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Report

Natural Selection Drives Extremely Rapid Evolution in Antiviral RNAi Genes

Darren J. Obbard,[1,*] Francis M. Jiggins,[1] Daniel L. Halligan,[1] and Tom J. Little[1]
[1] Institute of Evolutionary Biology
University of Edinburgh
Kings Buildings
West Mains Road
Edinburgh EH9 3JT
United Kingdom

Summary

RNA interference (RNAi) is perhaps best known as a laboratory tool. However, RNAi-related pathways represent an antiviral component of innate immunity in both plants and animals [1]. Since viruses can protect themselves by suppressing RNAi [2–4], interaction between RNA viruses and host RNAi may represent an ancient coevolutionary "arms race." This could lead to strong directional selection on RNAi genes, but to date their evolution has not been studied. By comparing DNA sequences from different species of *Drosophila*, we show that the rate of amino acid evolution is substantially elevated in genes related to antiviral RNAi function (*Dcr2*, *R2D2*, and *Ago2*). They are among the fastest evolving 3% of all *Drosophila* genes; they evolve significantly faster than other components of innate immunity and faster than paralogous genes that mediate "housekeeping" functions. Based on DNA polymorphism data from three species of *Drosophila*, McDonald-Kreitman tests showed that this rapid evolution is due to strong positive selection. Furthermore, *Dcr2* and *Ago2* display reduced genetic diversity, indicative of a recent selective sweep in both genes. Together, these data show rapid adaptive evolution of the antiviral RNAi pathway in *Drosophila*. This is a signature of host-pathogen arms races and implies that the ancient battle between RNA viruses and host antiviral RNAi genes is active and significant in shaping RNAi function.

Results and Discussion

The interaction between host and parasite is a dynamic relationship with broad evolutionary implications (e.g., [5]). Understanding this process at the molecular level can answer key questions about coevolutionary mechanisms: is the process largely due to selective sweeps in which genetic polymorphism is transient, or is frequency-dependent selection the norm, where common genotypes are disfavored and rare genotypes never go extinct? Here we show that one of the most ancient weapons in the defensive armoury of eukaryotic cells, RNA interference (RNAi) [1], appears to evolve through the former process. Specifically, we found that three antiviral RNAi genes showed evidence of extremely rapid adaptive evolution in response to strong directional selection, supporting the idea that RNAi-related genes may be engaged in an arms race with viral suppressors of RNAi [2, 3].

A molecular arms race occurs when newly arising amino acid variants (i.e., new mutations) prove advantageous to the host, but this advantage has a brief evolutionary lifespan owing to counteradaptations that subsequently arise in pathogens. This reciprocal antagonism causes repeated selective sweeps in the host and parasite genes. In DNA sequences, such strong directional selection can be detected by testing for an elevated rate of nonsynonymous nucleotide substitutions (K_A) compared to the rate of synonymous nucleotide substitutions (K_S).

We found that the antiviral RNAi genes *Dcr2*, *R2D2*, and *Ago2* are evolving among the fastest 3% of *Drosophila* genes (Figure 1). This is significantly faster than other components of the *Drosophila* immune system (pathogen-recognition proteins, signal transduction proteins, or antimicrobial peptides), which, although they evolve faster than the genome as a whole, do not in general show exceptional levels of amino acid substitution (Figure 1) [6–8].

siRNA-Pathway Genes Show Elevated Rates of Amino Acid Change, but miRNA-Pathway Genes Do Not

To test whether the elevated rate of amino acid evolution in RNAi-pathway genes is associated with antiviral function, we compared RNAi genes with antiviral function to RNAi genes whose function is primarily "housekeeping." Housekeeping RNAi pathways constitute a fundamental control mechanism for gene expression through degrading mRNA, inhibiting translation, and targeting DNA methylation [9]. Broadly speaking, housekeeping and antiviral functions differ in that antiviral function is mediated by short interfering RNA molecules (siRNAs) derived from the target sequence, while control of gene expression is mediated by micro RNAs (miRNAs), which are derived from host-expressed RNA genes. However, the mechanisms are closely related; both siRNAs (antiviral) and miRNAs (housekeeping) are processed by *Dicer* genes and both are incorporated into an effecter complex containing *Argonaute* proteins [9].

In *Drosophila*, there is functional separation between these pathways [10], allowing pairwise comparisons between paralogous genes whose functions have diverged into housekeeping and antiviral functions. For example, *Argonaute-1* (*Ago1*) is required for miRNA maturation and *Ago2* is not, but *Ago2* is required for a successful siRNA response [10]. Similarly, *Dicer-1* (*Dcr1*), but not *Dcr2*, is required in the miRNA pathway, while *Dcr2* is required for siRNA function [11]. Additionally, the dsRNA binding protein *R2D2* is required for efficient transferral of siRNAs from *Dcr2* to the effecter complex [12], and

*Correspondence: darren.obbard@ed.ac.uk

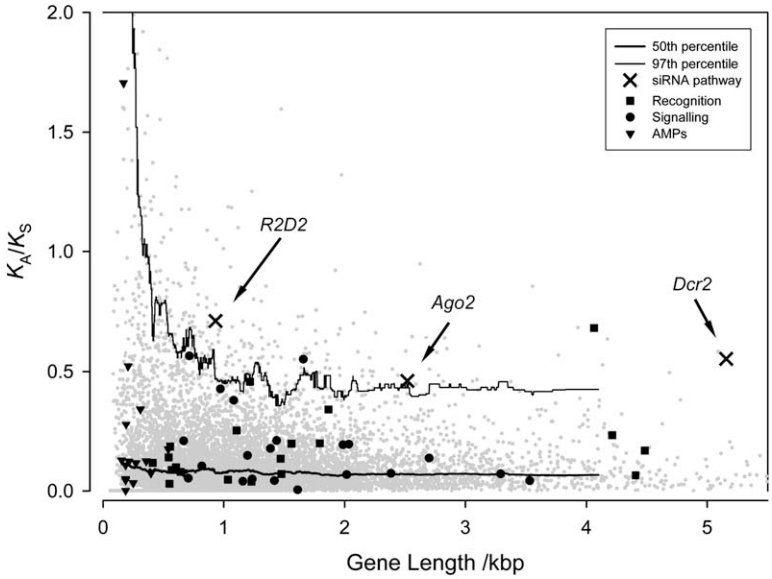

Figure 1. Rapid Evolution in Antiviral RNAi Genes, Compared to the Genome Average and to Innate Immunity Genes

The ratio of the rate of nonsynonymous (K_A) to synonymous (K_S) substitutions in antiviral RNAi genes (crosses) was high when compared to the genome as a whole (~8300 genes, gray circles) or innate immunity genes (filled shapes). As a group, *Dcr2*, *R2D2*, and *Ago2* have significantly higher K_A/K_S ratios than pathogen-recognition proteins (filled squares, two-tailed Mann-Whitney U-test, $p = 0.011$), signal transduction proteins (filled circles, $p = 0.019$), or antimicrobial peptides (filled triangles, $p = 0.025$). 97% of all genes (based on a 500 gene sliding-window) lie below the upper line and 50% below the lower line. In their size classes (class size 1000 genes), *R2D2* evolves amongst the fastest 1% and *Dcr2* the fastest 2% of genes. All K_A/K_S values are pairwise maximum likelihood estimates from PAML [14].

R3D1 (*Loquacious*) fulfils a similar role for *Dcr1* in the miRNA pathway [13].

We therefore compared the siRNA pathway genes *Dcr2*, *R2D2*, and *Ago2* to their paralogs in the miRNA pathway, *Dcr1*, *R3D1*, and *Ago1*, which act in a non-host-pathogen context. K_A/K_S was estimated in two phylogenetically independent comparisons, between *D. melanogaster* and *D. simulans* and between *D. yakuba* and *D. erecta*. In all six comparisons, we found an elevated K_A/K_S ratio in the antiviral siRNA-pathway genes relative to their housekeeping miRNA paralogs (Figure 2). In addition, a phylogeny-based test [14] also identified significantly elevated K_A/K_S ratios in the siRNA-pathway genes (see Table S1 in the Supplemental Data available with this article online). These results show that the elevated rate of evolution displayed by *Dcr2*, *Ago2*, and *R2D2* could be due to their role in an antiviral context and is not associated with RNAi function per se.

siRNA-Pathway Genes Are Positively Selected, but miRNA-Pathway Genes Are Not

Although K_A/K_S ratios were elevated in siRNA genes, none of the observed ratios were greater than 1, which would provide unequivocal evidence of positive selection. This raised the possibility that these antiviral RNAi genes experience relaxed selective constraints rather than positive selection. Therefore, to test for positive selection in the siRNA genes, we used McDonald-Kreitman (MK) tests [15]. These test for a departure from the neutral expectation that the ratio of nonsynonymous to synonymous fixed differences between species will be the same as the ratio for polymorphism within species. To obtain polymorphism data for these tests, we sequenced the genes *Dcr2*, *R2D2*, and *Ago2* from African populations of *D. melanogaster*, *D. simulans*, and *D. yakuba*, representing 8–12 alleles for each gene from each species. As a control, we also sequenced the homologous miRNA-pathway genes *Dcr1*, *R3D1*, and *Ago1* from the same *D. melanogaster* and *D. simulans* individuals (Table S2, Experimental Procedures).

In all *Drosophila* species examined, there was an excess of amino acid substitution compared to amino acid polymorphism in the siRNA-pathway genes (Table 1). The MK tests were significant in all four comparisons of *R2D2* ($p < 0.05$), indicating that *R2D2* has been consistently under positive selection in the *Drosophila melanogaster* subgroup. Two of four comparisons were significant for *Dcr2* (Table 1, $p < 0.001$, $p = 0.008$), while a third comparison was marginally nonsignificant ($p = 0.097$). For *Ago2*, only the most closely related species pair, *D. yakuba* and *D. santomea*, gave a significant result (Table 1, $p = 0.011$). In contrast, MK tests on the housekeeping miRNA-pathway genes *Dcr1*, *R3D1*, and *Ago1* did not identify a significant excess of amino acid change in any gene (Table 1).

Positive Selection Is Focused outside of Conserved Domains

Although sequence conservation between species is often an indicator of functional importance, identifying conserved regions may be unhelpful for rapidly evolving functions—for example, a site of antagonistic interaction between a host and parasite. Instead, it may be most informative to identify rapidly changing regions, which, for example, could represent targets of parasite immune evasion strategies. As shown above, *Dcr2*, *Ago2*, and *R2D2* all evolve rapidly, identifying them as candidate genes for such interaction with viral suppressors. We extended this argument to identify regions within genes that are under positive selection by applying MK tests separately to regions with known function and regions with unknown function in antiviral siRNA genes. For example, *Ago2* was split into two data sets, one comprising the PAZ and PIWI domains, a second comprising the remainder of the coding sequence (see Experimental Procedures). In the antiviral genes *Dcr2*, *Ago2*, and *R2D2*, we found more amino acid changes have been adaptively fixed in regions without known function than in regions with known function (Table 1). Indeed, MK tests did not show significant evidence of positive selection for any of the regions of known

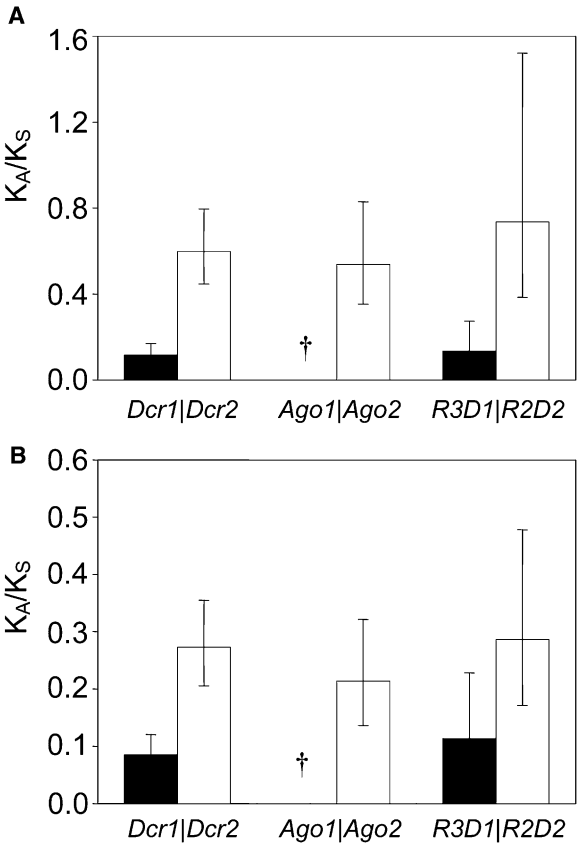

Figure 2. Rapid Evolution in Antiviral RNAi Genes, Compared to Their Housekeeping Paralogs

The ratio of the rate of nonsynonymous (K_A) to synonymous (K_S) substitutions was higher in antiviral RNAi genes (open bars) than in their paralogs with housekeeping functions (filled bars). Comparisons between *D. melanogaster* and *D. simulans* are shown in (A), and comparisons between *D. yakuba* and *D. erecta* are shown in (B). Error bars are 99% percentiles from 20,000 simulations assuming the observed K_A and K_S (see Experimental Procedures). K_A was zero for comparisons of *Ago1* (marked with a dagger).

Table 1. McDonald-Kreitman Tests for Positive Selection

	Polymorphic		Fixed			
Gene	Syn	Non	Syn	Non	% Adaptive	p Value
D. melanogaster versus *D. simulans*						
R3D1	5	2	26	11	5.5	1.000
R2D2	15	14	24	61	63.3	0.040
Known	5	5	13	18	27.8	0.724
Unknown	10	9	11	43	77.0	0.016
Dcr1	241	81	79	33	19.5	0.384
Dcr2	37	23	124	221	65.1	<0.001
Known	15	11	50	78	53.0	0.081
Unknown	22	12	74	143	71.8	<0.001
Ago1	152	0	21	0	0	n/a
Ago2	7	4	71	116	65.0	0.115
Known	2	2	39	41	4.9	1.000
Unknown	5	2	32	75	82.9	0.035
D. yakuba versus *D. erecta*						
R2D2	13	2	70	64	83.2	0.013
Ago2	11	4	107	73	46.7	0.412
Dcr2	42	16	233	199	55.4	0.008
D. yakuba versus *D. teissieri*						
R2D2	13	2	29	27	83.5	0.018
Ago2	11	4	45	21	22.1	0.768
Dcr2	56	17	111	58	41.9	0.097
D. yakuba versus *D. santomea*						
R2D2	13	2	5	10	92.3	0.008
Ago2	13	4	7	14	84.6	0.011
Dcr2	51	24	34	16	0	1.000

"Polymorphic" is a count of synonymous and nonsynonymous polymorphisms within species; "fixed" is a count of fixed synonymous and nonsynonymous nucleotide differences between species. "Known" and "unknown" refer to annotated functional domains (see main text). "% adaptive" is the estimated percentage of substitutions driven by selection [25]. p values were calculated with Fisher's exact test. Sample sizes for *D. melanogaster*, *D. simulans*, and *D. yakuba* are between 8 and 12, exact numbers are given in Table S2.

function, but were significant for the regions of unknown function. This indicates that positive selection in these genes is focused in coding regions that are currently without described function.

siRNA Genes Display Reduced Diversity

Directional selection, such as that associated with an arms race, not only inflates divergence between species but can also reduce polymorphism within species. This may explain the low level of polymorphism seen in the siRNA-pathway genes; *D. melanogaster* average pairwise diversity for silent sites (π_S) in the three siRNA pathway genes ranged from 0.42% to 1.43% (Table S2), compared to a genome average of 1.65% (autosomal loci in African populations [16]), and in *D. simulans*, π_S ranged from 0.07% to 0.89% (Table S2), versus an average of 3.2% (autosomal loci in African populations [16]). It is possible to test whether this effect is significant by means of a Hudson-Kreitman-Aguadé (HKA) test [17], based on the prediction that neutral polymorphism and divergence will be correlated across loci. By using synonymous sites only, we individually compared the antiviral RNAi genes *Dcr2*, *R2D2*, and *Ago2* and housekeeping RNAi genes *Dcr1, R3D1*, and *Ago1* to a control locus comprising a cluster of six *Drosomycin* genes (neighboring genes *Dro1-6*, excluding *Drs*), which have been shown previously not to differ from the neutral expectation for these individuals [7]. We found that none of the housekeeping genes differed from the *Drosomycins* (though *R3D1* was marginal, p = 0.052), while the antiviral genes *Ago2* and *Dcr2* did differ significantly from the *Drosomycins* (Table 2). The siRNA genes *Ago2* and *Dcr2* also differed from their housekeeping paralogs *Ago1* and *Dcr1* (Table 2). This indicates a significant reduction in diversity and suggests that both may have recently undergone a selective sweep.

Conclusions

Our data show that genes of the antiviral siRNA pathway are evolving extremely rapidly (Figure 1) in response to directional selection (Table 1), and in particular that they are evolving faster than their housekeeping miRNA paralogs (Figure 2, Tables 1 and 2). However, the division between housekeeping and antiviral RNAi function in *Drosophila* is not complete. For example, *Ago2* appears to have a housekeeping role in centromeric

Table 2. Hudson-Kreitman-Aguadé Tests for Reduced Genetic Diversity at Synonymous Sites

	Polymorphisms			p Value versus	p Value versus
	simulans	*melanogaster*	Differences	*Dros*	Paralog
Dros	21	10	29		
Ago1	103	54	31	0.150	
Ago2	2	5	71	<0.001	<0.001
Dcr1	133	112	162	0.670	
Dcr2	21	16	132	0.026	0.006
R3D1	4	1	28	0.053	
R2D2	6	9	25	0.446	0.181

p values were obtained by coalescent simulation (conditioned on θ) as implemented the computer program HKA (see Experimental Procedures), by means of either the *Drosomycin* (*Dros*) gene cluster as a control locus or the paralogous miRNA-pathway gene.

heterochromatin formation [18], and some functions of *Dcr1* are required for siRNA formation [11]. This does not detract from our conclusions because these effects would be conservative; i.e., housekeeping roles for *Ago2* would be expected to lead to a reduced rate of amino acid change, and antiviral functions for *Dcr1* would be expected to lead to elevated rates of change. It has also recently been suggested that hosts might have evolved miRNAs that target viral genomes [19], but our results suggest that these are likely to play only a minor role in *Drosophila*, as evolution in the miRNA pathway appears not to be driven by rapid host-parasite evolution.

Viruses are the most likely candidates for driving this rapid evolution in genes of the siRNA pathway. First, they are important natural pathogens of *Drosophila*; nearly 40% of *D. melanogaster* are infected with horizontally transmitted viruses [20], and vertically transmitted viruses are also common (10%–20% of *D. melanogaster* in France are infected with the Sigma virus [20]). Second, suppressors of RNAi are widely expressed by viruses [2–4], and although the functional mechanisms remain largely unknown, there is evidence (from noninsect systems) that some may directly or indirectly target *Dicer*- and *Argonaute*-related components of RNAi.

To date, the widespread occurrence of such viral suppressors has been some of the best evidence that host RNAi represents a long-term cost to virus fitness in the wild. Our data, through taking a different approach from functional studies, show that the antiviral RNAi (siRNA) pathway is evolving in a qualitatively different way than the housekeeping (miRNA) pathway. This supports the contention that the siRNA pathway is responding to antagonistic interactions in the field and constitutes additional evidence that RNAi is an important defense against viruses in natural animal systems.

It is striking that, with some notable exceptions, most of the *Drosophila* immune system does not seem to be evolving as fast as theory might predict (Figure 1). Indeed, many components of innate immunity, such as the antimicrobial peptides [7, 21] and pathogen recognition proteins [8], primarily show evidence of strong purifying selection. With the exception of *ref(2)p*, a *Drosophila* gene with alleles that confer resistance to Sigma virus [22], little is known about the evolution of antiviral genes in invertebrates. It is therefore all the more striking that we find all three siRNA-pathway genes evolve more rapidly than other immune system components, suggesting that viral interaction may be one of the strongest drivers of *Drosophila* innate immune system evolution. However, it should be noted that RNAi also targets transposable elements [4, 23], and we cannot rule out the possibility that interaction with transposable elements might drive evolution in RNAi genes.

Although functional studies of RNAi are common, there has previously been no information regarding the rate or mode of evolution in these genes—data that are likely to be essential for a full understanding of RNAi function. Studies of polymorphism and divergence can reveal the evolutionary history of genes through the imprint that natural selection leaves upon the DNA sequence. By searching for regions of rapid evolution, this approach has the power to inform functional studies by identifying potential sites of antagonistic interaction. We currently have a very incomplete picture of which regions of the immune-related genome are the primary targets of parasite-mediated selection. This knowledge gap may have arisen in part because much bioinformatics and genome characterization, while extremely valuable for some questions, often focuses on conserved regions of sequence that are the regions least likely to be subject to host-pathogen interaction. Our data on the genes associated with RNAi show that even the most ancient of interactions may still be engaged in ongoing adaptation and counteradaptation.

Experimental Procedures

Origin of Samples

All flies were originally collected as isofemale lines from natural populations: *D. santomea* (São Tomé and Principe Island, 1998) and *D. teissieri* (Lake Awing, Cameroon, 2003) were provided by the *Drosophila* species stock center (University of Arizona, Tucson, AZ); *D. melanogaster* and *D. yakuba* lines were collected in Gabon; and *D. simulans* was collected from Kenya. The *D. simulans* and *D. yakuba* lines were inbred by sib-mating for 6–9 generations. The second and third chromosomes of the *D. melanogaster* stocks were made isogenic by standard crosses to the balancer stock TM6/*Sb*. We then crossed the *D. melanogaster* isogenic chromosomes to an inversion-free stock and inspected the salivary gland chromosomes of the F_1 progeny for inversion loops; chromosomes containing inversions were discarded. All other sequences were derived from the publically available databases of completed or draft genomes: *D. anannassae* and *D. erecta* (Smith, D.R., 2004, Agencourt Bioscience Corporation Beverley, MA); *D. simulans* and *D. yakuba* (Wilson, R.K., 2004, Washington University Genome Sequencing Center, St Louis, MO), *D. pseudoobscura* [24].

DNA Sequence Data

PCR primers were designed from the published or preliminary genome sequences of *D. melanogaster*, *D. simulans*, and *D. yakuba.* Primers are available from the authors on request. Where possible, the entire coding sequence was amplified in multiple overlapping amplicons of 500–850 bp. The PCR products were treated with exonuclease I and shrimp alkaline phosphatase to digest unused PCR primers and dNTPs, and the PCR products were then sequenced in both directions with BigDye reagents and an ABI capillary sequencer. The sequence chromatograms were inspected by eye to confirm the validity of all differences within and between species, and they were assembled by SeqManII (DNAstar Inc., Madison, WI). Some alleles could not be amplified from the 3′ end of *Dcr2* in *D. simulans*, and therefore this region is excluded from the MK and HKA analyses. In *D. melanogaster* and *D. yakuba*, we found extensive length polymorphism in the glutamine-rich repeats at the 5′ end of *Ago2*, making sequencing and alignment problematic.

Therefore, this region was excluded from all analyses. Where heterozygosity remained after inbreeding in *D. simulans* and *D. yakuba*, we report pseudohaplotypes, derived by randomly assigning unphased heterozygosity data from direct sequences.

Elevated K_A/K_S

For genome-wide estimates of K_A/K_S (Figure 1), sequences were selected for analysis if exons were identifiable as best reciprocal BLAST hits between known *D. melanogaster* genes and the April 2005 release of the *D. simulans* genome. Genes were rejected if the coding sequence was invalid in either *melanogaster* or *simulans*. Codeml (PAML) [14] was used to provide maximum-likelihood estimates of K_A/K_S for all genes (runmode = −2). Because estimates of K_A/K_S will be poor for exceptionally short genes, 72 genes for which the total number of synonymous or nonsynonymous sites was less than 20 were excluded from further analysis, resulting in a final set of 8375 genes.

For pairwise comparisons of the K_A/K_S ratio between housekeeping and antiviral genes (Figure 2), we used K-estimator (J. Comeron, http://www.biology.uiowa.edu/comeron/index_files/Page322.htm), allowing the calculation of error bars representing 99% percentiles from 20,000 simulations assuming the observed K_A and K_S.

The phylogeny-based analysis of K_A/K_S in housekeeping and antiviral genes (Table S1) constituted a "fixed sites" analysis with Codeml (PAML) via concatenated gene sequences [14], with additional sequences from *D. pseudoobscura* and *D. anannassae*. Two models were compared: model 1, in which transition/transversion ratio and K_A/K_S were shared between the genes in each paralogous pair (parameter Mgene = 0), and model 2, in which each these ratios were estimated separately for each gene (parameter Mgene = 3). Significance was assessed by the difference in log-likelihood ($2\Delta l$), which is expected to follow a chi-squared distribution with 2 d.f.

McDonald-Kreitman Tests

MK tests are based on the premise that if divergence and polymorphism are due solely to genetic drift acting on neutral mutations, then the ratio of nonsynonymous to synonymous fixed differences between species will be the same as the ratio for polymorphism within species. Therefore, a two-by-two contingency table of polymorphisms and fixed differences at synonymous and nonsynonymous sites can be tested for independence, with a significant excess of amino acid substitutions between species providing strong evidence that positive selection has acted in one or both lineages since their most recent common ancestor. For the MK analysis of known-function and unknown-function regions, known-function regions were taken to be those Pfam-recognized domains or motifs (http://www.sanger.ac.uk/Software/Pfam/) annotated in the *D. melanogaster* genome (BDGP 4, April 2005, FlyBase September 2005) on the Ensemble website (http://www.ensembl.org). Known-function regions for each gene were as follows: *R2D2*, double-stranded RNA binding domain; *Ago2*, PAZ domain and PIWI domain; *Dcr2*, DEAD/DEAH box Helicase, Helicase conserved C-terminal domain, PAZ domain, and RNase III domain. All statistics and tests were performed with DNAsp (version 4.10.3, J. Rozas, J.C. Sánchez-DelBarrio, X. Messeguer, and R. Rozas, http://www.ub.es/dnasp/)

Hudson-Keitman-Aguadé Tests

If genes are evolving neutrally, it is expected that polymorphism and divergence will be correlated across loci. By comparing the focal locus to control loci, HKA tests [17] identify a significant departure from this expectation. HKA analyses were performed with the program HKA (J. Hey, 2004, http://lifesci.rutgers.edu/~heylab/HeylabSoftware.htm#HKA), with the *Drosomycin* genes [7] plus miRNA-pathway homologs *Ago1*, *R3D1*, and *Dcr1* as controls. Significance was assessed on the basis of 10,000 coalescent simulations with θ estimated from the data, as implemented in the program HKA.

Supplemental Data

Supplemental Data include two tables and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/6/580/DC1/.

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T.J.L. and F.M.J. initiated this study of dipteran immune system genes. D.J.O. conceived the RNAi-gene project and performed PCR/sequencing. F.M.J. cultured the flies and screened them for inversions. D.L.H. estimated genome-wide K_A/K_S ratios. D.J.O. and F.M.J. performed the other sequence analyses. D.J.O. and T.J.L. wrote the paper.

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Accession Numbers

Sequences have been submitted to GenBank as aligned sets, and accession numbers are DQ252338-DQ252360, DQ228766-DQ228816, and DQ398885-DQ398932.