

Genetic robustness and selection at the protein level for synonymous codons

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Keywords:

codon usage;
error minimization;
genetic code;
genetic robustness;
molecular evolution;
soft selection.

Abstract

Synonymous codons are neutral at the protein level, therefore natural selection at the protein level should have no effect on their frequencies. Synonymous codons, however, differ in their capacity to reduce the effects of errors: after mutation, certain codons keep on coding for the same amino acid or for amino acids with similar properties, while other synonymous codons produce very different amino acids. Therefore, the impact of errors on a coding sequence (genetic robustness) can be measured by analysing its codon usage. I analyse the codon usage of sequenced nuclear and cytoplasmic genomes and I show that there is an extensive variation in genetic robustness at the DNA sequence level, both among genomes and among genes of the same genome. I also show theoretically that robustness can be adaptive, that is natural selection may lead to a preference for codons that reduce the impact of errors. If selection occurs only among the mutants of a codon (e.g. among the progeny before the adult phase), however, the codons that are more sensitive to the effects of mutations may increase in frequency because they manage to get rid more easily of deleterious mutations. I also suggest other possible explanations for the evolution of genetic robustness at the codon level.

Introduction

Genetic robustness is the capacity of genotypes to minimize the effects of mutations at the phenotypic level. Mechanisms of genetic robustness are known at different levels (De Visser et al., 2003): diploidy, for example, masks recessive deleterious mutations in the whole genome; dominance and gene duplication can be seen as special cases of genetic robustness at the level of single genes.

The evolution of genetic robustness is still a debated question. The three main evolutionary explanations (deVisser et al., 2003) mirror the positions of Fisher, Haldane and Wright in their famous controversy over the evolution of dominance (a special case of genetic robustness at the level of a single gene). Robustness may evolve as an adaptation that decreases the phenotypic effect of mutations, which are usually deleterious (the *adaptationist* explanation – mirroring

Fisher); as a by-product of complex character adaptation itself (the *intrinsic* explanation – mirroring Wright); or as a side effect of the evolution for environmental robustness (the *congruent* explanation – mirroring Haldane). The relative importance of these explanations is unresolved.

The very structure of the genetic code seems to reduce errors at the protein level because amino acids with similar chemical properties are coded by similar codons (Woese, 1965; Epstein, 1966), though it is still debated whether selection for robustness is actually the main cause of the evolution of the code (Di Giulio, 2000; Freeland et al., 2000; Archetti, 2004a).

Here I discuss genetic robustness at the DNA sequence level. The different codons, even if they are synonymous, differ in their capacity to minimize the effects of mutations at the protein level: some codons, after mutation, will keep on coding for the same or for a similar amino acid, while other codons will code for very different amino acids. Therefore, synonymous coding sequences differ in their sensitivity to mutations, and genetic robustness at the DNA sequence level can be measured by analysing codon usage (Archetti, 2004b).

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The degree of genetic robustness of a coding sequence is a direct consequence of its codon usage, but codon usage itself may be due to different causes. I envisage at least three possibilities:

1. It is possible that genetic robustness is the by-product of codon usage bias that is produced by mutation bias. If codons ending with C and G are the ones that reduce more the impact of errors, (only because of the structure of the code: Archetti, 2004b) then it is possible that a mutation bias towards CG produces a higher degree of genetic robustness. In this scenario both codon usage bias and genetic robustness would be nonadaptive, and since the genetic code is almost universal, the different degrees of error minimization would simply be the by-product of different mutation biases.
2. It is possible that genetic robustness is a nonadaptive consequence of codon usage bias that evolved for other adaptive reasons. Codon usage may have evolved, for example, to match tRNA abundance for improved speed or efficiency of translation (Ikemura, 1981, 1985; Akashi, 1994). In this scenario the different degrees of genetic robustness would simply be the side effect of different codon usage patterns (that evolved to match tRNA abundance), but a strong codon bias would not necessarily correspond to a high degree of genetic robustness.
3. It is possible that the very force that shapes codon usage is selection for error minimization at the protein level: preference for robust codons evolved through selection for the conformation of the protein, that is because robust sequences reduce the impact (the effects, not the frequency) of mutations that are usually deleterious (Archetti, 2004b). In this scenario genetic robustness would be adaptive and differences in genetic robustness would be due to differences in selective pressures at the protein level.

I will call these hypotheses (to mirror the three general explanations for the evolution of genetic robustness), respectively, *intrinsic* (1 – genetic robustness depends on fluctuations of the mutation bias: it is the by-product of a nonadaptive codon usage bias), *congruent* (2 – genetic robustness depends on codon usage bias that evolved for reasons other than error minimization at the protein level: it is the by-product of a codon usage bias that is adaptive for other reasons) and *adaptationist* (3 – genetic robustness is an adaptation that reduces the impact of errors at the protein level). *Drosophila melanogaster* and rodents seem to prefer robust codons (Archetti, 2004b); and it is possible that this is an adaptation to reduce the impact of errors at the protein level (adaptationist explanation), but this is not necessarily the case for other genomes. It is possible that other species have no preference for robust codons or, even if they have a prevalence of robust codons, this may be just a by-product of mutation bias (intrinsic explanation) or codon

usage bias that evolved for other reasons (congruent explanation).

A theoretical foundation for the adaptationist explanation is lacking. Synonymous codons do not differ in fitness at the protein level, therefore natural selection is expected to produce differences in their frequencies only because of different rates of back mutations from their respective mutants: the codons whose mutants reduce more the impact of errors at the protein level will receive more back mutations and increase in frequency over time (Archetti, 2004b; Plotkin et al., 2004). It is not clear, however, whether this force is strong enough to produce a significant variation in codon usage.

My purpose here, therefore, is two-fold. First, I will try to understand whether there is variation in genetic robustness at the DNA sequence level in different genomes, and to unravel the relative importance of the three hypotheses. Second, I will develop a theoretical model to calculate the frequencies of the codons at equilibrium under different kinds of selection at the protein level, to test whether the adaptationist explanation is possible.

Materials and methods

Genetic robustness of individual codons

The basic idea (Archetti, 2004b) is to calculate the mean dissimilarity (MD) between the amino acid coded by each codon and its possible mutants, using a matrix (George et al., 1990) in which a similarity score is tabulated for each pair of amino acids. A transition/transversion bias as well as a CG/AT mutation bias may be allowed. For a complete explanation of the method to calculate the MD values, the reader is referred to Archetti (2004b).

I use a matrix based on chemical similarity (McLachlan, 1971). Although matrices based on observed substitutions are usually considered better for sequence analysis, they already include the very bias in codon usage that I want to study, and therefore may not provide a fair measure; using different matrices however does not change the results drastically (Archetti, 2004b).

I assume no transition/transversion bias (which seems not to lead to very different results – Archetti, 2004b) and either no CG/AT mutation bias ($\beta = 1$) or a CG/AT mutation bias calculated according to the per cent content (P_{CG}) of C and G in the genome: ($\beta = 100/P_{CG} - 1$; this is therefore the value expected if the observed codon usage bias was due entirely to mutation bias, not a true, measured mutation bias). This allows measuring genetic robustness under the two extreme assumptions that mutation bias does not affect at all, or is completely responsible for, codon usage bias. The similarity score of each amino acid with the termination signal is set to -100 (while the other similarity scores in McLachlan's matrix vary between 0 and 9) and multiple generations (10) are allowed as in Archetti (2004b).

Codon usage bias

I use the measures of codon usage tabulated in the Codon Usage Database (www.kazusa.or.jp/codon, described by Nakamura et al., 2000). These are codon frequencies relative to whole genomes or individual coding sequences. I analyse the mean codon usage of 2108 eukaryotes, 1901 bacteria (Eubacteria and Archaea), 2211 viruses and 463 organelles. I exclude other available species with less than 1000 sequenced codons. Since mitochondria use many different genetic codes, I analyse only organelles that use the standard code. I analyse species of the following genera with alternative nuclear genetic codes: *Mycoplasma*, *Spiroplasma*, *Oxytricha*, *Stylonychia*, *Paramecium*, *Tetrahymena*, *Acetabularia*, *Candida*; other species with known alternative codes are excluded because less than 1000 codons are available. For the analysis of individual genes I analyse only coding sequences with more than 100 codons, to exclude short or incomplete genes.

I measure codon usage bias as follows: for each codon, I take the distance (that is the absolute value) between its expected frequency in case all codons were used equally (within its synonymous family; that is 1/2 for two-fold degenerate amino acids; 1/4 for four-fold degenerate amino acids; 1/6 for six-fold degenerate amino acids; 1/3 for Ile) and its observed frequency (within its synonymous family). The sum of these distances for all the codons is called deviation from equal usage (DEU). The maximum values of the sum of the distances is 1 for two-fold degenerate amino acids, 1.33 for Ile, 1.5 for four-fold degenerate amino acids and 1.65 for six-fold degenerate amino acids; therefore, for the standard genetic code, DEU ranges between 0 and 22.78. It is highly correlated (data not shown) with another measure, the effective number of codons (ENC – Wright, 1990) that I have used in a previous analysis (Archetti, 2004b), but it has not the problems of ENC with short sequences and with sequences in which some codons are missing.

Genetic robustness of genes and genomes

Genetic robustness is measured by the degree of error minimization. To calculate the degree of error minimization of a coding sequence, the correlation between the MD values and the corresponding codon frequencies is calculated for each synonymous codon family; if N is the number of degenerate synonymous codon families on which the correlation is calculated (N depends on the genetic code and on the assumptions about mutation rates; for the standard code with no transition/transversion bias and multiple mutations, $N = 18$ if $\beta \neq 1$, while $N = 12$ if $\beta = 1$, unless some amino acids have no variance for the MD values or for the frequencies of their synonymous codons), and R is the sum of the correlations (R ranging between $-N$ and $+N$), the degree of error

minimization is measured by $R_N = R/N$ (R_N ranging between -1 and 1).

The R_N measures genetic robustness with the assumption that all amino acids are weighted equally, irrespective of their frequency on the protein. If the value of each correlation is weighted (multiplied) by the frequency of the corresponding amino acid, then the measure is denoted by wR_N . Since MD is a measure of dissimilarity, for both R_N and wR_N the lower the value the higher the degree of error minimization. A 'robust' codon usage will be one with a very low wR_N (or R_N), while an 'anti-robust' codon usage will have a very high wR_N (or R_N).

Random sequences

I use three different kinds of randomized usage patterns as null models to study the relationship of genetic robustness with mutation bias and codon usage bias:

1. 'Random' sequences are sequences in which codons are simply chosen at random with equal probability.
2. 'Inverted' sequences are generated by taking the real sequences and switching C with G, and A with T, at four-fold degenerate sites, that is at third codon positions that do not alter the amino acid coded by the codon. These 'inverted' sequences keep the same CG content and the same degree of codon usage bias as the real genes.
3. 'Rearranged' sequences are generated by switching at random the real frequencies among synonymous codons. These codon usage patterns have exactly the same degree of codon usage bias as the real sequences (but not the same CG content).

Theoretical model

The theoretical model is developed in the second part of the results. All the calculations were performed using the software MATHEMATICA (Wolfram Research, Champaign, IL, USA). Numerical simulations performed using a FORTRAN programme to check the equilibria gave identical results.

Results – genome analysis

Mean genetic robustness of sequenced genomes

I measured the degree of error minimization of all the genomes available in the Codon Usage Database (available as Supplementary Material, Table S1 and at users.ox.ac.uk/~zool0643/codon). It is evident that, while some genomes seem to prefer robust codons like *Drosophila* and rodents (Archetti, 2004b), there is an extensive variation in genetic robustness among species, and some genomes seem to prefer anti-robust codons (Fig. 1). The transition/transversion ratio, the 'similarity' score with the termination signal and the number of

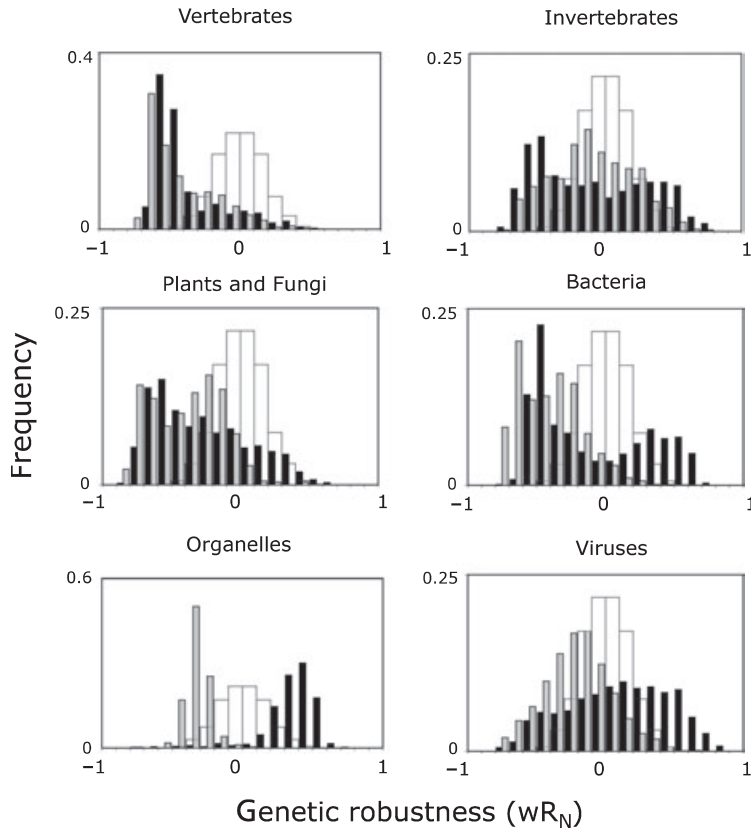


Fig. 1 The distribution of wR_N values for the n genomes with more than 1000 sequenced codons available in the Codon Usage Database: bacteria (Eubacteria and Archaea, $n = 1901$), plants and fungi ($n = 1031$), invertebrates (animals excluding vertebrates and including protozoa, $n = 634$), vertebrates ($n = 443$), viruses ($n = 2211$), organelles ($n = 463$). High wR_N values indicate anti-robustness, low wR_N values indicate robustness. Black: no mutation bias ($\beta = 1$); grey: mutation bias $\beta = 100/P_{CG} - 1$. White: distribution of wR_N values for 'random' codon usage patterns.

generations on which MD values are calculated, do not seem to change these results drastically (not shown, see also Archetti, 2004b).

With no mutation bias ($\beta = 1$) the variation is more extreme than when a mutation bias is considered ($\beta = 100/P_{CG} - 1$). Since we do not know the true mutation bias and the two cases correspond to the two extreme possibilities, it seems reasonable to assume that the real figure is intermediate to the ones shown in Fig. 1. The extensive variation in genetic robustness therefore seems unambiguous. The causes of genetic robustness, and of this variation, however are more difficult to understand. Some indirect observations are possible.

1.1. Genetic robustness is correlated with CG content.

There is a correlation between CG content and the degree of genetic robustness. It is worth noting that it is stronger (Table 1) in the case of no mutation bias ($\beta = 1$). If mutation bias was the cause of genetic robustness (intrinsic explanation) then CG content should be correlated with the degree of error minimization; however a correlation between wR_N and P_{CG} could also mean that different degrees of error minimization, originated for other reasons, can explain the CG content of different species. While this could be explained by the adaptationist explanation (because, due to the structure of the code, C and G are usually better at minimizing errors

Table 1 The correlation (r^2) between CG content and wR_N for the six groups shown in Fig. 1.

	No. of genomes*	r^2	
		$\beta = 1$	$\beta = 100/P_{CG} - 1$
Bacteria	1901 (658)	0.778 (0.784)	0.332 (0.310)
Plants and fungi	1031 (201)	0.589 (0.726)	0.381 (0.413)
Invertebrates	634 (153)	0.676 (0.683)	0.161 (0.101)
Vertebrates	443 (94)	0.504 (0.232)	0.434 (0.329)
Viruses	2211 (526)	0.611 (0.680)	0.041 (0.107)
Organelles	463 (54)	0.430 (0.600)	0.069 (0.075)

*The values in parentheses indicate the results limited to the genomes with more than 10 000 codons.

– see Archetti, 2004b), coevolution with tRNA abundance (congruent explanation) does not necessarily lead to a correlation between genetic robustness and CG content, unless tRNA abundance itself is biased towards C and G for all synonymous families (which is unlikely).

1.2. Genetic robustness depends on the specific codons used.

Codons ending with C and G (and A and T) have different degrees of error minimization (MD values), and inverting C with G (and A with T) at four-fold degenerate

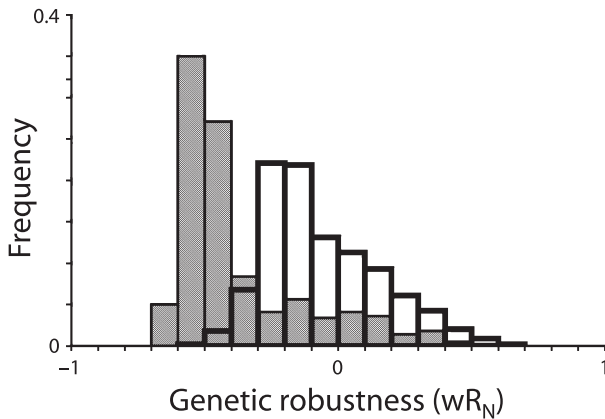


Fig. 2 The distribution of wR_N values for vertebrates (in grey). High wR_N values indicate anti-robustness, low wR_N values indicate robustness. White: the 'inverted' patterns (C–G and A–T switching at four-fold degenerate sites). No mutation bias ($\beta = 1$).

a sites (see *Materials and methods*) produces different wR_N values. The 'inverted' sequences are less biased in their degree of error minimization than the real sequences (Fig. 2 shows the case of vertebrates). Therefore, there is a difference between C- and G-ending codons and between A- and T-ending codons. While this could be explained by the adaptationist explanation (because of asymmetries in the structure of the genetic code, C- and G-ending codons, as well as A- and T-ending codons, do not have the same MD values) and by the congruent explanation (C- and G-ending codons, as well as A- and T-ending codons, do not necessarily have the same abundance of tRNAs), it seems more difficult to explain with the intrinsic explanation (C and G, as well as A and T, should have the same mutation bias if this is equivalent in the two strands).

1.3. Genetic robustness is correlated with the degree of codon usage bias.

It seems clear that the highest degrees of codon usage bias correspond to the highest and lowest degrees of error minimization. Figure 3 shows the example of Bacteria (similar plots are obtained for the other groups – see Table 2). If genetic robustness was the by-product of codon usage bias originated through coevolution with tRNA abundance (congruent explanation) this would be unlikely, because matching tRNA abundance does not necessarily lead to an extreme degree of error minimization; this would be the case only if the preferred codons (the most abundant tRNAs) were the ones with the lowest MD value for all synonymous families, which is unlikely. The 'rearranged' genomes, in fact, while maintaining the same degree of codon usage bias, have wR_N values that are not correlated with the degree of codon usage bias (Table 2). It is possible, however, that different degrees of codon usage bias are due to different

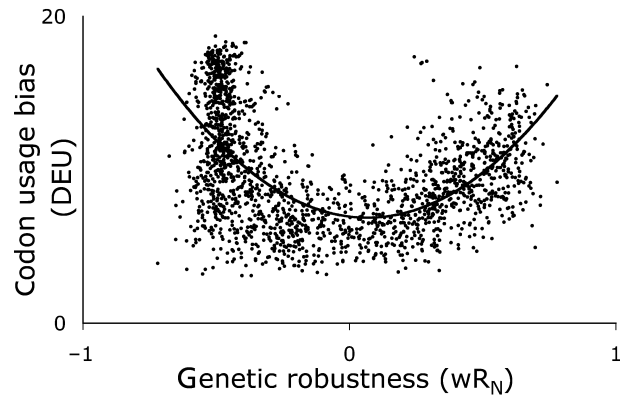


Fig. 3 The degree of codon usage bias (DEU) plotted against the degree of error minimization (wR_N) for the 1901 bacterial genomes. High wR_N values indicate anti-robustness, low wR_N values indicate robustness. High DEU values indicate strong codon usage bias. No mutation bias ($\beta = 1$). The polynomial (second order) regression line is shown.

Table 2 The correlation (r^2 , polynomial second order) between DEU and wR_N for the six groups shown in Fig. 1, for the real sequences and for the 'rearranged' sequences ($\beta = 1$; similar results are obtained with $\beta = 100/P_{CG} - 1$).

	No. of genomes*	r^2	
		Real sequences	Rearranged
Bacteria	1901 (658)	0.272 (0.332)	0.043 (0.071)
Plants and Fungi	1031 (201)	0.171 (0.208)	0.029 (0.010)
Invertebrates	634 (153)	0.200 (0.233)	0.021 (0.042)
Vertebrates	443 (94)	0.275 (0.319)	0.049 (0.062)
Viruses	2211 (526)	0.207 (0.227)	0.015 (0.011)
Organelles	463 (54)	0.060 (0.028)	0.016 (0.010)

*The values in parentheses indicate the results limited to the genomes with more than 10 000 codons.

degrees of genetic robustness that evolved for the minimization of errors at the protein level (adaptationist explanation) or for mutation bias (intrinsic explanation).

1.4. Cytoplasmic genomes are anti-robust.

While there is an extensive variation in genetic robustness among nuclear genomes, organelles are generally anti-robust (see Table S1), even if their corresponding nuclear genome is robust. Figure 4 shows the example of *Oryza sativa*. The differences between nuclear and cytoplasmic genome could be explained both by the adaptationist explanation (organelles and nuclear genomes have different transmission modes and may be under different kinds of selection – see below) and by the congruent explanation (if tRNAs used by organelles are very different in frequency from tRNAs used by the nuclear genome), and perhaps by the intrinsic explanation (if the polymerases that are responsible for errors

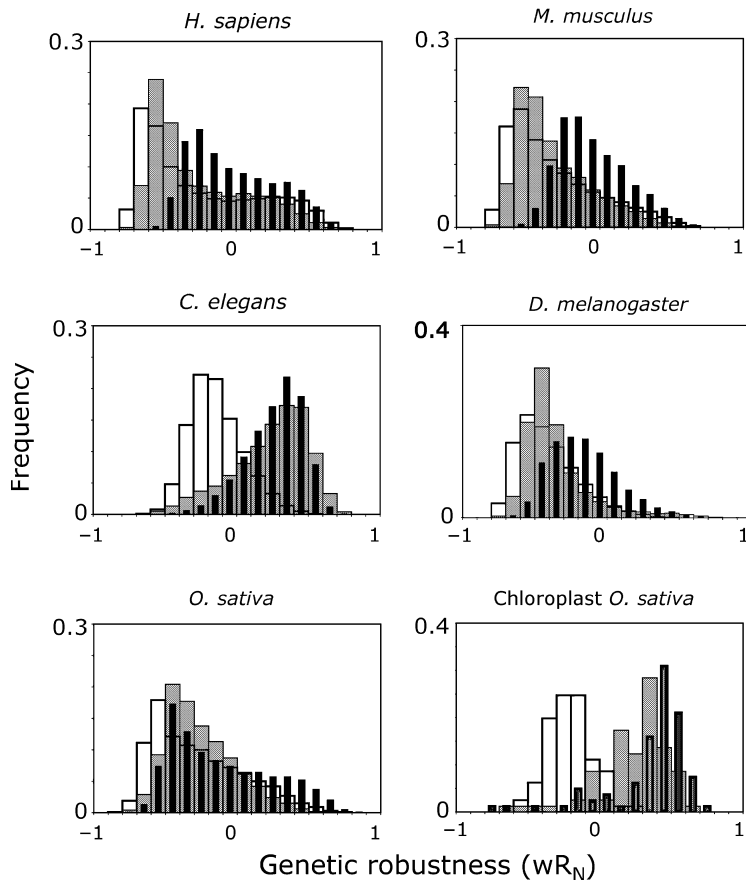


Fig. 4 The distribution of wR_N values for n sequences (nuclear genome) of *H. sapiens* ($n = 62\,378$), *M. musculus* ($n = 32\,141$), *C. elegans* ($n = 22\,487$), *D. melanogaster* ($n = 34\,139$) and *O. sativa* ($n = 38\,807$) and for the chloroplast of *O. sativa* ($n = 81$). High wR_N values indicate anti-robustness, low wR_N values indicate robustness. Grey: no mutation bias ($\beta = 1$); white: mutation bias ($\beta = 100/P_{CG} - 1$); black: 'inverted' sequences, no mutation bias ($\beta = 1$). For the *O. sativa* chloroplast: large grey bars: no mutation bias ($\beta = 1$); white: $\beta = 100/P_{CG} - 1$ (chloroplast P_{CG}); thin grey bars: $\beta = 100/P_{CG} - 1$ (nuclear P_{CG}).

are different in the nuclear and in the cytoplasmic genomes). The adaptationist explanation, however, seems to explain more easily the fact that organelles are almost always anti-robust (see the *Discussion* on the theoretical model).

Genetic robustness of individual genes

So far I have discussed the average codon usage of whole genomes, but codon frequencies may be different even among genes of the same genome. The overall codon usage, and therefore the overall wR_N value, arguably depends on the average codon preference among the genes, and it would be interesting to know if there is variation among individual genes. In fact, a wide variation seems to exist among genes belonging to the same genome (Fig. 4). Sampling different groups of 1000 random genes does not seem to change the distribution of wR_N values (not shown).

For some species, e.g. *Homo sapiens*, *Mus musculus*, and *O. sativa*, the CG/AT mutation ratio does not drastically change the absolute values of error minimization, while for other species, e.g. *Caenorhabditis elegans*, the mutation bias has important effects on the absolute degree of error minimization, while there is always extensive variation

among genes, that is in the relative values. Note, again, that the mutation bias ($\beta = 100/P_{CG} - 1$) has been deduced from the CG content of the genome and that, as for the genomic values, the real situation is probably intermediate between the two extremes shown in Fig. 4. I will discuss *D. melanogaster* here but these results are similar for other species.

2.1. Genetic robustness is correlated with CG content.

In *D. melanogaster* there is a correlation between CG content and wR_N , which is slightly stronger ($r^2 = 0.310$) with no mutation bias ($\beta = 1$) than with a mutation bias deduced from the CG content ($\beta = 100/P_{CG} - 1$) ($r^2 = 0.292$). As for the case of the genomic values, this could be explained by the adaptationist explanation but it seems more difficult for the congruent explanation; it may be explained by the intrinsic explanation if extensive differences in mutation bias exist within the genome.

2.2. Genetic robustness depends on the specific codons used.

As for the genomic values, there is a difference between the distribution of wR_N values of the real genes and the 'inverted' sequences (Fig. 4), and there-

fore a difference between C- and G-ending codons and between A- and T-ending codons. This could be explained, again, by the adaptationist explanation (because C- and G-ending codons, as well as A- and T-ending codons, do not have the same MD values) and by the congruent explanation (because tRNA frequencies may differ between tissues and genes expressed in different tissues may have different codon preferences), but it seems more difficult to explain with the intrinsic explanation (unless C and G, as well as A and T, have different mutation bias).

2.3. Genetic robustness is correlated with the degree of codon usage bias.

In *D. melanogaster*, there is a correlation ($r^2 = 0.128$) between the degree of codon usage bias (DEU) and genetic robustness (wR_N) (while for the rearranged sequences $r^2 = 0.059$). As for the genomic values, this could be explained by the adaptationist explanation and by the intrinsic explanation (provided that there exist differences in mutation bias within the genome), but would be unlikely under the congruent explanation, because matching tRNA abundance does not necessarily lead to an extreme degree of error minimization (indeed for difference between genes of the same genome this seems even more unlikely if tRNA abundance differs between different tissues).

2.4. Genes under strong purifying selection are not necessarily robust.

In *D. melanogaster* the genes with the highest wR_N values that is the most anti-robust genes (Table 3) do not seem to be under positive selection. Indeed for some of them it is rather likely that they are under strong negative selection. Therefore, robustness does not seem to be necessarily linked with negative selection. Under the intrinsic and under the congruent explanation there should be no clear relationship between gene function (selective pressure acting on the gene) and its robustness. Under the adaptationist explanation, instead, gene function should be related with the degree of genetic robustness. It is not necessary, however, that the most robust genes are those under strongest selection (as Plotkin et al., 2004 suggest). In the following theoretical analysis I will show why.

Results – theoretical analysis

Can selection on protein structure lead to codon usage bias?

Synonymous codons do not differ in fitness at the protein level, however they have different rates of back mutations from their respective mutants: ‘robust’ codons, those whose mutants reduce more the impact of errors at the protein level, will receive more back mutations and increase in frequency over time. This force, however, is

Table 3 The *D. melanogaster* genes with the highest wR_N values (not considering transposons and genes with unknown function) and their function.

wR_N	Gene	Function
0.804	fu2	Transcription factor activity (C2H2 and C2HC zinc fingers)
0.761	fl(2)d	Germ-line sex determination, regulation of alternative splicing
0.712	CG5384	Hydrolase activity, ubiquitin-specific protease activity
0.709	Trp1	SRP-dependent cotranslational membrane targeting
0.709	CG4747	3-hydroxyisobutyrate dehydrogenase activity
0.699	Ephrin	Ephrin receptor binding
0.694	ctp	Cytoplasmic dynein complex, microtubule motor activity
0.691	hep	Protein kinase activity
0.691	eIF-4G	Translation initiation factor 4F complex
0.689	pan	Transcription factor activity, embryonic pattern specification

No mutation bias ($\beta = 1$)

arguably weak. Here I calculate the theoretical equilibrium frequencies of the codons under mutation and selection for protein conformation, to understand whether selection at the protein level is strong enough to produce a significant variation in synonymous codon usage.

Under certain circumstances another mechanism can produce variation in synonymous codon frequencies: soft selection. In this case certain codons may increase in frequency because their mutants are eliminated more easily from the population, and not because of more back mutations. This is a rather different scenario and will be developed in the second part of the model.

The basic model

Consider a locus *A* (three base pairs) with 64 possible alleles (the 64 codons of the standard genetic code). The fitness conferred by a generic codon *i*, coding for amino acid a_i , at this locus, is

$$W_{A/i} = 1 - s[(\omega_{A/A} - \omega_{A/a(i)})/\omega_{A/A}],$$

where $\omega_{A/a(i)}$ is the similarity score of amino acid *A* with amino acid a_i and $\omega_{A/A}$ is the similarity score of amino acid *A* with itself (in McLachlan’s matrix $0 \leq \omega_{A/a(i)} < 9$ and $\omega_{A/A} = 9$); *s* is the selection coefficient against amino acids other than *A* ($0 \leq s \leq 1$). Therefore, for example, if $a_i = A$ then $\omega_{A/a(i)} = 9$ and $W_{A/i} = 1$, while if $\omega_{A/a(i)} = 0$ then $W_{A/i} = 1 - s$, and if $\omega_{A/a(i)} = 4$ then $W_{A/i} = 1 - (5/9)s$. If *i* is a termination codon I assume that $W_{A/i} = 1 - \sigma$ (with $s < \sigma \leq 1$). If m_{ji} is the mutation rate from codon *j* to codon *i*, then we have the following possibilities:

$$\begin{aligned}
 m_{ji} &= 1 - 3\mu & \text{if } i = j \\
 m_{ji} &= \mu/3 & \text{if } i \text{ is obtained by point mutation from } j \\
 m_{ji} &= 0 & \text{in other cases,}
 \end{aligned}$$

where μ is the mutation rate per nucleotide. This is an approximation, excluding mutation bias and double- or multiple-nucleotide mutations. Taking into account double mutations (with a frequency up to $100 \mu^2$) however does not drastically change the results (not shown).

The change in frequency x_i of codon i can be described by the recurrence equation

$$Tx'_i = \sum_{j=1}^{64} x_j W_{Aij} m_{ji},$$

where T is a normalizing factor obtained by summing the right-hand side over all i . The equilibrium frequencies of the 64 codons can be found numerically by calculating the leading eigenvector of the matrix $W_{Aij} m_{ji}$ for the 20 amino acids A . Assuming multiplicative fitness these frequencies correspond to the codon frequencies in the genome.

Let us look at the equilibrium frequencies of the six codons for Leucine, for example (Fig. 5 – see Table S2 for the other amino acids). Two of these codons produce termination codons after a point mutation (that is, they are ‘pretermination’ codons), and are the ones that maximize the impact of mutations (Archetti, 2004b): TTA and TTG. These two codons are actually the less frequent at equilibrium. This trend (lower frequency of pretermination codons) is also evident in the other synonymous families with pretermination codons (Ser, Arg and Gly) and the frequencies of the codons are generally correlated with their capacity to reduce the impact of errors (see also Archetti, 2004b).

Similar results are obtained for different values of μ . Given a certain μ , if $s \gg \mu$, the same, or very similar equilibria are obtained for any value of s . The results for $s \ll \mu$ are also independent from s ; for realistic values of μ , however, this case is relevant only for species with a large effective population size, e.g. viruses and bacteria. For small populations drift will prevail and these equilibria are not appropriate to describe what happens in nature.

For some synonymous families under some circumstances (e.g. many four-fold degenerate amino acids with $s \gg \mu$, see Table S2) strong selection does not lead to codon usage bias. This is because the codons of these synonymous families have exactly the same one-mutation neighbours, and differences among these synonymous codons are due solely to codons two or more mutations away. When selection is strong the contribution of these multiple-mutation neighbours is negligible, while it has some effect with weak selection (because the one-mutation neighbours survive at appreciable frequency).

We must keep in mind however that we are assuming here exactly no double-nucleotide mutations and no

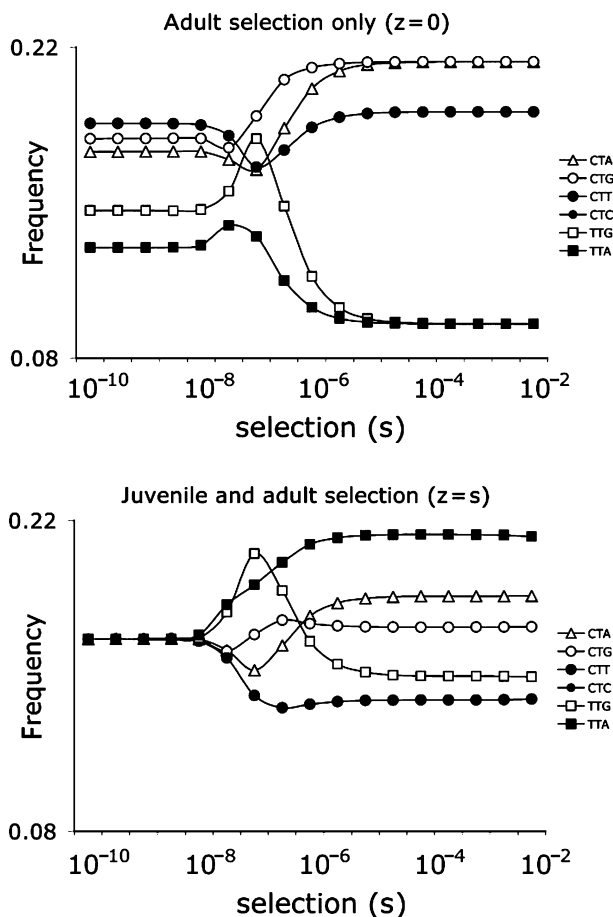


Fig. 5 The equilibrium frequencies of the six codons of Leucine predicted by the theoretical model for different values of selection coefficients (s) with ($z = s$) or without ($z = 0$) juvenile selection; mutation rate per nucleotide, $\mu = 10^{-8}$; selection coefficient for a termination codon, $\sigma = 1$; no mutation bias ($\beta = 1$).

transition/transversion bias – relaxing these assumptions leads to codon usage bias for all the amino acids even after one mutation (data not shown).

Soft selection

One assumption of the previous model was that selection occurs only in the adult phase, just before reproduction. However this is not always the case in nature. If a gene is expressed in an early phase of the organism, for example, and if the reproductive strategy is such that only few juvenile individuals go on to the adult phase in any case, then discarding the mutant part of the progeny does not lead to a loss of fecundity. This concept is often called ‘soft selection’ and is well known in ecology (Buchholz, 1922; Klekowski, 1988).

If soft selection is applied to our previous model, codons with more deleterious neighbours can eliminate a

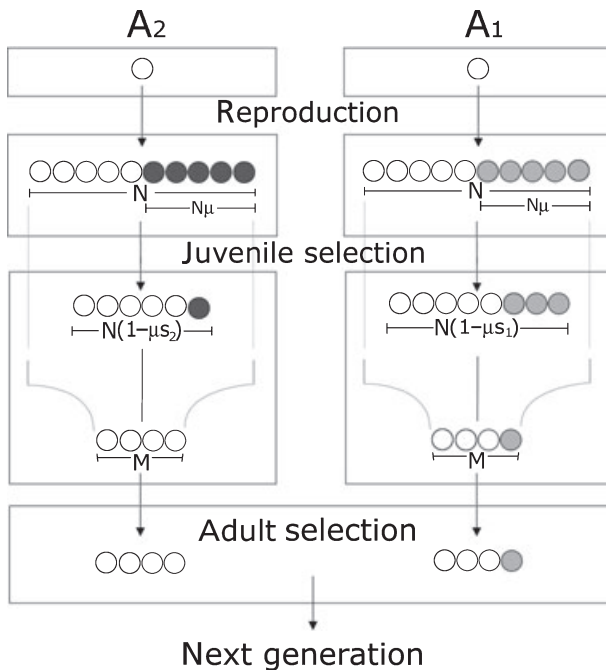


Fig. 6 A simplified example of soft selection during the juvenile phase. Each circle represents an individual. A_1 and A_2 are two synonymous codons at the same locus. Individuals with codon A_1 and individuals with codon A_2 produce the same number (N) of offspring and have the same fitness (1) and the same mutation rate (μ); mutants (grey) of individuals with codon A_1 , however, have higher fitness ($1 - s_1$) than mutants (black) of individuals with codon A_2 ($1 - s_2$); therefore, by definition, A_1 is more robust than A_2 ($s_2 > s_1$). If only a part of the progeny (M) goes on to the adult phase in any case, then codon A_2 manages to get rid more easily of its mutant alleles (with no loss of fecundity) than codon A_1 and will increase in frequency in the adult population. Note that the model described in the text is a continuous (infinite population) version of this discrete example (finite population).

higher fraction of their mutational load through juvenile selection that does not actually reduce the fitness of the codon (see Fig. 6).

With soft selection among the (N) offspring (but not with other individuals of the population), if only M ($\leq N$) individuals of the progeny go on to the adult phase (where competition among individuals of the population occurs), then the parameter $\phi = M/N$ ($0 < \phi \leq 1$) can be viewed as the 'buffering' potential of the growth phase (as ϕ approaches 0 only an infinitesimal number of the N juvenile individuals will go on to the adult phase in any case, and discarding part of the progeny will have no effect on fecundity).

The fitness conferred by a generic codon i at this locus in the juvenile phase is

$$U_{A/i} = 1 - z[(\omega_{A/A} - \omega_{A/a(i)})/\omega_{A/A}],$$

where z is the selection coefficient against the amino acid with the lowest similarity with A . If i is a stop codon, then

$U_{A/i} = 1 - \zeta$ (with $z < \zeta \leq 1$). The change in frequency x_i of codon i expressed in the juvenile and adult phase can be described by

$$Tx'_i = \sum_{j=1}^{64} [x_j W_{A/j} m_{ji} U_{A/i} / \alpha_{A/j}],$$

where T is a normalizing factor obtained by summing the right-hand side over all i , and $\alpha_{A/j}$ is a normalizing factor of the offspring codon frequencies, that is the sum of the frequencies, not normalized, of the offspring of codon j just after juvenile selection

$$\alpha_{A/j} = \sum_{h=1}^{64} (m_{jh} U_{A/h})$$

provided that this is $\geq \phi$ (this condition can be considered generally true for realistic values of μ ; if it is not true then there is loss of fecundity and $\alpha_{A/j} = \phi$). In this way the frequencies of the offspring are normalized (within the surviving offspring, not within the population) before the adult phase. In the adult phase, then, selection among all the individuals of the population occurs as in the basic model.

I assume for simplicity that if selection occurs both in the juvenile and adult phase then $s = z$ and $\sigma = \zeta$ (while with no juvenile selection, $z = 0$, $\zeta = 0$ and $\alpha_{A/i} = 1$). The equilibrium frequencies of the 64 codons can be found numerically by calculating the leading eigenvector of the matrix $W_{A/j} m_{ji} U_{A/i} / \alpha_{A/j}$.

Looking again at the equilibrium frequencies of the six codons for Leucine it is clear that if selection occurs also in the juvenile phase, codon frequencies are completely different from the case of no juvenile selection, and pretermination codons may increase in frequency (Fig. 5).

I have assumed that selection coefficients are the same in the adult and in the juvenile phase, but this assumption could be relaxed and would produce further different equilibrium frequencies.

Discussion

I have shown empirically that there is an extensive variation in genetic robustness at the codon level in available sequenced genomes, both among species and among genes of the same genome. The existence of this variation seems unambiguous and it is independent from its causes: even if codon usage was entirely due to coevolution with tRNA abundance, for example, the genes using the robust codons would still undergo less dramatic effects after mutation than genes using anti-robust codons. It is clear, however, that merely measuring genetic robustness does not allow us to understand the causes.

I have then shown theoretically that selection on the conformation of proteins can lead to differences in synonymous codon frequencies, and these frequencies

can be different depending on the kind of selection. I have suggested that, among other possibilities, codon usage bias may have evolved because certain codons reduce the impact of mutations and therefore increase in frequency, or because (with soft selection) they manage to get rid more easily of deleterious mutations.

I think it is worth pointing out that these two results are independent and I am not claiming that genetic robustness for all the genes and genomes I have listed is adaptive. I have only provided (i) a measure of genetic robustness of the available genomes and (ii) a demonstration that the adaptationist explanation is possible. The intrinsic and the congruent explanations remain entirely possible though (but see below for further discussion).

Moreover there are other factors that need to be taken into account. I have not analysed positive (diversifying) selection, for example. Positive selection would probably lead to a preference for codons that are anti-robust (as suggested by Plotkin et al., 2004) and the equilibrium frequencies in this case might be similar to those obtained under soft (negative) selection.

Another factor that I have not taken into account is population size. My theoretical results are relevant only for species with a large population size. With small effective population sizes one would need to take into account the nearly neutral theory. Again, I have measured genetic robustness for many species but for some of them (e.g. vertebrates) the results of the theoretical model might not be relevant.

Therefore I cannot draw any final conclusion on the causes of genetic robustness at the codon level on the basis of the present study. However I think it is worth noting the following results.

Intrinsic explanation

If mutation bias were the only cause of codon usage bias then the differences in genetic robustness would be a random effect of different mutation biases acting upon the same genetic code. In this scenario, C and G should be used with approximately the same frequencies (as should be A and T) unless there is a difference in the mutation bias for the two DNA strands.

Antezana & Kreitman (1999) already suggested that the preferred codons might have intrinsic, functional advantages over the minor codons, advantages unrelated to mutation bias. For instance, the major codon within each degenerate family is identical in *D. melanogaster* and *M. musculus* (the only exception is the Arg4 family CGN in *M. musculus*, in which both G and C are similarly preferred – see tables in the CUTG database, Nakamura et al., 2000). This is remarkable given that the major codon alternates from G-ending to C-ending across four-fold-degenerate families, making a simple GC content-oriented explanation untenable (according to Antezana & Kreitman, 1999).

I can add, as a result of the present study, that if codon usage bias was a result of mutation bias alone, irrespective of selection for genetic robustness, then inverting C with G (and A with T) at four-fold degenerate sites should produce similar wR_N values, which in fact I have shown is not the case (see results 1.2 and 2.2).

Mutation bias could explain, however, the correlation between CG content and robustness (see results 1.1 and 2.1) and between the extent of codon usage bias and robustness (see results 1.3 and 2.3), though in both cases this would require, at the level of the individual genes, that there is a variation in mutation bias across the genome.

Furthermore, within the same species, in the nuclear genome there is often a prevalence of robust genes, while in the organelles there is, in almost every case, a prevalence of anti-robust genes (see result 1.4). Sueoka (1988) suggested that mutation bias derives from inter-specific differences in the DNA polymerases. If this were the case, then the difference between nuclear and cytoplasmic genomes would be possible only assuming that the differences between DNA polymerases are also intraspecific, between nucleus and cytoplasm. The fact that organelles are almost always anti-robust, however, seems to suggest that there is something intrinsic in their structure or reproductive mode that is responsible for this constancy. This is more easily explained by the adaptationist explanation rather than by the intrinsic explanation.

Congruent explanation

The observation that inverting C with G and A with T at four-fold degenerate site does affect genetic robustness (see results 1.2 and 2.2) is compatible with the *congruent* explanation.

If genetic robustness is due to codon usage bias that evolved for coevolution with tRNA abundance, however, there should be no clear relationship between codon usage bias and the degree of error minimization, which in fact seems to exist (see results 1.3 and 2.3), and the correlation between CG content and genetic robustness (see results 1.1 and 2.1) would also be unexpected. As I have noted (see also Archetti, 2004b), a high degree of codon usage bias originated for other reasons (e.g. tRNA abundance) does not necessarily lead to an extreme degree of error minimization; intermediate values of the degree of error minimization may correspond to a low degree of codon usage bias, but a high degree of codon usage bias could lead to all the possible degrees of error minimization, including intermediate values, unless codon usage is biased (for other reasons independent of the structure of the code) in all synonymous families towards the codons with the lowest MD values, or in all synonymous families towards the codons with the highest MD values, which is very unlikely if tRNA abundance is arbitrary (independent on MD values). A similar argument is valid for CG content.

Differences in tRNA abundance for nuclear and cytoplasmic genes could lead to different degrees of robustness, within the same species, between nucleus and organelles (see result 1.4) but could hardly explain why there is an extensive interspecific variation in genetic robustness among nuclear genomes, while organelles in almost all species are anti-robust.

Adaptationist explanation

If genetic robustness is an adaptation due to selection for error minimization at the protein level, then C and G (and A and T) should not be used with equal frequencies, because they have different degrees of error minimization (MD values), and inverting C with G (and A with T) at four-fold degenerate sites should produce higher wR_N values, which is what I have actually shown (see results 1.2 and 2.2). Because C and G reduce the impact of errors more than A and T (Archetti, 2004b), the correlation between CG content and robustness is also expected (see results 1.1 and 2.1). The adaptationist explanation could also explain the correlation between degree of codon usage bias and genetic robustness (see results 1.3 and 2.3) and the differences between nuclear and cytoplasmic genomes (see result 1.4).

Under the adaptationist explanation, it may seem reasonable to think that robust genes are under strong negative (purifying) selection, while anti-robust genes are under positive (diversifying) selection. For example, it would not be surprising that genes involved in host-parasite interactions prefer anti-robust codons. This argument has been assumed recently by Plotkin et al. (2004). In a previous study (Archetti, 2004b) I had shown, in *Drosophila* and in rodents, a correlation between wR_N and substitution rates. The limited number of genes analysed had not allowed me to notice the presence of such large variation in robustness.

Indeed, as I have shown here, the genes with a preference for anti-robust codons are not necessarily under diversifying selection (see result 2.4). The results of Plotkin et al. (2004) in fact have been questioned by other recent analyses (Dagan & Graur 2004; Friedman & Hughes, 2004; Sharp, 2004; Chen et al., 2005; Hahn et al., 2005; Nielsen & Hubisz, 2005; Plotkin et al., 2005; Zhang, 2005). The relationship between selection and robustness is discussed in more detail below.

Theoretical results

I have shown in the theoretical results that selection at the protein level can actually lead to differences in the frequencies of synonymous codons. With a simple model of adult selection for the conformation of proteins, robust codons are expected to increase in frequency because they receive more back mutations from their mutants (which survive with higher probability).

In this model, however, the genes under strong selective pressure are expected to be more robust only if one does not take into account the possibility of soft selection. With soft selection certain codons may increase in frequency not because of more back mutations but because codons with more deleterious neighbours manage to eliminate a higher fraction of their mutational load, for example, through juvenile selection that does not reduce the fitness of the original codon itself.

Soft selection (not at the codon level) is well known in ecology and may occur in many species (Klekowski, 1988). In my model, elimination of the deleterious mutants can occur with no loss of fitness if the deleterious mutations are expressed in an early phase of the organism, in which there is competition only with relatives (mutant alleles) because discarding the mutant part of the progeny does not lead to a loss of fecundity.

The same scenario may apply to organelle populations within a cell, for example: they may also go through a bottleneck before being transmitted to the next generation, and discarding the mutants at this stage will lead to virtually no loss of fecundity for the organelle itself. This might explain why organelles show a preference for codons that maximize errors. In principle anti-robustness could also be possible for viruses, bacteria and other parasites, and even in tissues capable of cellular selection, where mutant cells can be discarded (through apoptosis for example) with no damage for the body.

My model requires only a phase of selection in which individuals compete solely with their sibs (their copies and their mutants); a metapopulation model like that of Levene (1953) would probably give similar results and might be more widely applicable.

With soft selection therefore using anti-robust genes may lead to easier elimination of the mutant alleles, and anti-robust codons may increase in frequency. Indeed other known cases of anti-robustness (not at the codon level) are strategies to get rid more easily of deleterious mutation, by discarding mutant individuals, (e.g. haploidy) or mutant cells (e.g. apoptosis). Overlapping reading frames (Krakauer, 2000) is another example of hypersensitivity to mutations at the sequence level. Under the adaptationist explanation, anti-robustness at the DNA sequence level may be also a strategy to get rid more easily of deleterious mutations.

In my model I have assumed either that there is no soft selection or that selection coefficients are equal in the adult and in the juvenile phase, but all the intermediate cases are possible. This diversity of possibilities might explain, or contribute to explain, the diversity of codon usage patterns.

The three hypotheses

I have stressed the importance of considering an adaptationist explanation for genetic robustness because the other two possible explanation rely on two possible

explanations for codon usage bias (mutation bias and coevolution with tRNA abundance) that have been extensively studied, while the adaptationist explanation relies on the concept of error minimization at the protein level, which has been considered a possible explanation for codon usage bias itself only recently (Archetti, 2004b).

I have shown that the adaptationist explanation is reasonable from a theoretical point of view. The intrinsic and the congruent explanation, however, remain possible. It is entirely possible that all these explanation contribute to explain genetic robustness. In species with very large population size selection at the protein level may be more effective while in small populations mutation bias may prevail and robustness may not be adaptive. The causes of genetic robustness at the codon level must still be studied in details and at the moment I cannot put forward any definitive argument in favour of the adaptationist hypotheses, though I have suggested that the it may explain more easily some asymmetries.

It is clear, however, that selection at the protein level cannot be ignored and studies that assume as null hypothesis the absence of differential selection at the protein level (e.g. the study of coevolution with tRNA abundance of Akashi, 1994) must be reconsidered.

Genetic robustness does not measure selection coefficients

The whole concept of soft selection may rule out the method developed by Plotkin et al. (2004) as an alternative to comparative analysis to detect selection from DNA sequences. Plotkin et al. (2004) suggest that genes with anti-robust ('volatile' in their terminology) codons are under positive (diversifying) selection, while robust genes are under negative (purifying) selection. Because soft negative selection may lead to a preference for anti-robust codons (as I have shown in the theoretical results) it would be difficult to distinguish a gene under positive selection and one under soft negative selection on the basis of codon usage alone. This adds to other concerns put forward by others on using codon usage to detect selection (Dagan & Graur, 2004; Friedman & Hughes, 2004; Sharp, 2004; Chen et al., 2005; Hahn et al., 2005; Nielsen & Hubisz, 2005; Plotkin et al., 2005; Zhang, 2005). I have shown that selection at the protein level can lead to synonymous codon usage bias but genes under purifying selection are not necessarily robust.

Evolvability

Whatever the causes, I have shown that an extensive variation exists in genetic robustness at the codon level, both between and within genomes. This variation may have important implication on the rate of evolution (evolvability) of the different species.

Genetic robustness (in general, not restricted to the codon level), reducing the phenotypic variation produced by the genotype, should lead to a reduction of evolvability (future rate of evolution) and this is actually observed in some cases (e.g. Ancel & Fontana, 2000). However a robust trait may produce a mutationally connected network of genotypes exhibiting the same phenotype (a neutral network) that allows, by drift, evolution towards new phenotypes that would not be otherwise accessible through small mutations. Therefore, genetic robustness may show increased rather than decreased adaptive potential. Neutral networks and their consequences have been studied in computational models of evolving RNA populations (Fontana, 2002), but the impact of robustness on evolvability and evolutionary dynamics in general remains an important open issue that has yet to be explored (deVisser et al., 2003).

Since the degree of error minimization at the codon level is easy to measure with the method used here (and in Archetti, 2004b), and genetic robustness in general is otherwise difficult to measure (deVisser et al., 2003), studying codon usage bias might prove an opportunity for future comparative studies on genetic robustness.

Acknowledgments

Alan Grafen provided many useful suggestions and helped me in the calculation of the eigenvector. Francisco Ubeda provided useful comments on the theoretical model. Tad Kawecki provided a fundamental suggestion on soft selection at the beginning of the theoretical study. Dieter Ebert helped me initially with the statistics and constantly with his advices. This work started at the Department of Biology, University of Fribourg while I was partially supported by the Roche Research Foundation and by the Swiss National Science Foundation. I am supported by a Long-Term Fellowship of the Human Frontier Science Program.

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Supplementary Material

The following supplementary material is available for this article online:

Table S1. The degree of genetic robustness of sequenced genomes.

Table S2. Equilibrium frequencies of the 61 sense codons without ($z = 0$) or with ($z = s$) juvenile selection for different values of s ; $\mu = 10^{-8}$.

This material is available as part of the online article from <http://www.blackwell-synergy.com>

Received 20 July 2005; revised 1 September 2005; accepted 1 September 2005