

Population genetics and substitution models of adaptive evolution

Mario dos Reis

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mario.barros@ucl.ac.uk

The MRC National Institute for Medical Research, Mill Hill, London, UK, NW7 1AA

Current address: Department of Genetics, Evolution and Environment, University College London, London, UK,
WC1E 6BT

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Abstract

The ratio of non-synonymous to synonymous substitutions $\omega (= d_N/d_S)$ has been widely used as a measure of adaptive evolution in protein coding genes. Omega can be defined in terms of population genetics parameters as the fixation ratio of selected vs. neutral mutants. Here it is argued that approaches based on the infinite sites model are not appropriate to define ω for single codon locations. Simple models of amino acid substitution with reversible mutation and selection are analysed, and used to define ω under several evolutionary scenarios. In most practical cases $\omega < 1$ when selection is constant throughout time. However, it is shown that when the pattern of selection on amino acids changes, for example after an environment shift, a temporary burst of adaptive evolution ($\omega \gg 1$) can be observed. The fixation probability of a novel mutant under frequency dependent selection is calculated, and it is used to show why $\omega > 1$ can be sometimes expected for single locations at equilibrium. An example with influenza data is discussed.

Introduction

Imagine a population with N individuals. In one particular generation an individual with a novel, selectively neutral mutation is born. Initially there is only one copy of the novel mutant allele in the population. If the organism is diploid, the initial frequency of the allele is $1/2N$. Stochastic fluctuations (random drift) will govern the fate of the mutant in the population. A classical result from population genetics theory is that the probability (after infinitely many generations) that *all* individuals will eventually carry the mutation is exactly $1/2N$. In this case we say the mutant allele has become fixed. If μ neutral mutations appear per genome per generation, then in a single generation, $2N\mu$ mutant alleles would be produced, each with a $1/2N$ chance of ultimately spreading throughout the population. Thus $2N\mu^{1/2N} = \mu$ is the rate at which neutral mutant alleles become fixed in the population each generation. This is a classical result from the neutral theory of molecular evolution, which maintains that most changes at the molecular level in populations are due to the random fixation of selectively neutral mutants (Kimura 1968, 1983).

Natural selection has an important effect on the probability of ultimate fixation of a novel mutant (Kimura 1962). If the mutant is advantageous, its probability of fixation will be larger than that of neutral mutants. Thus, regions of a genome generating advantageous mutants will be substituted at a faster rate than equivalent regions that evolve neutrally. On the other hand, if novel mutants are deleterious, their probability of fixation will be lower than that of neutral mutants, and regions of a genome that produce deleterious mutants will be substituted at a slower rate than equivalent regions that evolve neutrally. This provides the theoretical basis to detect adaptive evolution in protein coding genes. If we assume that synonymous sites evolve neutrally and that non-synonymous sites are under the influence of natural selection, then the ratio of non-synonymous to synonymous substitutions $\omega (= d_N/d_S)$ will reflect the dominant type of selection acting on the protein. Under this interpretation, $\omega > 1$, $\omega = 1$ and $\omega < 1$ indicate proteins under positive (diversifying) selection, neutral evolution and negative (purifying) selection respectively.

Very sophisticated methods have been developed to estimate ω from sequence alignments, methods that take into account the fact that synonymous and non-synonymous sites are present in unequal numbers and that the mutation rates for both types of sites are different (synonymous mutations are usually transitions, which occur at a faster rate than transversions, the dominant type of non-synonymous mutation). The literature describing statistical techniques to estimate ω is extensive (e.g. Miyata and Yasunaga 1980; Nei and Gojobori 1986; Goldman and Yang 1994; Ina 1995; for reviews see Yang and Bielawski 2000; and ch 8 in Yang 2006) and a large number of works have been published detecting positive selection in, for example, viral coating proteins (Fitch et al. 1997; Yang 2000; Yang et al. 2003), in the human histocompatibility complex (Hughes and Nei 1988; Yang and Swanson 2002), primate lysozymes (Messier and Stewart 1997; Yang 1998), etc (Endo et al. 1996).

For most protein coding genes, only a few locations are expected to be under positive selection, with the rest of the locations expected to be under strong purifying constraints. Hence, statistical methods that aim to detect positive

selection and that consider a single ω value for a protein are expected to be conservative. Considerable work has thus been aimed at models that consider variable ω ratios among sites (Nielsen and Yang 1998; Yang et al. 2000; Huelsenbeck et al. 2006), and tests have been developed to detect single codon locations where $\omega > 1$. Here there is a subtle but important point about the biological meaning of a codon site where $\omega > 1$. The theoretical justification discussed above actually assumes that genomes are composed of an infinite number of sites, and mutations do not occur more than once at any given location. The substitution rate of a genomic region under positive selection can only be larger than the substitution rate of a neutral region if advantageous mutants are persistently generated. The problem is that, when the number of sites is finite, the substitution rate per site would eventually decay to the level seen at sites under purifying selection. This is because once an advantageous mutant has become fixed, any new mutations at the location would then be deleterious. Under a finite sites model with reversible mutation further assumptions about the nature of selection are needed to understand $\omega > 1$.

The methods developed so far do not seem to have attempted to construct ω in terms of population genetic parameters. In my opinion, there is some weakness between the theoretical interpretation of ω (such as in the discussions by Bustamante 2005, or Nielsen and Yang 2003) and the development of codon substitution models to detect positive selection. In this work I explore the population genetics interpretation of ω under simple models of amino acid substitution with reversible mutation and selection. Several evolutionary scenarios, such as time non-homogeneous selection and frequency dependent selection are discussed. Some simple examples with viral data are presented, and the contribution of different types of selection to the evolution of flu viruses is discussed.

Theory

In the following discussion we assume a Fisher-Wright model of random genetic drift (e.g. Wright 1931). We work with idealised populations where the effective and the real population numbers are the same. Locations in a gene (or genome) are assumed to evolve independently, and they do not interfere with each other. We assume genic selection and that the selection coefficients involved in the models are small, so that some simplifying approximations about fixation probabilities can be made (Kimura 1962). It is also assumed that mutation rates are sufficiently small so that polymorphism is negligible and locations remain fixed most of the time. This is particularly important for the reversible mutation models described below (Bulmer 1991). The evolutionary process is viewed over long periods so the time from appearance to fixation of a novel mutant is nearly instantaneous. Issues such as selection on codon usage or non-homogeneous mutation patterns are ignored. These assumptions are necessary to simplify the mathematical treatment of the models discussed below.

Infinite sites model

We can use Kimura's infinite sites model (Kimura 1969; p. 46 in Kimura 1983) to find an analytical expression for ω . Let's consider a large genome where the mutation rate per haploid genome per generation is μ . Because the genome is very large, we assume that all new mutants always appear at new locations, effectively assuming that there is no reversible mutation. Of all the new mutants produced every generation a fraction $f_0\mu$ are neutral, and a fraction $f_s\mu$ are selected with selection coefficient s ($f_0 + f_s = 1$). There are N haploid genomes in the population and the probability of ultimate fixation of a newly arisen mutant is given by $u(S) = S/N(1 - e^{-S})$, where $S = 2Ns$ is the confounded selection coefficient (Fisher 1930; Wright 1931; Kimura 1962). We note that the fixation probability of a neutral mutant is $u(0) \equiv \lim_{S \rightarrow 0} u(S) = 1/N$. Thus each generation, $k_s = Nf_s\mu u(S)$ selected, and $k_0 = Nf_0\mu/N$ neutral mutants are produced that will become ultimately fixed. Thus the expected substitution (fixation) rate per generation is given by

$$k = k_s + k_0 = Nf_0\mu u(0) + Nf_s\mu u(S).$$

We are interested in defining ω in terms of the relative fixation rates of selected vs. neutral mutants, and this is

$$\omega = \frac{k_s}{k_0} \times \frac{f_0}{f_s} = \frac{\mu f_s u(S)}{\mu f_0 u(0)} \times \frac{f_0}{f_s} = \frac{S}{1 - e^{-S}}, \quad (1)$$

where the f_0/f_s term is included to normalise ω (Nielsen and Yang 2003; Bustamante 2005). The right hand side of equation 1 is simply the relative fixation probability of selected vs. neutral mutants: $h(S) \equiv u(S)/u(0) = S/(1 - e^{-S})$. Thus $\omega > 1$, $= 1$, and < 1 represent positive ($S > 0$), neutral ($S = 0$) and negative ($S < 0$) selection. Note that for large S we have that $h(S) \approx S$.

Equation 1 defines ω in terms of the fraction of neutral and selected *mutants* that appear in a genome each generation. We can also define ω in terms of the numbers of neutral and selected *sites* present. If there are fractions f_s^* of selected and f_0^* neutral sites, each producing mutants at rates μ_s and μ_0 respectively, then the total number of mutant alleles produced per generation is $\mu \equiv f_s^*\mu_s + f_0^*\mu_0$. The fraction of selected mutants is then $f_s = f_s^*\mu_s/(f_s^*\mu_s + f_0^*\mu_0)$ and the fraction of neutral mutants is $f_0 = 1 - f_s^*$. It follows that $\mu f_s = \mu_s f_s^*$ and $\mu f_0 = \mu_0 f_0^*$. Thus, an equivalent definition of ω is

$$\omega = \frac{\mu_s f_s^* u(S)}{\mu_0 f_0^* u(0)} \times \frac{\mu_0 f_0^*}{\mu_s f_s^*} = h(S),$$

where the $\mu_0 f_0^*/\mu_s f_s^*$ normalising term takes into account the fact that there are different numbers of neutral and selected sites, each producing mutants at different rates.

We can imagine a protein coding gene as being composed of a fraction f_0^* of synonymous, neutrally evolving sites and a fraction f_s^* of non-synonymous sites constrained by natural selection. If the protein is composed of thousands of sites, and if we observe the evolution of this protein during a short period of time, so that backward mutations are

unlikely, then equation 1 summarises the evolutionary dynamics and the selective pressure acting on the protein.

As noted in the introduction, this interpretation of ω as a measure of positive selection is conservative, especially since in real proteins we might have fractions f_{s_-} and f_{s_+} of negatively and positively selected mutants arising each generation ($s_- < 0$, $s_+ > 0$; $f_{s_-} + f_{s_+} + f_0 = 1$). Defining ω in terms of f_{s_-} and f_{s_+} we have that

$$\omega = \frac{k_{s_-} + k_{s_+}}{k_0} \times \frac{f_0}{f_{s_-} + f_{s_+}} = \frac{f_{s_-} h(S_-) + f_{s_+} h(S_+)}{f_{s_-} + f_{s_+}}, \quad (2)$$

which is simply the weighted average of the relative fixation probabilities of both types of mutants. Thus, if there is a large fraction of negatively selected mutants, we might expect $\omega < 1$ even if $S_+ \gg 1$ (formally $\omega < 1$ if $f_{s_-}(1 - h(S_-)) > f_{s_+}(h(S_+) - 1)$). Equation 2 can be easily extended to any arbitrary number of classes of selected mutants.

Given the conservative nature of equation 2, it would be better if we could explicitly define ω for each codon location in a gene. The infinite sites model specifically ignores reversible mutation. Under this model a new mutant codon is either fixed or lost, and if it becomes fixed, it will remain in that state, rendering the site specific substitution rate effectively equal to zero. Equation 1 is a useful approximation for interpreting the relative evolutionary rates of different proteins, or even to compare the rates of the same protein along different lineages in a phylogenetic tree, however, it is not appropriate to understand ω for single locations. An explicit finite sites model with reversible mutation is needed. The reader can consult Bustamante (2005) for a discussion of ω under the infinite sites model.

A simple amino acid substitution model with selection and reversible mutation

We now focus our attention to defining ω for a single amino acid location in a protein. The tertiary structure of a protein imposes constraints on which amino acids can be accommodated at a particular location. We focus our interest in locations where only two amino acids, c_1 and c_2 , are observed. Any mutant different from c_1 and c_2 is lethal and hence can never become fixed in the population. The neutral mutation rate per genome per generation from c_1 to c_2 is α , and β is the rate in the opposite direction ($\alpha, \beta > 0$). The selection coefficient in favour of c_1 is $s (> 0)$ and against c_2 is $-s$. When the population is fixed for c_1 , the substitution rate (q_{12}) from c_1 to c_2 is $N\alpha u(-S) = \alpha h(-S)$. Similarly $\beta h(S)$ is the rate (q_{21}) from c_2 to c_1 when the population is initially fixed for c_2 . The substitution rates (q_{ij}) can be accommodated into the rate matrix

$$\mathbf{Q} = \begin{pmatrix} -\alpha h(-S) & \alpha h(-S) \\ \beta h(S) & -\beta h(S) \end{pmatrix}, \quad (3)$$

where the diagonal elements satisfy $q_{ii} = -\sum_{j \neq i} q_{ij}$. The probability that the location, currently fixed for c_i , will become fixed for c_j after a certain time t (in generations) has elapsed is given by the transition probability matrix $\mathbf{P}_t = \{p_{ij}(t)\} = e^{t\mathbf{Q}}$. Equation 3 describes the pattern of amino acid substitution at the location as a time continuous Markov process.

During its evolutionary history, the location will spend a fraction of time π_1 fixed at c_1 and a fraction π_2 at c_2 ($\pi_1 + \pi_2 = 1$). At equilibrium the balance equation

$$\pi_1 \alpha h(-S) = \pi_2 \beta h(S) \quad (4)$$

holds. Solving for π_1 and π_2 we have $\pi_1 = \beta h(S) / (\beta h(S) + \alpha h(-S))$ and $\pi_2 = \alpha h(-S) / (\beta h(S) + \alpha h(-S))$. Parameters π_1 and π_2 can also be interpreted as the expected equilibrium frequencies of amino acids c_1 and c_2 for a collection of locations under the same substitution (selection + mutation) pattern.

Setting $v = \beta h(S) + \alpha h(-S)$ is easy to show that \mathbf{Q} can be written as

$$\mathbf{Q} = \begin{pmatrix} -v\pi_2 & v\pi_2 \\ v\pi_1 & -v\pi_1 \end{pmatrix}, \quad (5)$$

which is the simplest 2×2 reversible amino acid substitution matrix. Parameter v would then represent the ‘exchangeability’ of c_1 and c_2 .

The expected substitution rate at equilibrium for the location is given by

$$\begin{aligned} k_s &= \pi_1 \alpha h(-S) + \pi_2 \beta h(S) \\ &= \frac{2\alpha\beta h(S)h(-S)}{\beta h(S) + \alpha h(-S)}. \end{aligned}$$

For a location where $S = 0$, $\pi_1^* = \beta / (\alpha + \beta)$ and $\pi_2^* = \alpha / (\alpha + \beta)$ are the equilibrium amino acid frequencies, which are solely determined by the mutation pattern. The expected rate at equilibrium for a neutral location is given by

$$k_0 = \pi_1^* \alpha + \pi_2^* \beta = \frac{2\alpha\beta}{\alpha + \beta}. \quad (6)$$

We can now define the site specific ω as

$$\begin{aligned} \omega &= \frac{k_s}{k_0} = \frac{\pi_1 \alpha h(-S) + \pi_2 \beta h(S)}{\pi_1^* \alpha + \pi_2^* \beta} \\ &= \frac{(1 + \frac{\alpha}{\beta})h(S)h(-S)}{h(S) + \frac{\alpha}{\beta}h(-S)}. \end{aligned} \quad (7)$$

This equation (7) describes a typical site under purifying selection. In most cases $\omega < 1$ unless the mutation rate away from the optimal amino acid is larger than the backward rate (i.e. $\alpha/\beta > 1$). In this case $\omega > 1$ might be observed (figure 1), and the location might spend a longer fraction of time at c_2 (i.e. $\pi_2 > \pi_1$) despite this being the suboptimal

amino acid. This later scenario seems unlikely to occur with real data since α and β are usually within the same order of magnitude (*e.g.* table 6.1 in Lynch 2006). Note that describing the site as evolving under either ‘positive’ or ‘negative’ selection might be inappropriate. This is because at equilibrium the number of positively selected mutant substitutions equals the number of negatively selected ones (equation 4).

A time non-homogeneous selection model

It is clear that if selection is constant throughout time no adaptive evolution can take place. When selection is constant, locations in a protein will reach a stationary state where the amino acid frequencies at each location will depend on a balance between random drift, mutation and selection. We are interested in modelling the case where the selection coefficient s in favour of amino acid c_1 is a function of time. The simplest case is when there is an environment shift, such as when an organism colonises a new habitat. In this scenario the previously suboptimal amino acid c_1 would become optimal, and the substitution pattern at the location would change.

Let’s write s_a for the selection coefficient acting on c_1 in environment a before the shift, and s_b for the coefficient after a shift towards environment b . The pattern of evolution for the location under each environment can be described by the appropriate rate matrices:

$$\mathbf{Q}_a = \begin{pmatrix} -\alpha h(-S_a) & \alpha h(-S_a) \\ \beta h(S_a) & -\beta h(S_a) \end{pmatrix} \text{ and } \mathbf{Q}_b = \begin{pmatrix} -\alpha h(-S_b) & \alpha h(-S_b) \\ \beta h(S_b) & -\beta h(S_b) \end{pmatrix} \quad (8)$$

where $S_a = 2Ns_a$.

Let’s analyse the simple case where $\gamma \equiv \alpha = \beta$, $s_a = -s$ and $s_b = s$ ($s > 0$). That is, c_1 is the suboptimal amino acid in a and becomes preferred after the shift towards b . Let’s now assume the location has been evolving in a for a very long time, so it can be considered stationary. The expected substitution rate immediately after the environment shift ($t = 0$) is $k_{s_b}(0) = \pi_{1_a} \gamma h(-S) + \pi_{2_a} \gamma h(S)$, where π_{i_a} is the expected equilibrium frequency of i in a . The expected substitution rate in b at equilibrium (*i.e.* $t \rightarrow \infty$) is $k_{s_b}(\infty) = \pi_{1_b} \gamma h(-S) + \pi_{2_b} \gamma h(S)$. In general, we can find the expected substitution rate at any time point t after the shift, and this is given by

$$\begin{aligned} k_{s_b}(t) &= \pi_{1_a} p_{11_b}(t) q_{12_b} + \pi_{1_a} p_{12_b}(t) q_{21_b} \\ &+ \pi_{2_a} p_{22_b}(t) q_{21_b} + \pi_{2_a} p_{21_b}(t) q_{12_b} \end{aligned}$$

where the $p_{ij_b}(t)$ are the transition probabilities, which can be easily found by solving $\mathbf{P}_{t_b} = e^{t\mathbf{Q}_b}$ (*e.g.* p. 39 in Yang 2006). For a neutral mutant, the expected substitution rate is $k_0 = \gamma$ (equation 6), which is independent of t and of whether the population is in a or b . So ω can now be defined as a function of t by $\omega(t) = k_{s_b}(t)/k_0$. With some algebraic

manipulation it is easy to show that

$$\omega(t) = \omega(\infty) + (\omega(0) - \omega(\infty))e^{-\gamma_s t} \quad (9)$$

where $\gamma_s = \gamma(h(S) + h(-S))$. For large S we note that $\omega(0) \approx S$.

Equation 9 indicates that immediately after an environment shift, the substitution rate is accelerated, and $\omega(0) > 1$. As time passes, the system moves towards equilibrium and $\omega(t)$ decays exponentially with rate γ_s towards $\omega(\infty) < 1$ (figure 2).

Despite its apparent simplicity, this model has practical applications. We can imagine a virus that infects and spreads in a certain host a . After a host shift, the intracellular environment in the new host b might be substantially different, and the viral proteins would be subjected to novel selective pressures. We shall see later an actual example with influenza viruses evolving in birds and humans, and the varying selective pressures involved.

Frequency dependent selection

The previous models show that for a single location, ω is usually less than one unless the mutation bias favours the suboptimal amino acid (equation 7) in which case there is no adaptive evolution involved, or ω is temporarily larger than one when there is a shift in the selection pattern acting on the location (equation 9). Several studies, for example those concerned with the evolution of viral coating proteins, have shown ω values for single locations that are consistently larger than one for large phylogenies (Yang 2000; Yang et al. 2003). Here we seek to describe a population genetics model that can justify $\omega > 1$ at equilibrium.

We start with a model where the fitness of a mutant allele is a function of the frequency of the allele in the population. We could imagine, for example, a virus with a novel mutation in its coating protein that would allow it to escape the host population immune system. As the virus would become more common, and host individuals become resistant, further spreading of the virus would be hampered. Thus, the virus would lose its selective advantage as its frequency in the population increases. We can construct a simple model where the fitness (w) of a novel mutant allele, in a haploid organism, decays exponentially with the frequency (q) of the allele: $w(q) = w_0 e^{-rq}$ where w_0 is the initial fitness and r is the rate of decay. The fitness can be defined in terms of the selection coefficient as $w = 1 + s$, so the previous equation can be written as

$$s(q) = (1 + s_0)e^{-rq} - 1, \quad (10)$$

where $s_0 (> 0)$ is the selective advantage of the yet non-existent mutant (*i.e.* when $q = 0$). We are interested in the special case when $s(1) = 0$ (*i.e.* the fitness of the fixed allele is indistinguishable from that of the previous wild type), and this is equivalent to setting $r = \log(1 + s_0)$. Under this condition, equation 10 becomes

$$s(q) = (1 + s_0)^{1-q} - 1. \quad (11)$$

Let's write q_t for the frequency of the allele in the current generation t . In a finite population, q_{t+1} is a binomial random variable with probability

$$p(q_{t+1} = i/N) = \binom{N}{i} \rho_t^i (1 - \rho_t)^{N-i}, \quad (12)$$

where N is the population number, $\rho_t = q_t w(q_t) / \bar{w}(q_t)$ and $\bar{w}(q_t) = 1 + q_t s(q_t)$ is the average fitness (p. 406 in Crow and Kimura 1970). Thus, equation 12 can be used to construct the stochastic matrix $\mathbf{P} = \{p_{ij}\}$ where p_{ij} is the probability that $q_t = i/N$ will become $q_{t+1} = j/N$ in one generation ($i, j \in \{0, 1, 2, \dots, N\}$). For sufficiently large t , repeated matrix multiplication ($\mathbf{P}_t = \{p_{ij}(t)\} = \mathbf{\Pi}^t \mathbf{P}$) can be used to obtain a numerical approximation to the ultimate fixation probability, $g(S_0) \equiv p_{1,N}(t \rightarrow \infty)$, of a novel mutant with initial frequency $q_1 = 1/N$ and initial selective advantage $s(1/N)$. It is assumed that N is constant throughout generations, which might not be a realistic assumption for the case of a virus.

Figure 3 shows the relative fixation probabilities $g(S_0)/u(0)$ and $h(S_0) \equiv u(S_0)/u(0)$ as functions of S_0 when both are computed numerically (assuming $N = 100$). As should be expected, for $S_0 > 0$ we have that $h(S_0) > g(S_0)/u(0)$. For small positive s_0 , we find numerically that $g(S_0)/u(0) \approx 0.261 + 0.697h(S_0)$ (figure 3, inset). We can define numbers $\zeta \equiv \zeta(s_0)$ and $Z = 2N\zeta$ so that

$$h(Z) = Z/(1 - e^{-Z}) \approx g(S_0)/u(0).$$

This is, an allele with constant selection coefficient ζ would have the same ultimate fixation probability as another with frequency dependent selection with parameter s_0 . Using the linear relationship between $h(S_0)$ and $g(S_0)/u(0)$ we get that, roughly, $Z \approx 0.261 + 0.697S_0$. I leave the exact computations of $g(S_0)$ and Z to skillful mathematicians. The message here is that, when the selective advantage decays as a function of the allele frequency, the probability of fixation of the allele is a function of some value Z such that $0 < Z < S_0$. This is all we need to construct a substitution model under frequency dependent selection.

Let's consider again two amino acids c_1 and c_2 at a particular protein location, with mutation rates α and β . Initially, the population is fixed for c_2 . The selective advantage of a novel c_1 mutant is $s(q)$ where q is the frequency of c_1 and s is given by equation 11. When c_1 becomes fixed, its selective advantage is 0 and remains at 0 until it becomes lost. Returning to our virus example, c_1 is advantageous when rare, but once it becomes fixed, the host population has become immunologically resistant, and will remain so even if the frequency of c_1 decreases when a new mutant c_2 appears. When c_2 re-appears, the host population has become again sensitive to c_2 (*i.e.* the host population loses its 'immune memory' to a virus once this disappears), and c_2 has now selective advantage $s(1 - q)$. The substitution rates at the location are thus given by

$$\mathbf{Q} = \begin{pmatrix} -\alpha h(Z) & \alpha h(Z) \\ \beta h(Z) & -\beta h(Z) \end{pmatrix}. \quad (13)$$

The stationary frequencies are $\pi_1 = \beta h(Z)/(\alpha h(Z) + \beta h(Z)) = \beta/(\alpha + \beta)$ and $\pi_2 = \alpha/(\alpha + \beta)$, so they are independent of the selective pressure acting on the location. The expected substitution rate at equilibrium is $k_s = \pi_1 \alpha h(Z) + \pi_2 \beta h(Z)$. For a neutral mutant $k_0 = \pi_1^* \alpha + \pi_2^* \beta$, but in this model $\pi_i = \pi_i^*$ so ω is simply

$$\omega = \frac{k_s}{k_0} = h(Z), \quad (14)$$

which is similar to equation 1.

Setting $v = \alpha + \beta$ it can be shown that

$$\mathbf{Q} = \begin{pmatrix} -v\omega\pi_2 & v\omega\pi_2 \\ v\omega\pi_1 & -v\omega\pi_1 \end{pmatrix},$$

which is equivalent to the simplest 2×2 codon substitution model when there are no synonymous codons. So in this model we have that, at equilibrium, $\omega > 1$ and ω is independent of the mutation pattern.

Numerical examples with influenza virus data

Time non-homogeneous selection

We analyse a set of 401 influenza A polymerase subunit (PB2) sequences from avian and human isolates. The PB2 alignment and the tree topology are described by Tamuri et al. (2009). The PB2 human isolates are monophyletic, and they are thought to be the product of a shift from an avian (the natural reservoir) to a mammalian host dating back to around 1882–1913, before the Spanish flu pandemic of 1918 (dos Reis et al. 2009). The PB2 gene codes for a subunit of the polymerase complex, which is composed of three different subunits (PB2, PB1 and PA). The polymerase proteins seem to be involved in host adaptation, and there is evidence of several amino acid substitutions after the host shift (Taubenberger et al. 2005).

In this example we focus on amino acid location 627 of the PB2 protein. Glutamate (E) and lysine (K) are observed in avian isolates, with glutamate being the predominant amino acid. Lysine is exclusively observed in the monophyletic human lineage analysed. There is experimental evidence that lysine at location 627 is related to adaptation to the mammalian host (Subbarao et al. 1993). Glutamate is encoded by GAA and GAG codons while lysine is encoded by AAA and AAG codons. Hence $G \rightleftharpoons A$ nucleotide transitions are responsible for $E \rightleftharpoons K$ mutations. We apply Yang and Nielsen's (2008) FMutSel model of codon substitution to the data above to estimate the background frequencies

of G and A nucleotides in the PB2 gene. These are $\hat{\pi}_A^* = 0.400$ and $\hat{\pi}_G^* = 0.196$ under a HKY85 model of nucleotide substitution (Hasegawa et al. 1985). The estimated transition transversion rate parameter is $\hat{\kappa} = 8.00$. Thus we estimate the (non-scaled) neutral mutation rates E→K as $\hat{\alpha} = \hat{\kappa}\hat{\pi}_A^*$ and K→E as $\hat{\beta} = \hat{\kappa}\hat{\pi}_G^*$.

We can now construct three substitution models. In the first model there is a single substitution matrix describing the evolution of location 627 throughout the tree. The matrix is given by equation 3 and there is no selection ($S = 0$). This is the neutral model (M0). In the next model the substitution matrix is also given by equation 3 but $S(\neq 0)$ is now the selective advantage of glutamate. This is the simple selection model (M1). The final model considers two substitution matrices, each describing the evolution of the location in either the avian or the human clades. The background mutational parameters are the same for both hosts but the selective pressures in each host are different. We write s_{av} and s_{hu} for the selective advantage of glutamate in the avian and human hosts respectively. The substitution matrices have the form shown in equation 8. The branch linking the human and avian clades is the host shift branch, where the shift in substitution pattern occurred. This is the non-homogeneous selection model (M2).

Under each model we can calculate the likelihood of site 627 for the given tree topology using the pruning algorithm (Felsenstein 1981; Yang 2006). We use maximum likelihood to estimate the confounded selection coefficients (S, S_{av}, S_{hu}) for models M1 and M2 for the fixed tree topology. The branch lengths and mutational parameters (α and β) are considered fixed, and they are scaled in terms of neutral mutant substitutions as suggested by Halpern and Bruno (1998). Since models M0, M1 and M2 are nested, we use the likelihood ratio test to select the best model. The estimated parameters for the three models are shown in table 1. Model M2 is by far the best model, so we conclude that there are different selective pressures acting at the location in each host. Note that because S is a confounded parameter of the population number (N) and the selection coefficient (s), the differences observed between S_{av} and S_{hu} could be due to differences in N as well as in s . However, because there is a change in the *sign* of S_{hu} , we conclude that glutamate is indeed ill-favoured in the human host.

A brief comparison of the $\pi_a \neq \pi_b$ vs. $\omega > 1$ criteria to detect adaptive evolution

Tamuri et al. (2009) performed an analysis of selective constraints in human and avian isolates of influenza. They used a sitewise non-homogeneous model to test whether amino acid composition in each location of the influenza proteome would differ between avian and human isolates. Their model is equivalent to equation 8, but the model was parametrised in terms of equilibrium frequencies and exchangeabilities, as in equation 5, and the number of amino acids observed per location was usually larger than two. To account for multiple testing, they used a false discovery rate (FDR) approach to correct for false positives. For the PB2 gene they identified 13 locations (FDR= 5%, 22 locations for FDR= 20%) where $\pi_{hu} \neq \pi_{av}$ ($\pi_a = \{\pi_{i_a}\}$). This approach is equivalent to testing if $s_{hu} \neq s_{av}$.

The objective now is to compare Tamuri's et al. (2009) $\pi_a \neq \pi_b$ criteria to detect whether a site has undergone

adaptive changes, with the classical criteria that $\omega > 1$ for the PB2 data. First, a classical codon model where ω is constant throughout sites and along the tree was fitted (Yang 1998). A second model, where ω is allowed to vary between the two host lineages was also assessed. Allowing ω to vary between viruses evolving in human and avian hosts significantly improved the model fit ($2\Delta\ln\ell = 127$, $p \ll 0.001$). There is a conspicuous increase in amino acid substitution rates in human virus isolates compared to avian isolates ($\hat{\omega}_{av} = 0.0493$ and $\hat{\omega}_{hu} = 0.1036$). In the traditional sense, no adaptive evolution has been detected because $\hat{\omega}_{hu} < 1$. Intuitively, the increase in substitution rates in human viruses would agree with the notion that these viruses are undergoing adaptive evolution in the novel host.

Next, we seek to identify locations in the PB2 protein where $\omega > 1$. We use Yang's et al. (2000) M7 and M8 models to test for ω variation among codons. In the M7 model, ω varies following a beta distribution, thus ω is bounded between 0 and 1. In the M8 model, ω also varies according to a beta distribution but there is an extra category of sites with $\omega > 1$. Models M7 and M8 are nested, and M8 has two additional parameters. The likelihood ratio test can be used to decide whether there are sites under adaptive evolution, and an empirical Bayes approach can then be used to identify those sites where $\omega > 1$. Fitting M7 and M8 to the human PB2 subtree give essentially the same likelihood, so the inclusion of a class of sites with $\omega > 1$ is not justified. Under M7, $\hat{\omega}$ varies between 0.0270 and 0.4410 among locations with a mean value of 0.1076. When M7 and M8 are fitted to all eight gene segments from human viruses (not shown), the inclusion of positively selected sites ($\omega > 1$) is only justified for the two surface proteins (HA and NA) and for the nucleoprotein (NP).

The M8 model averages ω over a phylogeny, so it might be conservative when the adaptive event has occurred at a particular branch. It is possible that most of the adaptive changes for the PB2 protein occurred along the host shift branch. We apply the branch-sites test of Zhang et al. (2005) to the PB2 protein, with the host shift branch linking human and avian viruses as the 'hot' or 'foreground' branch, and the rest of the tree comprising the 'background' branches. A model allowing a class of sites with $\omega > 1$ in the foreground branch is compared with a model where ω is capped at one (Zhang et al. 2005). The inclusion of a class of sites with $\omega > 1$ is not justified for this branch, as it has the same likelihood as the alternative neutral model.

Discussion

The meaning of ω

Sawyer and Hartl (1992) provided a theoretical derivation of the expected fixation rate of synonymous and non-synonymous substitutions (table 1 in their paper). Although they did not explicitly define ω , this is perhaps the first work where a formal theoretical interpretation for this parameter can be found. Nielsen and Yang (2003) provided an explicit link between a population genetics interpretation of ω and Markov models of codon substitution. Using various

models of ω variation among sites (Yang et al. 2000) they used equation 1 to map the distribution of selection coefficients from the distribution of ω ratios in real sequence data. Nielsen and Yang (2003) assumed a finite sites model, but the only way to reconcile equation 1 with this assumption is if the fitnesses at a location are reassigned every time a novel mutant appears, so that a novel mutant has the same selective advantage (or disadvantage) of the previously fixed allele at the location. In the case of positively selected mutants, this assumption is similar to the frequency dependent selection model described here (equations 13 and 14). For negatively selected mutants, this assumption seems unreasonable. Defining ω under a purifying selection model (equation 7) would seem more appropriate. Kryazhimskiy and Plotkin (2008) refer to the reassignment of fitnesses as the ‘continual selection’ model. In my opinion, Nielsen and Yang (2003) ‘reassignment of fitnesses’ seem to be an *ad hoc* justification to use equation 1 to estimate the distribution of selection coefficients, rather than a prior assumption of their model.

As pointed out by Kimura (1983) “One should expect in this case [positive selection] that the rate of evolution would depend strongly on the environment, being high for a species offered a new ecologic opportunity but low for those kept in a stable environment.” In the influenza example described here, the novel ecologic opportunity is given by the introduction of the virus into a new host. In the novel environment, several locations are suddenly found to be fixed for suboptimal amino acids and novel mutants have a good chance to be positively selected. As advantageous mutants appear, spread and become fixed, the evolutionary rate decays. Eventually, an equilibrium is reached where most locations become fixed for the optimal state. Sometimes there might be cyclical changes in the environment, and locations might undergo recurrent changes in selective pressure. In the influenza example, the cyclical environment is given by the increasingly hostile immune system within the host. In this case, a model such as frequency dependent selection gives a more reasonable interpretation of the evolutionary rate.

The environment shift model predicts a burst of adaptive evolution where ω is initially high (> 1) and then decays with time until it reaches a stationary value (< 1). For the simple 2×2 amino acid case, the accelerated substitution rate would be due to an excess of substitutions occurring at several different locations. The implications for this are important: although we are likely to detect an increase in ω for a clade of interest (such as in the influenza PB2 example), we might be unable to detect the individual locations responsible since mostly, all that is observed are single, one-off substitutions. To detect sites where $\omega > 1$, recurrent substitutions along a branch are necessary. Tamuri et al. (2009) identified a total of 172 locations throughout the influenza proteome that show evidence of adaptive evolution in the human and avian hosts. It is clear that one-off adaptive substitutions are a widespread phenomenon in influenza. We can imagine the genome of this virus as composed of three classes of sites. The first class represents those locations under strong structural constraints at the protein level, locations where the optimal amino acid is independent of the host environment. These locations are described by the constant selection model of equation 3 and represent the largest fraction of the genome. The second class of locations are those involved in host adaptation, where the substitution pattern depends on the intracellular environment provided by the particular host. These locations evolve

according to the non-homogeneous selection model of equation 8. Finally, the third class represents those sites under recurrent changes in selective pressure and they evolve according to the frequency selection model of equation 13. I expect the first two models to account for most of the evolutionary pattern observed in proteins in most organisms, the second model accounting for episodic periods of evolution, such as the colonisation of new habitats or large scale environmental changes. Recurrent selection models are a special case that would apply to a fraction of locations in particular proteins.

Gradual change in selective advantage

Sometimes it might be reasonable to think that the fitness of an allele would change gradually, perhaps in a correlated manner with some environmental variable. Kimura and Ohta (1970) studied the fixation probability of a mutant allele when the selective advantage decreases as a function of time as $s(t) \propto e^{-\nu t}$. They showed that the probability of fixation can be approximated as $Y/N(1 - e^{-Y})$ where $Y = 2Ny$ is a parameter with a very similar meaning to that of Z in the frequency dependent selection case. The model of Kimura and Ohta (1970) could be used to generalise equation 8 for the case of gradual environmental changes. However, if the change in selective pressure is fast compared to expected time between fixations ($1/\mu$) then model 8 should provide an adequate approximation.

Pseudo genes and duplicated genes

The model described in equation 8 can also be used to analyse the substitution rate of a recently generated pseudo gene. Imagine a gene that becomes duplicated, where the novel copy lacks a promoter. This situation is equivalent to a sudden environment shift where $s_a > 0$ and $s_b = 0$ (equation 8), then \mathbf{Q}_a represents the substitution pattern of a location in the functional copy, and \mathbf{Q}_b the pattern of the pseudo gene. With an argument similar to that used to derive equation 9, it can be shown that the expected value of ω for the location after the duplication event ($t = 0$) is

$$\omega(t) = 1 + (\omega(0) - 1)e^{-\nu t},$$

where $\nu = \alpha + \beta$ and $\omega(0) = (h(S_a) + h(-S_a))(\alpha + \beta)/2\nu_s$ ($\nu_s = \alpha h(-S_a) + \beta h(S_a)$). When $\alpha \neq \beta$ and $S > 0$, we have that $\omega(0) > 1$; when $S < 0$ then $\omega(0) < 1$; and when $\alpha = \beta$ then $\omega(0) = 1$ whatever the value of S (figure 4). As the time after the duplication approaches infinity, then $\omega(t \rightarrow \infty) \rightarrow 1$. Depending on the degree of mutational bias and the strength of selection at each location, average $\omega(0)$ for a whole pseudo gene might exceed one (figure 4).

If the novel duplicated gene does have a promoter, the organism would find itself with two functional copies of the same gene. One of the copies could become dysfunctional if it was to suffer a disruptive mutation (for example, an enzyme that suffers a critical mutation at its active site). Since the organism still has a functional copy, its fitness might be relatively unchanged (for example if the gene is haplosufficient or double recessive; Kondrashov and Koonin 2004;

Innan 2009). Then the mutated copy would effectively become a pseudo gene. During this period the dysfunctional copy would evolve rapidly with $\omega \approx 1$, with some locations reaching $\omega > 1$. If due to the serendipitous nature of evolution this copy was to acquire a novel function, given its carrier a definite selective advantage, then the substitution pattern would revert to the case of purifying selection and eventually $\omega < 1$. This observation could explain why $\omega > 1$ has been observed for internal branches of phylogenetic trees purporting gene duplications. Rather than invoking adaptive evolution, I suspect that a period of neutral evolution would explain the accelerated rate. This could be the case, for example, for the primate lysozymes (Messier and Stewart 1997; Yang 1998).

Models with more than two amino acids

The 2×2 models described here can be easily extended to the full set of 20 amino acids or 61 codons. For a codon location under purifying selection, the 61×61 substitution matrix could be defined as

$$q_{ij} = \begin{cases} 0 & \text{if } i \text{ and } j \text{ differ at more than one position} \\ \mu_{ij} & \text{if } i \text{ and } j \text{ are synonymous} \\ \mu_{ij}h(S_{ij}) & \text{if } i \text{ and } j \text{ are nonsynonymous} \end{cases},$$

where μ_{ij} is the neutral mutation rate from codon i to j , $S_{ij} = 2Nf_j - 2Nf_i$ is the confounded selection coefficient and f_j is the fitness of the amino acid encoded by j (Yang and Nielsen 2008). This is very similar to Halpern and Bruno's (1998) location specific model to estimate evolutionary distances among coding sequences. The non-synonymous substitution rate at equilibrium is $k_N = \sum_i \sum_j \pi_i \mu_{ij} h(S_{ij})$ where $i \neq j$ and $aa_i \neq aa_j$. The expected synonymous rate is $k_0 = \sum_i \sum_j \pi_i \mu_{ij}$ where $i \neq j$ and $aa_i = aa_j$. Similarly, when there is no selection both expected rates are given by $k_N^* = \sum_i \sum_j \pi_i^* \mu_{ij} h(0)$ and $k_0^* = \sum_i \sum_j \pi_i^* \mu_{ij}$. Then ω can be defined as

$$\omega = \frac{k_N}{k_0} \times \frac{k_0^*}{k_N^*}. \quad (15)$$

Terms k_N , k_0 , k_N^* and k_0^* are equivalent to ρ_N , ρ_S , ρ_N^1 and ρ_S^1 in Yang's (2006) notation. Note the similarity of 15 with equation 1. In real proteins, structural and functional constraints restrict the number of amino acids that can be accommodated into a location, and thus for several amino acids we would have that $h(S_{ij} \rightarrow -\infty) \rightarrow 0$. A consequence of this is that the numerator in 15 is reduced and ω would tend to be smaller than one. If there is some form of recurrent selection acting at the location then some of the q_{ij} terms would be of the form $\mu_{ij}h(Z_j)$. Because $Z_j > 0$ the inclusion of these terms increases the numerator in 15 and the value of ω would increase, possibly over one.

If the time t (in generations) is known, the number of non-synonymous and synonymous substitutions that have occurred along a lineage are tk_N and tk_0 respectively. The *scaled* non-synonymous and synonymous distances are

$d_N \equiv tk_N/k_N^*$ and $d_S \equiv tk_0/k_0^*$. It is clear that $\omega = d_N/d_S$, however, because d_N and d_S are corrected for the relative contributions of non-synonymous and synonymous mutant substitutions, they should be interpreted with caution when considered as evolutionary distances.

Future challenges and conclusions

The models presented here are an approximation to understand ω at the population genetics level when the substitution process is considered over long periods of time. Equation 15 can be used to construct more realistic evolutionary models that would include selection as a function of time, as a function of allele frequencies, or under other models not explored here such as over dominant selection. Huelsenbeck et al. (2006) point out that “there is [currently] little population genetics theory to inform us of the appropriate probability distribution for among-site variation in the non-synonymous rate of substitution”. Working out this theory is an important challenge for both population geneticists and phylogeneticists. If we can understand the contributions of different modes of selection among locations, and if we can find an appropriate distribution for the fitnesses of codons within locations, then a reasonable distribution of ω values among locations could be worked out. The sitewise approach exemplified here is computationally expensive and highly parametrised, and currently is only practical for large phylogenies (such as influenza) where the pattern of amino acid substitution and the location specific equilibrium frequencies can be reasonably estimated. Models that use average, protein wide codon frequencies and that use empirical distributions for ω are currently the best approach.

Note

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Table 1: Adaptive evolution at location 627 of the PB2 protein of influenza A.

Model	π	S (95% CI)*	np	ℓ	p -value
M0	$\hat{\pi}_E = 0.328$	$S = 0^{**}$	0	-34.7	
M1	$\hat{\pi}_E = 0.668$	$\hat{S} = +1.4 (+0.16, +2.9)$	1	-32.2	2.5×10^{-2}
M2	$\hat{\pi}_{E_{av}} = 0.783$ $\hat{\pi}_{E_{hu}} = 0.000$	$\hat{S}_{av} = +2.0 (+0.30, +4.1)$ $\hat{S}_{hu} = -940 (-\infty, -5.5)$	2	-27.4	1.9×10^{-3}

* Confidence interval calculated using the LRT statistic (p. 25-26 in Yang 2006). ** fixed. np: number of parameters estimated by maximum likelihood.

NOTE: It is assumed that only two amino acids are allowed at position 627. In reality, other amino acids could be present at very low frequencies, and are not observed. A consequence of this is that the true number of parameters in the models are not known, and the likelihood ratio tests are thus approximate.

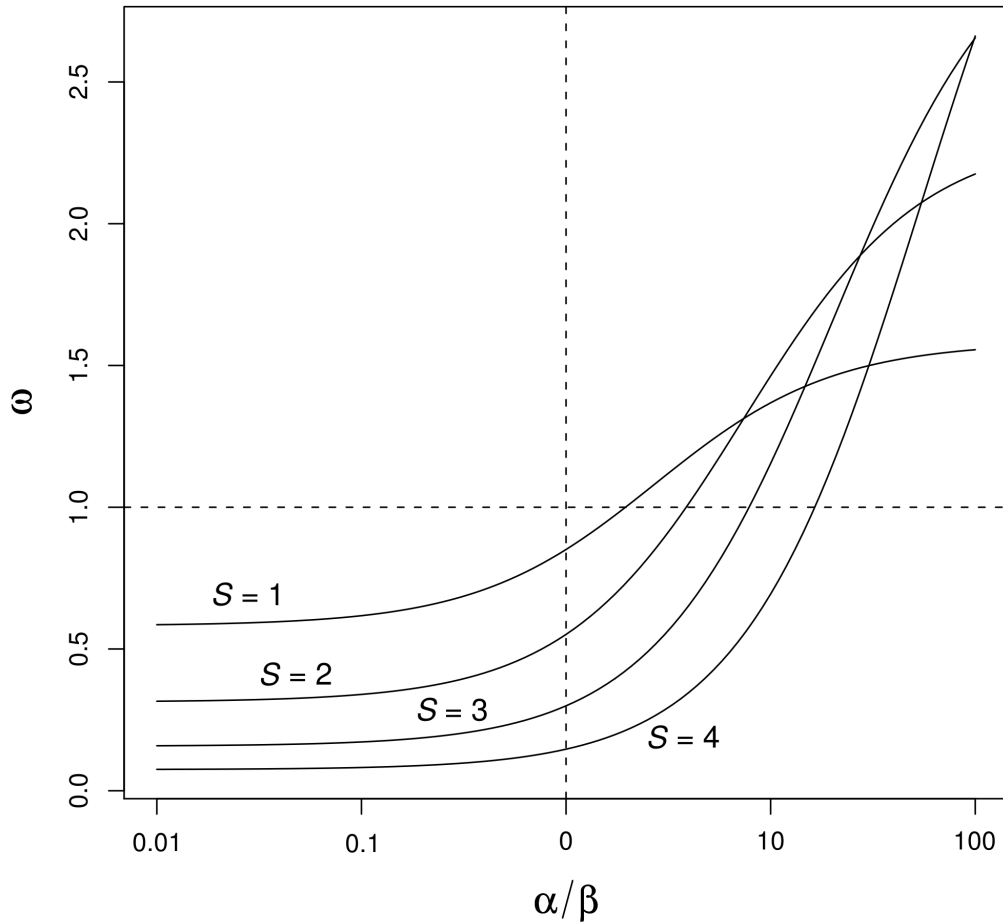


Figure 1: The expected value of ω in a simple amino acid substitution model with selection and reversible mutation.

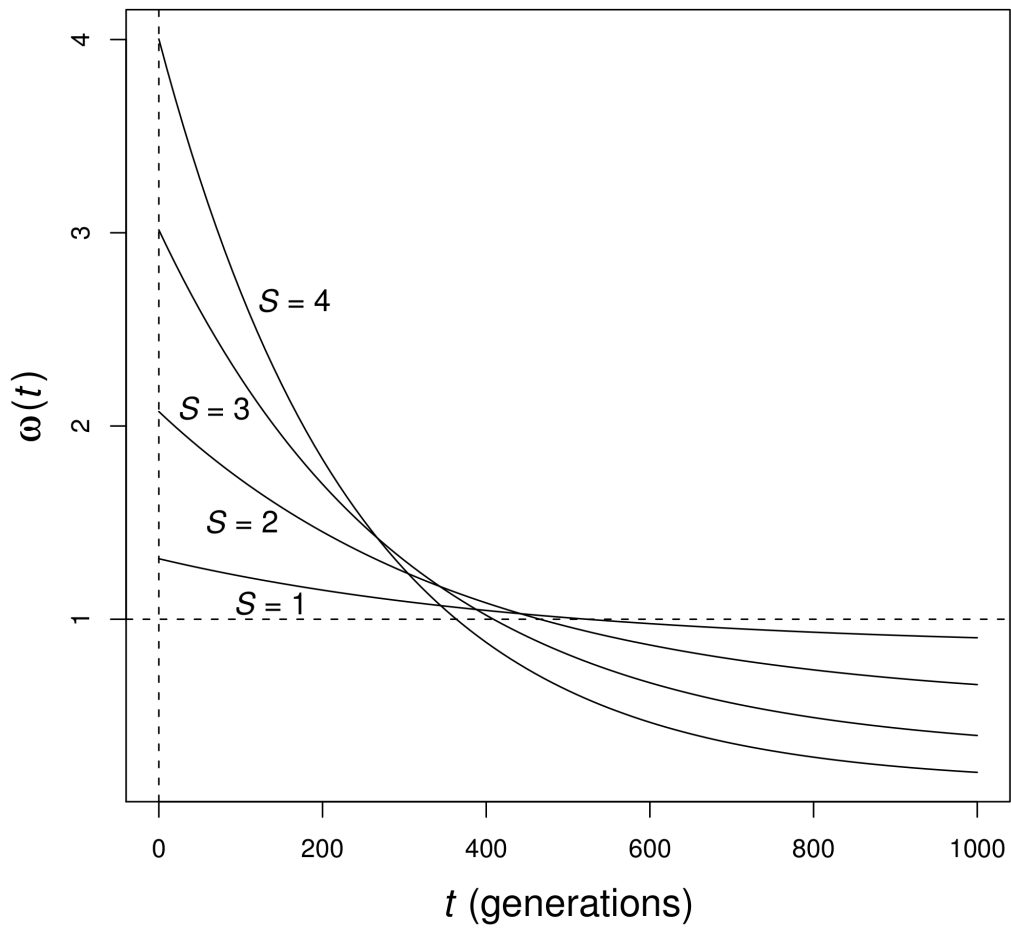


Figure 2: The expected value of ω after an environment shift.
 The neutral mutation rate is $\gamma = 1 \times 10^{-3}$ per generation.

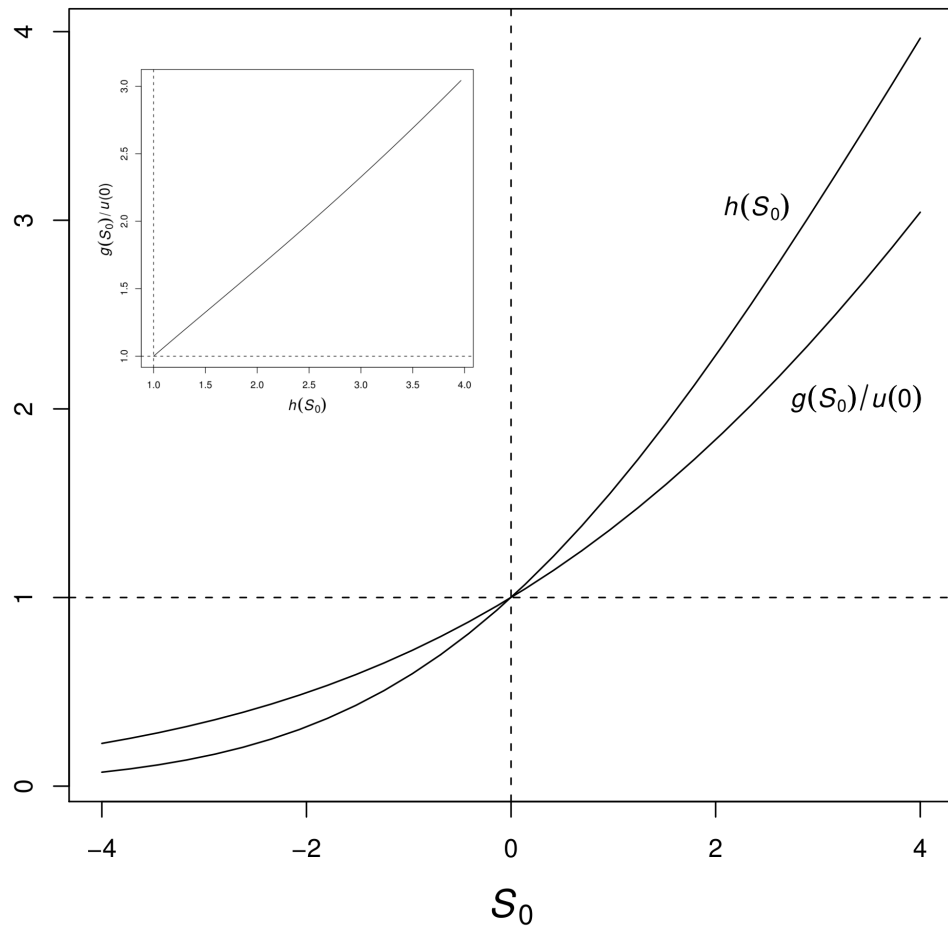


Figure 3: The relative fixation probabilities of a novel mutant under frequency dependent and constant selection. Inset: $g(S_0)/u(0)$ is approximately a linear function of $h(S_0)$ for small positive s_0 .

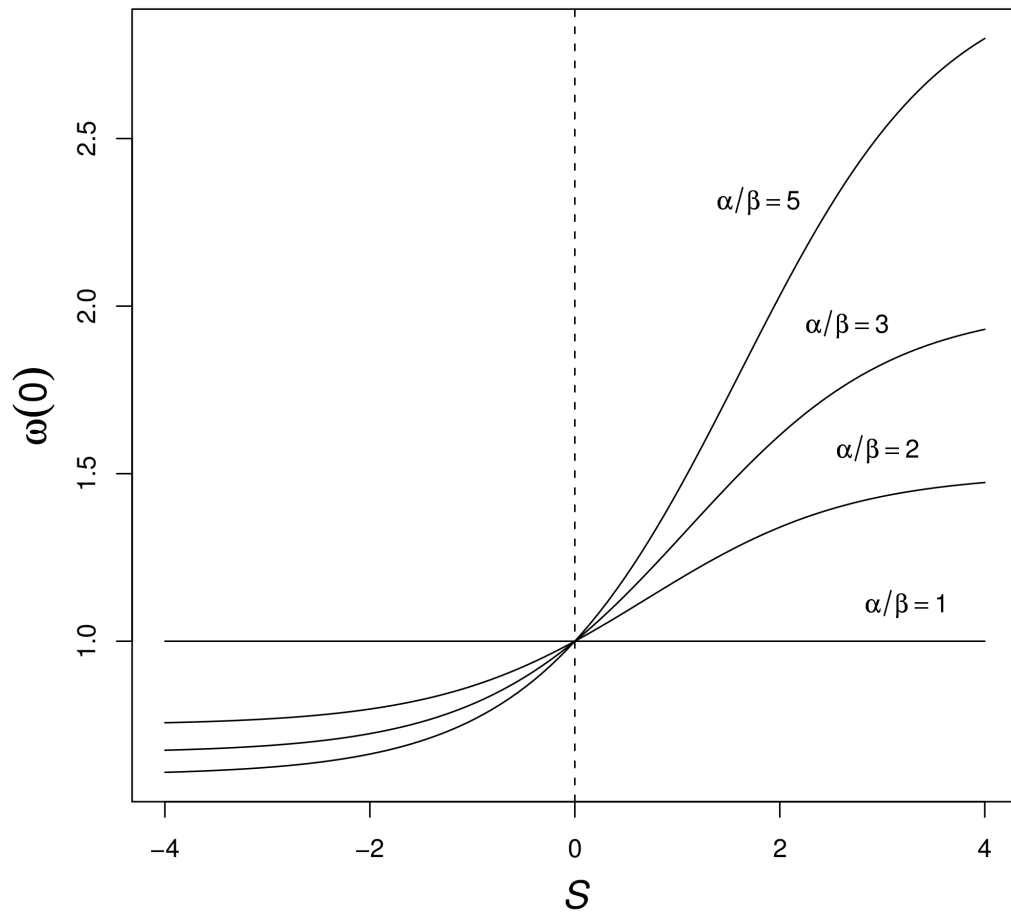


Figure 4: The initial value of ω for a codon location in a recently generated pseudo gene. For example, mutational biases $A/T \rightarrow G/C$ (α/β) of around 4.54 have been observed for some organisms (table 6.1 in Lynch 2006), considering $S = 2.5$, that would mean a codon location with $\omega(0) = 2.18$. On the other hand, if $S = -2.5$, then $\omega(0) = 0.65$. A pseudo gene equally composed of both type of codon locations would evolve at an average $\omega(0) = 0.5 \times 2.18 + 0.5 \times 0.65 = 1.42$, that is, substantially faster than the neutral rate at equilibrium.