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Abstract

Understanding the relationship between robustness and evolvability is key to understand how living things can withstand mutations, while producing ample variation that leads to evolutionary innovations. Mutational robustness and evolvability, a system's ability to produce heritable variation, harbour a paradoxical tension. On one hand, high robustness implies low production of heritable phenotypic variation. On the other hand, both experimental and computational analyses of neutral networks indicate that robustness enhances evolvability. I here resolve this tension using RNA genotypes and their secondary structure phenotypes as a study system. To resolve the tension, one must distinguish between robustness of a genotype and a phenotype. I confirm that genotype (sequence) robustness and evolvability share an antagonistic relationship. In stark contrast, phenotype (structure) robustness promotes structure evolvability. A consequence is that finite populations of sequences with a robust phenotype can access large amounts of phenotypic variation while spreading through a neutral network. Population-level processes and phenotypes rather than individual sequences are key to understand the relationship between robustness and evolvability. My observations may apply to other genetic systems where many connected genotypes produce the same phenotypes.
On the relationship between robustness and evolvability in RNA structures

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To understand the relationship between robustness and evolvability is key to understand how living things can withstand mutations, while producing ample variation that leads to evolutionary innovations. Mutational robustness and evolvability, a system’s ability to produce heritable variation, harbor a paradoxical tension. On one hand, high robustness implies low production of heritable variation. On the other hand, both experimental evidence and analyses of neutral networks in genotype space indicate that robustness enhances evolvability. I here resolve this tension, using RNA genotypes and their secondary structure phenotypes as a study system. To resolve the tension, one must distinguish between robustness and evolvability for genotypes on one hand, as well as for phenotypes on the other hand. I confirm that genotype (sequence) robustness and evolvability share an antagonistic relationship. In stark contrast, phenotype (structure) robustness promotes structure evolvability. A consequence is that finite populations of sequences with a robust phenotype can access large amounts of phenotypic variation while spreading through a neutral network. Population-level processes and phenotypes rather than individual sequences are key to understand the relationship between robustness and evolvability. My observations may apply to all genetic systems where many connected genotypes produce the same phenotypes.
Introduction

Robustness and evolvability are fundamental properties of biological systems. They determine a system’s persistence and its potential for future evolutionary change. Multiple definitions of robustness and evolvability have been proposed (Aharoni et al. 2005; Ancel & Fontana 2000; Bedau & Packard 2003; Bloom et al. 2006; Bloom et al. 2004; Calabretta 2007; Carter et al. 2005; Deem 2004; Earl & Deem 2004; Feder et al. 2002; Gardner & Zuidema 2003; Gilchrist & Lee 2007; Griswold 2006; Hansen 2003; Hermida et al. 2002; Husimi et al. 2002; Kaneko 2003; Kirschner & Gerhart 1998; Lee & Cho 2003; Masel & Bergman 2003; Michod et al. 2003; Nijhout et al. 2003; O'Loughlin et al. 2006; Pal 2001; Pepper 2003; Poole et al. 2003; Reader 2006; Rutherford 2003; Smith et al. 2002; Sniegowski & Murphy 2006; Tanay et al. 2005; Van Belle & Ackley 2003; Volkert 2003; Wagner 2005; Yamauchi et al. 2002; Yamauchi et al. 2003). For my purpose, I call a biological system mutationally robust if its function or structure persist after mutations in its parts. The system is evolvable if mutations in it can produce heritable phenotypic variation. Both robustness and evolvability are quantitative properties, that is, a system can be more or less robust or evolvable, as a response to a given number of mutations. Both definitions apply to systems on different levels of organization, including RNA and protein molecules, small genetic circuits, genome-scale networks such as metabolic networks, and even whole organisms. The appropriate nature of a mutation depends on the level of organization one focuses on. It may correspond to amino acid change for proteins, regulatory mutations in genetic circuits, or changes in enzymatic reactions for a metabolic network. The appropriate notion of phenotype also depends on this level of organization. Examples include the spatial conformation or catalytic activity of a macromolecule, and a gene expression pattern for a genetic circuit.

At first sight, the above definitions imply an antagonistic relationship between robustness and evolvability: The more robust a system is, the less phenotypic variation a given number of mutations generates, and hence the less evolvable the system is (Ancel & Fontana 2000; Sumedha et al. 2007). However, from different perspectives, robustness and evolvability may go hand in hand. First, multiple experiments showed that many organisms harbor cryptic genetic variation – reflecting robustness – which can become visible in certain environments or genetic backgrounds, and thus lead to enhanced evolvability (Dun & Fraser 1959; Queitsch et al. 2002; Rutherford & Lindquist 1998; Waddington 1953; Waddington 1959). Second, a biological system with a given phenotype typically has many alternative genotypes that can produce this phenotype (Wagner 2005). These genotypes are often connected, that is, they can be reached from each other through series of single mutations, as has first been shown for RNA (Schuster et al. 1994). Importantly, this connectedness implies at least some degree of robustness, because for a typical genotype, some mutations must leave the phenotype unchanged. This connectedness also suggests evolvability, because many new phenotypes might be produced by single mutations, if a phenotype has many different genotypes that adopt it (Huynen 1996; Sumedha et al. 2007).

Thus, from one perspective robustness hinders evolvability. From another perspective, robustness promotes evolvability. I here show how this apparent paradoxical tension can be resolved. To this end, I use RNA secondary structure as a study system. RNA secondary structure is an important phenotype in its own right, because it is
required for the biological function of many RNA molecules (Baudin F et al. 1993; Dayton E et al. 1992; Powell et al. 1995). Because computationally efficient algorithms exist to predict RNA secondary structure from an RNA sequence (Hofacker et al. 1994; Tacker et al. 1996; Zuker & Sankoff 1984), RNA secondary structure has proven an important computational model to understand how genetic variation maps into phenotypic (structural) variation (Fontana 2002; Fontana & Schuster 1998a; Reidys et al. 1997; Schuster et al. 1994). The biological relevance and computational tractability of RNA structure allow one to explore the relationship between robustness and evolvability. The insights thus obtained can inform our thinking for many other biological systems that are less tractable.

One might argue that the above notion of evolvability, focusing only on variation is not sufficiently ambitious. A more expansive definition might involve the ability of a phenotype or genotype to produce new and desirable phenotypes, evolutionary innovations. In reality, evolutionary innovations are obvious only in hindsight. However, one can specify a closely related measure of evolvability in a computational analysis by asking how easily a blind random walk starting from a given phenotype can find a pre-defined but otherwise arbitrary “target” phenotype. I show that this notion of evolvability is independent of robustness, and may depend very little on properties of the starting phenotype.

Results

Genotype and phenotype robustness. To resolve the above tension between robustness and evolvability, I first recast definitions of robustness and evolvability in terms of RNA genotypes and phenotypes. The relevant genotype space is the set of all $4^n$ possible RNA sequences of some given length $n$. Two sequences are 1-mutant neighbors or simply neighbors in this space if they differ in one nucleotide. The relevant phenotypes are the set of all possible RNA structures, whose number scales approximately as $1.8^n$ (Gruner et al. 1996; Schuster 2003). The set of all genotypes forming the same structure is often called a neutral network (Schuster et al. 1994). A neutral neighbor of a sequence is a neighbor that has the same structure. I will refer to the 1-neighborhood of a structure or, equivalently, the 1-neighborhood of a neutral network as the set of sequences that differ from sequences that fold into the structure by exactly one nucleotide. With these notations, I now introduce two different definitions of robustness and evolvability.

Genotype (sequence) robustness: The number $R_G$ (or fraction $r_G$) of neutral neighbors of a genotype $G$.

Phenotype (structure) robustness: The number $R_P$ (or fraction $r_P$) of neutral neighbors averaged over all genotypes $G$ with a given phenotype. I will refer to this quantity also as the number of neutral neighbors of the structure.

Genotype (sequence) evolvability: The number $E_G$ of different structures found in the 1-neighborhood of a sequence $G$. 

Phenotype (structure) evolvability: The number \( E_P \) of different structures found in the 1-neighborhood of a structure \( P \).

Below, I will show that the tension between robustness and evolvability disappears with the distinction just introduced. Specifically, genotypic robustness is negatively associated with genotypic evolvability, whereas phenotypic robustness is positively associated with phenotypic evolvability. Note that sequence robustness and structure robustness cannot be meaningfully compared to one another, and neither can sequence evolvability and structure evolvability. Note also that the above definitions of evolvability are special cases of the more general definition in the introduction. A sequence that is more evolvable has more structural variants in its 1-neighborhood. Similarly, a structure that is more evolvable has more structural variants in its 1-neighborhood.

High sequence robustness means low sequence evolvability. For reasons detailed in Methods, I focus throughout on random RNA sequences of length \( n=30 \), which strike a medium between structural richness and computational tractability. Biological RNAs are currently not suitable for this analysis, because of the necessity to analyze many different structures of the same length, and because of the unsolved problem of estimating the frequency of a given structure. This frequency is defined as the number of sequences adopting the structure. In protein engineering, it is also known as a structure’s designability.

It is well-known that the distribution of structure frequencies is highly skewed (Hofacker et al. 1998; Schuster 2003). That is, there are relatively few structures adopted by many sequences, but many structures adopted by few sequences. Figure 1a shows the distribution of structure frequencies for a sample of \( 10^6 \) RNA sequences. Structure frequencies in this sample vary over a factor 1600, from less than \( 1.06 \times 10^{-6} \) (structures found only once) to \( 1.7 \times 10^{-3} \).

A sequence \( G \)'s mutational robustness \( R_G \) and the number of structures in the 1-neighborhood of the sequence that are different from \( G \)'s structure are trivially and inversely related, i.e., this number is equal to \( 3n-R_G \). But what is the relationship between \( R_G \) and the number of structures in the 1-neighborhood of a sequence that are unique, i.e., different from both the structure of \( S \) and from each other? To find out, I determined the number \( U \) of unique structures that are different from one another in the neighborhood of \( G \) by counting all structures in the neighborhood, but counting structures that occurred twice or more in this neighborhood only once. By the above definition of sequence evolvability, \( E_G=U \). To normalize for sequence length, one might also determine \( r_G=R_G/3n \) and an appropriately normalized analog \( e_G \). Starting from \( U \), however, there are two different ways of determining the proportion of unique structures. The first is to divide \( U \) by the number \( 3n(1-r_G) \), i.e., by the total number of structures in the neighborhood of \( G \) that are different from the structure of \( G \). To do so reduces the influence of mutational robustness on evolvability, as indicated by the factor \( (1-r_G) \) in this expression. The statistical association between \( r \) and \( U/3n(1-r) \) is then weak, although highly significant in a large sample of sequences (Spearman’s \( s=-0.06 \), \( P<10^{-17} \); \( N=7.5 \times 10^4 \)). It is also negative, suggesting that the proportion of structures that are different from each other is reduced for sequences of high robustness. A second approach
to determine the proportion of unique structures is to divide $U$ simply by $3n$, the total number of sequences in the neighborhood of $G$. Arguably, this approach is more sensible, because it reflects the likelihood that a new structure is encountered in a blind evolutionary exploration of the neighborhood of $G$. Figure 1b shows the association between this measure of evolvability $e_G=U/3n$ and $r_G$ for $7.5 \times 10^8$ sequences whose structures span three orders of magnitude in frequency. The two quantities are highly negatively correlated with mutational robustness (Spearman’s $s=-0.64$, $P<10^{-17}$; $N=7.5 \times 10^8$). The dashed line in the figure indicates $1-r_G$, the fraction of structures different from that of $G$. It indicates that many structures different from that of $G$ are not different from each other, otherwise the data would fall on the dashed line. The negative association between $r_G$ and $e_G$ persists if one controls for structure frequency in a partial correlation analysis (Partial product-moment correlation coefficient $r=-0.65$; $P<0.01$; $N=7.5 \times 10^8$). This underscores a previous finding that sequences folding into the same structure (Ancel & Fontana 2000) also show a negative association between mutational robustness and the structural repertoire found in a sequence neighborhood. In sum the greater a sequence’s robustness, the lower its evolvability.

**High structure robustness means high structure evolvability.** By the above definition, the robustness $R_P$ of a phenotype (structure) $P$ is the average number of neutral neighbors of all sequences with phenotype $P$, or their proportion $r_P=R_P/3n$. The corresponding measure of evolvability $E_P$ is the total number of structures different from $P$ in the 1-neighborhood of $P$.

Because neutral networks are so vast, neither robustness $R_P$ nor evolvability $E_P$ can be determined exactly. For example, even the rarest structures in a sample of $10^6$ sequences of $n=30$ nucleotides may be adopted by approximately $10^6 \times 4^n \approx 10^{12}$ sequences.

One can, however, take a sampling approach to address this problem. That is, one can inversely fold a sample of sequences with phenotype $P$ and determine $R_P$ in this sample. Similarly, one can determine for such a sample the number $U$ of unique structures, that is, structures different from each other and from $P$. Because structure evolvability depends on the size of a neutral network, this number $U$ then still needs to be multiplied by an estimate of the total size of a neutral network, such as the structure frequency $f$. In other words $e_G$ will be proportional to $Uf$. Figure 2a shows the relationship between phenotype robustness $r_P$ and $Uf$ ($e_P$) for $2.5 \times 10^4$ structures, and for 100 randomly sampled sequences from each structure’s neutral network. Structure robustness and evolvability show a strong positive association (Spearman’s $s=0.55$, $P<10^{-17}$; $N=2.5 \times 10^4$).

To understand this positive association, several observations are germane. First, the higher a structure’s frequency, the higher is also its robustness (Spearman’s $s=0.64$, $P<10^{-17}$; $N=2.5 \times 10^4$; Figure S1). When a neutral network is viewed as a graph, this observation simply states that the average number of neutral neighbors of a typical genotype $G$ on the network increases with the size of the neutral network. It also means that the greater $f$ is, the more sequences in the 1-neighborhood of any one sequence $G_i$ will have the same structure as $G_i$. Conversely, the greater $f$ is, the smaller the proportion of the neighbors of any one $G_i$ that will have unique and different structures.

Second, the structures found in the neighborhoods of two or more different sequences are typically very different from each other. To see this, consider the total number of different structures found in the 1-neighborhood of a set of sequences
$(G_1, \ldots, G_k)$ sampled from a neutral network with structure frequency $f$. Denote the set of structures that are different from each other in the 1-neighborhood of $G_i$ as $\{U_i\}$, and the size of this set as $|U_i|$. When one compares two different sets, say $\{U_i\}$ and $\{U_j\}$, there are two extreme possibilities and a wide spectrum in between: All of these sequences could be identical, i.e., $\{U_i\} \cap \{U_j\} = \{U_i\} = \{U_j\}$, or all of these sequences could be different, i.e., $\{U_i\} \cap \{U_j\} = \emptyset$. The truth is closer to the second extreme, as illustrated by the following analysis. Consider the quantity

$$\frac{1}{k} \sum_{i=1}^{k} \frac{|U_i|}{3n}$$

(1)

where $k$ is some number of sequences sampled from the neutral network of a given structure. This quantity is the proportion of unique structures in the 1-neighborhood of $G_i$, averaged over all $k$ sampled sequences. Consider next the quantity

$$\frac{\bigcup_{i=1}^{k} U_i}{3nk}$$

(2)

which is the total number of structures different from each other found in the neighborhood of all $k$ sampled sequences, divided by the total number of sequences examined, that is, $k$ times $3n$, because each of the $k$ sequences has $3n$ neighbors. Of interest is the ratio of (1) and (2), that is

$$Q := \frac{\bigcup_{i=1}^{k} U_i}{\sum_{i=1}^{k} |U_i|}$$

If the structures found in the neighborhood of two sequences are very similar, then $Q \ll 1$, because in that case $\bigcup_{i=1}^{k} U_i \approx |U_i|$ for every $i$, which does not depend on $n$, and (2) would thus approach zero with increasing $k$. For example, for the $k=100$ sequences sampled here, one would then expect that $Q \approx 0.01$. If, however, all the structures found in the neighborhood of two sequences are different from each other, then $Q$ would be of order 1.

Figure 2b shows the distribution of $Q$ for $2.5 \times 10^4$ structures. The median of this distribution is equal to 0.6, or 60-fold greater than if the structures found in the neighborhoods of two sequences were identical. This means that most structures that occur in the neighborhoods of different sequences along a neutral network are different from each other. For sequences that are not randomly sampled, but encountered along a random walk along a neutral network, this has been shown previously (Huynen 1996; Sumedha et al. 2007).
Because most structures in the 1-neighborhoods of different sequences in a neutral network are different from each other, the size of a neutral network has an important influence on structure-based evolvability. Specifically, even though the number of structures found in the 1-neighborhood of any one sequence decreases modestly with increasing neutral network size (Figure S1), this decrease is more than compensated for by the increased number of different structures accessible from a much larger neutral network. In addition, the ratio $Q$ increases modestly with structure frequency (Figure 2c; Spearman's $s=0.11$, $P<10^{-17}$; $N=2.5\times10^4$). This means that the larger a neutral network, the more distinct are the structures found in the 1-neighborhoods of sequences sampled from the neutral network.

Another observation useful to understand the relation between phenotype robustness and evolvability emerges from a comparison between structure frequencies and sequence robustness. For any sequence of length $n$, the number of neighbors with the same mfe structure can vary between 0 and $3n$. In contrast, the number of sequences folding into a given structure can vary over a much broader range, from zero to a fraction of a percent of the total number of sequences, i.e., of the order of the number $4^n$ of sequences itself. This discrepancy appears even in modestly sized samples of sequences. For example, Figure 2d shows the mutational robustness, normalized to (0,1), for a sample of $7.5\times10^4$ sequences whose structure frequencies vary over the same range as that in Figure 1, i.e., by a factor 1600. In contrast, mutational robustness in this sample varies only by a factor 37. In other words, even in this modest sample of sequences, structure frequencies are more than 40 times more variable than mutational robustness.

In sum, the positive association between phenotype robustness and evolvability can be explained by two observations. First, the number of genotypes folding into any one structure can vary by many orders of magnitude, whereas mutational robustness among sequences of similar lengths varies more modestly. Second, most structures found near two or more sequences sampled from the same neutral network are different from each other. Thus, even though structure robustness increases modestly with structure frequency, this increase is more than compensated by the vastly increased number of different structures found near larger neutral networks.

Populations evolving on large neutral networks can access greater amounts of variation.

Neutral networks are vast in size and a finite population may take a very long time to explore such networks and all the structures in their neighborhoods. It is thus important to show that robustness also affects evolvability on short time scales in finite populations. To determine whether this is the case, I first chose two different structures, one with high frequency ($f\approx10^{-3}$) and thus high robustness, and another one with low frequency and low robustness ($f\approx10^{-6}$). I then inversely folded 20 sequences for each of these structures. For each of the $40(=2\times20)$ sequences, I then established a population of $P=500$ identical sequences. Each population then underwent repeated rounds of mutation (one nucleotide per sequence generation) and selection that confined the population to the neutral network. That is, in each generation mutants that were no longer on the neutral network were eliminated and replaced by randomly sampled mutants (with replacement) that still resided on the network. After each such round of mutation and selection, I determined the total number of unique structures found in the neighborhood of the entire population. The
results show that the more robust phenotypes can access much more variation in their evolution on a neutral network (Figure 3a). For example, after a mere 10 generations, the neighborhoods of the population on the large network contains 2118 (±362 s.e.m.) unique structures. In contrast, the neighborhood of the populations on the small network contains merely 874 different (±148 s.e.m.) structures.

How can an evolving population with a robust phenotype access more variation, despite the fact that each individual typically has fewer unique structures in its neighborhood? The answer is that the populations with the highly robust phenotype are more diverse, and this increased diversity more than compensates for the lower diversity around any one sequence. Figure 2b shows, for each population, the number of different sequences, and Figure 2c shows the mean Hamming distance of the sequences from each other. For both measures, the population with the robust phenotype rapidly accumulates greater diversity. But why is this increased sequence diversity observed in the first place? The reason is simply that in each generation, mutations kill fewer sequences with a more robust phenotype. Assume, for example, that in a population sequences have average robustness $r_G$. Then, a number of individuals proportional to $(1 - r_G)$ will be eliminated in every generation as a result of mutations. Populations of sequences with greater robustness (lower $1 - r_G$) can thus accumulate greater diversity.

These observations are not peculiarities of the structures I used. I repeated this approach with inversely folded sequences derived from $4 \times 10^3$ different structures, each of which was used to seed an evolving population. Figure 4d shows the number of unique structures in the neighborhood of these $4 \times 10^3$ populations after 10 generations of mutations and selection. There is a modest but highly significant positive association between structure frequency and the amount of phenotypic variation accessible to these structures. Populations with more frequent and thus more robust phenotypes thus have access to more new variation.

I note that these results would be qualitatively the same if I had not used “soft” selection, where population sizes are held constant but “hard” selection, in which population sizes are allowed to fluctuate. The reason is that in this case, populations evolving on small neutral networks would simply shrink faster over time than populations on large neutral networks, and show lower diversity of sequence for this reason.

An evolutionary search’s ability to find a target structure is only weakly correlated with robustness.

A definition of evolvability that focuses only on the variation directly accessible from a given genotype $G$ or phenotype $P$ may seem limited. It does not address how a blind evolutionary search driven by mutations would find a phenotype (structure) that is not in the immediate neighborhood of $G$ or $P$, but an arbitrary distance away from it. Some genotypes $G$ or phenotypes $P$ might be more amenable to finding such arbitrary target phenotypes, and thus be more evolvable in this sense. If so, how is this kind of evolvability related to genotypic or phenotypic robustness?

To address this question, I pursued an approach that started with a set of $7.5 \times 10^4$ random RNA structures that span three orders of magnitude in structure frequency. I drew pairs of structures $(S, T)$ at random from this set (with replacement). For each such pair, I inversely folded a sequence $G$ with structure $S$. From the starting sequence, I then
performed a random walk towards the “target” structure $T$. Specifically, each step of this random walk consisted in a random change of a single nucleotide. If the mutation had not increased the Hamming distance to $T$ then the random walk was continued with the mutated sequence; otherwise, the original sequence was mutated again. This process was repeated until a sequence with mfe structure $T$ was obtained. The number of mutational steps needed to get to the target structure $T$ can be used as a measure of evolvability.

Figure 4a shows that sequence robustness is only marginally associated with evolvability in this sense (Spearman’s $r=0.01; P=0.016; N=3.7 \times 10^4$). What other factors might influence the length of this random walk? One candidate factor is the frequency of the starting structure $S$. This frequency is associated with the size of the sequence space that can be explored while staying on a neutral network. However, it is not associated with the length of this random walk either (Spearman’s $r=0.006; P>0.05; N=3.7 \times 10^4$).

Another candidate factor is the distance between the starting structure and the target structure. It might take longer to reach a given target structure if this structure is very dissimilar from the starting structure. However, two different structure distance measures are only weakly associated with the length of this random walk (Hamming distance: Spearman’s $r=0.03; P=8 \times 10^{-7}; N=3.7 \times 10^4$; base pair distance, the number of base pairs that need to be opened or closed to transform one structure into the other: Spearman’s $r=-0.07; P<10^{-17}; N=3.7 \times 10^4$). The only variable that is moderately associated with walk length is the frequency of the target structure (Figure 4b; Spearman’s $r=-0.21; P<10^{-17}; N=3.7 \times 10^4$). This means that regardless of the starting structure $S$, it is more difficult for a blind evolutionary search to get to a target structure $T$ if this structure is rare.

Evolvability defined as the length of a random walk starting from a given sequence is a form of sequence evolvability. An analogous measure of structure evolvability can be defined as the average length of a random walk starting from a given structure $S$ to a target structure $T$. This measure of evolvability, however, is also not associated with mutational robustness, when estimated for $k=100$ inversely folded sequences with structure $S$ (Spearman’s $r=-0.04; P>0.05; N=910$). It is also not associated with the structure frequency of $S$ (Spearman’s $r=-0.04; P>0.05; N=910$). The association between the length of this random walk with distance between $S$ and $T$ is weak and depends on the distance measure used (Hamming: $r=-0.096; P=0.004$; base pair distance: $r=-0.04; P>0.05; N=910$). Again, the only feature of some relevance is the frequency of the target structure $T$ ($r=-0.34; P<10^{-17}; N=910$).

The reason why the starting sequence may be irrelevant for the length of this random walk becomes obvious if one asks how many different structures the random walk encounters between $S$ and $T$. A histogram of this distribution is shown in Figure 5c. The median (mean) of the distribution is 63 (121) with a 10th percentile at 19 structures. Thus, an evolutionary search starting at $S$ traverses many other structures before arriving at $T$. Arguably, during this search the properties of the starting structure may matter much less than the properties of the structures encountered during the search. Robustness and evolvability are also not associated if one restricts the analysis to random walks that traverse fewer than 10 (Spearman’s $r=0.04; P>0.05; N=745$) or fewer than 5 structures (Spearman’s $r=0.11; P>0.05; N=84$) before arriving at $T$. This implies that the properties of the starting point are rapidly “forgotten” in an evolutionary search.
Discussion

In sum, a highly robust RNA genotype has low evolvability. In contrast, a highly robust phenotype has high evolvability. This positive association is caused by (i) the large neutral networks (many sequences) associated with mutationally robust phenotypes, and (ii) the different structures occurring in the neighborhoods of two or more sequences sampled from a neutral network. This synergism between robustness and evolvability manifest itself in populations of genotypes spreading on a neutral network. On a large neutral network, such populations have on average higher robustness. They thus suffer lower losses through mutations, which allows them to accumulate greater genotypic diversity. In consequence, they can access greater phenotypic diversity in their neighborhood.

I have explored robustness and evolvability for a specific genotype and phenotype, RNA and its secondary structure. However, the genotypic and phenotypic notions of robustness I use here can be applied to systems on all levels of organization, ranging from molecules to whole organisms. The reason is that for many systems, the same phenotypes can be achieved by vast numbers of genotypes (Wagner 2005). Take the example of gene regulation networks, like that of Hox genes guiding axial development in many animals (Carroll et al. 2001). They can be characterized according to a regulatory genotype that indicates which network genes interact (Ciliberti et al. 2007). This genotype is encoded by DNA sequences that comprise both the gene coding regions and their regulatory regions on DNA, promoters and enhancers. The phenotype of such a network corresponds to a spatiotemporal expression pattern of network genes in response to some “input” from genes upstream of the network. Clearly, any one phenotype can be adopted by many genotypes, partly because regulatory DNA is very flexible in its organization. Recent work suggests that in such network the same phenotypes can also be realized by vastly different numbers of genotypes (Ciliberti et al. 2007). As in the case of RNA, genotypic robustness and evolvability are properties of one specific genotype, whereas phenotypic robustness and evolvability are properties of all genotypes with the same phenotype.

Whether to focus on genotype or phenotype when studying robustness and evolvability is to some extent a matter of taste. There is only one reason to prefer phenotypes. On evolutionary time scales, genotypes change constantly, and are thus a moving target for studies of robustness and evolvability. Phenotypes, however, can stay invariant over short and intermediate evolutionary time scales, if their conservation is important to the organism. To study evolvability of phenotypes – RNA and protein conformations, network gene expression patterns, etc. – may thus be of greater relevance for processes that take place on evolutionary time scales.

I note that structure evolvability is related to the amount of information and thus the information entropy associated with a sequence that folds into a given structure. To specify any sequence of length $n$, one needs $\log_2(4^n)=2n$ bits. In contrast, to specify a sequence that folds into a given structure, one needs merely $\log_2(4^n/(4^n))=-\log_2(f)$ bits, where $f$ is the proportion of the $4^n$ sequences that fold into the structure, or an average of $-\log_2(f)/n$ bits per residue. Entropically favored phenotypes are structures with large frequency $f$, which are also structures with high evolvability. In other words, structure evolvability is associated with low information entropy of a phenotype.
This work leaves two important open questions. First, how robust and evolvable are biologically important phenotypes, such as RNA structures? To answer this question is currently impossible, because it requires the ability to estimate phenotype frequencies for many phenotypes. Partly because of the vastness of genotype space, no reliable and tractable method to do this is currently available. Second, this work does not ask about the evolutionary forces that might cause high evolvability, of which there may be several (Bloom et al. 2006; Gerhart & Kirschner 1998; Schlosser & Wagner 2004). There are two principal possibilities. High evolvability might be an adaptation in its own right, or a by-product of other selective pressures. A good candidate for such a selective pressure is natural selection for mutational robustness or thermodynamic stability, which are positively associated (Ancel & Fontana 2000). A small number of studies suggest that biological evolution has produced RNA molecules with high genotypic or phenotypic robustness (Borenstein & Ruppin 2006; Meyers et al. 2004; Sanjuan et al. 2006; Wagner & Stadler 1999). Although high phenotypic evolvability could be a by-product of selection for high phenotypic robustness, the question whether this is generally the case remains to be resolved.

Methods

Choice of structures

To study the relationship of robustness and evolvability requires the analysis of many RNA structures. This renders biological RNA sequences poor objects of analysis: although many biologically important RNA molecules are known, their sizes $n$ are different (and thus incommensurable for my purpose). Conversely, for molecules of any given size $n$, an insufficient number of biological RNAs are known, with the possible exception of micro-RNA precursors that have only simple hairpin structures. In addition, although it is straightforward to generate a sample of structures with different frequencies, it is very difficult to estimate the frequency of a given structure, unless the structure is very short. For these reasons, I here focus on structures generated by random sequences. Sequences much shorter than $n=30$ nucleotides have a limited repertoire of structures, because of the requirement that any loop that terminates a stem must have at least three unpaired nucleotides. In sequences much longer than that, even a large random sample leads only to unique structures, that is, structures of the same frequency. For instances, in a sample of $10^6$ random sequences of length $n=75$ one typically finds only unique structures, structures that occur only once in the sample. I thus focus on sequences of modest length ($n=30$), because they occupy a middle ground of being structurally diverse, yet allow me to explore a broad range of structure frequencies with computationally feasible sample sizes. I refrain from study coarse-grained structures that only contain information about the number and order of stems and loops, but not their size. Such coarse-graining has been both necessary and highly successful in some analyses (Fontana & Schuster 1998b). However, biological properties of RNA molecules may depend on the sizes of stems and loops, and coarse-graining does not reflect the full diversity of structures.
RNA structure determination

I used the Vienna RNA package (http://www.tbi.univie.ac.at/~ivo/RNA/ Hofacker et al. 1994) for all analyses. Specifically, I determined the minimum free energy (mfe) structure of a sequence using the routine fold (with default parameters) of the Vienna RNA package (http://www.tbi.univie.ac.at/~ivo/RNA/ Hofacker et al. 1994). To determine the mutational robustness $R_G$ of a sequence $G$ of length $n$, I generated all its $3n$ mutational neighbors and determined the number of neighbors with the same mfe structure as $G$. I define $r_G$ as the fraction of neighbors that have the same mfe structure as $G$, i.e., $r_G=R_G/3n$. The number of $G$'s neighbors that adopt a different structure is then, by definition, equal to $3n(1-r)$. Some of the $3n(1-r)$ structures are identical to another structure in the same neighborhood. I determined the number $U$ of unique structures that are different from one another in the neighborhood of $G$ by counting all structures in the neighborhood, but counting structures that occurred twice or more in this neighborhood only once. By definition, $E_G=U$. I define the fraction of unique structures in a neighborhood as $e_G=U/3n$, and the proportion $u_d$ of unique structures among all different structures in the neighborhood as $u_d=U/(3n(1-r))$. Note that phenotypic robustness and evolvability harbor an important asymmetry that does not permit interchangeable use of absolute numbers and fractions in defining phenotypic robustness and evolvability. Briefly, there is only one way for two structures to be the same, but many ways for them to be different. Put differently, while it is appropriate to calculate the average number of neutral neighbors for sequences with a given phenotype to determine robustness, it would not be appropriate to calculate the average number of unique different structures in the neighborhood of these sequences to determine evolvability. The reason is that different sequences can harbor completely different structures, such that one needs to add the unique structures encountered in different neighborhoods.

To generate sequences folding into a given mfe structure, I used the routine inverse_fold, which creates sequences folding into a given minimum free energy structure, using a guided random walk through sequence space that begins with a randomly chosen sequence to sample sequences from a random network. Past work (Schuster et al. 1994; Sumedha et al. 2007) has shown that inverse_fold effectively samples the space of sequences folding into a given structure at random. The routine occasionally fails to arrive at a sequence folding into a given structure. For reasons of computational limitations, the maximum number of such unsuccessful inverse foldings for every sequence to be sampled from a neutral network was limited to 10.

Random walk towards a target structure

To determine the association between robustness and evolvability, when evolvability is defined as the length of a random walk from a starting sequence with a given structure $S$ to a target sequence with a given structure $T$, I pursued the following approach. I started with a set of $10^6$ randomly generated sequences ($n=30$), and eliminated from this set those sequences that did not have a mfe structure, i.e., one in which all bases were unpaired. I ranked the remaining $9.4\times10^5$ structures according to their frequency, i.e., the number of times they occurred in the set of structures. This yielded $1.5\times10^5$ different structures with frequencies ranging over 3 orders of magnitude from less than $1.06\times10^{-2}$
(structures found only once) to $1.7 \times 10^{-3}$ (structures found 1700 times). $7.9 \times 10^4$ of the structures had the lowest frequency, that is, they occurred only once in the sample of $10^6$ sequences. For reasons of computational tractability, I used only the first 75,000 structures in the ranked structure list in further analyses. This number of structures contains the full range of structure frequencies, including more than 3000 structures of the lowest frequency. From these 75,000 structures, I then randomly sampled pairs of structures (without replacement) where the first member of the pair was designated as the starting structure $S$, and the second member of the pair was designated as the target structure $T$. I recorded the Hamming distance between $S$ and $T$ in their dot-parenthesis representation, their base-pair distance (Hofacker et al. 1994), as well as the frequencies of both $S$ and $T$. I then determined a sequence $G$ folding into $S$, using inverse_fold. From this sequence, I initiated a random walk in sequence space that was biased towards $T$ as follows. I changed an arbitrary nucleotide in $G$ to arrive at a sequence $G'$ and determined its mfe structure $S'$. If $d(S', T) \leq d(S, T)$, where $d(.,.)$ denotes the Hamming distance, then the mutated sequence was kept and a new sequence $G''$ was generated from it to continue the random walk. If this condition was not met, that is, if $d(S', T) > d(S, T)$, then $G'$ was discarded, and a new mutated $G, G'$, was generated, until the condition was met, at which time a new sequence $G'''$ was generated from $G'$ to continue the random walk. This process was repeated until a sequence was reached that had the mfe structure $T$, or until a number of $10^6$ steps in the random walk had been reached. In the latter case, the walk was considered unsuccessful, and a new inversely randomly chosen structure pair was considered. For random walks that successfully reached $T$, the length of the walk, as well as the number of different structures that the random walking sequence visited between $S$ and $T$ were recorded. Note that the random walk as defined here allows a sequence to drift on a neutral network until it reaches a sequence whose structure is equally distant or closer to $T$.

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Figure Captions

Figure 1: a) The number of sequences folding into one structure has a highly skewed distribution. Structures found in a random sample of 10^6 sequences were ranked according to their frequency, defined as the number of sequences that adopt a structure divided by 10^6. The plot shows structure rank (horizontal axis) plotted against structure frequency (vertical axis). Note the logarithmic scale on the vertical axis. Structure frequencies vary by more than a factor 10^3 in this sample. b) High genotype robustness implies low genotype evolvability. Data shown are based on 7.5×10^4 different RNA structures (n=30 nucleotides) whose frequencies span 3 orders of magnitude, and on one RNA sequence inversely folded for each structure. Robustness (r_G) and evolvability (e_G) were calculated for these inversely folded RNA sequences. Lengths of error bars indicate one standard error. Bars are too short to be visible for most of the data points. The dashed line indicates points where e_G=1-e_R. Note that 1-e_R is the fraction of sequences in the 1-neighborhood of a sequence G that have a mfe secondary structure different from that of G.

Figure 2: a) High phenotype robustness implies high phenotype evolvability. For any one structure, the estimate of evolvability (e_P) used here is the total number U of structures different from each other that were found in the 1-neighborhood of k=100 inversely folded sequences, multiplied by the structure frequency f. b) Histogram of the ratio Q (see main text) indicating how many structures in the 1-neighborhoods of k (=100) sequences are different from each other. Q ranges from Q=1/k if the k neighborhoods are identical in their structure content, to a value of Q=1 if no two structures in any two 1-neighborhoods are identical. Q is greater than ½, where k=100, indicating that the majority of structures in different 1-neighborhoods are different. c) The ratio Q increases with structure frequency, indicating that the neighborhood of a sequence folding into a structure with a larger neutral network contains a greater number of structures unique to this neighborhood. Data shown are based on the 2.5×10^4 different RNA structures (n=30 nucleotides) with the highest ranking from Figure 1, and on k=100 inversely folded RNA sequences for each structure. Error bars indicate one standard error. d) Mutational robustness r_G varies widely among sequences inversely folded from different structures. Data shown are based on 7.5×10^4 different RNA structures (n=30 nucleotides) whose frequencies span 3 orders of magnitude, and on one inversely folded RNA sequence for each structure.

Figure 3. Populations evolving on a large neutral network with robust phenotypes have access to greater amounts of phenotypic variation. Panels a)-c) show a) numbers of unique structures in the 1-neighborhood of evolving populations, b) numbers of different sequences in the population, and c) pairwise Hamming distance among sequences in the population, as a function of the number of generations of evolution (horizontal axes) on a neutral network. Open and closed circles in a)-c) correspond to populations with lowly and highly robust phenotypes, respectively. Data are based on 20 inversely folded sequences per structure and on populations of size P=500. d) Mean (circles) and standard errors (bars) of numbers of unique structures (vertical axis) in the 1-neighborhood of
populations that have evolved for 10 generations on a neutral network associated with structures whose frequency is shown on the horizontal axis. Data in d) is based on 4,000 different structures ranging in frequency from $3.3 \times 10^{-5}$ to $1.7 \times 10^{-3}$, and on one inversely folded sequence per structure that is used to seed a population of size $P=100$. Dots and bars indicate means and one standard error.

**Figure 4:** a) Mutational robustness (horizontal axis) is not associated with the length of a random walk to a target structure (vertical axis). b) The frequency $f$ of a target structure shows a weak negative association with the length of the random walk. c) Distribution of the number of different secondary structures encountered during a random walk beginning from a sequence folding into a structure $S$ to a sequence folding into a structure $T$. All data based on $3.7 \times 10^3$ random structure pairs (S,T).

**Figure S1.** Phenotypic robustness increases with structure frequency. Data based on $2.5 \times 10^4$ structures and $k=100$ inversely folded sequences per structure. Error bars indicate one standard error.
Literature Cited


Feder, M. E., Bedford, T. B. C., Albright, D. R. & Michalak, P. 2002 Evolvability of Hsp70 expression under artificial selection for inducible thermotolerance in


Figure 1a
Spearman's $s = -0.64; P < 10^{-17}; n = 7.5 \times 10^4$
Phenotype robustness ($r_P$)

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**Phenotype evolvability ($U_f$)**

Spearman's $s = 0.55$; $P < 10^{-17}$; $n = 2.5 \times 10^4$

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**Figure 2a**
Figure 2b
Figure 2c
Figure 2d

Mutational robustness

Minimum: 0.02
Maximum: 0.74

Number of structures
Figure 3a
Figure 3b

Number of different sequences in population

Structure frequency: $10^{-3}$
Structure frequency: $10^{-6}$

Number of generations
Number of generations

Sequence diversity
(average Hamming distance)

0 1 2 3 4 5 6 7 8 9 10

Structure frequency: $10^{-3}$
Structure frequency: $10^{-6}$

Figure 3c
Spearman's $s=0.27$; $n=4000$; $P<10^{-17}$

Figure 3d
Figure 4a

Spearman's $s=0.01; P=0.02; N=3.7 \times 10^4$
Spearman's $r = -0.2$; $P < 10^{-17}$; $N = 3.7 \times 10^4$

Figure 4b
Figure 4c

Number of structures traversed

Number of random walks
Phenotype robustness ($r_P$)

Structure frequency ($f$)

Spearman's $s=0.64$; $P<10^{-17}$; $n=2.5\times10^4$

Figure S1