: *D. simulans*, D. sec: *D. sechellia*, D. ere: *D. erecta*.

(B) Construction of miR-983 and miR-984 knock out lines. For *dme-mir-983-1/-2* in *D. melanogaster*, the grey region shows the 186bp deletion which spans the tandem duplications (see also Fig. S2A). For *dme-miR-984-5p*, an 8bp fragment was inserted in combination with several substitutions. For *dsi-miR-983a-5p*, three disparate KO lines were constructed and termed KO-1/-2/-3, which contain only small deletions or deletion with substitutions.

(C) miR-983 and miR-984 expression in different miRNA KO lines in *D. melanogaster*. n.d., miRNA expression not detectable.

(D) miR-983 expression in miR-983 KO lines in *D. simulans*. n.d., miRNA expression not detectable.

Figure 3 Phenotypes of miRNA deletions

(A) Male fertility of miR-984 KO and control *D. melanogaster* flies. Hatched larvae or emerged adults were counted, respectively. All numbers are relative to the mean of offspring from controls (m = 29.3 and 79.7 for 1st instar larvae and adults, respectively). The numbers of biological replicates are indicated at the bottom (below the same).

(B) Male fertility of miR-983 KO and control *D. melanogaster* flies. Two consecutive rounds of mating were performed. Two genetic backgrounds (w^{1118} and Oregon-R) are indicated on the x axis. The mean values of offspring for w^{1118} and Oregon-R control flies in the first round of mating are 154.4 and 65.8, respectively. All numbers of offspring are relative to the corresponding control in the first round of mating.

*: $P < 0.05$, two tailed Student's t test.

(C) Male fertility of flies after *dme-miR-983-5p* AMO or mock injection in *D. melanogaster*. All numbers are relative to the mean of offspring from males injected with the mock oligonucleotide (m = 152.4).

(D) Male fertility of miR-983 KO and control *D. simulans* flies. All numbers are relative to the mean of offspring from controls in the first round of mating (m = 148.5). ***: $P < 0.0001$, one-way ANOVA with Bonferroni correction.

Figure 4 Target derepression and mis-regulated genes in miR-983 mutant flies

(A) Empirical cumulative distributions of expression changes in *D. melanogaster*. Red lines: miR-983-5p, blue: miR-983-3p targets, and black: non-targets. Target number is indicated in parentheses. Kolmogorov-Smirnov test, $P < 0.001$ for miR-5p targets.

(B) Empirical cumulative distributions of expression changes in *D. simulans*. Line colors are the same as above. Kolmogorov-Smirnov test, $P < 0.001$ for both 5p and 3p targets.

(C) Significantly mis-regulated genes in *D. melanogaster*. Grey dots show all genes expressed in testes, while red dots show the significantly mis-regulated genes detected by DEseq2 ($P < 0.05$).

(D) Significantly mis-regulated genes in *D. simulans.* Dot colors are the same as above.

Table:

A

	Mis-regulated genes	Other genes	Fisher's exact test
Autosomes	1073	4618	<0.0001
X	118	842	

B

	Up-regulated	Down-regulated	Fisher's exact test
Male-biased	158	250	<0.0001
Female-biased	217	87	

C

	Mis-regulated genes	Other genes	Fisher's exact test
Spermatogenesis related	55	175	0.0127
Others	904	4346	

D

	Mis-regulated genes	Other genes	Fisher's exact test
Testes specific expressed	31	91	0.0291
Others	928	4430	

Table 1 Mis-regulated genes in *D. simulans* miR-983 KO flies

(A) Mis-regulated genes are highly enriched on autosomes. Fisher's exact test, $P < 0.0001$.

(B) Male-biased genes tend to be down-regulated while the trend for female-biased genes is reversed. Fisher's exact test, $P < 0.0001$.

(C) Mis-regulated genes are enriched in spermatogenesis-related genes. Fisher's exact test, $P = 0.0127$.

(D) Mis-regulated genes are enriched in testes-specific genes. Fisher's exact test, $P = 0.0291$.

Figures:

Figure 1

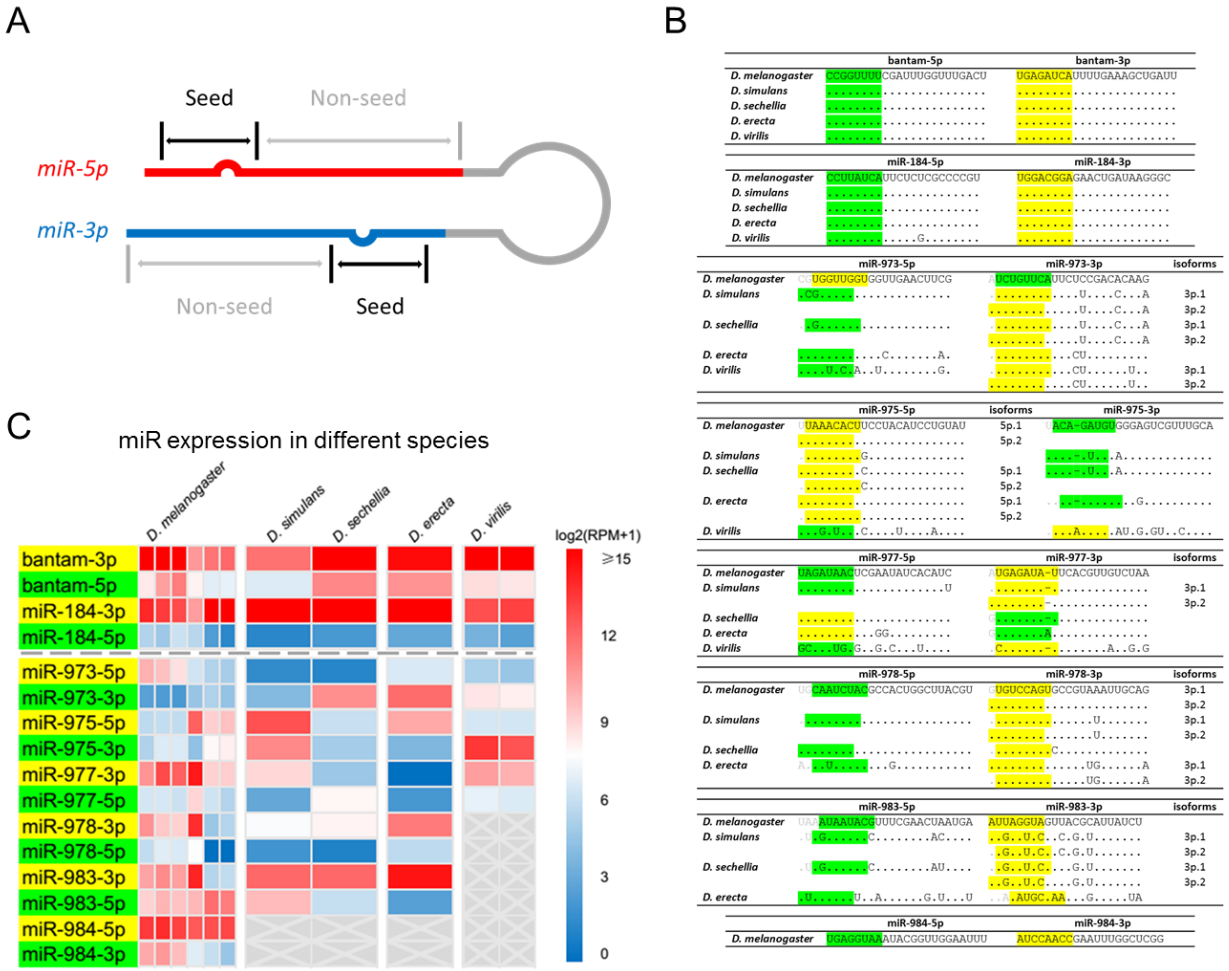


Figure 2

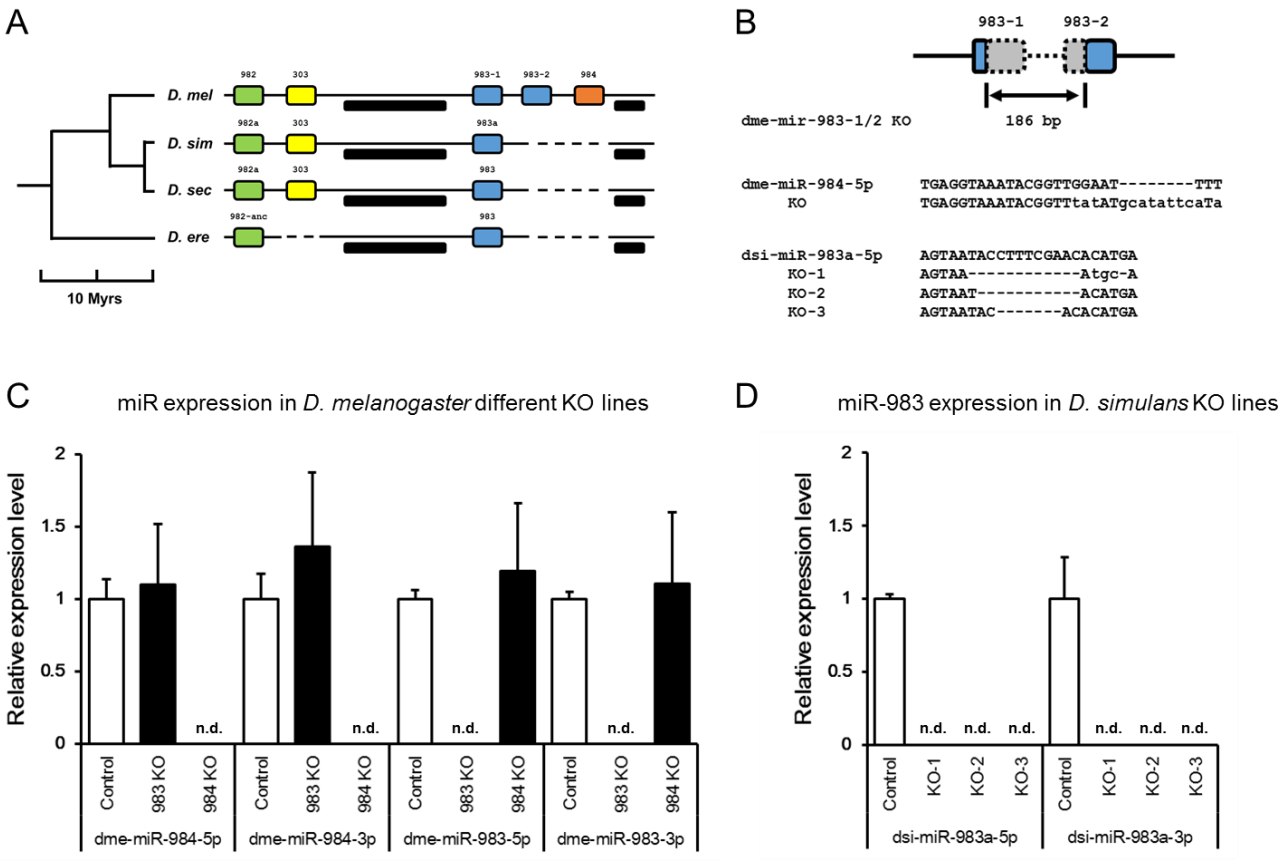

A
D. mel
D. sim
D. sec
D. ere
982
303
983-1
983-2
984
982a
983a
983
982-anc
10 Myrs
B
983-1
983-2
dme-mir-983-1/2 KO
186 bp
dme-miR-984-5p TGAGGTAAATACGGTTGGAAT--------TTT
KO TGAGGTAAATACGGTTtatATgcatattcaTa
dsi-miR-983a-5p AGTAATACCTTTCGAACACATGA
KO-1 AGTAA----------Atgc-A
KO-2 AGTAAT----------ACATGA
KO-3 AGTAATAC-------ACACATGA
C
miR expression in D. melanogaster different KO lines
Relative expression level
n.d.
Control
983 KO
984 KO
dme-miR-984-5p
dme-miR-984-3p
dme-miR-983-5p
dme-miR-983-3p
D
miR-983 expression in D. simulans KO lines
Relative expression level
n.d.
Control
KO-1
KO-2
KO-3
dsi-miR-983a-5p
dsi-miR-983a-3p

Figure 3

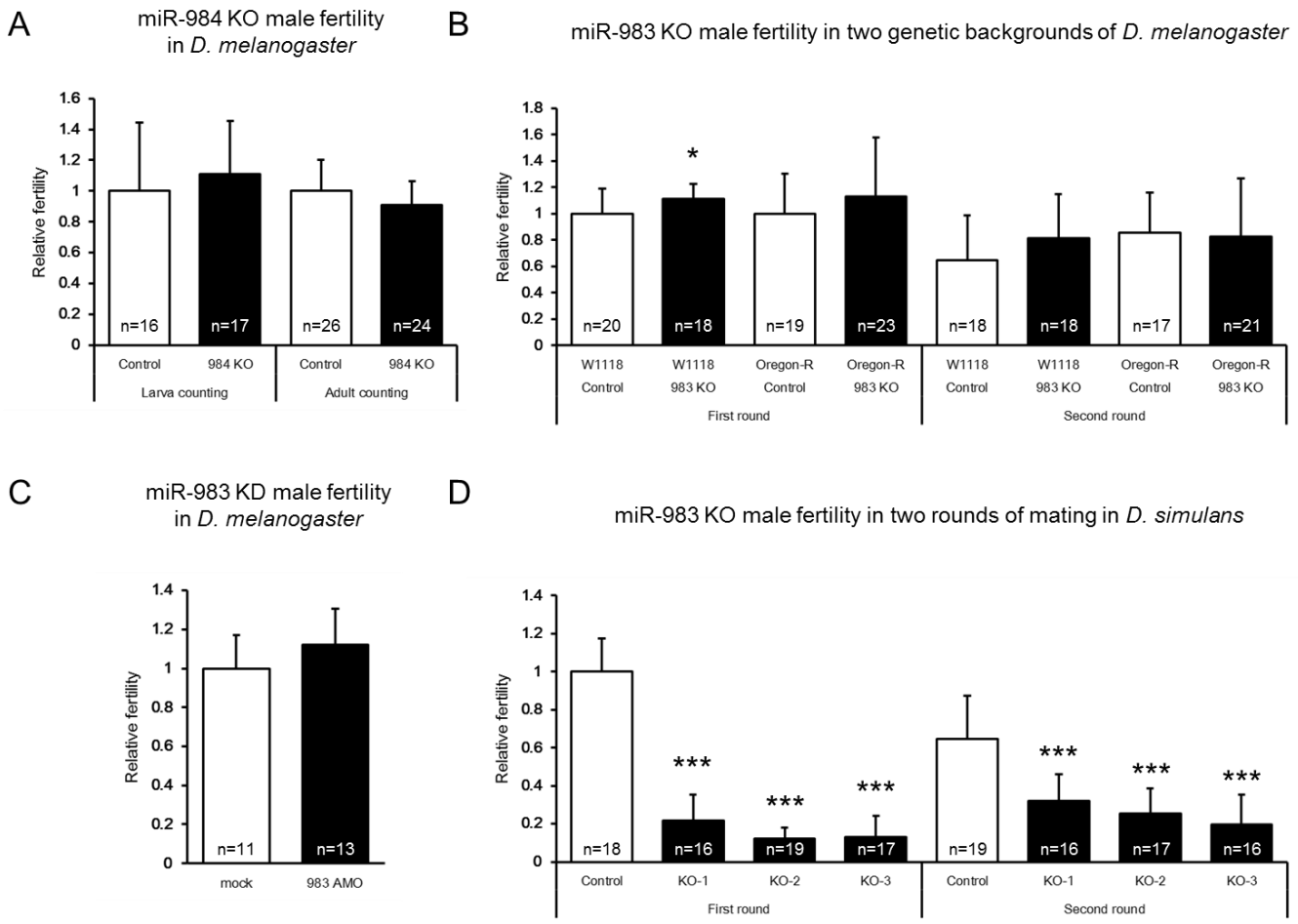
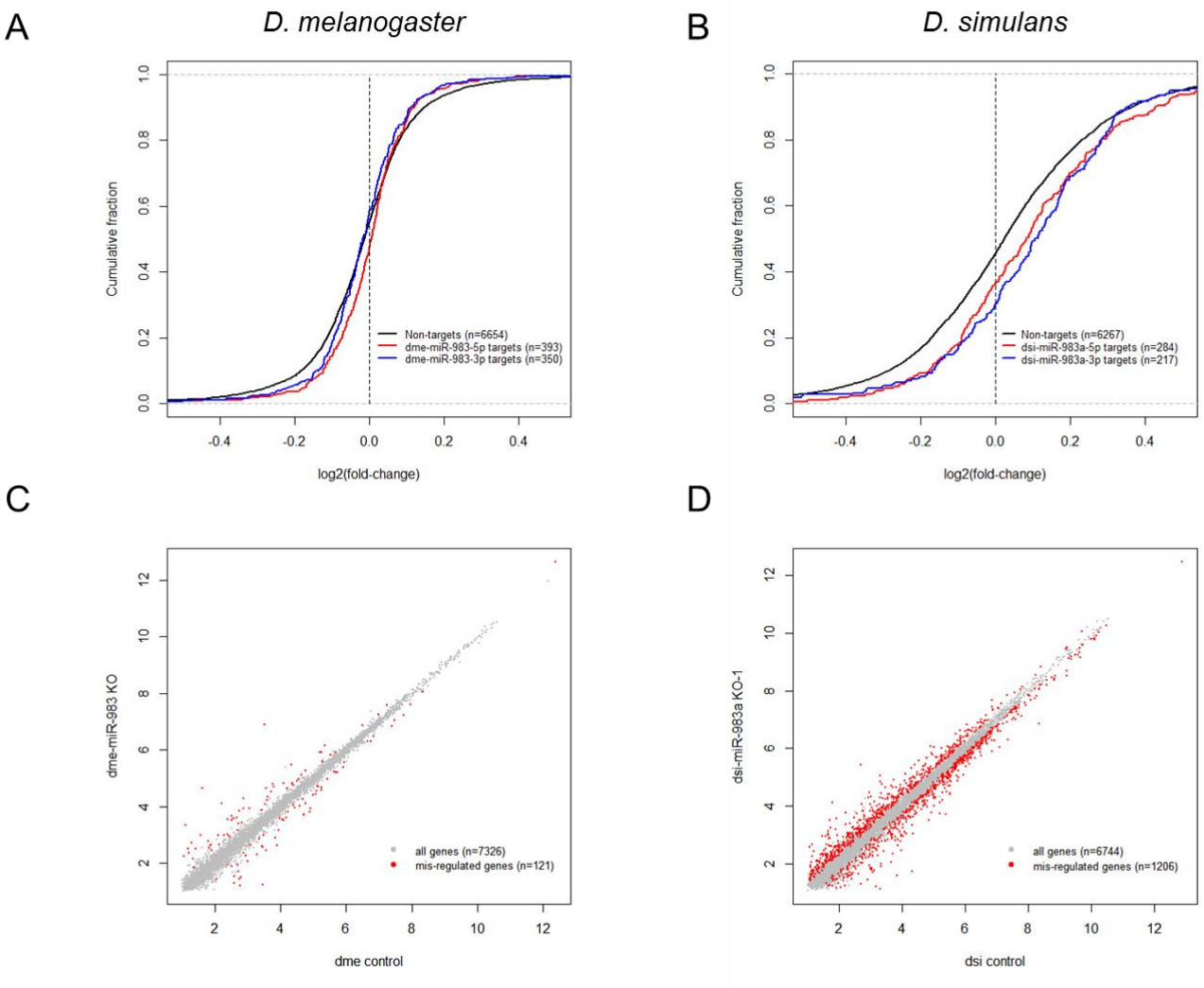

A
miR-984 KO male fertility in D. melanogaster
Relative fertility
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
n=16
n=17
n=26
n=24
Control
984 KO
Control
984 KO
Larva counting
Adult counting
B
miR-983 KO male fertility in two genetic backgrounds of D. melanogaster
Relative fertility
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
n=20
n=18
n=19
n=23
n=18
n=18
n=17
n=21
W1118 Control
W1118 983 KO
Oregon-R Control
Oregon-R 983 KO
W1118 Control
W1118 983 KO
Oregon-R Control
Oregon-R 983 KO
First round
Second round
C
miR-983 KD male fertility in D. melanogaster
Relative fertility
1.4
1.2
1
0.8
0.6
0.4
0.2
0
n=11
n=13
mock
983 AMO
D
miR-983 KO male fertility in two rounds of mating in D. simulans
Relative fertility
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
***
***
***
***
***
n=18
n=16
n=19
n=17
n=19
n=16
n=17
n=16
Control
KO-1
KO-2
KO-3
Control
KO-1
KO-2
KO-3
First round
Second round

Figure 4


A
D. melanogaster
Cumulative fraction
log2(fold-change)
Non-targets (n=6654)
dme-miR-983-5p targets (n=393)
dme-miR-983-3p targets (n=350)
B
D. simulans
Cumulative fraction
log2(fold-change)
Non-targets (n=6267)
dsi-miR-983a-5p targets (n=284)
dsi-miR-983a-3p targets (n=217)
C
dme-miR-983 KO
dme control
all genes (n=7326)
mis-regulated genes (n=121)
D
dsi-miR-983a KO-1
dsi control
all genes (n=6744)
mis-regulated genes (n=1206)