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Mini review

Can RNA selection pressure distort the measurement of Ka/Ks?

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Abstract

Recently, an interesting question has emerged in the evolutionary interpretation of sequence substitution data as evidence of amino acid selection pressure. Specifically, the Ka/Ks metric was designed to measure selection pressure on amino acid substitutions, assuming that the synonymous substitution rate Ks reflects the neutral nucleotide substitution rate. However, there is increasing evidence for selection pressure at silent sites due to constraints of RNA splicing. Is Ka/Ks an appropriate metric for selection pressure on amino acid substitutions, in the presence of other selection pressures acting only at the RNA level (such as selection for exonic splicing enhancers)? Or can the resulting decreases in Ks from such selection pressures introduce bias into the Ka/Ks metric, so that it no longer gives an accurate measure of amino acid level selection pressure? In this review, we present both mathematical models and empirical evidence for these divergent points of view.

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Keywords: Evolution; Splicing; RNA; Synonymous substitutions; Nonsynonymous substitutions**1. Background: the Ka/Ks metric of amino acid selection pressure**

The Ka/Ks metric was first proposed as a means of distinguishing amino acid selection pressure ω from the background nucleotide mutation rate π (Li et al., 1985; Nei and Gojobori, 1986; Yang and Bielawski, 2000; Yang and Nielsen, 2000; Hurst, 2002). This approach took advantage of the fact that synonymous sites (nucleotides where substitutions produce no change in the encoded amino acid) are subject to the background nucleotide mutation rate π , but not to the amino acid selection pressure ω . By contrast, non-synonymous sites are subject to both processes. Thus, the rate of observed mutations at non-synonymous sites (Ka) vs. the rate at

synonymous sites (Ks) can be combined to obtain an estimate of the amino acid selection pressure ω :

Non-synonymous sites:	$K_a = \omega\pi$
Synonymous sites:	$K_s = \pi$
	$K_a/K_s = \omega$

Typically, Ka and Ks are normalized by the numbers of non-synonymous sites vs. synonymous sites in a gene, respectively, so that a Ka/Ks value of 1 represents the ratio of non-synonymous vs. synonymous mutations expected under a random mutation model and neutral amino acid selection pressure (i.e. $\omega=1$). Since its introduction, the Ka/Ks metric has been very widely used as a measure of amino acid level selection pressure (Nei and Kumar, 2000; Yang and Bielawski, 2000; Hurst, 2002).

However, amino acid sequence is not the only feature of a gene that can experience selection pressure during evolution. Other selection processes can also act on a gene and affect its rate of evolution. In most genomes, synonymous codon usage shows bias towards a set of major codons (Akashi and Eyre-Walker, 1998; Akashi, 2001, 2003). Codon usage bias is proposed to reflect selection on optimal codons for a higher translational efficiency (often referred to as “translational selection”). Analyses of codon usage in the human genome

Abbreviation: ESE, exonic splicing enhancer.

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indicate a weak positive correlation between gene expression level and frequencies of optimal codons (Lavner and Kotlar, 2005). The importance of codon usage bias and translational selection has been reviewed extensively elsewhere (Akashi and Eyre-Walker, 1998; Akashi, 2001, 2003; Duret, 2002). It is well known that selection on codon usage reduces the synonymous divergence rate, and it can introduce bias into the Ka/Ks metric. Methods have been developed to adjust for such bias in measuring Ka/Ks (Hirsh et al., 2005).

In this review, we focus on a distinct mechanism of selection pressure in coding regions, which is associated with regulation of pre-mRNA splicing. We will refer to such selection pressure as “RNA selection pressure” throughout our manuscript. Recently, there is a new wave of evidence for widespread RNA selection pressure in the eukaryotic genomes (Cartegni et al., 2002; Buratti and Baralle, 2004; Fairbrother et al., 2004; Pagani and Baralle, 2004; Baek and Green, 2005; Carlini and Genut, 2005; Ohler et al., 2005; Pagani et al., 2005; Parmley et al., 2006; Xing and Lee, 2005). Sequence motifs that are important for transcript processing (e.g. splice sites or exonic splicing enhancers) are observed to experience strong selection pressure (Fairbrother et al., 2004; Pagani and Baralle, 2004; Parmley et al., 2006), even for mutations that do not alter the amino acid sequence and have no effect on translation. Many silent mutations are believed to disrupt splicing regulatory elements within exons and to be strongly selected against (Cartegni et al., 2002; Pagani and Baralle, 2004). Genome-wide analyses of alternatively spliced exons in mammals have also indicated strong RNA selection pressure associated with alternative splicing (Baek and Green, 2005; Xing and Lee, 2005). In a pivotal study, Pagani and colleagues systematically introduced 19 synonymous mutations to the exon 12 of *CFTR*, and found that ~30% of the mutations disrupted the splicing of *CFTR* and resulted in an inactive protein (Pagani et al., 2005). This study provides molecular evidence that synonymous mutations can affect splicing and are not neutral in evolution (Pagani et al., 2005). Other types of functional constraints on the RNA level, such as RNA secondary structure requirements (Chamary and Hurst, 2005), might affect synonymous sites as well. In fact, recent studies indicate that RNA secondary structure is under selection for the proper splicing of a number of genes (Buratti and Baralle, 2004; Krehling and Graveley, 2005). It should be emphasized that RNA selection pressure (i.e. selection pressure on RNA functional features such as splicing regulatory motifs or secondary structure) is mechanistically distinct from translational selection, since the latter is associated with protein translation instead of pre-mRNA splicing. Does the presence of widespread RNA selection pressure distort the Ka/Ks metric, so that it no longer gives an accurate measure of ω ?

Since amino acid selection pressure and RNA selection pressure arise from distinct cellular processes (e.g. the effect of a Ala → Val substitution on an enzyme’s activity, vs. the effect of an A → G substitution on the binding of a specific splicing factor to this site), we must introduce an additional, independent variable ρ to represent the RNA selection pressure. In addition,

we reinterpret the variable π to represent not the true, neutral rate of mutation (which we will now symbolize as μ), but instead as the product of the mutation rate (μ) and RNA selection pressure (ρ). Considering the three processes of (1) background nucleotide mutation μ (can act at all sites), (2) RNA selection pressure ρ (can act at all sites), and (3) amino acid selection pressure ω (can only act at non-synonymous sites), the extended definition of Ka/Ks becomes:

Non-synonymous sites:	$Ka = \omega\rho\mu$
Synonymous sites:	$Ks = \rho\mu$
	$Ka/Ks = \omega$

On the one hand, this suggests that Ka/Ks remains a valid measure of amino acid level selection pressure even in the presence of RNA selection pressure. Specifically, ρ is present in both the numerator and the denominator of the Ka/Ks metric, so even though it may cause decreases in Ks, this should not cause an increase in the Ka/Ks ratio. Because of the name “Ka/Ks,” intuitively any decrease in Ks should cause an increase in Ka/Ks. However, this is not the case: both of the factors ρ and μ that constitute Ks are also present in the numerator Ka, so they cancel. Indeed, that is the reason why Ks was included in the denominator of the Ka/Ks metric in the first place — to remove the effect of the background nucleotide substitution density $\pi = \rho\mu$ that is present in Ka. On the other hand, this also shows why it is hard to prove this definitively: in effect, we are trying to estimate three independent variables (ω , ρ , and μ) from only two observed values (Ka and Ks).

There are some empirical data on the relationship of Ks and Ka/Ks from genome-scale analyses. One systematic study of Ka/Ks has been performed recently by Zhang and Li, for a set of 1580 human–mouse orthologous genes (Zhang and Li, 2004). If low levels of Ks indeed cause systematic overestimation of Ka/Ks, one would expect to observe a trend of increasing Ka/Ks for genes with low values of Ks, especially

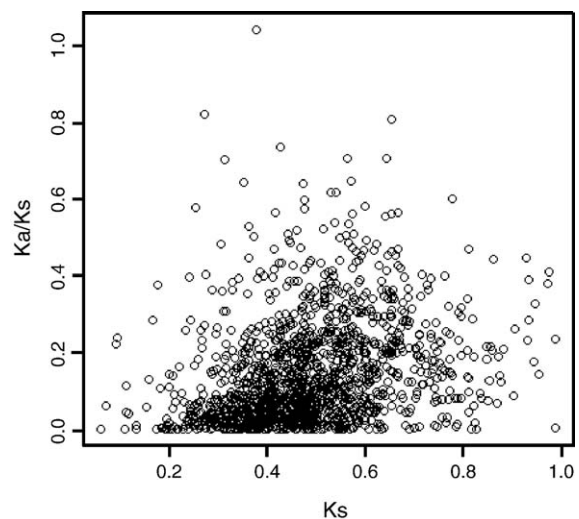


Fig. 1. Ka/Ks and Ks for 1580 human–mouse orthologs. Ka/Ks and Ks values were obtained from a recent analysis (Zhang and Li, 2004) on 1580 housekeeping and tissue-specific genes between human and mouse. The data does not indicate increased Ka/Ks for genes with low Ks.

when K_s is quite low (e.g. $K_s < 0.2$). However the K_a/K_s vs. K_s data of Zhang and Li show no such a trend over this large set of genes (Fig. 1). For the reasons discussed above, this result should be considered more suggestive than conclusive.

2. *BRCA1*: can decreases in K_s cause increased K_a/K_s ?

Often, researchers who observe increased K_a/K_s values in a specific dataset check that it is due to an increase in K_a (observed amino acid changes) while expecting K_s to remain constant, consistent with its traditional interpretation as a measure of the background nucleotide substitution rate (Thomas et al., 2003; Zhang and Li, 2004). Thus, it was with some surprise that Hurst and colleagues reported a region of the *BRCA1* gene with greatly increased K_a/K_s , in which K_a was approximately the same as in the rest of the protein, but K_s was significantly decreased (Hurst and Pal, 2001). Subsequently, Orban and colleagues identified the likely cause of the decrease in K_s : this region corresponds to exon 11, which is observed to be alternatively spliced, and contains specific splicing enhancer sequences that are likely under selection pressure to maintain the proper splicing of *BRCA1* (Orban and Olah, 2001) (without these splicing enhancers, splicing of the exon might fail).

Hurst et al. performed careful analysis seeking evidence for a mechanism by which the decrease in K_s could be considered to cause the observed increase in K_a/K_s . For example, codon usage bias might be able to produce such an appearance (which is specifically directed at silent sites and can cause reduced K_s , discussed in detail below). The authors examined several tests for codon usage bias, including the effective number of codons (ENC) (Wright, 1990) and the χ^2/L method (Shields et al., 1988). However, they observed no evidence for codon usage bias that could explain the simultaneous increase in K_a/K_s and decrease in K_s in this exon.

Recent studies of alternative splicing have shed light on this interesting observation in *BRCA1*. A variety of studies have shown that alternative splicing is associated with a significant relaxation of selection pressure (e.g. selection pressure against exon creation/loss (Modrek and Lee, 2003), tolerance for exonic Alu sequences (Sorek et al., 2002) or premature termination codons (Lewis et al., 2003; Xing and Lee, 2004), etc.). Thus, the fact that this exon is alternatively spliced in *BRCA1* would predict that it would indeed have increased K_a/K_s (reflecting relaxed selection pressure against amino acid mutations). At the same time, alternatively spliced exons often are observed to have increased conservation of nucleotide sequence, reflecting the importance of exonic splicing enhancers or other motifs that regulate splicing of the exon (Sorek and Ast, 2003; Itoh et al., 2004). This stronger RNA selection pressure (ρ) predicts a decrease in K_s . Thus, alternative splicing can explain both observations in a simple way. Indeed, both patterns appear to be general, observed in alternatively spliced exons throughout eukaryotic genomes (Iida and Akashi, 2000; Xing and Lee, 2005).

However, these studies rely on K_a/K_s as a metric of amino acid selection pressure (ω). Is it possible that the decreased K_s

values caused by RNA selection pressure (ρ) in such exons distort K_a/K_s so that it grossly overestimates the true value of ω ?

3. How might RNA selection pressure bias K_a/K_s ?

The potential source of bias is the possibility that the RNA selection pressure is different at synonymous vs. non-synonymous sites. For example, if the RNA selection pressure at the first and second positions of each codon were different from the RNA selection pressure at the third codon, then measurements of RNA selection pressure at synonymous sites may not be applicable to non-synonymous sites.

To look into this possibility further, it would be helpful to briefly discuss other mechanisms that are known to cause such a systematic bias. One distinct mechanism that could cause such divergence is codon usage bias. By definition, codon usage bias only affects synonymous sites. If codon usage bias was strong enough to render a substantial fraction of synonymous mutations unacceptable, and furthermore the strength of this selection pressure varied greatly between two different samples, it could give rise to an artifactual appearance of a large difference in K_a/K_s between the samples. Defining the selection pressure due to codon usage bias in sample 1 as χ_1 and in sample 2 as χ_2 ,

Non-synonymous sites, sample 1:	$K_a = \omega\rho\mu$
Synonymous sites, sample 1:	$K_s = \chi_1\rho\mu$
Sample 1:	$K_a/K_s = \omega/\chi_1$
Non-synonymous sites, sample 2:	$K_a = \omega\rho\mu$
Synonymous sites, sample 2:	$K_s = \chi_2\rho\mu$
Sample 2:	$K_a/K_s = \omega/\chi_2$

In this case a large decrease in K_s in one of the samples ($K_{s2}/K_{s1} = \chi_2/\chi_1$) can indeed induce an artifactual increase in K_a/K_s [$(K_a/K_s)_2/(K_a/K_s)_1 = \chi_1/\chi_2$], even when the actual amino acid selection pressure ω remains constant.

Thus it is essential to test a given dataset for evidence of strong changes in codon usage bias, when comparing K_a/K_s values with another dataset (Zhang and Li, 2004; Baek and Green, 2005). A number of metrics have been developed for measuring codon bias, including the codon adaptation index (CAI) (Sharp and Li, 1987), the effective number of codons (ENC) (Wright, 1990), the χ^2/L method (Shields et al., 1988), the codon bias index (CBI) (Morton, 1994), the intrinsic codon deviation index (ICDI) (Freire-Picos et al., 1994), the frequency of optimal codons (Fop) (Ikemura, 1981), etc.

What are the general requirements for RNA selection pressure to exhibit systematic differences between adjacent synonymous and non-synonymous sites? To do so, RNA selection pressure would have to be periodic, with a periodicity of exactly 3 nt, and moreover would have to keep a fixed, constant relation to the protein reading frame in different exons and different genes, in order to produce a net bias that would not be averaged out when data from multiple exons were combined. As we have seen, codon usage bias can produce such an effect, but other sources of bias are also possible. It is conceivable that nucleotide sequence features such as binding sites for splicing factors or other proteins could be “phased” to

position their most constrained nucleotides in synonymous sites, and to avoid non-synonymous sites, so that the separate requirements of the amino acid sequence and nucleotide sequence could both be fulfilled without interfering with each other.

4. Empirical data on “synonymous phasing” of splicing factor sites

Some recent studies have shed light on how RNA selection pressure might affect K_a/K_s . Krainer and colleagues have characterized the binding sites for the splicing factors SF2/ASF, SC35, SRp40 and SRp55 by RNA SELEX (Systematic Evolution of Ligands by EXponential enrichment) experiments, and have constructed a position specific scoring matrix for each motif (Liu et al., 1998; Cartegni et al., 2003) (also see: <http://rulai.cshl.edu/tools/ESE/ESEmatrix.html>). These data indicate the maximum possible effect that ESE motifs could have on synonymous sites, relative to non-synonymous sites. For example, for the SF2/ASF motif, positioning its strongly constrained G (position 6) at a synonymous site yields a 54% reduction of substitution probability at synonymous sites relative to nonsynonymous sites. The maximum reductions for the SC35, SRp40 and SRp55 motifs were 24%, 21%, and 21%, respectively. Thus in theory SF2/ASF motifs could cause an up to 2-fold K_a/K_s artifact (i.e. an actual value of $\omega=0.1$ might be measured as a K_a/K_s value of 0.2). However, this maximum can only be achieved if (1) every SF2/ASF motif is positioned so its G at position 6 is in a synonymous site; (2) every synonymous site is four-fold redundant, which is not the case at all sites; (3) every nucleotide in the region being measured is covered by an SF2/ASF motif (i.e. a minimum of 21 SF2/ASF 7 nt motifs to cover a 150 nt exon). The effects of realistic numbers of such motifs over a typical exon or entire protein sequence would be much less.

A handful of other studies suggest that experimentally verified SF2/ASF sites do not exhibit such strong phasing. Studies by Dirksen et al. (2000), Pollard et al. (2002), and Rooke et al. (2003) have demonstrated the presence of six functional SF2/ASF sites in three genes: GH1 (Dirksen et al., 2000), GNAS (Pollard et al., 2002), and c-Src (Rooke et al., 2003). Their data show that 3 of the 6 sites place the conserved G at the 1st codon position (non-synonymous), while the remaining three sites put it at the 3rd (synonymous) codon position (but even these sites were only two-fold redundant, halving their impact on K_s).

5. Challenges: more experimental data are needed for validated splicing factor sites

It would be useful to test whether important RNA regulatory motifs (such as exonic splicing enhancers or silencers) exhibit a strong 3 nt periodicity for their strongly constrained nucleotide positions, and whether there is a significant bias for these positions to be matched to synonymous sites (and to avoid non-synonymous sites). To measure this bias in a meaningful way requires a “gold standard” set of experimentally validated nucleotide motifs; i.e. motif sites shown to be biologically active in

real genes. The general weakness of such motif sequence patterns leads to very high false positive rates when searching genomic sequence, so each candidate site would have to be experimentally tested for its putative activity (e.g. via site-directed mutagenesis) (Cartegni et al., 2003). This question cannot really be resolved solely by bioinformatics, and would require a significant experimental effort. Great care would be required in the design of the motif identification analysis and experimental validation, to avoid introducing possible bias. For example, validating motifs by making site-directed mutations at synonymous sites would clearly be biased, as would any validation protocol that depended on measuring the activity of the protein product.

6. Concluding remarks

Recent studies of RNA splicing indicate widespread RNA selection pressure in eukaryotic genomes (Pagani and Baralle, 2004). This challenges a basic assumption of the K_a/K_s metric, that synonymous substitutions are selectively neutral. Is K_a/K_s still an appropriate metric for selection pressure on amino acid substitutions? If RNA selection pressure indeed introduces systematic bias into the K_a/K_s metric, what is the magnitude of the bias? Much additional work is needed on the distribution of experimentally validated RNA regulatory motifs in real genes.

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