Functional Divergence of the miRNA Transcriptome at the Onset of Drosophila Metamorphosis

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Associate editor: Patricia Wittkopp

Abstract

MicroRNAs (miRNAs) are endogenous RNA molecules that regulate gene expression posttranscriptionally. To date, the emergence of miRNAs and their patterns of sequence evolution have been analyzed in great detail. However, the extent to which miRNA expression levels have evolved over time, the role different evolutionary forces play in shaping these changes, and whether this variation in miRNA expression can reveal the interplay between miRNAs and mRNAs remain poorly understood. This is especially true for miRNA expressed during key developmental transitions. Here, we assayed miRNA expression levels immediately before (≥18BPF [18 h before puparium formation]) and after (PF) the increase in the hormone ecdysone responsible for triggering metamorphosis. We did so in four strains of Drosophila melanogaster and two closely related species. In contrast to their sequence conservation, approximately 25% of miRNAs analyzed showed significant within-species variation in male expression levels at ≥18BPF and/or PF. Additionally, approximately 33% showed modifications in their pattern of expression bias between developmental timepoints. A separate analysis of the ≥18BPF and PF stages revealed that changes in miRNA abundance accumulate linearly over evolutionary time at PF but not at ≥18BPF. Importantly, ≥18BPF-enriched miRNAs showed the greatest variation in expression levels both within and between species, so are the less likely to evolve under stabilizing selection. Functional attributes, such as expression ubiquity, appeared more tightly associated with lower levels of miRNA expression polymorphism at PF than at ≥18BPF. Furthermore, ≥18BPF- and PF-enriched miRNAs showed opposite patterns of covariation in expression with mRNAs, which denoted the type of regulatory relationship between miRNAs and mRNAs. Collectively, our results show contrasting patterns of functional divergence associated with miRNA expression levels during Drosophila ontogeny.

Key words: Drosophila, evolution of expression profiles, miRNAs, metamorphosis, miRNA–mRNA associations.

Introduction

Precise regulation of gene expression is instrumental for proper execution of the majority of biological processes, including cell differentiation and homeostasis. A key parameter of this regulation is mRNA abundance, which is influenced posttranscriptionally by microRNAs (miRNAs) (Filipowicz et al. 2008; Axtell et al. 2011). miRNAs are small (~22 nt) noncoding RNA transacting factors that induce mRNA decay or translation inhibition by base pairing with complementarity regions on the mRNA molecule (Carrington and Ambros 2003; Ambros 2004; Bartel 2004). This complementarity involves Watson–Crick pairing with particular motifs at the 3′-untranslated region (UTR) of the mRNA, although it can also occur at the 5′-UTR or coding region (Duursma et al. 2008; Forman et al. 2008; Orom et al. 2008; Tay et al. 2008; Schnall-Levin et al. 2010). Importantly, miRNAs found in distantly related taxa tend to exhibit a remarkable degree of sequence conservation, especially in the so-called “seed” motif, which is close to the 5′-end of the miRNA and is critical for the interaction with the targeted mRNAs (Bartel 2009; Wheeler et al. 2009).

miRNAs are thought to ameliorate expression noise in expression networks (Horhammer and Shomron 2006; Wu et al. 2009; Ebert and Sharp 2012) and, consequently, increase the robustness of developmental systems contributing to phenotypic stability (Waddington 1959). It is therefore of special interest to determine the limits to which changes in miRNA expression attributes such as the expression level can be accommodated over evolutionary time. Changes in miRNA abundance can contribute to interindividual variation in expression of miRNA-regulated targets ultimately impacting on protein levels and distribution (Baek et al. 2008; Selbach et al. 2008; Borel et al. 2011). Some changes in miRNA abundance have been shown to account for variability in platelet reactivity and drug sensitivity in humans (Huang et al. 2011; Nagalla et al. 2011), elicit common disorders such as cancer (Chen et al. 2012), or underlie the intraspecific variation of morphological characters (Arif et al. 2012).
2013). Among closely related species, divergence in miRNA abundance has also been linked to important functional and phenotypic consequences. In the natural occurring hybrid of two sister Arabidopsis species, miRNA expression diversity from the parental species results in novel phenotypes that contribute to adaptation (Ha et al. 2009). In primates, it has been proposed that miRNA-mediated differences in mRNA abundance underlie partly the evolution of human cognitive functions (Hu et al. 2011). To date, the malleability in miRNA expression levels during key developmental transitions requiring precise regulation of gene expression remains largely unexplored both at the intra- and interspecific levels.

Metamorphosis is an intricate biological process in which large-scale tissue remodeling and organogenesis are orchestrated (Thummel 2001). In Drosophila, fluctuation of 20-hydroxycydsone level induces the transition from larva to immodulate pupa (Baehrecke 1996; Thummel 1996, 2001). This transition is accompanied by multiple changes in mRNA abundance (White et al. 1999; Arbeitman et al. 2002; Beckstead et al. 2005), with some occurring in a sex-dependent fashion (Lebo et al. 2009). Evolutionary changes in miRNA abundance have been reported both within and between closely related species of the Drosophila melanogaster species subgroup at the onset of metamorphosis (Rifkin et al. 2003). miRNAs play critical roles during insect metamorphosis (Varghese and Cohen 2007; Caygill and Johnston 2008; Sokol et al. 2008; Gomez-Orte and Belles 2009) and in fact their expression profiles have also been characterized at the onset of D. melanogaster metamorphosis (Bashirullah et al. 2003; Sempere et al. 2003; Ruby et al. 2007; Berezikov et al. 2010). However, neither the extent to which miRNAs can accommodate intra- and interspecific changes in abundance nor the interplay of these evolved changes with fluctuation in target mRNA abundance has been elucidated. This is especially relevant when considering the contrast between the two stages that define this transition: Late third instar larval stage, mostly characterized by the transition into a postfeeding stage, wandering, and finding a place to glue; and puparium (at puparium formation; PF) in both sexes of D. melanogaster, Oregon-R and in males of its close relatives D. simulans and D. yakuba.

### Results and Discussion

**miRNA Expression Profiles at the Onset of Metamorphosis**

**Expressed miRNAs at the Onset of Metamorphosis**

We surveyed miRNA expression at late third instar larvae (18 h before puparium formation; ≥18BPF) and white prepuparium (at puparium formation; PF) in both sexes of D. melanogaster Oregon-R and in males of its close relatives D. simulans and D. yakuba using Illumina RNA-seq (Materials and Methods; supplementary table S1 and data set S1, Supplementary Material online). These two species shared ancestor with D. melanogaster approximately 5.4 and 12.8 Ma respectively (Tamura et al. 2004). We found reads corresponding to sequences of approximately 76% (130 of 171) of the miRNAs registered in miRBase release 15 for D. melanogaster (Kozomara and Griffiths-Jones 2011), which is comparable to other surveys (Ruby et al. 2007; Berezikov et al. 2010) (supplementary fig. S1A, Supplementary Material online). Of the miRNAs with evidence of expression, approximately 73% (94 of 130) were supported by sequence reads both in our Illumina data set and in previously generated sequence reads by 454 (Ruby et al. 2007 and Ranz JM and Ashburner M, unpublished data; supplementary table S2 and alignments S1, Supplementary Material online). Interestingly, approximately 38% (41 of 107) and approximately 35% (120 of 350) of the miRNA genes with sequence reads in the ≥18BPF and PF Illumina data sets, respectively, do so in one sex only, suggesting some potential sex-biased expression (supplementary fig. S1B, Supplementary Material online).

In the case of D. simulans and D. yakuba, we documented the expression of 81 and 95 orthologous miRNAs, respectively (supplementary data set S1, Supplementary Material online). Among these orthologs, 9 in D. simulans and 33 in D. yakuba were either not listed in miRBase release 15 or listed with an associated hairpin sequence for which we found no support due to nucleotide differences relative to the reference genome sequence of these species (e.g., miR-277-3p and miR-305-5p in D. simulans; supplementary fig. S2, Supplementary Material online).

As a preamble to our analyses on polymorphism and divergence (see below), we assessed levels of miRNA expression at ≥18BPF and PF in males using a microarray platform that included: 1) Reporters for miRNAs registered in miRBase release 15 across insect species, 2) 100 additional reporters based on previous deep-sequencing results from several Drosophila species, and 3) several controls (Materials and Methods; supplementary fig. S3 and table S3, Supplementary Material online). We examined four strains of D. melanogaster, including one African strain to better reflect the recent demographic history of the species (Li and Stephan 2006), and one strain of each of its relatives D. simulans and D. yakuba (Materials and Methods; supplementary table S1, Supplementary Material online). A total of 280 reporters representing 132 and 148 sequences of the 5’- and 3’-arms, respectively, of different miRNA genes relevant to the six strains of the D. melanogaster species subgroup were subject to downstream analyses. The potential impact of nucleotide mismatches on expression estimates across D. melanogaster strains was found to be limited to two reporters (supplementary text, table S4, and fig. S4, Supplementary Material online). Within the subset of reporters considered, 120 were identical in sequence for the three species whereas the rest provided reliable information for two or one of the species only. Per species, we found similar counts of expressed miRNA reporters: 77 in D. melanogaster, 73 in D. simulans, and 70 in D. yakuba. Among the 67 miRNA reporters that are
conserved in sequence across the three species and could be assayed in our arrays, 55 (83%) were confirmed to be expressed at either ≥18BPF, PF, or both in all four D. melanogaster strains (supplementary data set S1, Supplementary Material online). The reliability of our expression measures using an array platform was supported by the high positive correlations between biological replicates (supplementary fig. S3, Supplementary Material online), by replicating some experiments with quantitative reverse-transcription polymerase chain reaction (qRTPCR) (supplementary text, figs. S5 and S6, and table S5, Supplementary Material online), and by the good agreement between the expression estimates obtained between microarrays and deep-sequencing experiments (Spearman’s ρ = 0.6171, P < 0.0001; supplementary fig. S7, Supplementary Material online).

miRNA Expression Variability during Male Development

Microarray profiling across males from six Drosophila strains revealed the miRNA reporters differentially expressed between ≥18BPF and PF (i.e., ≥18BPF- and PF-enriched; fig. 1A). Concordant with early single-strain studies (Bashirullah et al. 2003; Sempere et al. 2003; Caygill and Johnston 2008), known edcsyne-induced miRNAs let-7-5p, miR-125-5p, and miR-100-5p (Sempere et al. 2002; Garbuzov and Tatar 2010; Chawla and Sokol 2012) were PF-enriched across D. melanogaster strains, as they were in D. simulans and D. yakuba. Conversely, miR-34-5p, which is downregulated by the transcription factor Broad in high edcsyne titer conditions, was found to be ≥18BPF-enriched across strains, a pattern also displayed by miR-8-5p (Thummel 2001; Sempere et al. 2003; Jin et al. 2012). Nevertheless, the precise fraction of developmentally regulated miRNA reporters varied from strain to strain ranging from 32% in D. yakuba to 62% in the D. melanogaster strain Zimbabwe-109, being the median 42% (table 1; supplementary fig. S8, Supplementary Material online).

Among D. melanogaster males, we confirmed several types of variation associated with miRNA expression levels. Approximately 25% (15 of 60) of the expressed miRNAs suitable for comparison across the four D. melanogaster strains showed significant differences in expression levels at ≥18BPF (6), at PF (4), or at both (5) (P_adj < 0.01; Materials and Methods). Further inspection revealed that only approximately 66% (42 of 64) of the miRNA reporters with detectable level of expression in at least three strains showed consistency in the type of developmental expression pattern, for example, PF enrichment across all the strains (supplementary table S6, Supplementary Material online). The remaining 33% of miRNAs (22 of 64) harbored differences in their developmental expression pattern among strains. This is the case of miR-956-3p, which is ≥18BPF-enriched in all strains of D. melanogaster but in Zimbabwe-109 (fig. 1B). Only the reporter corresponding to miR-289-5p showed opposite developmental regulation, that is, ≥18BPF or PF enrichment depending on the strain. No difference in the ratio of developmentally to nondevelopmentally regulated miRNAs (21:21) was observed among reporters categorized as consistent.

Among the strains assayed, Zimbabwe-109 showed the largest proportion of PF-enriched miRNAs among those expressed (supplementary fig. S8, Supplementary Material online), a difference that is statistically significant (randomization test of goodness-of-fit, P_adj = 2.9 × 10⁻² when Zimbabwe-109 is included and P_adj = 3.1 × 10⁻¹ when excluded; P_adj < 0.05 when any other strain is omitted). To determine whether the lineage leading to Zimbabwe-109 or that leading to the other three strains of D. melanogaster accumulated the most alterations in developmental expression pattern, we included the strains of D. simulans and D. yakuba to phylogenetically polarize the differences recorded (supplementary fig. S9, Supplementary Material online). Assuming maximum parsimony, the branch associated with Zimbabwe-109 appears to have accumulated more differences in developmental expression pattern than that leading to the other three D. melanogaster strains.

Consistency in the developmental expression pattern across strains does not preclude more subtle changes in miRNA abundance. For example, miR-1012-5p was categorized as nondevelopmentally enriched across all strains; however, it entailed statistically significant differences at PF among some of them (analysis of variance [ANOVA], P_adj < 0.01; fig. 1B). To evaluate the extent of these more subtle changes in expression, we focused on those miRNAs showing consistent patterns of expression not only within D. melanogaster but also across D. simulans and D. yakuba. Thirty such miRNA reporters were documented (8 ≥18BPF-enriched, 5 PF-enriched, 18 nondevelopmentally enriched) of which 17% (2 ≥18BPF-enriched, 2 PF-enriched, and 1 nondevelopmentally enriched) showed significant differences at ≥18BPF, PF, or both (P_adj < 0.01; supplementary data set S2, Supplementary Material online). Taken together, all these forms of variation pointed toward a malleable miRNA transcriptome at the onset of metamorphosis.

Gender Differences in miRNA Expression

We assayed miRNA expression levels in females from two strains of D. melanogaster and one of D. simulans finding a similar fraction of developmentally regulated miRNAs to that in males (table 1; supplementary fig. S10, Supplementary Material online). Seventy-eight percent (54 of 69) of miRNAs with detectable level of expression in females of the two D. melanogaster strains showed a consistent expression pattern between developmental stages with 24 of them (45%) exhibiting developmental regulation (11 ≥18BPF-enriched and 13 PF-enriched). Unlike in males, the proportion of miRNAs displaying different developmental expression patterns did not differ across strains (supplementary fig. S8, Supplementary Material online; randomization test of goodness-of-fit, P_adj = 1.5 × 10⁻¹; males, including the same three strains as in females, P_adj = 6.0 × 10⁻³).

Sex-biased gene expression for the protein-coding fraction of the genome has been examined at PF (Lebo et al. 2009), but no equivalent analysis has been performed for miRNAs. Although limited, we did find evidence of miRNA sex-biased expression occurring as early as ≥18BPF (supplementary fig. S11A, Supplementary Material online). Most miRNAs
though, whether developmentally regulated or not, showed no evidence of sex-bias in expression (supplementary fig. S12A and B, Supplementary Material online). At $P_{\text{adj}} < 0.01$, ten miRNA reporters showed significant sex-biased expression in at least one of the six strains by sex combinations assayed, a number that increased up to 22 at $P_{\text{adj}} < 0.05$ (supplementary fig. S11B and data set S2, Supplementary Material online). Among miRNA reporters showing sex-bias in expression, we found cases such as miR-964-5p, which displayed differences in expression between the genders at a single developmental stage, and others like miR-312-3p, which did at both $\geq 18$BPF and PF (supplementary fig. S12C and D, Supplementary Material online). No case involving reversal in the pattern of developmental enrichment between the sexes, for example, from $\geq 18$BPF enrichment in males to PF enrichment in females, was detected.

Among the miRNA reporters showing sex-biased gene expression, the cluster miR-310 to miR-313 stood out. Each of the four constituent miRNA genes showed statistically significant male-biased expression in at least two of the six strains by developmental stage combinations assayed (supplementary fig. S12D, Supplementary Material online). Although the sequence similarity of some of the miRNA genes in the cluster (miR-310-3p, miR-311-3p, and miR-312-3p) could give rise to some apparent coexpression, inspection of the deep-sequencing data for the strain Oregon-R (supplementary alignments, Supplementary Material online) and the inferred coexpression of the mir-310 cluster based on deep-sequencing data

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Developmental changes in miRNA expression at the onset of metamorphosis across six Drosophila males. (A) Differences in expression levels between $\geq 18$BPF and PF. $x$ axis, difference in normalized log$_2$-transformed expression levels between $\geq 18$BPF and PF; $y$ axis, significance of the difference as $-\log_{10}(P \text{ value})$. Statistically significant differences were determined using a one-way ANOVA. Red dotted line, $P_{\text{adj}} = 0.01$. (B) Examples of variation in miRNA abundance in males. The average expression level and the standard error of the mean are shown. Left, interstrain difference affecting the developmental expression pattern. miR-956-3p is $\geq 18$BPF-enriched in all strains but in Zimbabwe-109. In this strain, there is no significant difference in expression level between stages due to an increase in the level of expression at PF ($P_{\text{adj}} = 2 \times 10^{-4}$). Right, interstrain difference not associated with differences in developmental expression patterns. miR-1012-5p is nondevelopmentally enriched in all strains but still statistically significant differences in expression level were confirmed at PF ($P_{\text{adj}} = 7.1 \times 10^{-4}$). Canton-S, Oregon-R, Samarkand, and Zimbabwe-109 are strains of D. melanogaster.
Table 1. List of miRNAs Showing Differential Expression between ≥18BPF and PF Stages.

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(continued)
(Ryazansky et al. 2011) ruled out this possibility. Therefore, the observed sex-biased expression pattern strongly suggests a sex-dependent coregulation of the mir-310 cluster at PF. This interpretation agrees well with the influence of this cluster on male fertility by modulating the Wingless signaling pathway, which is required for cell differentiation of the somatic and germline tissues in testis (Pancratov et al. 2013). Intriguingly, the expression of mir-313-5p could represent a case of sex-dependent arm-switching.

Evolution of miRNA Expression Profiles

**Differentiation of Expression Levels at ≥18BPF and PF**

The multiple developmental changes occurring during early pupation compared with late instar larvae parallel changes in miRNA abundance of many genes during this developmental transition (Arbeitman et al. 2002). Expression profiles of regulatory genes at PF should be especially refractory to change due to potential detrimental effects. We tested whether this hypothesis was reflected in miRNA expression levels by examining the way significant differences have accumulated over evolutionary time and by comparing the magnitude of expression differences between stages.

We calculated expression distances among the males of the six strains surveyed at ≥18BPF and PF separately as well as the divergence time for each strain pair (Materials and Methods; supplementary text and table S7, Supplementary Material online). At PF, unlike at ≥18BPF, expression divergence in miRNA abundance did correlate with divergence time (fig. 2A and supplementary fig. S13, Supplementary Material online). This result suggests that the way changes in miRNA abundance accumulate over evolutionary time differs between the stages.

We subsequently estimated the magnitude of miRNA expression differences across the six strains as the coefficient of variation (CV), that is, the ratio of the standard deviation (SD) to the mean. We compared the log10-transformed CV at ≥18BPF and PF finding no significant difference (average CV: ≥18BPF, 0.761; PF, 0.729; one-way ANOVA, $P = 0.630$). Nevertheless, absence of significant differences in the global levels of variation does not rule out more subtle patterns of differentiation among particular groups of miRNAs based on their expression attributes. According to this, we tested for differences in expression levels among miRNAs showing consistent developmental expression patterns in D. melanogaster. Both at ≥18BPF (one-way ANOVA, $P = 0.012$) and PF (one-way ANOVA, $P = 0.005$), we found the same rank of differentiation in miRNA expression levels: ≥18BPF-enriched > PF-enriched > nondevelopmentally enriched miRNAs (fig. 2B).

Post hoc tests underscored the difference between ≥18BPF- and nondevelopmentally enriched miRNAs (Tukey–Kramer HSD; ≥18BPF, $P = 0.019$; PF, $P = 0.003$). Collectively, these results suggest that the two developmental stages sampled might be subject to different evolutionary dynamics, which affect the pace at which expression changes accumulate over evolutionary time and the global levels of differentiation of particular groups of miRNAs.

**Evolutionary Mode and Developmental Expression Pattern**

We determined the mode of evolution (i.e., stabilizing selection, genetic drift, or directional selection) of miRNA expression levels at ≥18BPF and PF. We used a two-step ANOVA-
based approach in which differences in expression levels were tested first within and then between species (supplementary text, Supplementary Material online). Approximately 75% (39 of 48) and 80% (47 of 55) miRNAs showed a mode of evolution consistent with the action of stabilizing selection at ≥18BPF and PF, respectively (fig. 3A), a result reminiscent of previous observations for mRNA levels (Rifkin et al. 2005). Our results suggest that directional selection has acted on the expression level of a few miRNAs: miR-34-5p and miR-956-3p at ≥18BPF, and miR-34-5p, miR-312-3p, and miR-995-3p at PF. The remaining miRNAs exhibited patterns of variation within and between species compatible with genetic drift alone or with other evolutionary scenarios such as relaxation of constraints in a lineage-dependent manner or combinations of genetic drift and some form of lineage-dependent selection (fig. 3B).

If miRNAs with different developmental expression patterns differ in their degree of variation in expression levels (fig. 2B), they should also show a different propensity to evolve under stabilizing selection. We confirmed this nonrandom association especially at PF (supplementary fig. S14, Supplementary Material online). At this developmental stage, nondevelopmentally enriched and PF-enriched miRNAs largely evolve under stabilizing selection (~83% in both cases) whereas only 50% of the ≥18BPF-enriched miRNAs follow this mode of evolution. The difference in

**FIG. 3.** Evolutionary modes of miRNA expression at the onset of male metamorphosis in the *Drosophila melanogaster* species subgroup. (A) Counts of miRNAs with patterns of intra- and interspecific variation compatible with particular evolutionary modes. (B) miRNAs illustrating four main evolutionary modes. Box plots are used to show the dispersion around the median level of expression for each strain by miRNA combination. CS, Canton-S; ORR, Oregon-R; Sam, Samarkand; Zw, Zimbabwe-109; and sim, *D. simulans*. Within and between species differences in expression levels were tested, respectively, by one-way ANOVA. Whether evidence of significant intra- and interspecific variation was found is indicated on the x and y axes, respectively. See supplementary text, Supplementary Material online, for the rationale followed to categorize each miRNA under a particular evolutionary mode. miRNAs showing nonsignificant and significant differences in expression between *D. melanogaster* and *D. simulans* are shown on top and bottom, respectively. Depending on the combination of significant intra- and interspecific differences in expression levels, miRNAs are categorized as evolving under stabilizing selection (top left), directional selection (bottom left), genetic drift (bottom right), or associated with complex scenarios (top right). y axis, log₂-tranformed expression values in an arbitrary scale. Examples shown correspond to miRNAs expressed at the PF stage.
the proportion of miRNAs evolving under stabilizing selection is statistically significant between ≥18BPF- and nondevelopmentally enriched miRNAs (two-tailed Fisher’s exact test, FET; ≥18BPF, \( P = 0.040 \); PF, \( P = 0.011 \)). Why the expression level of ≥18BPF-enriched miRNAs is more variable overall than that of nondevelopmentally enriched miRNAs is unclear at this time. Nevertheless, this pattern reinforces the notion that miRNAs with different developmental expression patterns are exposed to different evolutionary pressures.

**Evolutionary Mode and miRNA Evolutionary Age**

We examined whether the evolutionary mode in miRNA expression is related to evolutionary age, that is, the moment at which we can parsimoniously date the emergence of a miRNA. Presumably, more ancient miRNAs should be more stably integrated into regulatory networks than younger miRNAs and thus should more likely evolve under stabilizing selection (Chen and Rajewsky 2007). We dated the emergence of miRNAs assuming a maximum parsimony framework (supplementary text and data set S3, Supplementary Material online) distinguishing between two main age classes. The first class included miRNAs inferred to have emerged during the evolution of the subgenus Sophophora after the split with the subgenus Drosophila—and therefore less likely to evolve under stabilizing selection, and the second class included miRNAs inferred to have been present in the common ancestor of both subgenera—and therefore more likely to be already stably integrated into the regulatory network. Subsequently, we examined whether these two age classes differ in the extent to which they evolve under stabilizing selection versus other evolutionary modes. miRNAs inferred to have emerged during the evolution of the subgenus Sophophora (6 expressed at ≥18BPF and 8 at PF) do evolve less often under stabilizing selection than more ancient miRNAs (46 expressed at ≥18BPF and 51 at PF), which is confirmed at PF but not at ≥18BPF (FET; ≥18BPF, \( P = 0.157 \); PF, \( P = 0.046 \)). Although this result should be taken cautiously due to the limited count of the Sophophora-specific miRNAs present in the analysis, it suggests that the expression levels of more recently evolved miRNAs have not had enough time to be shaped by natural selection to the extent the levels of expression of more ancient miRNAs have been.

**Determinants of miRNA Expression Polymorphism in D. melanogaster Males.** The regulatory role of recently emerged miRNAs is more likely to be still evolving compared with that of miRNAs found in many phyla. Because of the potentially detrimental effects on organismal fitness of recently evolved miRNAs if expressed to a high level or across multiple tissues/organisms, these miRNAs should exhibit lower expression levels and narrower spatiotemporal expression profiles than ancient miRNAs (Chen and Rajewsky 2007). Consistent with this notion, recently evolved miRNAs have been shown to be expressed at a low level in primates and Drosophila species (Berezikov et al. 2006; Lu, Shen, et al. 2008; Liang and Li 2009). Further, the introduction of the relatively young mir-310 family from D. pseudoobscura into the D. melanogaster genome resulted in misexpression of numerous genes and in lower organismal fitness (Tang et al. 2010). Based on these premises, miRNAs that are either present across most metazoans, exhibit a detrimental phenotype if misexpressed, or are more ubiquitously expressed across tissues and/or developmental stages should possess lower expression polymorphism.

We tested for a negative relationship between the intraindividual log_{10}CV in expression of 62 miRNAs expressed at least in one of two studied developmental stages in D. melanogaster and evolutionary age, having associated a gain-of-function phenotype (Schertel et al. 2012), and expression breadth across developmental stages and tissues (Shen et al. 2011). We did so for each developmental stage separately. For the evolutionary age, we distinguished broadly between Drosophila-evolved miRNAs versus miRNAs inferred to have been present in the ancestor to the Drosophila genus and Anopheles gambiae (age classes “young” and “ancient” in fig. 4A), finding evidence of a significant negative association with the level of expression polymorphism (one-way ANOVA; ≥18BPF, \( P = 0.086 \); PF, \( P = 0.008 \)). When the number of age classes considered was further divided into 4 based on additional phylogenetic partitions (supplementary text, Supplementary Material online), the trend was similar (one-way ANOVA; ≥18BPF, \( P = 0.069 \); PF, \( P = 0.034 \)). In this case, pairwise post hoc tests revealed, at least for PF, that the diametrically opposed relationship between the expression polymorphism of miRNAs originated during the evolution of the subgenus Sophophora versus that of the most ancient miRNAs is the main factor contributing to the pattern found (supplementary fig. S15A and table S8, Supplementary Material online). Considering age as a continuous variable did not alter this observation (supplementary fig. S15B, Supplementary Material online).

Phenotypic effects upon inducing miRNA misexpression or being ubiquitously expressed were associated similarly with miRNA expression polymorphism in D. melanogaster. miRNAs displaying gain-of-function phenotypes were found to harbor significantly lower CVs in expression than miRNAs with no phenotype especially at PF (one-way ANOVA; ≥18BPF, \( P = 0.089 \); PF, \( P = 0.017 \)) (fig. 4B). Furthermore, for the expression breadth, we found a significant negative correlation between expression ubiquity and log_{10}CV, a pattern confined to the PF stage (\( r^2 = 0.228 \), \( P = 0.004 \); ≥18BPF, \( r^2 = 0.021 \), \( P = 0.426 \)) (fig. 4C). These results confirm that miRNAs that are more necessary for obtaining a wild type phenotype and miRNAs with more ubiquitous expression profiles, which are likely to be exposed to conflicting functional requirements across tissues (Khaitovich et al. 2005), are more constrained in their capability to accommodate segregating expression variance during population differentiation. Importantly, these functional constraints are more apparent at PF than at ≥18BPF.

The negative correlations observed between miRNA expression polymorphism and evolutionary age, gain-of-function phenotypes, and expression ubiquity are unlikely to be independent from one another. This would be the case if more recently originated miRNAs occupy less relevant
positions in the regulatory network and have narrower spatiotemporal expression profiles, which can result in better accommodating higher levels of expression polymorphism. We did find statistical evidence of the association among these variables pointing to this parsimonious view (supplementary text, Supplementary Material online), which is consistent with a higher association of ancient miRNAs with disease phenotypes and with broader expression profiles across tissues, compared with recently originated miRNAs, reported in humans and Diptera, respectively (Lu, Zhang, et al. 2008; Lyu et al. 2014).

**Using Intraspecific Variation in Expression Levels to Uncover the Landscape of the miRNA–mRNA Regulatory Network**

To uncover the interface between miRNAs and mRNAs at the expression level at the onset of metamorphosis, we leveraged

![Graph](image-url)
the intraspecific variation in expression levels for both molecules. We identified developmentally regulated miRNAs upon pooling the miRNA expression data from the males of the four strains of *D. melanogaster* (*P* adj < 0.05; supplementary text, Supplementary Material online). Then, we assayed levels of mRNA abundance from the same biological samples and used a mixed-effects linear model to estimate the expression association between developmentally regulated miRNAs, and the transcripts for which the microarray platform had distinctive probesets, that is, the so-called mRNA exemplars (Materials and Methods; supplementary fig. S3 and text S1, Supplementary Material online). An mRNA exemplar might correspond to one or more transcripts. Permutation tests helped determine whether the observed miRNA–mRNA association values were higher than expected by chance alone (*P* adj < 0.05; supplementary text, Supplementary Material online).

We identified 617 mRNA exemplars, representing 545 genes, as significantly associated with 43 developmentally regulated miRNAs (supplementary data sets S4 and S5, Supplementary Material online). The number of significant associations with mRNA exemplars varied remarkably among miRNAs (average SD/median; ~217 ~133/212; supplementary fig. S16, Supplementary Material online), with *miR-34-3p* displaying the highest number—612. These statistically significant associations between expression levels of miRNAs and mRNAs may reflect concurrent coregulation by a common upstream factor in the transcriptional hierarchy or a bona fide causal regulatory relationship, which can result from either direct or indirect targeting, the latter as it might occur between an miRNA regulating a transcription factor and the battery of genes under the transcription factor’s control. We investigated the interplay between expression associations and their potential causal regulatory nature by considering the sign of the association, that is, positive or negative, the link with miRNA developmental expression patterns, and the associated biological coherent patterns.

We distinguished between positive and negative associations in expression for each miRNA–mRNA exemplar pair. Importantly, when a miRNA covaried in expression with an mRNA exemplar, it was more likely to show a negative than a positive association (supplementary fig. S16, Supplementary Material online). As reported in other organisms such as primates (Hu et al. 2011; Lu and Clark 2012; Parts et al. 2012; Lappalainen et al. 2013), positive expression associations were also abundant, which highlights the ambiguous nature of the interplay between expression levels of miRNAs and mRNAs (Ameres and Zamore 2013). Intriguingly, developmentally regulated miRNAs showed a bimodal distribution for the proportion of negative significant associations over the total exhibited by each miRNA (fig. 5A). We investigated whether this bimodal distribution was related to miRNA developmental expression patterns in a nonrandom manner. Specifically, we calculated the correlation between the proportion of negative significant associations and the propensity of a miRNA in showing a particular type of developmental expression pattern across the four strains assayed (fig. 5B). We found that the higher is the number of strains showing miRNA expression enrichment at PF, the higher is the proportion of negative miRNA–mRNA expression associations (Spearman’s *p* = 0.687, *P* < 0.0001). Accordingly, ≥18BPF-enriched miRNAs across *D. melanogaster* strains were more likely to exhibit positive expression associations with mRNAs whereas PF-enriched miRNAs were more likely to exhibit negative associations.

These patterns could denote distinctive relationships with the covariating miRNAs (fig. 5C). Downregulation of a miRNA alone does not result directly in an increase of its targets’ abundance unless that miRNA is involved in a feedback loop to inhibit the transcription of its targets. Therefore, positive miRNA–mRNA expression associations may often reflect the concurrent downregulation of both molecules at PF denoting no causal regulatory relationship (fig. 5C, left panel). Conversely, the upregulation of a miRNA at PF may be important to facilitate the degradation of truly regulated targets that are not necessary at this stage, resulting in a decreasing abundance (fig. 5C, right panel). This second pattern is reminiscent of the degradation of maternally deposited mRNAs by a set of zygotically expressed miRNAs in the *Drosophila* embryo (Bushati et al. 2008). In consequence, negative associations of PF-enriched miRNAs and mRNAs should more likely represent bona fide causal regulations by miRNAs.

To test this, we examined whether PF-enriched miRNAs showing negative expression associations with predicted targets among the 617 mRNA exemplars were significantly over-represented relative to ≥18BPF-enriched miRNAs displaying equivalent properties. Indeed, we found that there are more predicted targets among mRNAs negatively associated with PF-enriched miRNAs than among those with ≥18BPF-enriched miRNAs, a pattern not shown in positive expression associations (Randomization test of goodness-of-fit; *P* = 0.013 and *P* = 0.246, respectively; supplementary table S9, Supplementary Material online). This difference reinforces the possibility that positive expression associations are less likely than negative expression associations in denoting bona fide causal miRNA regulation at the onset of metamorphosis.

We further examined the biological properties of the miRNA–mRNA exemplar associations by searching for biological coherent patterns in genes grouped by their patterns of expression association with the 43 developmentally regulated miRNAs. Briefly, ten clusters of mRNA exemplars were identified by hierarchical cluster analysis based on the sign of their expression associations (supplementary fig. S17, Supplementary Material online). Subsequently, functional enrichment for functional rubrics in each cluster was tested with DAVID under several degrees of stringency (Materials and Methods). We found enrichment for biological processes and other functional rubrics unambiguously related to the onset of metamorphosis in nine of the ten clusters (supplementary table S10, Supplementary Material online). Nucleotide biosynthesis and energy production pathways, structural and regulatory genes related to muscle formation, and genes involved in molting formation were enriched among those downregulated at PF in Clusters 1, 3, 4, and 8–10. On the other hand, histolysis upon tissue apoptosis...
and innate immune response pathway related genes were found to be overrepresented among those upregulated at PF in Clusters 5 and 7.

A closer inspection of several clusters unveiled the complexity of the regulatory interactions that occur during the transition from larva to pupa. For instance, 66 mRNA exemplars in Cluster 8 were negatively associated with up to 27 miRNAs and positively associated with up to 14 miRNAs. This cluster is enriched for genes involved in the functional rubric "molting cycle" and the cellular component rubric "muscle myosin complex" (supplementary table S11, Supplementary Material online). Among the seven genes in Cluster 8 annotated as part of the functional rubrics relevant to muscle development (muscle myosin complex or "muscle protein"), five were predicted to harbor binding sites in their 3′-UTRs according to TargetScan. Three of these genes showed negative associations in expression with miRNAs whereas the other two showed positive associations. The remaining two genes in Cluster 8 (Tm2 and Mlc2) did not have any predicted mRNA binding site. Similar patterns were found for the constituent genes of Cluster 10, which are annotated as part of the functional rubric "contractile fiber" (supplementary table S11, Supplementary Material online).

An intriguing aspect is that none of the genes that are a part of functional rubrics related to muscle development in Clusters 8 and 10 possesses binding sites for let-7 complex miRNAs in their 3′-UTRs or open reading frames (ORFs). Nevertheless, the expression levels of these genes are negatively associated with those of the let-7 complex miRNAs. The let-7 complex is required for the maturation of neuromuscular junction and deformation of abdominal neuromusculature, which at least in part is achieved by downregulating the BTB-zinc finger transcription factor Ab (Caygill and Johnston 2008; Sokol et al. 2008). Several nonmutually exclusive explanations may account for this observation. First, miRNAs other than let-7 may contribute to the regulation of these genes during metamorphosis (supplementary table S11, Supplementary Material online). Second, the let-7 complex may regulate these genes indirectly through the control of their upstream transcription factor(s) during metamorphosis. Third, the expression levels of these genes are not regulated posttranscriptionally by miRNAs denoting false positives.

The innate immune response of Drosophila at the onset of metamorphosis is well represented in the significant expression associations found. This response is regulated by ecdysone and juvenile hormone and mainly consists of two
components: Localized melanization and antimicrobial peptides production (Beckstead et al. 2005; Flatt et al. 2008; Fullaondo and Lee 2012). We found that Cluster 7 is enriched for genes involved in these two components. The genes Dat, e, and ple were found to participate in the functional rubric “dopamine metabolic process,” which contributes to melanization among other biological functions (Hsouna et al. 2007; Wittkopp and Beldade 2009; Takahashi 2013). Three other genes encode antimicrobial peptides: Drsl2, and Drsl5. The expression levels of these six genes are significantly higher at PF as expected (Graveley et al. 2011 and this work). Interestingly, positive expression associations were mostly found between genes and miRNAs that are predicted to bind their ORFs whereas the only two negative associations involve genes (Drsl2 and ple) presumably bound at their 3’-UTRs by miRNAs (supplementary table S12, Supplementary Material online). Further, the putative miRNAs regulating upstream genes of the innate immune pathway have been studied in silico (Fullaondo and Lee 2012). Some of these miRNAs have been identified in our miRNA–mRNA exemplars association list such as the miR-2 family, miR-9a (both arms), miR-125-5p, miR-279-3p, and miR-281-2-5p. Thus, the expression levels of these innate immune response genes may be miRNA-regulated directly or through their upstream regulators in those same immune pathways.

Our characterization of the landscape of miRNA–mRNA exemplars associations is limited in two ways. First, it is dependent on the differential miRNA expression between the developmental stages compared. Second, some miRNAs are expressed in tissue- or cell-type-specific manners and therefore expression associations may happen in multiple organs but in different directions resulting in a blurry association signal if any. It is remarkable therefore that we are still able to capture significant expression associations from our whole-body assays, which in some cases are suggestive of bona fide causal regulation.

We have generated a portrait of the intra- and interspecific differences in expression levels of sequence conserved miRNAs at the onset of Drosophila metamorphosis. In spite of the documented phylogenetic differentiation in expression levels, we find that the evolution of miRNA abundance is driven mainly by stabilizing selection. This agrees well with the stabilizing role that miRNAs play by repressing leaky expression or fine-tuning transcript levels. Notably, the type of developmental expression pattern of a miRNA appears to be an excellent predictor of the degree to which a miRNA can accommodate variation in expression level during the evolutionary process. The expression levels of some miRNAs are still evolving, which in a few cases seems compatible with the optimization of their functional role by directional selection. Whether this functional optimization goes beyond canalizing expression levels during metamorphosis contributing as well to the phenotypic diversification in the genus Drosophila remains to be established. Overall, we find distinct patterns of differentiation among miRNA expression levels between late third instar larva and white prepupa, which is also evidenced in how these evolved changes are linked to relevant proxies for the integration of miRNAs into the regulatory network. In addition, miRNAs with different developmental expression patterns exhibit marked differences in how they covariate with miRNAs in expression, which might reflect the type of regulatory relationship between both kind of molecules. Taken together, the profound differences in the biological requirements of these two developmental stages seem to dictate the properties in miRNA expression levels at the onset of Drosophila metamorphosis.

Materials and Methods

Fly Stocks and Husbandry

Six strains representing three species of the D. melanogaster species group were used (supplementary table S1, Supplementary Material online). Flies were grown in standard corn meal medium, constant lighting conditions, and at 25°C. Third instar larvae were identified as previously reported (Maroni and Stamey 1983; Andres and Thummel 1994). Briefly, larvae were raised under noncrowded conditions in medium with 0.05% bromophenol blue. Wandering larvae exhibiting dark blue gut, which corresponds to approximately 18 h before puparium formation, were collected, rinsed with water, and separated by sex. For the 0–1 h white prepuparia, wandering larvae with light blue to white gut were separated by sex, placed in a Petri dish on damp light-wipe tissue until appropriate moment for collection (Bainbridge and Bownes 1981). The presence of visible male gonads was used for sex identification; trial collections were performed to assure process accuracy. Samples were snap frozen in liquid nitrogen and stored at −80°C until RNA isolation.

RNA Extractions

Two rounds of RNA collection were performed: the first, for small RNA-seq; and the second, for the remaining expression profiling approaches used (see below). For each collection, approximately 120 mg (60–80 individuals depending on the strain) of frozen flies for each biological replicate was grinded using motorized pestles and the total RNAs extracted and purified with miRVana miRNA isolation kit (Ambion Inc.), which allows the optional separation of mRNAs from small RNAs. In the first collection, the enriched small RNAs were isolated according to the manufacturer indications for subsequent small RNA library preparation. In the second collection, both total RNAs and small RNAs from four biological replicates for each strain/sex were isolated and used in all three expression profiling approaches. Concentration, quality, and integrity of the RNA samples were assessed using the NanoDrop 8000 Spectrophotometer and the RNA 6000 Nano and Small RNA kits (Agilent Technologies) in an Agilent 2100 Bioanalyzer. RNA extractions for different strains were performed independently to avoid cross-contamination.

Small RNA-seq

Adaptor-ligated cDNAs were prepared according to Illumina small RNA preparation protocol (Preparing Samples for
Analysis of Small RNA Using the Oligo Only Kit, http://www.illumina.com, last accessed February 1, 2014). Briefly, 16–28 nt small RNAs were size selected from a denaturing polyacrylamide gels, 5'-adaptors with 4-n index and 3'-adaptors were ligated using T4 RNA ligase (Ambion 2140) and the resulting molecules were purified by size selection from denaturing polyacrylamide gels after each ligation reaction. Four samples with different indexes were pooled for 3'-adaptor ligation and further preparation steps. The ligated small RNAs were reversed transcribed using Superscript II reverse transcriptase (Invitrogen 18064), subsequently PCR amplified with a Phusion high fidelity PCR DNA polymerase (Finnzymes F-506) through ten cycles, and purified from denaturing polyacrylamide gels. Library preparation for different strains was performed independently to avoid cross-contamination. The quality of the cDNAs was evaluated with the High Sensitivity DNA kit (Agilent Technologies) using an Agilent 2100 Bioanalyzer before high-throughput sequencing using a Genome Analyzer II (Illumina) at the Genome Center of the University of California, Davis. Twelve libraries, named as L5–L16 in supplementary table S2, Supplementary Material online, were sequenced in three lanes. Reproducibility of sequencing results was evaluated at biological and technical levels.

Reads were sorted by distinctive indexes before their 5'– and 3'-adaptor sequences were trimmed. Reads matching yeast rRNAs and D. melanogaster 25 S RNA were discarded. The remaining reads were aligned against the stem-loop sequences of known miRNAs according to miRBase release 15 using Bowtie 0.12.5 (Langmead et al. 2009). Because of the potential errors in the reference genome of D. simulans and D. yakuba, we also used the D. melanogaster genome sequence as a reference in the characterization of the libraries of these two species. Alignments and read numbers were recorded. Four libraries sequenced by 454, named as L1–L4 insupplementary two species. Alignments and read numbers were recorded. as a reference in the characterization of the libraries of these

miRNA Microarray Profiling

The miRNAarthropoda_15_UC_100610 array from LC Sciences based on the annotations of miRBase release 15 was used. In addition, 100 custom probes were added to the array (supplementary table S3, Supplementary Material online). Custom probes include previously dubbed passenger sequences of known D. melanogaster miRNAs, putative miRNAs found in deep-sequencing experiments by us and others (Ruby et al. 2007; Lu, Shen, et al. 2008), and control reporters for the 25 rRNA gene harboring nucleotide differences in number and position to evaluate the impact of mismatches on hybridization kinetics. Probes for annotated and predicted miRNAs were present in triplicates. Internal quality controls included 43 spikes and 6 additional positive controls replicated either 4 or 16 times on the array.

Four biological replicates per developmental stage per strain were used in competitive hybridizations. Labeling, hybridization, and image acquisition were performed by LC Sciences. For a given array, the Cy3 and Cy5 fluorescent intensity values of each array were first adjusted by subtracting local background and then normalized according to a locally weighted regression approach (Bolstad et al. 2003). The adjusted values were further log2-transformed and normalized across arrays using the quantile method implemented in JMP Genomics 5.0 (Rao et al. 2008). The expression values for the 280 reporters relevant to the six strains of interest were obtained by averaging over the three technical replicates spotted on separate blocks of the array. A mean intensity value lower than 32 was not considered to be reflective of expression above the background. A given miRNA was not considered to be expressed if it lacked evidence of expression across all sex by strain by developmental stage combinations assayed.

A linear model was used to test for differences in expression levels between developmental stages in any given strain by sex combination and for differences in expression levels between the sexes in any given strain by developmental stage combination. For this purpose, a linear mixed-effects gene model that takes into account both array and dye-specific effects (Jin et al. 2001; Wolfinger et al. 2001) was considered

\[ y_{ijklm} = \mu + A_i + T_j + S_k + D_l + Z_m + S_kD_l \\
+ S_kZ_m + D_lZ_m + S_kD_lZ_m + \epsilon_{ijklm}, \]

where \( y_{ijklm} \) denotes the miRNA expression for the \( i \)th array, \( j \)th dye, \( k \)th strain, \( l \)th developmental stage, and \( m \)th sex. Also, \( \mu \) is the baseline expression, \( A_i \) is the effect of the \( i \)th array, \( T_j \) is the effect of the \( j \)th dye, \( S_k \) is the effect of the \( k \)th strain, \( D_l \) is the effect of the \( l \)th developmental stage, and \( Z_m \) is the effect of the \( m \)th sex. \( A \) was implemented as a random effect whereas \( T, S, D, Z \), and the interaction effects were implemented as fixed effects in JMP Genomics 5.0. Next, the appropriate contrasts were made to obtain the differences of interest. The differences of interest were considered to be statistically significant at a false discovery rate (FDR) of 0.01.

For a given strain, to test the null hypothesis that the difference in magnitude of developmental change was the same in both sexes, a linear mixed-effects model was developed as follows:

\[ y_{ijlm} = \mu + A_i + T_j + D_l + Z_m + D_lZ_m + \epsilon_{ijlm}, \]

where \( y_{ijlm} \) denotes the miRNA expression for the \( i \)th array, \( j \)th developmental stage, and \( m \)th sex. Also, \( \mu \) is the baseline expression, \( A_i \) is the effect of the \( i \)th array, \( T_j \) is the effect of the \( j \)th dye, \( D_l \) is the effect of the \( l \)th developmental stage, and \( Z_m \) is the effect of the \( m \)th sex. \( A \) was implemented as a random effect whereas \( T, D, Z \), and the interaction effect were implemented as fixed effects. Because there were two developmental stages and two sexes, the interaction term corresponded to the difference of interest. The difference in magnitude of developmental change in expression across
sexes was considered to be statistically significant at an FDR of 0.05.

Quantitative Reverse-Transcription Polymerase Chain Reaction

Triplicate total RNA samples for each strain by developmental stage by sex analyzed were polyadenylated and reverse-transcribed using oligo-dT as a primer following manufacturer conditions (Exiqon Universal cDNA Synthesis Kit, 203300). The qRT-PCR step was performed in a CFX-96 real-time instrument (BioRad) using locked nucleic acid primers (Exiqon; [supplementary table S5, Supplementary Material online]) and SYBR Green chemistry (Exiqon SYBR Green Master Mix, 203450). Expression levels of the miRNAs analyzed were estimated relative to the reference genes miR-1-3p and miR-995-3p, which were chosen based on two criteria: 1) Expression uniformity in microarray experiments across all strain by developmental stage by sex combinations, and 2) because they cover two differentiated levels of expression (miR-1-3p is substantially more expressed than miR-995-3p according to the small-RNA sequencing experiments performed in this study). Estimates were calculated using the $-2^{\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) implemented in the Bio-Rad CFX manager software and statistically significant differences among samples were interrogated in JMP Genomics 5.0 (SAS Institute Inc.).

mRNA Microarray Profiling

We assayed levels of mRNA abundance for D. melanogaster males at ≥188PF and PF ([supplementary table S1, Supplementary Material online]). Three of four biological samples used for assaying miRNA levels of expression were randomly chosen for this purpose. Per sample, 10 μg of total RNA was reverse transcribed into cDNA using the SuperScript Double-Stranded cDNA Labeling Kit (Invitrogen). The quality of the cDNAs was evaluated with the DNA 12000 kit (Agilent Technologies) using an Agilent 2100 Bioanalyzer. Probe labeling, hybridization, array scanning, and data extraction were performed by Roche NimbleGen Service Group in Iceland. Single color hybridizations were performed onto species-specific 12 × 135k NimbleGen oligonucleotide arrays corresponding to the FlyBase release 5.7 for D. melanogaster.

The raw mRNA expression data were preprocessed using NimbleGen’s DEVA software suite, which includes background correction, quantile normalization (Bolstad et al. 2003), and summarization of probeset expression using the NimbleGen expression data have been deposited in the NCBI GEO database under accession numbers GSE57438, GSE55562, and GSE55398, respectively. Sequence data for protein-coding and miRNA loci have been deposited in NCBI GenBank under accession numbers KJ774561–KJ774633.

Accession Numbers

Supplementary Material

Supplementary text, alignment, figures S1–S17, tables S1–S12, and data sets S1–S5 are available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).
Acknowledgments

The authors thank Vera Chan for technical help; Kevin Thornton for providing the Zimbabwe-109 strain; Yang Shen for providing the expression breadth values; and Bryan Clifiton, Anthony Long, Hsiu-Ching Ma, Antonio Marco, Andrei Tatarenkov, John True, and Zhaoxia Yu for discussions on different aspects of the manuscript. This work was supported by the National Science Foundation grants (DEB-0949365 and MCB-1157876 to J.M.R.) and the Foundation for Polish Science (to M.V.G.).

References
