The chromosomal distribution of sex-biased microRNAs in *Drosophila* is non-adaptive

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ABSTRACT

Genes are often differentially expressed between males and females. In *Drosophila melanogaster*, the analysis of sex-biased microRNAs (short non-coding regulatory molecules) has revealed striking differences with protein-coding genes. Mainly, the X chromosome is enriched in male-biased microRNA genes, although it is depleted of male-biased protein-coding genes. The paucity of male-biased genes in the X chromosome is generally explained by an evolutionary process called demasculinization. I suggest that the excess of male-biased microRNAs in the X chromosome is due to high-rates of de novo emergence of microRNAs (mostly in other neighboring microRNAs), a tendency of novel microRNAs in the X chromosome to be expressed in testis, and to a lack of a demasculinization process. To test this hypothesis I analysed the expression profile of microRNAs in males, females and gonads in *D. pseudoobscura*, in which an autosome translocated into the X chromosome effectively becoming part of a sex chromosome (neo-X). I found that the pattern of sex-biased expression is generally conserved between *D. melanogaster* and *D. pseudoobscura*. Also, orthologous microRNAs in both species conserve their chromosomal location, indicating that there is no evidence of demasculinization or other inter-chromosomal movement of microRNAs. *D. pseudoobscura*-specific microRNAs in the neo-X chromosome tend to be male-biased and particularly expressed in testis. In summary, the apparent paradox resulting from male-biased protein-coding genes depleted in the X chromosome and an enrichment in male-biased microRNAs is consistent with different evolutionary dynamics between coding genes and short RNAs.

INTRODUCTION

Gene expression is tightly regulated by mechanisms that make gene products to be expressed in specific organs at specific times. This spatiotemporal control of gene expression determines the development of fertilized eggs into adult organisms (Davidson 2006; Wolpert et al. 2006). In organisms with two sexes a large fraction of genes are also differentially expressed between males and females (Parisi et al. 2003; Ranz et al. 2003). Also, most male or female specific expression occurs in the gonads, alhough other tissues also show sex-expression bias (Parisi et al. 2004; Chang et al. 2011). Female biased genes tend to be located in the X chromosome (in XY systems) and male-biased genes tend to be depleted in the X chromosome (Parisi et al. 2003; Ranz et al. 2003; Khil et al. 2004).

In the model species *Drosophila melanogaster* the study of sex-biased expression indicates that male-biased genes often appear in the X chromosome (Arguello et al. 2006; Levine et al. 2006; Begun et al. 2007; Chen et al. 2007), but they are later retroposed (copied) to an autosome and the original copy is eventually lost (Betran et al. 2002; Zhang, Vibranovski, Krinsky, et al. 2010). This process, called 'demasculinization', was predicted already by early theoretical models (Rice 1984), and recent works seems to support it (Jiang and Machado 2009; Bachtrog et al. 2010; Zhang, Vibranovski, Landback, et al. 2010; Zhang, Vibranovski, Krinsky, et al. 2010), although there is still some controversy around, and alternative models should be taken into consideration (Meiklejohn and Presgraves 2012). However, these conclusions cannot be extrapolated to all gene products, as most work on sex-biased expression has been focused on a specific type of gene: protein-coding genes. Characterising the distinct evolutionary dynamics of different types of genes is paramount in evolutionary biology, as it can reveal how genetic and genomic features influence, and ultimately determine, the fate of genes (Lynch 2007).

MicroRNAs, short RNA post-transcriptional regulators [reviewed in (Bartel 2004; Axtell et al. 2011; Marco, Ninova, and Griffiths-Jones 2013)], are also expressed differently in males and in

females (Marco, Kozomara, et al. 2013; Marco 2014; Marco 2015; Warnefors et al. 2017; Fowler et al. 2019). Although an early investigation suggested that they were also subject to demasculinization (Zhang, Vibranovski, Krinsky, et al. 2010), a more recent work indicates that this is probably not the case (Marco 2014). Indeed, male microRNAs tend to be enriched in the X chromosome (Mishima et al. 2008; Li et al. 2010), contrary to what is observed in protein-coding genes. In *D. melanogaster* novel microRNAs in the X chromosome are often expressed in testis and most are evolutionarily young (Marco 2014). To investigate the evolutionary dynamics of microRNAs in the X chromosome I characterised the sex-biased microRNA complement of the species *Drosophila pseudoobcura*, which diverged from *D. melanogaster* ~37 Myr ago (Hedges et al. 2015), and had an autosome translocated into an X-chromosome, becoming a newly evolved X-chromosome (or neo-X). This neo-X emerged about 13 Mys ago (Kaiser and Bachtrog 2010) which, despite being a short evolutionary period, has been enough to identify demasculinization affecting protein-coding genes in several studies (Zhang, Vibranovski, Krinsky, et al. 2010; Nozawa et al. 2014). Indeed, it has been estimated that, in *Drosophila*, one retrogene emerges every 2 Myr years at an approximate constant rate (Bai et al. 2007), and we would expect (at least in theory) a similar rate for microRNA genes. Hence, this system will shed light on the evolution of chromosomal composition of sex-biased microRNAs.

RESULTS

In order to characterize differentially expressed microRNAs between males and females in *D. pseudoobscura* I generated and sequenced small RNA libraries from males and young unfertilised females (see Methods). There are available small RNA datasets for males and females of this species (Mohammed et al. 2018). However, these datasets have either no biological replicates, or they have been sequencing using different library construction methods. Hence, I sequenced male and female samples in two paired sequencing reactions (one male sample and one female sample in each sequencing array) using the same protocols. The differential gene expression analysis was performed taking into account batch (paired) effects. In any case, the log2 fold-changes computed for both sets of experiments (ours and the already available) were similar, and a linear model to predict log2 fold-changes in the external dataset

from our values fitted very well, given the important technical differences ($p < 0.001$; $R^2 = 0.35$; Supplementary Figure 1).

The analysis identified 21 microRNAs with a clear sex-biased expression profile at a False Discovery Rate (FDR) of 10% and a expression difference of 20% or over: 10 overexpressed in females and 11 in males (Figure 1A, Supplementary Table 1), so we would expect about one false positive in each sex. For a more stringent cutoff of 50% expression difference the number of differentially expressed microRNAs is 18. There were 100 orthologous microRNAs conserved between *D. melanogaster* and *D. pseudoobscura*. The chromosome in which the microRNA is located (Muller element) is conserved for all studied sequences, although some evidence of translocations and inversion are observed within chromosomes (Figure 2). Importantly, there is not a single case of a microRNA in one of the *Drosophila* species that moved to a different chromosome in the other species. In other word, the analysis did not identify a microRNA that moved out of a sex chromosome to an autosome (or vice versa), a phenomena that is well described for protein-coding genes, and it is generally due to retroposition (see Discussion).

A potential bias in the chromosomal location analysis is that orthology may have been defined, partly, by using chromosomal context. To control for that I run reciprocal BLAST among all *D. melanogaster* and *D. pseudoobscura* annotated microRNAs (see Methods). Clusters of similar sequences were aligned and similarity trees were built (Supplementary File 1). Only one microRNA in *D. pseudoobscura* remained unpaired to an ortholog: dps-mir-92c. Mir-92 is one of the most conserved microRNA families in animals, and it has two copies in *D. melanogaster* (mir-92a and mir-92b) and three (mir-92a, mir-92b and mir-92c) in *D. pseudoobscura*. Recent work has shown that mir-92c is deeply conserved in in insects and it is not associated with a recent duplication of any of the other mir-92 in the pseudoobscura lineage (Ninova et al. 2014)

In addition, the *D. pseudoobscura* genome was scanned for potential homologous microRNAs annotated so far only in *D. melanogaster* (see Methods), to identify copies that may have not

been detected in earlier works (either because of limited genome sequence information or by the use of strict criteria to filter putative microRNAs). The BLAST searches identified 48 putative one-to-one previously unknown microRNA orthologs between both species (Table 1). Only two newly identified microRNAs were in different chromosomes between the two species: dme_424 and dme-mir-4645. In the case of dme_424 this is due to the proximity to the X centromere in *D. pseudoobscura*, which makes it close to the telomere of the *D. melanogaster* X chromosome which is in fact also derived from the Muller element A, where the microRNA in located in *D. melanogaster*. In any case, dme_424 is placed in an X chromosome in both species. For dme-mir-4645, independently of whether this is a bona fide microRNA (it is lowly expressed and poorly conserved), it would be an autosome to autosome translocation.

There were 4 microRNAs in *D. melanogaster* that have two copies in *D. pseudoobscura* with at least one of those not previously annotated (Table 2). Of those, two had one copy located in the putative orthologous position and another elsewhere, which may indicate copies to other chromosomes. For mir-1006, both copies are in autosomes. For dme_86, the conserved copy was in an autosome and the derived copy in the X chromosome (Table 2). Experimentally validating this novel microRNA is out of the scope of this work. However, even if true, that would potentially indicate a copy from an autosome to the sex chromosome, further supporting the hypothesis here presented that there is no demasculinization of the X chromosome for microRNAs.

Finally, I considered those *D. melanogaster* microRNAs with multiple copies in the *D. pseudoobscura genome*. There were 8 microRNAs in this category. Six of them (dme_208, dme_417, dme_455, dme_5, dme_7 and dme-mir-10404) are microRNAs derived from rRNA sequences and similar sequences are found associated to rRNA genes in insects (Chak et al. 2015). For another one, mir-4973, 3 out of the 4 hits were spurious hits at the 3' low-complexity end (CGT repeats). Last, mir-2281 had 5 hits. None of those expanded the two arms of the putative homolog (Supplementary Figure 2) and had multiple mismatches and indels in the mature sequences. Indeed, the central part not covering the mature microRNAs is found in

hundreds of copies in *D. pseudoobscura* and other species of the pseudoobscura group, not in other drosophilids, and mostly near heterochromatic regions, indicating that this is probably a novel repetitive element, yet to be characterized. In any case, mir-2281 is located in chromosome 3R in *D. melanogaster* so it will not be useful to identify X to autosome microRNA movements. In summary, these analyses did not reveal any microRNA that may have moved out or copied from the X to any other autosomal chromosome.

To investigate whether the expression profile of microRNAs change in a different chromosomal context, I compared the sex-bias in expression of orthologogous microRNAs between both *Drosophila* species. A scatter plot of the log2 fold-change values for pairs of orthologs reveals that the expression bias is roughly conserved between both species (Figure 1B, Supplementary Table 1). The female/male expression bias is also conserved in microRNAs that are autosomal in *D. melanogaster* but that are in the new-X chromosome in *D. pseudoobscura* (Figure 1B; fitted linear model $p = 0.007$ $R^2=0.3$), showing evidence that the chromosomal context (sex versus autosomal) does not change the sex-biased expression pattern. There are, however, three notable exceptions of microRNAs that are strongly male biased in *D. pseudoobscura* but female biased in *D. melanogaster* (Figure 1B): mir-286, mir-311 and mir-92c (see Discussion).

To characterize the expression of novel and conserved microRNAs depending on the chromosomal context, I compared the expression bias (log2 fold-change) of novel and conserved microRNAs in both autosomes and the X chromosome in *D. pseudoobscura*. Novel microRNAs that emerged in the X chromosome tended to be male expressed compared to the other microRNAs, although the difference was small (Figure 1C). However, when I compare the testis to ovary expression ratio for microRNAs expressed in the gonads, it is clear that novel microRNAs in the X tend to be overexpressed in testis (Figure 1D; $p = 0.00018$ Mann-Whitney test).

A caveat in this analysis is the non-independence of expression profiles among clustered

microRNAs, as they tend to be produced from the same transcript. To evaluate the impact of microRNA clusters, the analysis was repeated and only the highest expressed microRNA in each cluster (microRNAs within 10Kb to each other) was considered. Overall the log2 fold change in expression is similar in both species (Figure 3A) with the main exception of the microRNAs already mentioned above. For novel X-linked microRNA clusters, there were four loci, all highly expressed in testis (Figure 3B). This also suggests that novel X-linked microRNAs tend to emerge within other microRNA clusters/loci (see Discussion).

DISCUSSION

In this work I characterised the sex expression pattern of microRNAs in *Drosophila pseudoobscura* to investigate the evolutionary dynamics of male expressed microRNAs. By comparing the expression profile of *D. pseudoobscura* and *D. melanogaster* it was evident that the sex-bias of microRNAs is largely conserved between both species. This is also the case for microRNAs that were located in an autosome which became the neo-X arm after a translocation, supporting that the relative expression between males and females does not depend on the chromosomal context. In other words, male-biased microRNAs may acquire their expression profile early during evolution, but then their expression is maintained even if the chromosome acquires a sex chromosome status. The conservation of the expression bias as well as the conservation of the chromosome in which microRNAs are located provides strong evidence against demasculinization having any impact in microRNAs.

There were three exceptional cases of microRNAs strongly male-biased in *D. pseudoobscura* but strongly female-biased in *D. melanogaster*. These microRNAs belong to two microRNA clusters: mir-309~6 and mir-310~313. MicroRNAs of the cluster mir-310~313 are maternally deposited in the egg (Ninova et al. 2014; Marco 2015), and mir-309~6 is mostly expressed during the first hours of development (Ninova et al. 2014). In this study, to avoid the excessive contamination from egg/embryonic microRNAs in females, young virgin females were sequenced. That may explain the differences observed in these two particular clusters. In any

case, both microRNAs clusters are located in the homologous autosome in both species, so the observed difference is surely not a consequence of a different chromosomal context.

In the lineage of the species *Drosophila willistoni* there was an independent fusion of the Muller element D (chr3L in *D. melanogaster*) with the X chromosome, generating a neo-X similar to what happened in the pseudoobscura lineage. Of the male-biased *D. melanogaster* microRNAs, only two were located in the 3L chromosome: mir-274 and mir-276a. BLAST searches revealed that both microRNAs are conserved in *D. willistoni* as well as the neighbouring region, suggesting that the synteny is conserved. However, as far as I know, these scaffolds are not mapped into the Muller elements and a detailed analysis of any potential movelement out of the *D. willistoni* neo-X is not possible at the moment.

The demasculinization model described in the Introduction assumes that male-expressed (male-beneficial) genes are more likely to be evolutionarily lost if they are located in the X chromosome, as damaging mutations are often lethal in hemizygosity (Figure 4A). Hence, gene copies in the autosome are favored while the ancestral X located copy is eventually lost (Sturgill et al. 2007). The genes are copied by retroposition (Figure 4B), in which a reverse transcriptase makes a DNA copy of the processed transcript which is inserted (by a retroviral integrase) in the genome. The original copies of genes retroposed into the autosomes are eventually lost (Figure 4C). This mechanism is highly unlikely, if not impossible, in microRNAs since processed microRNA precursors leaving the nucleus (and therefore exposed to endogenous reverse transcriptases) are short (less than 100 nucleotides) RNA molecules with an internal hairpin structure (Bartel 2004), lacking a polyadenylation sequence that could be used as a primer by the endogenous reverse transcriptases (Wei et al. 2001). DNA-based gene relocation (that is, not involving retroposition) has also been described in X to autosome gene movement (Vibranovski et al. 2009). However, it was not detected in this analysis any microRNA X to autosome translocation, so DNA-based demasculinization has not been observed either. Nevertheless, in future works DNA-based transposition should be taken into account as the most likely mechanism of microRNA interchromosomal movement.

This work supports an alternative model of evolution. MicroRNAs have a high rate of turn-over. That is, novel microRNAs appear at high frequencies (Figure 4D), which is less common in protein-coding genes. Novel genes are often male-biased (Metta and Schlötterer 2008), and if they are in the X they are frequently highly expressed in testis (this work, see also (Mohammed et al. 2014)). New microRNAs in the X with male-biased expression can also serve as sources of novel linked microRNAs sharing the transcript, and hence the bias (Figure 1D and 3B (Marco, Ninova, Ronshaugen, et al. 2013)). This bias in gene expression of newly emerged microRNAs, together with the high rate of evolutionarily young microRNA loss (Figure 4E) eventually leads to an enrichment of male (testis) expressed microRNAs in the X chromosome (Figure 4F). Alternatively, if X-located male-biased microRNAs had an important function and, therefore, were under purifying selection, this selection would be significantly weaker than in protein-coding genes given the short length of the final functional product and the low probability of generating loss-of-function mutants. Either way, natural selection seems to have a limited role in the birth and maintenance of male-biased X-linked microRNAs. Future work on older neo-X chromosomes in *Drosophila* and other insects will shed further light on the validity of this model in other species and over significantly longer evolutionary periods.

It is also important to keep into consideration that male-biased expression does not necessarily mean beneficial for males, or that losing the gene males fertility will be compromised. Indeed, genes whose lack of function lead to infertility are male expressed, but the converse does not hold true, male-biased genes are not generally fertility genes (Lindsley et al. 2013). Putting all this together, this work strongly suggest that the X-chromosome is enriched in male-biased microRNAs as a consequence of mutation bias (tendency of novel microRNAs in the X to be male biased), lack of retroposition, and a conservation of the expression-bias independently of the chromosomal context. In summary, the evidence supports that the chromosomal location of sex-biased microRNAs is non-adaptive.

METHODS

Sample collection: The work was done with a *Drosophila pseudoobscura* stock kindly donated

by Tom Price (University of Liverpool) and Nina Wedell (University of Exeter), collected in Show Low (Arizona, USA). All flies were kept at 18ºC on cornmeal based media, with 12 hours light/dark cycles. Adult males and females were collected at age 7 days. Fertilized eggs were collected from virgin females in population cages (about 30-50 females) in cycles of 12 hours. Virgin females were allowed to lay eggs in control vials to ensure they were virgins. We discarded population cages with females coming from control vials having larvae within two weeks (in these experiments, it only happened once). Eggs were collected with a sieve and washed with wash solution (NaCl 100 mM, Triton X-100 0.7 mM) and water.

RNA extraction and sequencing: Total RNA from four samples (two males and two females) samples was extracted using TRIzol (Life Technologies) as recommended by the manufacturer. RNA was dissolved in RNase-free water. For small RNA sequencing I used the TruSeq Small RNA Sample Preparation Kit (Illumina) to generate the cDNA library using selected constructs of sizes 145 to 160 bp in a 6% PAGE gel, and precipitated in ethanol. DNA integrity was checked with TapeStation (Agilent). Samples were sequenced in-house with an Illumina MiSeq sequencing machine.

MicroRNA expression analysis: Reads were mapped to the *Drosophila pseudoobscura* genome sequence assembly 104 (Liao et al. 2021) with HISAT2 version 2.2.1 (Kim et al. 2019) with default parameters. Raw reads were deposited in the Gene Expression Omnibus (GEO) at NCBI with accession number GSE179989. Additionally, reads from other *D. pseudoobscura* samples were retrieved from GEO: GSE98013 (Mohammed et al. 2018) (two males and one female samples) and GSE48017 (Lyu et al. 2014) (one ovary and one testis samples) and also mapped to the same reference genome. Reads from *D. melanogaster* SRR016854, SRR018039, SRR069836 and SRR069837 (Chung et al. 2008; Roy et al. 2010) were mapped with the same method to the reference genome dm6 (Hoskins et al. 2015). Adapters were trimmed with Cutadapt 1.18 (Martin 2011) prior to mapping. Read counts were obtained with featureCounts 2.0.2 (Liao et al. 2014) using the annotation coordinates from miRBase release 21 (Kozomara et al. 2018) and the additional microRNAs annotated by (Mohammed et al. 2018), and microRNAs not supported by

this study were also removed from the analysis. Differential gene expression was conducted with DESeq2 version 1.24.0 (Love et al. 2014: 2) with local fitting, using the model `expression ~ batch + sex`, where batch is the sequencing run.

Homology analysis. Annotated microRNAs from both species were compared via BLAST 2.9.0 (Altschul et al. 1997) using the R wrapper rBLAST 0.992 (https://github.com/mhahsler/rBLAST/): blastn with word size 10 and E-value threshold of 0.1. Pairs of similar sequences between species were built into a graph with igraph 1.2.11 (https://igraph.org) and connected graphs were extracted as similarity (potentially homology) groups. For each similarity group a sequence alignment with MAFFT 2.9.0 (Katoh and Toh 2008) and a neighbor-joining tree (Saitou and Nei 1987) using uncorrected distances with ips 0.0.11 (https://www.rdocumentation.org/packages/ips) were built for visual inspection. To identify potential, and previously missed, microRNAs, *D. melanogaster* microRNA precursors were compared against the *D. pseudoobscura* genome with blastn (word size 10, E-value threshold of 0.01). Alignments of length 50 positions or over were further considered.

All data generated and scripts to reproduce the full analysis is available from GitHub: https://github.com/antoniomarco/miRpseudoobscura

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Table 1. Predicted newly discovered *D. pseudoobscura* microRNAs from similarity analysis.

MicroRNA	chromosome[1]	Start	End	Dmel Muller[2]	Dpse Muller[2]
dme_293	NC_046683.1	10364806	10364912	A	A (XL)
dme_165	NC_046683.1	22123368	22123423	A	A (XL)
dme-mir-4956	NC_046683.1	4407613	4407712	A	A (XL)
dme-mir-4959	NC_046683.1	7183132	7183227	A	A (XL)
dme_424	***NC_046683.1***	***37674259***	***37674347***	***A***	***D (XR)***
dme_410	NC_046681.1	10383429	10383522	B	B
dme_282	NC_046681.1	12960481	12960541	B	B
dme_439	NC_046681.1	15066171	15066236	B	B
dme-mir-4971	NC_046681.1	18771926	18771991	B	B
dme-mir-4984	NC_046681.1	20718387	20718460	B	B
dme_249	NC_046681.1	23301966	23302031	B	B
dme_436	NC_046681.1	25009425	25009501	B	B
dme-mir-4987	NC_046681.1	6472128	6472255	B	B
dme-mir-2495	NC_046681.1	7935176	7935255	B	B
dme_401	NC_046681.1	8052896	8052975	B	B
dme-mir-4979	NC_046680.1	13111227	13111316	C	C
dme_419	NC_046680.1	13538018	13538096	C	C
dme-mir-4978	NC_046680.1	15054874	15054942	C	C
dme-mir-11182	NC_046680.1	18533519	18533571	C	C
dme-mir-9376	NC_046680.1	21482721	21482779	C	C
dme-mir-4976	NC_046680.1	4622992	4623089	C	C
dme_430	NC_046680.1	4911946	4912000	C	C
dme_237	NC_046680.1	8875772	8875870	C	C
dme-mir-4981	NC_046680.1	9274706	9274755	C	C
dme_245	NC_046683.1	46892399	46892496	D	D (XR)
dme-mir-4968	NC_046683.1	52785417	52785474	D	D (XR)
dme_252	NC_046683.1	60707098	60707189	D	D (XR)
dme-mir-956	NC_046683.1	64904325	64904476	D	D (XR)
dme-mir-4941	NC_046683.1	64977320	64977421	D	D (XR)
dme-mir-4945	***NC_046680.1***	***13009749***	***13009804***	***E***	***C***
dme_378	NC_046679.1	12937728	12937828	E	E
dme_160	NC_046679.1	13576524	13576577	E	E
dme-mir-4952	NC_046679.1	14132980	14133092	E	E
dme-mir-9381	NC_046679.1	14684384	14684448	E	E
dme-mir-1014	NC_046679.1	15081742	15081809	E	E
dme_422	NC_046679.1	17038344	17038431	E	E
dme_234	NC_046679.1	1899750	1899799	E	E
dme-mir-1017	NC_046679.1	19276923	19276996	E	E
dme_166	NC_046679.1	22033947	22034020	E	E
dme_377	NC_046679.1	22813321	22813379	E	E
dme-mir-998	NC_046679.1	24781264	24781407	E	E
dme-mir-996	NC_046679.1	2566820	2566915	E	E
dme-mir-1003	NC_046679.1	2708636	2708694	E	E
dme-mir-9383	NC_046679.1	27090644	27090698	E	E
dme_421	NC_046679.1	28069076	28069174	E	E
dme-mir-999	NC_046679.1	6866309	6866409	E	E
dme-mir-4969	NC_046679.1	6866607	6866655	E	E
dme-mir-4944	NC_046679.1	7523386	7523519	E	E

[1]Chromosome accession number in NCBI; [2]Muller chromosomal element. In bold-italics: microRNAs located in different Muller elements between both species.

Table 2. Predicted newly discovered *D. pseudoobscura* with two copies.

MicroRNA	chromosome[1]	Start	End	Dmel Muller[2]	Dpse Muller[2]
dme_86	NC_046680.1	18533515	18533571	C	C
dme_86	NC_046683.1	25073378	25073430	C	A (XL)
dme-mir-1006	NC_046680.1	8618854	8618906	B	C
dme-mir-1006[3]	NC_046681.1	20903167	20903248	B	B
dme-mir-4942	NC_046683.1	3212290	3212347	D	A (XL)
dme-mir-4942	NC_046683.1	7624108	7624161	D	A (XL)
dme-mir-4948	NC_046679.1	1526563	1526662	E	E
dme-mir-4948	NC_046679.1	2671493	2671578	E	E

[1]Chromosome accession number in NCBI; [2]Muller chromosomal element. In bold-italics: microRNAs located in different Muller elements between both species. [3]Already known.

FIGURE LEGENDS

Figure 1. Sex-biased expression of microRNAs in *Drosophila pseudoobscura*. (A) Smear plot of expressed microRNAs. Black dots are microRNAs with a significant difference (see Methods). (B) Scatter-plot of log2 fold-change expression values of *D. melanogaster* and *D. pseudoobscura*. Black dots represent microRNAs located in the neo-X chromosome in *D. pseudoobscura*, and the other microRNAs are in grey, and black and grey straight lines represent the linear model fitted to both groups respectively. Dashed line is the 1:1 ratio. (C) Log2 fold-change of male versus female expression for conserved or *D. pseudoobscura* specific (novel) microRNAs, either autosomal (A) or in the X chromosome (X). (D) Log2 fold-change of testis versus ovary expression.

Figure 2. Comparison of the chromosomal location of microRNAs between *D. melanogaster* and *D. pseudoobscura*. Homologous chromosomes of both species are paired, and grey lines connect the location of orthologous microRNAs. Dark grey lines indicate overlap of two or more lines, the darker the more microRNAs: this is due to clustered microRNAs.

Figure 3. Sex-biased expression of microRNA clusters in *Drosophila pseudoobscura*. (A) Scatter-plot of log2 fold-change expression values of *D. melanogaster* and *D. pseudoobscura*. Black dots represent microRNA clusters located in the neo-X chromosome in *D. pseudoobscura*, and the other microRNAs are in grey, and black and grey straight lines represent the linear model fitted to both groups respectively. Dashed line is the 1:1 ratio. (B) Log2 fold-change of testis versus ovary expression for conserved or *D. pseudoobscura* specific (novel) microRNAs, either autosomal (A) or in the X chromosome (X).

Figure 4. Models of protein-coding and microRNA genes evolution. (A) Effect of deleterious mutations in male genes. (B) Retroposition out of the X chromosome. (C) Deletion of the original copy of a retroposed gene. (D) Emergence of novel microRNAs in the X chromosome highly-expressed in testis (in blue) and in autosomes. (E) Random deleterious mutations in microRNA genes. (F) Enrichment of male-biased microRNAs in the X chromosome. Details of the models are in the main text.

Figure 1

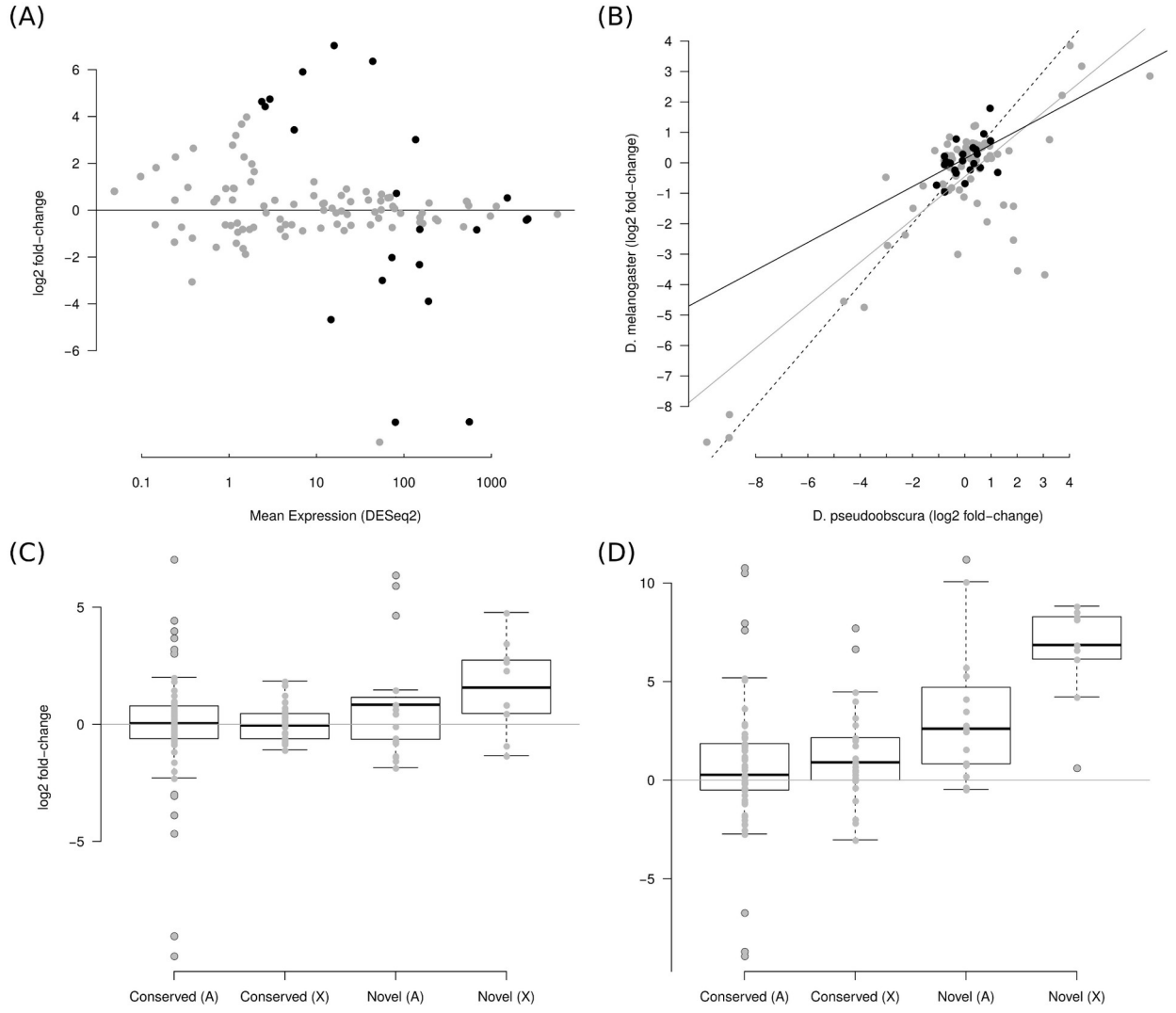

(A)
log2 fold−change
Mean Expression (DESeq2)
(B)
D. melanogaster (log2 fold−change)
D. pseudoobscura (log2 fold−change)
(C)
log2 fold−change
Conserved (A)
Conserved (X)
Novel (A)
Novel (X)
(D)
Conserved (A)
Conserved (X)
Novel (A)
Novel (X)

Figure 2

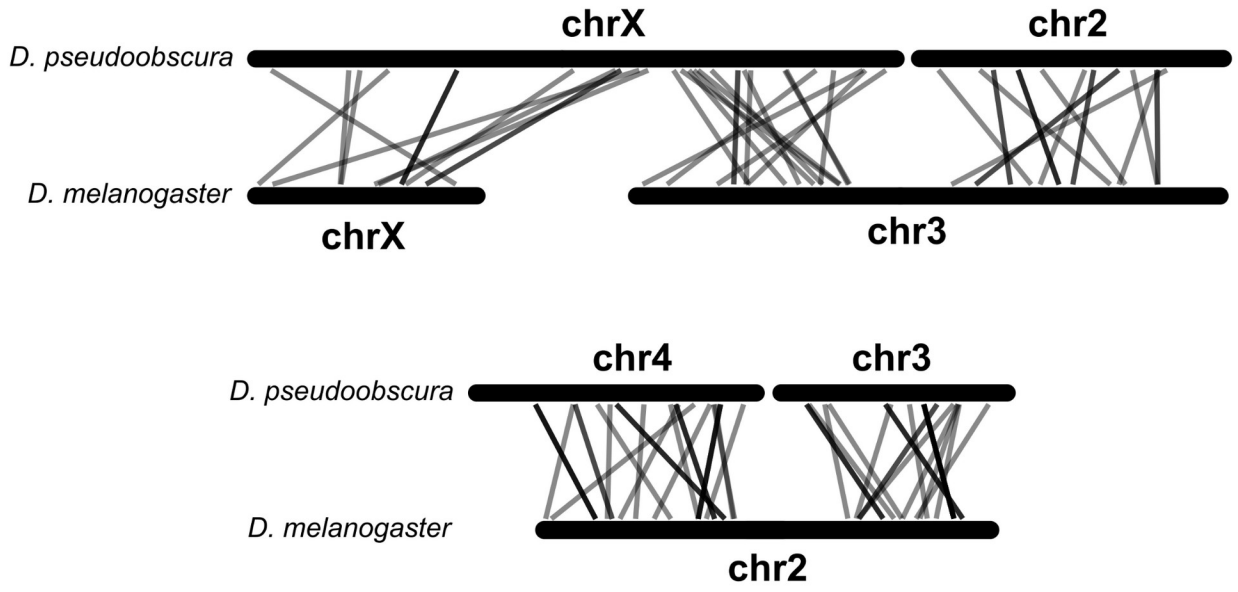

chrX
chr2
D. pseudoobscura
D. melanogaster
chrX
chr3
chr4
chr3
D. pseudoobscura
D. melanogaster
chr2

Figure 3

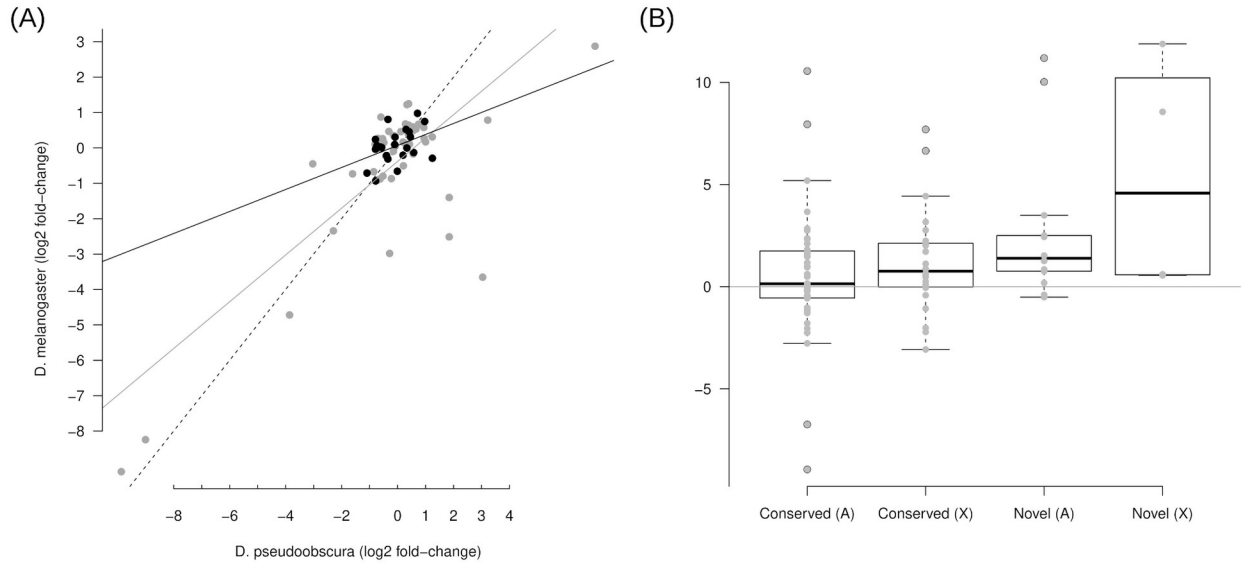
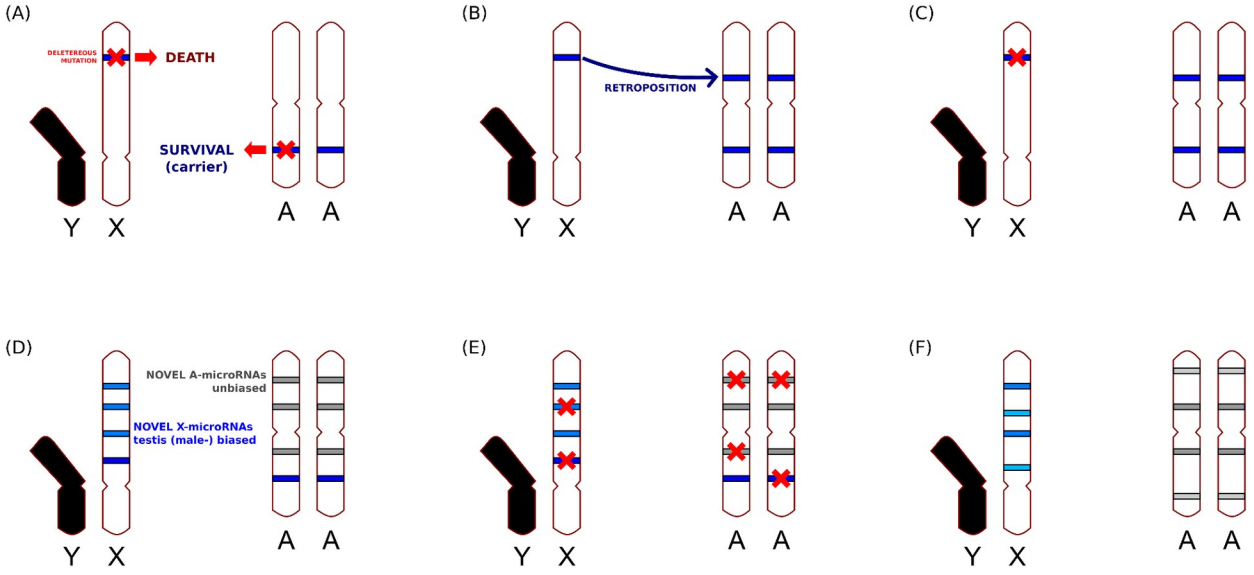

(A)
D. melanogaster (log2 fold−change)
D. pseudoobscura (log2 fold−change)
(B)
Conserved (A)
Conserved (X)
Novel (A)
Novel (X)

Figure 4


(A)
DELETEREOUS MUTATION
DEATH
SURVIVAL (carrier)
Y X
A A
(B)
RETROPOSITION
Y X
A A
(C)
Y X
A A
(D)
NOVEL A-microRNAs unbiased
NOVEL X-microRNAs testis (male-) biased
Y X
A A
(E)
Y X
A A
(F)
Y X
A A