Congruent Evolution of Genetic and Environmental Robustness in Micro-RNA

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Genetic robustness, the preservation of an optimal phenotype in the face of mutations, is critical to the understanding of evolution as phenotypically expressed genetic variation is the fuel of natural selection. The origin of genetic robustness, whether it evolves directly by natural selection or it is a correlated byproduct of other phenotypic traits, is, however, unresolved. Examining micro-RNA (miRNA) genes of several eukaryotic species, Borenstein and Ruppin (Borenstein E, Ruppin E. 2006. Direct evolution of genetic robustness in microRNA. Proc Natl Acad Sci USA. 103: 6593) showed that the structure of miRNA precursor stem loops exhibits significantly increased mutational robustness in comparison with a sample of random RNA sequences with the same stem-loop structure. The observed robustness was found to be correlated with traditional measures of environmental robustness—implying that miRNA sequences show evidence of the direct evolution of genetic robustness. These findings are surprising as theoretical results indicate that the direct evolution of robustness requires high mutation rates and/or large effective population sizes only found among RNA viruses, not multicellular eukaryotes. We demonstrate that the sampling method used by Borenstein and Ruppin introduced significant bias that lead to an overestimation of robustness. Introducing a novel measure of environmental robustness based on the equilibrium thermodynamic ensemble of secondary structures of the miRNA precursor sequences, we demonstrate that the biophysics of RNA folding induces a high level of correlation between genetic (mutational) and environmental (thermodynamic) robustness, as expected from the theory of plastogenetic congruence introduced by Ancel and Fontana (Ancel LW, Fontana W. 2000. Plasticity, evolvability, and modularity in RNA. J Exp Zool. 288: 242–283). In light of theoretical considerations, we believe that this correlation strongly suggests that genetic robustness observed in miRNA sequences is the byproduct of selection for environmental robustness.

Introduction

The magnitude of genetic effects on phenotype depends strongly on genetic background, the effects of the same mutation can be larger in one genetic background and smaller in another. The idea that wild-type genotypes are mutationally robust, that is, show invariance in the face of mutations (more generally heritable perturbations), goes back to Waddington and Kacser (1957), who originally introduced the concept as canalization. Although genetic robustness has been found across different levels of organization from individual genes, through simple genetic circuits to entire organisms (ca.,80% of yeast single knockouts have no obvious effect in rich medium; Hillenmeyer et al. 2008), the origin of the observed robustness has remained a source of contention. The three main hypotheses regarding the potential origin of genetic robustness predate the concept itself and fall along the lines of the famous debate between members of the modern synthesis (in particular Wright, Haldane, and Fisher) surrounding the origin of dominance (dominance can be understood as a simple case of robustness, a dominant phenotype being more robust against mutations) (Mayo and Bürger 1997; de Visser et al. 2003): 1) the most straightforward explanation, favored by Wright, was that robustness evolves “directly,” through natural selection (Fisher 1928); 2) an alternative congruent hypotheses, put forward in the context of dominance by Haldane, proposes that the evolution of genetic robustness is a correlated byproduct of selection for environmental robustness, that is, invariance in the face of nonheritable perturbations, for example, temperature, salinity, or internal factors such as fluctuations in the concentration of gene products during development (Haldane 1930); 3) although a third view holds that genetic robustness is “intrinsic,” arising simply because the buffering of a character with respect to mutations is the necessary or likely consequence of character adaptation, in the context of dominance Wright (1934) and later Kacser and Burns (1981) argued that it arises as an inevitable, passive consequence of enzyme biochemistry and selection for increased metabolic flux.

Recently, robustness has been a subject of renewed interest. Several theoretical and simulation studies have addressed robustness in a wide range of contexts ranging from gene redundancy (Krakauer and Plotkin 2002) to model regulatory networks (Siegal and Bergman 2002; Azevedo et al. 2007; Ciliberti et al. 2007a, 2007b; Crombach and Hogeweg 2008). In the first case building on evidence of excess mutational robustness present in RNA secondary structure (Wagner and Stadler 1999) and in the second case on the expectation that high mutation rates present among RNA viruses should favor mutational robustness (Wilke and Adami 2003) two pioneering studies by Monetville et al. (2005) and Borenstein and Ruppin (2006) have managed to step beyond computer simulations and through using, respectively, in vitro evolution experiments (Monetville et al. 2005) and miRNA sequences from diverse taxa found evidence to support the hypotheses that genetic robustness can evolve directly. Further work on in vitro evolution experiments has provided additional evidence showing that if a population is highly polymorphic, robustness can evolve directly (Bloom et al. 2007; Sán- juan et al. 2007).

The theoretical underpinnings of these studies is provided by the results of van Nimwegen et al. (1999), who through solving the quasispecies equations describing the evolution of a population on a network of phenotypically neutral sequences were able to demonstrate, that provided a sufficiently polymorphic population, mutational robustness can evolve directly. The necessary mutation rates and/or population sizes were found to be very large in
simulation studies using RNA secondary structure as a genotype–phenotype map (van Nimwegen et al. 1999; Forster et al. 2006; Szöllősi and Derényi 2008), direct evolution of increased neutrality requiring the product of the effective population size $N_e$, and the mutation rate per nucleotide $u$ to be well in excess of one. Such high mutation rates can only readily be found among RNA viruses, are extraordinary even among unicellular organisms (Prochlorococcus 2N$_e$u $\approx$ 2, Escherichia coli 2N$_e$u $\approx$ 0.2, and Saccharomyces cerevisiae 4N$_e$u $\approx$ 0.09), and completely unheard of among multicellular eukaryotes possessing RNA-silencing mechanisms and miRNA genes (Arabidopsis thaliana 4N$_e$u $\approx$ 0.012, Drosophila melanogaster 4N$_e$u $\approx$ 0.015, Caenorhabditis elegans 4N$_e$u $\approx$ 0.013, Ciona intestinalis 4N$_e$u $\approx$ 0.012, Mus musculus 4N$_e$u $\approx$ 0.001, and Homo sapiens 4N$_e$u $\approx$ 0.001) (Lynch and Conery 2003).

In their study, Borenstein and Ruppin examined miRNA (miRNA) precursor sequences from several eukaryotic species. miRNA are small endogenous noncoding RNAs that regulate the expression of protein-coding genes through the RNA interference (RNAi) pathway (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; Bartel 2004). Functionally relevant short (≈22 nt) mature miRNA sequences are excised from longer precursor sequences that fold into a stem-loop hairpin structure. The hairpin-like secondary structure of precursor stem loops plays a crucial role in the maturation process (Bartel 2004) and is under evolutionary constraint to conserve its structure. Borenstein and Ruppin used the novel and ingenious method of generating for each miRNA sequence a random sample of sequences with identical minimum free-energy (MFE) structure to uncover traces of adaptation. To compare the mutational robustness of miRNA precursor sequences with random sample sequences with identical MFE structure, they compared the single mutant neighborhood of a given miRNA precursor sequence with the single mutant neighborhood of the sample sequences. Calculating the average distance of the MFE structure of each single mutant sequence to the MFE structure of the original sequence for both stem loop and sample sequences (details on secondary structure calculations are presented below), they demonstrated that miRNA precursor sequences have single mutant neighborhoods with sequences that fold into more similar MFE structures compared with sequences in the single mutant neighborhoods of sample sequences with identical MFE structure. Although a similar comparison of the folding MFE showed a comparable, but lower bias, the finding that the two were only weakly correlated allowed the authors to conclude that the observed bias is a result of direct selection for mutational robustness. Their results were re-examined by Shu et al. (2007) who argued that mutational robustness among miRNA precursors may be the correlated byproduct of selection for environmental robustness but found only a moderately higher correlation using a different measure of mutational robustness.

In light of the consistently low value of $uN_e$ among multicellular eukaryotes, the results of Borenstein and Ruppin are highly surprising. There is no known mechanism that can explain the direct evolution of robustness that they observe. According to the classic results of Kimura and Maruyama, the average fitness of an asexually reproducing population (in the limit of very large populations) depends only on the mutation rate and is independent of the details of the fitness landscape (Kimura and Maruyama 1966). This result, however, only holds under the assumption that the fittest genotype does not have any neutral sites. Although the extension of these results to more general fitness landscapes by van Nimwegen et al. demonstrates that the presence of neutral genotypes can lead to selective pressure to evolve mutational robustness simulation studies using genotype–phenotype maps induced by RNA secondary structure have demonstrated that $uN_e > 1$ is a necessary condition (Forster et al. 2006) even in the presence of recombination (Szöllősi and Derényi 2008). The case for the direct evolution of genetic robustness rests on the parallel findings that a stronger bias for mutational robustness is present in miRNA precursor sequences than for environmental robustness and that the two are only weakly correlated. Introducing a new measure of environmental robustness in this paper, we endeavor to demonstrate that, indeed as previously also suggested by Shu et al. (2007), the exact opposite is true: The bias for environmental robustness is stronger, and it is highly correlated with mutational robustness. The correlated evolution of environmental and mutational robustness in RNA sequences under selection to retain secondary structure is expected as a corollary of a general casual link between environmental robustness and genetic robustness in RNA sequences proposed by Ancel and Fontana (2000). They argue that “plastogenetic congruence,” that is, the correlation between the set of structures thermally accessible to a sequence, its “plastic repertoire,” and the MFE structures of its “genetic neighborhood” will lead to the emergence of mutational robustness in the presence of selection for some predefined structure.

Materials and Methods

MiRNA Sequences and Sampling

MiRNA precursor sequences were downloaded from miRBase version 9.0 (Griffiths-Jones et al. 2006). All 4,361 miRNA genes were used, yielding 3,641 unique miRNA precursor sequences. For each miRNA precursor sequence, we produced a sample of random sequences by 1) using the stochastic optimization routine from the Vienna RNA package (Hofacker et al. 1994) to produce a sequence with MFE structure identical to that of the native sequence that is stored 2) and subsequently randomizing this sequence by attempting 4L random nucleotide substitutions in a miRNA precursor sequence of length L, accepting a substitution if the resulting sequence’s MFE structure remains unchanged. For each miRNA precursor sequence, on average >800 sample sequences with identical MFE structure were generated. Supplemental material, Supplementary Material online, accompanying our paper contains the robustness values for all 4,361 genes associated with 3,641 unique sequences we considered.

Measuring Thermodynamic Robustness

In order to calculate the thermodynamic robustness measure $\eta_r$, defined below, we sampled the equilibrium
thermodynamic ensemble of stem-loop and sample sequences using the stochastic backtracking routine from the Vienna RNA package producing $10^5$ suboptimal structures per sequence, using the default temperature of 310 K. The average distance from the MFE structure in the thermodynamic ensemble can be calculated exactly with the help of base-pairing probabilities, which are available as a by-product of partition function folding in the Vienna package, and were used to validate the sampling.

Statistics

Given a rank score $r$ and sample size $N$, a good estimate for the probability of observing an equal or lower rank score by chance is given by $(rN)/(N+1) \approx r$. Following Borenstein and Ruppin (2006), rank scores of $r < 0.05$ are considered significantly robust. To determine if the robustness of miRNA precursor sequences according to some measure $\eta$ has the same distribution as the robustness of sample sequences $\eta'$ for a group of sequences, following Borenstein and Ruppin (2006) we test against the null hypothesis that they are drawn from identical distributions using the nonparametric Wilcoxon signed rank test. In contrast to Borenstein and Ruppin (2006), however, we do not consider as paired values $\eta$ and the average of $\eta'$ over all $N$ sample sequences, $(\eta')$, as we found this to result in spuriously low $P$ values, but instead calculate the $P$ values for a given group of sequences by averaging over 1,000 different sets of $\{\eta, \eta'\}$ pairs where in each set the $\eta'$ values belong to a random sample sequence. As a complementary approach, we also tested the hypothesis that the distribution of rank scores of a group of sequences for a given robustness measure is uniform—as we would expect if miRNA precursor sequences were randomly sampled from the set of sequences with identical MFE structure—using a standard Kolmogorov–Smirnov goodness of fit test. We found the two significance analyses to be in good agreement indicating highly significant bias for higher values of $\eta_d$ and $\eta$, but mostly no or only nonsignificant bias for higher $\eta$. The supplemental information, Supplementary Material online, accompanying our paper contains species-level statistics and significance analyses.

Results

We assessed the environmental and mutational robustness of 3,641 unique miRNA precursor sequences and for each sequence compared them with a random sample of sequences with the same MFE structure. The idea of looking for signs of adaptation for increased robustness among miRNA precursor sequences by comparing the robustness of naturally occurring sequences with that of random sequences with the same secondary structure is conceptually similar to the approach used to support the argument that the genetic code has evolved to minimize mutational load (Di Giulio 1987; Haig and Hurst 1991; Szathmáry and Smith 1997). In the case of the genetic code, the authors took the common genetic code, and, for each codon, calculated the change in polarity of the encoded amino acid caused by replacing each of the three nucleotides, one after the other. In order to determine whether the genetic code is adapted to minimize mutational load, they proceeded by comparing the mean squared change caused by the replacement of a single nucleotide in the common genetic code with 10,000 randomly generated codes with the same redundancies. They found that only two of the random codes were more conservative than the common code with respect to polarity distances between neighboring amino acids.

We undertook a similar program in the case of miRNA precursor sequences. Each miRNA gene encodes a short ~22-nt sequence that is partially complementary to the mRNA of proteins regulated by the particular miRNA gene. For the proper short sequence to be excised by the protein Dicer, and hence for the miRNA gene to be functional, a larger part of the miRNA sequence, called the miRNA precursor sequence, must fold into the proper secondary structure. In order to determine whether a miRNA precursor sequence is adapted to minimize the effects of mutational and/or environmental perturbations, that is, to maximize mutational and/or environmental robustness, we compared the mutational and environmental robustness of each miRNA precursor sequence (robustness measures we used are defined below) with the mutational and environmental robustness of a random sample of sequences with identical structural phenotype (i.e., identical MFE structure).

To generate a random sample of sequences with a given MFE structure, we first used, starting from a random sequence, stochastic minimization of the free energy of the target structure to find a sequence with the desired MFE structure. This method by itself, however, yields a “biased” sample of sequences (see fig. 1a and b) and must be supplemented by an additional randomization step (see Materials and Methods). To measure the mutational robustness of a given sequence, we used the measures introduced by Borenstein and Ruppin (2006): 1) the structural distance–based mutational robustness measure $\eta_s$ of an RNA sequence of length $L$ is defined by $\eta_s = 1/(3L) \sum_{i=0}^{3L} |L - d_i|/L$, where $d_i$ is the base-pair distance between the secondary structure of mutant $i$ and the native sequence (given by the number of base pairs present in one structure but not the other), and the sum goes over all 3L single mutant neighbors and 2) the more stringent measure $\eta_n$ is simply defined as the fraction of neutral single mutant neighbors, that is, those that have an identical MFE structure to the original sequence. In order to quantify the level of excess mutational robustness among miRNA precursor sequences we counted, for each miRNA precursor sequence, the number of sample sequences that have higher mutational robustness according to a given measure (see Materials and Methods) and used this to calculate the rank scores $r_s$ and $r_n$, defined as the fraction of sample sequences with identical or higher robustness according to $\eta_s$ and $\eta_n$, respectively. To facilitate an overview of the extent of excess mutational robustness, we also calculated the average of the rank scores over all miRNA precursor sequences $r_s$ and $r_n$ as well as the fraction of miRNA precursor sequences with higher than average robustness (i.e., rank scores < 0.5) $S_s$ and $S_n$, and the fraction of sequences with statistically significantly increased robustness (i.e., rank scores <0.05, see Materials and Methods) $S_s$ and $S_n$, respectively, according to a give measure. The statistical
Fig. 1.—(a) Generating a random sample of sequences with a desired MFE structure by stochastic minimization of the free energy of the desired fold (the method employed the RNAInverse program used by Borenstein and Ruppin 2006 as well as Shu et al. 2007) results in a biased sample in which sequences with lower than average neutrality (higher than average number single mutant neighbors) are overrepresented. This can be avoided if after finding a sequence with the desired MFE structure, a random walk is performed among sequences with the desired MFE structure. This random walk on the neutral network associated with the MFE structure mimics the sequence drift of a sequence evolving under the constraint to fold into the desired MFE structure. (b) Rank-score distributions for two measures of mutational robustness (\(\eta_g\) and \(\eta_r\) see text). Comparing the distributions derived from sampling using only stochastic optimization (top, \(R^{\eta_g}_{\text{biased}}=0.25, R^{\eta_r}_{\text{biased}}=0.37, \) and \(R^{\eta_{\text{neutral}}}=0.66\) with that derived from sampling with subsequent randomization (bottom, \(r_g=0.29, r_r=0.78, r_{\eta_g}=0.44, \) and \(r_{\eta_r}=0.59\)) shows that increased neutrality is predominately an artifact of biased sampling, whereas the lower than average distance of MFE structures in the mutational neighborhood to the wild-type MFE structure becomes somewhat less pronounced but is still significant.

The presence of excess mutational robustness is, by itself, insufficient to determine whether mutational robustness has evolved as a result of direct selection or in congruence with selection for environmental robustness. As established previously (Borenstein and Ruppin 2006), there is evidence for excess thermodynamic robustness, robustness to thermal fluctuations, as evidenced by a significantly lower than chance minimum folding energy among miRNA precursor sequences. Defining the environmental robustness measure \(\eta_E\) simply as minus the minimum folding energy, we also find \(R_E=0.278, R_E=0.796, \) and \(S_E=0.220\) using unbiased sampling. The correlation between \(r_g\) and \(r_E\) across miRNA precursor sequences is, however, rather low with a Pearson’s correlation coefficient of \(c(r_g, r_E)=0.217\) and \(c(r_{\eta_g}, r_E)=0.071\). The minimum folding energy is a somewhat crude measure of thermodynamic robustness and does not even reflect the excess mutational robustness according to the measure \(\eta_E\). There is no good reason to assume that a low MFE in itself confers environmental robustness, as even for relatively high free energies a given sequence may nonetheless with high probability fold into structures sufficiently similar to the MFE structure to remain functional. The large number of miRNA precursor sequences that exhibit excess mutational robustness as measured by the structural similarity-based measure \(\eta_E\), suggests that a strict adherence to the MFE structure is not necessary to retain functionality—a sufficiently similar, but not necessarily identical, secondary structure is enough to guarantee the excision of the proper subsequence. This is further supported by the fact that folding free energy alone is not sufficient to discriminate miRNA precursors, as well as recent evidence that a diverse set of structural features are needed for successful cleavage of a miRNA precursor sequence (Ritchie et al. 2007).
To construct an appropriate measure of thermodynamic robustness that also reflects this observation, we would need to know the extent of similarity that is required to retain functionality—indeed we would require detailed knowledge of the interaction between the RNA substrate and the enzyme Dicer to establish an appropriate measure of structure similarity. As such information is not at present available, we chose to use the most simple and widely employed structure similarity measure, the base-pair distance used above. In order to determine the extent of similarity required to retain functionality, we defined the threshold thermodynamic robustness measure \( \eta(d_{th}) \) as a function of the threshold distance \( d_{th} \), by equating it with the probability in the equilibrium thermodynamic ensemble of structures that have base-pair distances equal to or less than a threshold \( d_{th} \), with respect to the MFE structure, that is,

\[
\eta(d_{th}) = \sum_{i \in \Omega} H(d_{th} - d_i) \frac{e^{-E_i/kT}}{Z},
\]

where the sum goes over the set of all possible structures \( \Omega \), \( d_i \) denotes the base-pair distance of structure \( i \) to the MFE structure, \( Z = \sum_{i \in \Omega} e^{-E_i/kT} \) is the partition sum, and \( H(x) \) is the unit step function, that is, \( H(x) = 0 \) if \( x < 0 \) and \( H(x) = 1 \) if \( x \geq 0 \).

Examining the thermodynamic robustness of miRNA precursor sequences in comparison to an unbiased sample of sequences with identical MFE structure, we found that miRNA precursor sequences have significantly more structures in their equilibrium thermodynamic ensemble that are similar to the MFE structure than sample sequences (see fig. 2a and b and table 1). In other words, miRNA precursor sequences tend to adopt more similar structures as a result of thermal fluctuations than random sample sequences with the same structure. Calculating the average rank score \( \bar{r}(d_{th}) \) and the fraction of robust \( R(d_{th}) \) and significantly robust \( S(d_{th}) \) miRNA precursor sequences, with respect to the measure \( \eta(d_{th}) \) (fig. 3a) and examining the distribution of structures as a function of the base-pair distance for individual miRNA precursor sequences (see, e.g., fig. 2a) indicates that above a threshold distance \( d_{th} \approx 20 \), the measures start to saturate, yielding an estimate of the required similarity to retain function. The correlation between the rank score of miRNA precursor sequences according to the distance similarity–based mutational robustness measure and the threshold thermodynamics measure is high for all threshold values. This is the direct result of the high degree of similarity between the distribution of structures in the thermodynamic ensemble and the mutational neighborhood (fig. 2). The average rank score \( \bar{r}(d_{th}) \) and the fraction of robust \( R(d_{th}) \) and significantly robust \( S(d_{th}) \) miRNA precursor sequences with respect to the threshold thermodynamic robustness measure indicate a markedly larger extent of excess robustness than their counterparts for mutation robustness, that is, \( r_s, R_s, \) and \( S_s \) (see fig. 3a and b and table 1) above \( d_{th} > 20 \).

### Discussion

The results presented above demonstrate the correlated presence of excess environmental (thermodynamic) and genetic (mutational) robustness among miRNA precursor sequences as measured according to, respectively, \( \eta_s \) and \( \eta_d(d_{th}) \). A rather general causality between environmental and genetic robustness in the context of RNA secondary structure has been suggested by Ancel and Fontana (2000), who studied the dynamics of an in silico population of RNA sequences evolving toward a predefined target shape. They found that a correlation exists between the set of shapes in the plastic repertoire of a sequence and the set of dominant (MFE) shapes in its genetic neighborhood. They argue that this statistical property of the RNA genotype–phenotype map, which they call plastogenetic congruence, traps populations in regions where most genetic variation is phenotypically neutral. In other words, RNA sequences explore a similar repertoire of suboptimal structures as a result of perturbations due to mutations and perturbations resulting from thermal fluctuations, and selection for a given target structure favors sequences with higher robustness to perturbations of both type.

Because in contrast to genetic robustness, environmental robustness does not require high values of \( uN_e \), as it is a property of the sequence and not its mutational neighborhood, we contend that the observed bias in mutational robustness is in fact the result of the “congruent” evolution of environmental and genetic robustness.

The correlation between the response to heritable (mutational) and nonheritable (thermodynamic) perturbation, and hence the congruent evolution of genetic and environmental robustness, may extend to other systems with

### Table 1

<table>
<thead>
<tr>
<th>Group/Species</th>
<th>( r_s )</th>
<th>( r_{25} )</th>
<th>( S_s )</th>
<th>( S_{25} )</th>
<th>( c(r_s, r_{25}) )</th>
<th># of seqs</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>0.44</td>
<td>0.17</td>
<td>0.31</td>
<td>0.74</td>
<td>0.28</td>
<td>0.73</td>
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<tr>
<td>Vertebrate</td>
<td>0.29</td>
<td>0.13</td>
<td>0.29</td>
<td>0.75</td>
<td>0.30</td>
<td>0.76</td>
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<tr>
<td>Invertebrate</td>
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<td>0.27</td>
<td>0.22</td>
<td>0.84</td>
<td>0.36</td>
<td>0.73</td>
</tr>
<tr>
<td>Landplant</td>
<td>0.41</td>
<td>0.21</td>
<td>0.40</td>
<td>0.63</td>
<td>0.19</td>
<td>0.68</td>
</tr>
<tr>
<td>Virus</td>
<td>0.38</td>
<td>0.18</td>
<td>0.21</td>
<td>0.85</td>
<td>0.32</td>
<td>0.65</td>
</tr>
<tr>
<td>Homo sapiens</td>
<td>0.48</td>
<td>0.11</td>
<td>0.28</td>
<td>0.76</td>
<td>0.33</td>
<td>0.74</td>
</tr>
<tr>
<td>Mus musculus</td>
<td>0.46</td>
<td>0.12</td>
<td>0.31</td>
<td>0.74</td>
<td>0.27</td>
<td>0.79</td>
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<tr>
<td>Drosophila melanogaster</td>
<td>0.40</td>
<td>0.24</td>
<td>0.23</td>
<td>0.82</td>
<td>0.35</td>
<td>0.74</td>
</tr>
<tr>
<td>Caenorhabditis elegans</td>
<td>0.30</td>
<td>0.23</td>
<td>0.23</td>
<td>0.82</td>
<td>0.34</td>
<td>0.75</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
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<td>0.19</td>
<td>0.43</td>
<td>0.60</td>
<td>0.15</td>
<td>0.75</td>
</tr>
<tr>
<td>Epstein-Barr virus</td>
<td>0.31</td>
<td>0.22</td>
<td>0.16</td>
<td>0.87</td>
<td>0.48</td>
<td>0.81</td>
</tr>
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</table>

Average rank scores that indicate significantly increased according to both measures discussed in the Materials and Methods section (\( p \) value < 10\(^{-13}\)) are given in bold.
genotype-phenotype maps different from RNA secondary structure. In particular, Xia and Levitt (2002) have found compelling evidence of the correlated evolution of increased thermodynamic stability and the number of neutral neighbors in lattice protein models. Understanding the relationship between sequence, structure, and function is, and will remain to be in the foreseeable future, a central theme in both molecular and evolutionary biology. A comprehensive view of how the relationship between sequence, structure, and function is shaped during the course of evolution must take into consideration both the potential correlations that arise from the physics of the structure-sequence relationship as well as the relevant population genetic conditions in the context of which it takes on the role of a genotype-phenotype map. In the context of computational miRNA gene discovery, our thermodynamic robustness measure potentially offers an improved structural feature that may perform better than the free-energy score of the hairpin or its ensemble diversity (which have proved uninformative; Freyhult et al. 2005).

**Fig. 2.**—In order to examine the robustness of miRNA precursor sequences to thermal fluctuations, we sampled the equilibrium thermodynamic ensemble of structures. Sampling $10^6$ structures for each miRNA precursor sequence and each member of the random sequence sample, we binned structures according to their distance from the MFE structure. (a) For example, for the *Monodelphis domestica* miRNA precursor sequence mdo-mir-1 examining the distribution of structures as a function of the base-pair distance shows that the averaged random sequence sample distribution (white bars) has a much larger fraction of structures that are drastically different from the MFE structure, compared with the distribution of structures for the original miRNA precursor sequence (black bars). (b) Examining the averaged distribution of stem-loop (black bars) and random corresponding random sequence sample distributions (white bars) shows that there is a general tendency among miRNA precursor sequences for increased thermodynamic robustness, that is, of avoiding structures that are highly dissimilar to the MFE structure. A strikingly similar effect can be observed if we examine the distribution of structures in the mutational neighborhood. Analogous to (a), in (c) we binned, according to their distance from the MFE structure of the wild type, the MFE structures of all (3L) single point mutants for the *M. domestica* miRNA precursor sequence mdo-mir-1 (black bars) as well as the MFE structures for each sequence in the single mutant neighborhood of sample sequences (white bars). The distribution of structures in both the thermodynamic ensemble (a) and the mutational neighborhood (c) of the mdo-mir-1 miRNA precursor sequence have a significantly smaller fraction of structures that are highly dissimilar than sample sequences with identical MFE structure. Comparing the averaged distribution of stem-loop (black bars) and random corresponding random sequence sample distributions (white bars) in the mutational neighborhood (d) with similar averaged distributions in the thermodynamic ensembles of the same sequences (b) shows that the tendency among miRNA precursor sequences for increased robustness is present both in the mutational neighborhood and the thermodynamic ensemble, that is, miRNA precursor sequences show excess robustness in the face of both thermal and mutational perturbation.
**Supplementary Material**

Supplementary information is available at Molecular Biology and Evolution online (http://www.mbe.oxfordjournals.org/).

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**Literature Cited**


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