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Evidence of Functional Selection Pressure for Alternative Splicing Events that Accelerate Evolution of Protein Subsequences

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Abstract

Recently, it was proposed that alternative splicing may act as a mechanism for opening accelerated paths of evolution, by reducing negative selection pressure, but there has been little evidence so far whether this could produce adaptive benefit. Here we employ metrics of very different types of selection pressures (e.g. against amino acid mutations (*Ka/Ks*); against mutations at synonymous sites (*Ks*); and for protein reading-frame preservation) to address this question via genome-wide analyses of human, chimpanzee, mouse, and rat. These data show that alternative splicing relaxes *Ka/Ks* selection pressure up to seven-fold, but intriguingly that this effect is accompanied by a strong *increase* in selection pressure against synonymous mutations, which propagates into the adjacent intron, and correlates strongly with the alternative splicing level observed for each exon. These effects are highly local to the alternatively spliced exon. Comparisons of these four genomes consistently show an increase in the density of amino acid mutations (*Ka*) in alternatively spliced exons, and a decrease in the density of synonymous mutations (*Ks*). This selection pressure against synonymous mutations in alternatively spliced exons was accompanied in all four genomes by a striking increase in selection pressure for protein reading-frame preservation, and both increased markedly with increasing evolutionary age. Restricting our analysis to a subset of exons with strong evidence for biologically functional alternative splicing produced identical results. Thus alternative splicing apparently can create evolutionary "hotspots" within a protein sequence, and these events have evidently been selected for during mammalian evolution.

Introduction

Alternative splicing has recently emerged as a major mechanism of functional regulation in the human genome and in other organisms (1-3), with up to 80% of human genes reported to be alternatively spliced (4). One question that has attracted much interest is comparing alternative splicing in different genomes. Several groups have sought to assess whether alternative splicing is more abundant in the human genome vs. other genomes (5-7). Another major focus has been to use sequence conservation (regions of high percent identity) to discover motifs that are important for regulation and alternative splicing (8-11). These data indicate that such regulatory motifs are clustered near splice sites, in both exonic sequence and the flanking introns. For example, measurements of conservation by percent identity between human and mouse show an approximately 20% increase in the 30nt of intron sequence immediately adjacent to alternatively spliced exons, relative to that for constitutive exons (8). The sequence of alternatively spliced exons also appears to have slightly higher conservation than constitutive exons, perhaps by a few percentage points of identity in comparisons of human vs. mouse (11).

It has also been proposed that alternative splicing can greatly increase the rate of certain types of evolutionary alterations, such as exon creation, by reducing negative selection pressure against such events (12-14). Evidence from many groups have shown associations between alternative splicing and increases in different types of evolutionary change, including exon duplication (15, 16); Alu element-mediated exonization (17); exon creation / loss (13, 18); and introduction of premature protein termination codons (19). In all of these cases, alternative splicing is associated with *reduced* levels of conservation during genome evolution. These lines of evidence suggest that alternative splicing has played a significant role during mammalian evolution, by opening neutral pathways for more rapid evolutionary change. However, at least superficially, these data would appear to be inconsistent with reports that alternative splicing is associated with *increased* levels of conservation (8, 11).

These data raise several questions about the role of alternative splicing in evolution. First, is the hypothesis that alternative splicing reduces negative selection pressure a general phenomenon? For example, does it hold true even for alternatively spliced exons that are clearly functional, or is it limited to alternatively spliced exons that have no biological function? Several groups have presented evidence for a stringent criterion that an alternative splicing event is functional, based on independent observations of that specific alternative splicing event in two different organisms (e.g. human and mouse) (20-22). For this dataset, evolutionary processes measured over this period have genuinely taken place under the influence of alternative splicing, and should reflect its effects. We have therefore performed a genome-wide analysis of exons observed to be alternatively spliced in both human and mouse transcripts, which we will refer to as 'ancestral alternative exons'.

Second, if alternative splicing does reduce selection pressure in a general way, is there any evidence that this phenomenon is *adaptive*, i.e. that such events have been selected for during evolution? Questions such as these require a transition from a single metric of evolutionary change (such as percent identity), to multiple metrics that can distinguish different types of selection pressure, e.g. selection pressure against amino acid amino acid mutations; selection pressure against synonymous nucleotide substitutions that disrupt important nucleotide motifs (e.g. binding sites for splicing factors), etc. We have therefore analyzed the well-known selection pressure metrics *Ka/Ks* and *Ks*, which give empirical measures of these two selection pressures (23, 24). Non-synonymous nucleotide sites experience the background nucleotide mutation level (whose density is symbolized by π), nucleotide selection pressure (which we will symbolize as ρ), and amino acid selection pressure (ω), while synonymous sites experience only the first two factors. Thus, in the standard formulation of *Ka/Ks*, the densities of observed mutations at non-synonymous sites (*Ka*), and synonymous sites (*Ks*) are

Eq. 1 $$Ka=\omega\rho\pi$$

$$Ks=\rho\pi$$

and $Ka/Ks=\omega$, with no dependence on π or ρ (23). *Ka/Ks* has been very widely used, because the normalization by *Ks* yields a metric of amino acid selection pressure that is independent of π (which varies enormously according to the total time of evolutionary divergence between a pair of genomes (25)). A *Ka/Ks* ratio of 1 indicates neutral evolution (absence of selection pressure); by contrast, in most protein coding regions *Ka/Ks* is significantly less than 1, indicating strong negative selection pressure against amino acid mutations (26).

In this paper, we analyze *Ka* and *Ks* both for ancestral alternative exons that have strong evidence of functional alternative splicing, and in genome-wide comparisons of four mammalian genomes (human, chimpanzee, rat, and mouse), to evaluate how alternative splicing affected selection pressure over different evolutionary timescales. We use a standard metric for alternative splicing – the exon inclusion level, defined as the fraction of a gene's transcripts that include an exon rather than skipping it (13) – and measure its impact on *Ka* and *Ks* selection pressures.

Methods

Alternative splicing analysis

We detected alternative splice forms in human and mouse by mapping mRNA and ESTs onto genomic sequences as previously described (27) using the following data: (i) UniGene EST data (28) from June 2003 for human and mouse (ftp://ftp.ncbi.nih.gov/repository/UniGene) (ii) genomic sequence data from June 2003 for human and mouse (ftp://ftp.ensembl.org/pub/current_human and ftp://ftp.ensembl.org/pub/current_mouse). Internal exons were identified as genomic regions flanked by two splices, and all exon boundaries were confirmed by checking consensus splice site motifs. We computed exon inclusion level for each alternatively spliced exon, defined as the number of ESTs that included an exon divided by total number of ESTs that either included or skipped this exon. Based on this ratio, we grouped alternatively spliced exons into three classes: major-form (inclusion level above 2/3), medium-form (inclusion level between 1/3 and 2/3) and minor-form (inclusion level below 1/3).

We identified orthologous human-mouse exons as previously described (13), using orthologous gene information from HOMOLOGENE (29) (ftp://ftp.ncbi.nih.gov/pub/HomoloGene/ July2003), including all orthologous pairs of genes that were successfully mapped onto genomic sequences during our splicing calculation. We defined a pair of human-mouse orthologous exons as 'ancestral alternative exons' if the exon was alternatively spliced in both human and mouse transcripts. Similarly we defined a pair of human-mouse orthologous exons as 'ancestral constitutive exons' if the exon was constitutively spliced in both organisms. Our dataset included 132 orthologous exon pairs in the ancestral alternative exon set, and 10190 pairs in the constitutive set.

***Ka/Ks* and *Ks* sequence divergence metrics**

We computed the *Ks* rate and *Ka/Ks* ratio between orthologous exon pairs following the approach of Li and colleagues (30). Briefly, orthologous exon sequences from human and mouse were both translated in all possible reading frames. Translations containing STOP codons were removed and the remaining protein sequences were aligned in all possible combinations. We computed sequence identities in all resulting alignments using the global sequence alignment program *needle* in EMBOSS software package (31). After excluding alignments between human and mouse protein sequences that were translated from different reading frames (indicated by a cut-off of 50% protein sequence identity), we selected the reading frame pair with the highest amino acid identity, and then aligned these two protein sequences using CLUSTALW (32) under default parameters. This protein alignment was used to re-align corresponding nucleotide sequences, and gaps in the alignment were trimmed. We estimated the *Ks* rate and *Ka/Ks* ratio from the codon-based nucleotide sequence alignment using both the Nei-Gojobori method and Yang-Nielsen method, implemented in the yn00 program of PAML package (33). These two methods yielded similar results. For each group of exons (constitutive, major-form, medium-form, minor-form), we calculated its mean *Ka* and *Ka/Ks*, and estimated a 95% confidence interval for the mean using nonparametric bootstrapping.

For each pair of orthologous exons, we aligned the entire exons as well as 250bp upstream and downstream intronic sequences, using the program *needle* in EMBOSS software package (31). We computed the observed nucleotide substitution density (number of observed substitutions per site) in the alignment.

Genome-wide analyses of conserved constitutive and alternative exons in human, chimpanzee, mouse and rat

We calculated *Ka*, *Ks* and *Ka/Ks* for constitutive and alternative exons conserved between the genomic sequences of human and chimpanzee, or mouse and rat, or human and mouse. The exon inclusion level was estimated based on human EST data (for human vs. chimpanzee analysis, and human vs. mouse), or based on mouse EST data (mouse vs. rat). We estimated *Ka* and *Ks* for each pair of orthologous exons between human and mouse using the Yang-Nielsen method as described above, summing up the total number of synonymous and nonsynonymous substitutions/sites for each group of exons (constitutive, major-form, medium-form, minor-form). For human vs. chimpanzee, we searched the entire chimpanzee genome (ftp://ftp.ensembl.org/pub/current_chimp May2004) with each human exon, using BLASTN (34), requiring an expectation score of 10^{-4} or less, and a match-length within at least 12nt of the human exon's length. Using the best hit from the chimpanzee genome, we identified the best reading frame pair as above, requiring 80% protein sequence identity. For mouse vs. rat, we searched the rat genome (ftp://ftp.ensembl.org/pub/current_rat July2004), for each mouse exon, and processed hits in the same way.

Frame preservation analysis

We defined an exon as "frame-preserving" if the length of the exon was a multiple of 3nt, and as "frame-switching" if not (35). Inclusion or exclusion of a "frame-preserving" exon by alternative splicing leaves the downstream protein reading frame unchanged; for this reason, frame-preservation has been proposed by several groups as evidence that an alternative splicing event is functional (21, 35-37). We calculated the frame preservation ratio for a given set of exons as the number of "frame-preserving" exons divided by the number of "frame-switching" exons (35).

Results

***Ka/Ks* analysis**: To understand in detail how alternative splicing affects selection pressure, we performed a genome-wide analysis of exons observed to be alternatively spliced in both human and mouse transcripts. Our results showed that ancestral alternative exons had much higher *Ka*/*Ks* values compared to ancestral constitutive exons. The average *Ka*/*Ks* estimated from the Yang-Nielsen method for the set of 132 ancestral alternative exons was 0.394, significantly higher than the average for the set of 10190 ancestral constitutive exons (0.114, $P= 6.6\times10^{-11}$). The Nei-Gojobori method yielded similar results.

To make our analysis more quantitative, we used a standard metric for alternative splicing — exon inclusion level (13, 38), defined as the number of transcripts observed to include the exon, divided by total number of transcripts that either include or skip it. We categorized ancestral alternative exons into three groups based on this ratio measured from human transcript data. We found a striking negative correlation between the exon inclusion level θ and mean *Ka*/*Ks* ratio (Fig. 1A). Exons with high inclusion levels (θ >

2/3, defined as major-form exons) had a low *Ka*/*Ks* ratio (0.262), while exons with low inclusion levels ($\theta < 1/3$, defined as minor-form exons) had a *Ka*/*Ks* ratio (0.814) more than 7-fold higher than constitutive exons. The difference in *Ka*/*Ks* ratio between major-form and minor-form exons was statistically significant (P=0.0015). Thus, alternative splicing appears to relax negative selection against amino acid changes, even when there is strong evidence that these alternative splicing events are functional (they were observed in both mouse and human transcripts). Moreover, the degree of relaxation depends quantitatively on the *amount* of alternative splicing in these exons.

***Ks* analysis**: The *Ka/Ks* metric divides the observed density of amino acid substitutions (*Ka*) against the observed density of synonymous nucleotide substitutions (*Ks*). In mammals, it has generally been assumed that synonymous substitutions are selectively neutral (39), i.e. that *Ks* simply reflects the background mutation rate of a gene. Consistent with this view, genes with relaxed selection pressure levels typically have been found to be associated with increases in *Ka*, without significant changes in *Ks* (40, 41), reflecting the ubiquitous importance of protein-level selection pressure.

However, contrary to this expectation, when we measured *Ka* and *Ks* rates separately for ancestral alternatively spliced exons, we found that increased *Ka/Ks* levels were associated with a large drop in the *Ks* rate in minor form exons (Fig. 1B). The average *Ks* rate (Yang-Nielsen estimates) for constitutive exons was 0.700, but dropped to 0.406 for major-form exons, and 0.133 for ancestral minor-form exons, a more than 5-fold reduction. The differences in *Ks* rate between these groups of exons were statistically significant ($P<2.2\times10^{-16}$ for ancestral constitutive vs. alternative exons; $P=3.6\times10^{-5}$ for ancestral major-form vs. minor-form exons).

Control tests vs. neighboring exons and introns: To control for gene-specific effects such as gene expression level, we also repeated our *Ks* analysis for constitutive exons within the same genes as these minor-form exons (Fig .1C). The average *Ks* rate for this subset of constitutive exons was 0.617, the same as that for other constitutive exons. Thus, ancestral alternative exons experience a significant reduction in the rate of synonymous divergence, even compared to neighboring exons within the same genes. This suggests that the *Ks* rate at these exons is no longer proportional to the background mutation rate. Instead these silent sites appear to be under purifying selection, and the degree of selection is strongest at ancestral minor-form exons.

Evidence of selection pressure on silent sites is often attributed to factors such as codon usage bias (42), which can cause reduced *Ks* and an artificial increase in *Ka/Ks*. Might this explain our results? Since intronic sequences, by definition, are not translated and are thus free from selection on codon usage, we sought to test this hypothesis by measuring the rate of nucleotide divergence at intronic sequences flanking alternative exons. Again we observed a striking reduction in the observed mutation frequency specifically for intron sequences flanking minor-form exons (Fig. 2). For the 50nt intronic region upstream of constitutive exons, the density of observed substitutions was 0.414, versus 0.334 for major-form exons and 0.198 for minor-form exons, a more than two-fold increase in selection pressure. The same trend was observed for the 50nt region downstream of each exon. This selection pressure diminished beyond 150nt from the exon, and beyond 250nt returned to the background level observed in constitutive exons.

Analysis of *Ka* and *Ks* in human, chimpanzee, mouse and rat genomes: In the standard formulation of *Ka*/*Ks*, *Ks* represents the baseline nucleotide substitution frequency π, and by definition can't affect the protein-level selection factor ω, which is an independent variable. The appearance of "*Ks*" in the denominator of the term "*Ka*/*Ks*" might seem to imply that changes in *Ks* can change the value of *Ka*/*Ks*, but this is not true in the standard formulation of *Ka*/*Ks*, because *Ks* is also present in the numerator of *Ka*/*Ks* (see equation 1, Introduction). Indeed *Ks* is included in the denominator of *Ka*/*Ks* solely to cancel its presence from the numerator, to obtain a measure of protein-level selection pressure separate from the baseline nucleotide substitution frequency (23).

To test our interpretation completely independent of this assumption, we have analyzed the observed density of amino acid substitutions (*Ka*) in several genome comparisons ranging in timescale from human vs. chimpanzee (5.4 my), to mouse vs. rat (41 my), to human vs. mouse (91 my) (43). For ancestral alternatively spliced exons (human vs. mouse), we observed a marginal increase (24%) in *Ka* for minor-form exons compared with major-form exons. In our genome-wide analyses, we observed no increase in human vs mouse, a 41% increase in mouse vs. rat, and a nearly three-fold increase in human vs. chimpanzee (Fig. 3 and Supplementary Data). Thus, even the absolute density of amino acid substitutions, without any correction made for the underlying nucleotide substitution density, shows a reproducible increase in alternatively spliced exons, and correlates with the level of alternative splicing for each exon (i.e. its exon skipping frequency).

Is the reduction in *Ks* observed in ancestral alternatively spliced exons reproducible across these multiple genome comparisons? In all cases, *Ks* showed a clear correlation with the exon inclusion level, with highest values for constitutive exons, and lowest values for minor-form exons (Supplementary Data). In all cases the difference between constitutive vs. minor-form exons was statistically significant, with the smallest difference in human vs. chimpanzee (a 58% difference, P=3.7x10^{-3}), and the largest difference in human vs. mouse ancestral alternatively spliced exons (a more than five-fold difference, P=6.6x10^{-16}).

These multiple genome comparison data also provide some basis for assessing whether our observed increase in *Ka/Ks* is real, or an artifact of decreasing *Ks*. Specifically, are these data consistent with the standard formulation of *Ka/Ks* (in which *Ka/Ks* is independent of *Ks*, because the nucleotide substitution density π is present in both the numerator (*Ka*) and denominator (*Ks*), as outlined above), or do they support an alternative model, in which decreases in *Ks* can cause increases in *Ka/Ks*? To assess this question in our alternative splicing dataset, we calculated the minor-form / major-form ratio for *Ks*, *Ka*, and *Ka/Ks* in the three different genome comparisons (Fig. 3). These different datasets display substantial shifts in *Ks* (shifts ranging from 37% to nearly four-fold), giving some opportunity to see the impact of changes in *Ks* on changes in *Ka/Ks*. Strikingly, the large shifts in *Ks* produced no corresponding shift in *Ka/Ks*, which remained approximately constant in all three datasets, because the observed shifts in *Ka* exactly followed the trend of shifts in *Ks*. These results are exactly what is expected under the standard formulation of *Ka/Ks*, and are not consistent with the hypothesis that decreasing *Ks* causes increased *Ka/Ks* in our data.

Minor-form exons display increased selection pressure for frame-preservation: We previously defined exons whose length is an exact multiple of 3nt as “frame-preserving”, because inclusion or skipping of the exon will not alter the protein reading-frame of subsequent exons (35). It has been previously observed that exons that were observed to be alternatively spliced in both human and mouse ESTs show an increased ratio of frame-preserving vs. non-frame-preserving exons (21, 35), implying selection pressure for frame-preservation. We have therefore measured evidence for such selection pressure as a function of exon inclusion level, across the genome-wide comparisons between human vs. chimpanzee, mouse vs. rat, and human vs. mouse (see Fig. 4). These data show a reproducible increase in frame-preservation ratio specifically in minor-form alternatively spliced exons, up to a maximum value of 2.6 (vs. an average value of 0.6 in constitutive exons).

Older alternatively spliced exons show increased evidence of RNA selection pressure: Over the wide range of evolutionary timescales we have analyzed (5 my – 90+ my), the effect of alternative splicing on *Ka/Ks* was strikingly consistent. For example, the ratio of *Ka/Ks* in minor-form vs. major-form exons was approximately constant in all of these genome comparisons (see Fig. 3). At least over this range of timescales, the effect of alternative splicing on *Ka/Ks* does not appear to be a sensitive function of time, or to have changed substantially over the last 100 my of mammalian evolution.

By contrast, the effect of alternative splicing on *Ks* showed a very clear increasing trend with increasing age of evolutionary conservation (Fig. 3), with the smallest difference between minor-form vs. major-form *Ks* observed in human vs. chimpanzee (37%), and the largest difference in human vs. mouse (3.8-fold). These data suggest that older alternatively spliced exons, conserved over longer periods of evolutionary history, display much stronger evidence of RNA selection pressure.

It is interesting to note that selection pressure for frame-preservation displayed a similar increasing trend as a function of increase age of evolutionary conservation (Fig.

4). The ratio between minor-form vs. constitutive frame-preservation was lowest in the human vs. chimpanzee comparison (1.6), intermediate in the mouse vs. rat comparison (3.6), and highest in the human vs. mouse comparison (4.0).

Discussion

These data suggest that alternative splicing reduces amino acid selection pressure, even for alternatively spliced exons that are functional, while simultaneously increasing nucleotide selection pressure. However, several alternative interpretations are possible. First, might this be a trivial result? The observed increase in *Ka/Ks* might itself be interpreted as evidence that these exons are newly created exons that have no biological function. Second, can we accept these *Ka/Ks* data as valid evidence of reduced amino acid selection pressure? The observed decrease in *Ks* raises some question of whether the increase in *Ka/Ks* could be an artifact. Third, might our results be an artifact of regions of unusually low mutation rate, rather than evidence of selection pressure? We will now consider each of these hypotheses.

Might our data simply reflect newly created, non-functional exons? This does not appear to be supported by the evidence. The age of creation of an exon does not seem to affect our results; we observed the same four-fold *Ka*/*Ks* increase in very recent genome comparisons (human vs. chimpanzee) as we did for exons that are over 80-100 my old (human vs. mouse). Might some of these exons be newly created, i.e. truly exonic only in one genome, and not the other? This also does not appear to explain our results. For example, in the human vs. mouse ancestral alternative splicing dataset, each exon was observed to be expressed both in human transcripts, and in mouse transcripts, yet we observed the same large increase in *Ka*/*Ks* in this dataset as in all the others. There are several independent lines of evidence that these exons are functional. Not only have they been conserved in the genome for over 80 my of evolution, but their pattern of alternative splicing is conserved as well (i.e. they were independently observed to be both included and skipped in human transcripts, and in mouse transcripts as well); this has been widely used as a criterion indicating that an alternative splicing event is functional (20-22). Furthermore, the observation of strong selection pressure on silent mutations in these exons (up to five-fold reduction in *Ks*) indicates that these exons are indeed functional in the sense of contributing to fitness. We have also observed in the same dataset that these minor-form exons display a four-fold increase in selection pressure for having an exon length that is an exact multiple of 3 nt (which preserves the protein reading frame when the exon is inserted or skipped), relative to constitutive exons. This striking increase in frame-preservation was observed for minor-form exons in all of the genome comparisons we performed, and furthermore followed the same trend as was observed for *Ks* (evolutionarily older exons showed the biggest increase in selection pressure).

Our observation of a simultaneous increase in *Ka/Ks* and decrease in *Ks* (for minor-form exons) raises questions about whether decreases in *Ks* could actually cause apparent increases in *Ka/Ks* (contrary to the standard formulation of *Ka/Ks*; see Results). However, multiple analyses of the human, chimpanzee, mouse and rat genomes showed that alternative splicing was associated with an increase in the observed density of amino acid mutations (*Ka*), even without taking into consideration the underlying density of nucleotide substitution (*Ks*).

Might our evidence of increased selection pressure in minor-form exons (e.g. reduced *Ks*) be instead interpreted as just a region of unusually low mutation rate? Such regions have been reported (44, 45), but do not appear to be consistent with our data. First, we have observed other types of selection pressure in these minor-form exons (e.g. a four-fold increase in the protein reading-frame preservation ratio, as described above), which simply cannot be explained by a low mutation rate. Second, regions of low mutation rate have been reported to be large, at least a megabase in size (44, 45). By contrast, our analysis shows that the zone of reduced *Ks* includes only the alternatively spliced exon and about 100 nt of flanking intron on either side; neighboring exons in the same gene showed no decrease in *Ks*. This zone of reduced *Ks* is thus over a thousand fold smaller than expected for reported regions of low mutation rate. Third, this hypothesis requires many improbable coincidences: the low probability that such regions' positions in the genome would coincidentally match a given set of exons; that they would hit only alternatively spliced exons, and not constitutive exons; and that their occurrence in the genome would correlate with the alternative splicing level of exons in mRNA (e.g. hitting minor-form exons but not major-form exons).

Our data suggest that alternative splicing can relax selection pressure in a strongly local fashion, without affecting neighboring constitutive exons in the same gene. Thus alternative splicing can create "evolutionary hotspots" in which one part of a protein sequence is allowed to accumulate amino acid mutations at a much higher rate than the rest of the protein. It is customary to view poor protein sequence conservation (i.e. neutral or near-neutral *Ka/Ks* values) as evidence of reduced functional importance. However, while it is natural to interpret a high *Ka/Ks* value for an entire gene sequence as evidence that it is not functional (e.g. a pseudogene), this assumption seems much less safe when the zone of high *Ka/Ks* is confined to a short segment of a protein. Recalling the definition of *Ka/Ks*, it should be emphasized that high values of *Ka/Ks* simply mean *rapid change*, not necessarily lack of function. For example, specific regions with high *Ka/Ks* have often been shown to be functionally very important (e.g. the antigen presentation cleft of major histocompatibility complex (MHC) proteins (46), and drug resistance mutations in human immunodeficiency virus (HIV) (47)). In many such cases the regions with highest *Ka/Ks* are the most important functional sites in the protein (such as the antigen binding site in MHC, or drug resistance mutations in HIV protease). Subsegments of elevated *Ka/Ks*, often corresponding to individual exons that are alternatively spliced, appear to have been important in both the evolution and function of many proteins, such as *BRCA1* (48) and *CD45* (49). Our *Ks* data and frame-preservation results provide systematic evidence that such rapid evolution of a protein subsequence is *not* necessarily indicative of loss of function. This suggests that such alternative splicing-accelerated evolution has produced adaptive functions that have been selected for during recent evolution.

Intuitively, it may seem surprising to suggest that alternative splicing can cause both a loss of selection pressure (*Ka/Ks*), and an increase in selection pressure (*Ks*). However, there is abundant support in the literature for both effects. Alternative splicing is associated with reduced selection pressure against exon creation / loss (13, 18); presence of *Alu* sequences in exons (17); premature protein termination codons (19); amino acid substitutions (this study); and splice site movement (data not shown). It has also been shown to *increase* selection pressure for protein reading frame preservation (35); and silent nucleotide substitutions (this study). Indeed, the observation that alternative splicing and splice-regulatory motifs are associated with increased percent identity in genome comparisons has been widely reported (8, 10, 11, 36, 37).

Our *Ks* data provide additional evidence of extensive "RNA-level" selection pressure that is distinct from protein-level selection. In alternatively spliced exons, *Ks* appears to behave very differently from amino acid selection pressure (*Ka*/*Ks*), and this effect extends into the flanking intronic sequence, as would be expected for selection pressure on splicing regulatory motifs. Moreover, the fact that the *Ks* reduction correlates strongly with the efficiency of the splicing reaction for that exon (i.e. its inclusion level), directly indicates that this reflects selection pressure on the splicing reaction itself. This conclusion is abundantly supported by reports of increased percent identity around alternatively spliced exons, attributed to splicing regulatory motifs (such as splicing enhancers and silencers) shown to be enriched in these exons (9, 10, 50, 51). Indeed there is a known case in *BRCA1* where alternatively spliced exons were found to have greatly increased *Ka*/*Ks* and reduced *Ks* where several splicing regulator elements were detected (48). Why don't major-form alternatively spliced exons show as high a level of RNA sequence selection pressure as minor-form exons? This may suggest that minor-form exons require more regulatory signals, and that their splicing may be more highly regulated, whereas major-form exons may represent a "default" splicing pattern.

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Figure Legends

Figure 1: *Ancestral alternative splicing: amino acid selection pressure and nucleotide selection pressure as a function of alternative splicing*
(**A**) *Ka/Ks* decreases as a function of increasing exon inclusion levels for alternatively spliced exons, and was lowest in constitutive exons. Two methods of estimating *Ka/Ks* are shown: Nei-Gojobori (blue); Yang-Nielsen (red). Error bars indicate the 95% confidence interval for the mean *Ka/Ks* ratio computed by nonparametric bootstrapping.
(**B**) *Ks* increases as a function of increasing exon inclusion level for alternatively spliced exons, and was highest in constitutive exons. Two methods of estimating *Ks* are shown: Nei-Gojobori (blue); Yang-Nielsen (red). Error bars represent the 95% confidence interval for the mean *Ks* ratio computed by nonparametric bootstrapping.
(**C**) *Ks* of alternatively spliced exons versus neighboring constitutive exons (blue bars) within the same gene. *Ks* was measured using the Yang-Nielsen method. Error bars indicate the 95% confidence interval for the mean *Ks* computed by nonparametric bootstrapping.

Figure 2: *Intronic nucleotide substitution density as a function of alternative splicing and distance to intron-exon junctions*
Intronic nucleotide substitution density increases as a function of increasing exon inclusion levels for alternatively spliced exons, and was highest in constitutive exons. The greatest difference in the intronic nucleotide substitution density between minor-form and constitutive exons was observed in the 50-nucleotide intronic regions immediately adjacent to the intron-exon junctions. (**A**) upstream introns. (**B**) downstream introns.

Figure 3: *Increased Ka/Ks and decreased Ks is a general phenomenon associated with alternative splicing during recent mammalian evolution*
The ratios for minor-form exons over major-form exons calculated for *Ka*, *Ks* and *Ka/Ks*. Reduced *Ks* and elevated *Ka/Ks* is observed in all three genome comparisons: human vs chimpanzee, mouse vs rat and human vs mouse. *Ka*, *Ks* and *Ka/Ks* were estimated using the Yang-Nielsen method.

Figure 4: *Protein reading-frame preservation as a function of alternative splicing.*
The frame-preservation ratio (ratio of frame-preserving exons over frame-switching exons) was highest in minor-form exons, and near the value expected by random chance (0.5) in constitutive exons.

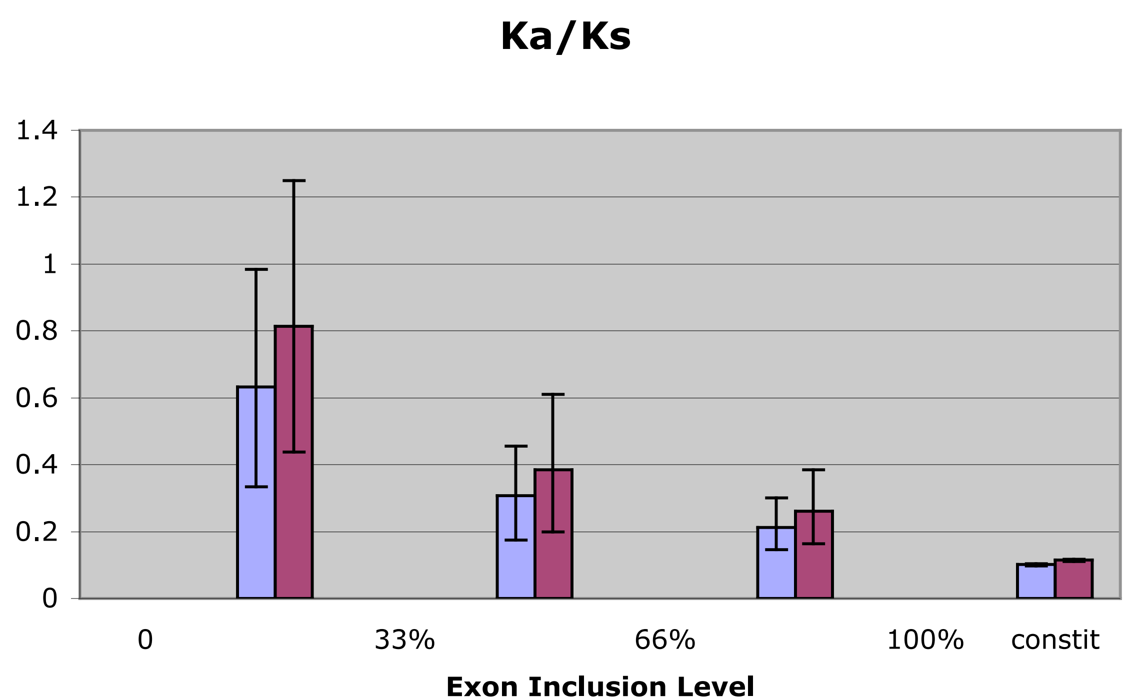

Ka/Ks
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
33%
66%
100%
constit
Exon Inclusion Level

Fig 1A

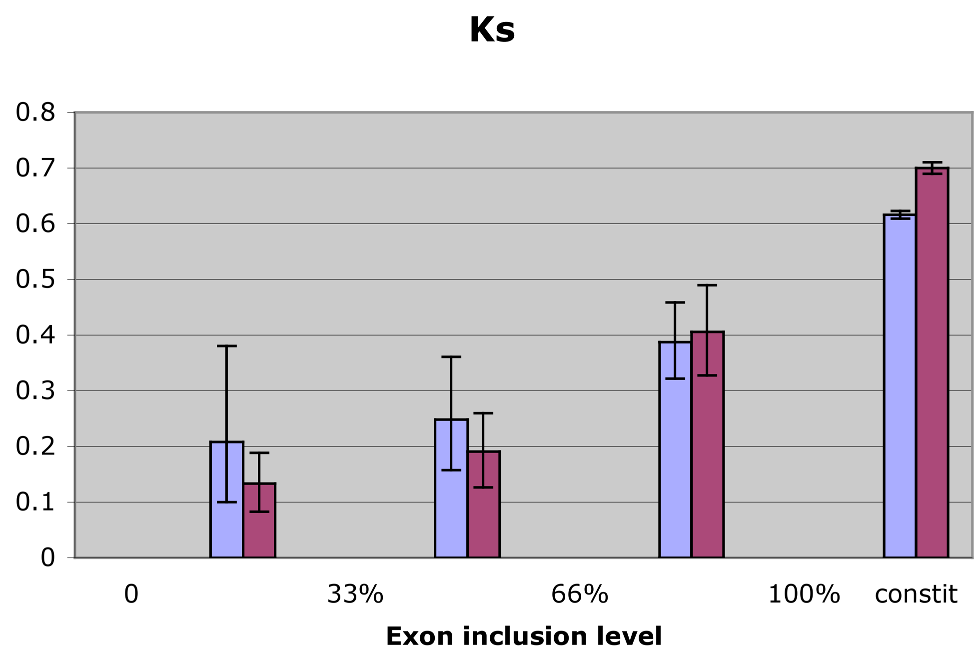

Ks
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
33%
66%
100%
constit
Exon inclusion level

Fig 1B

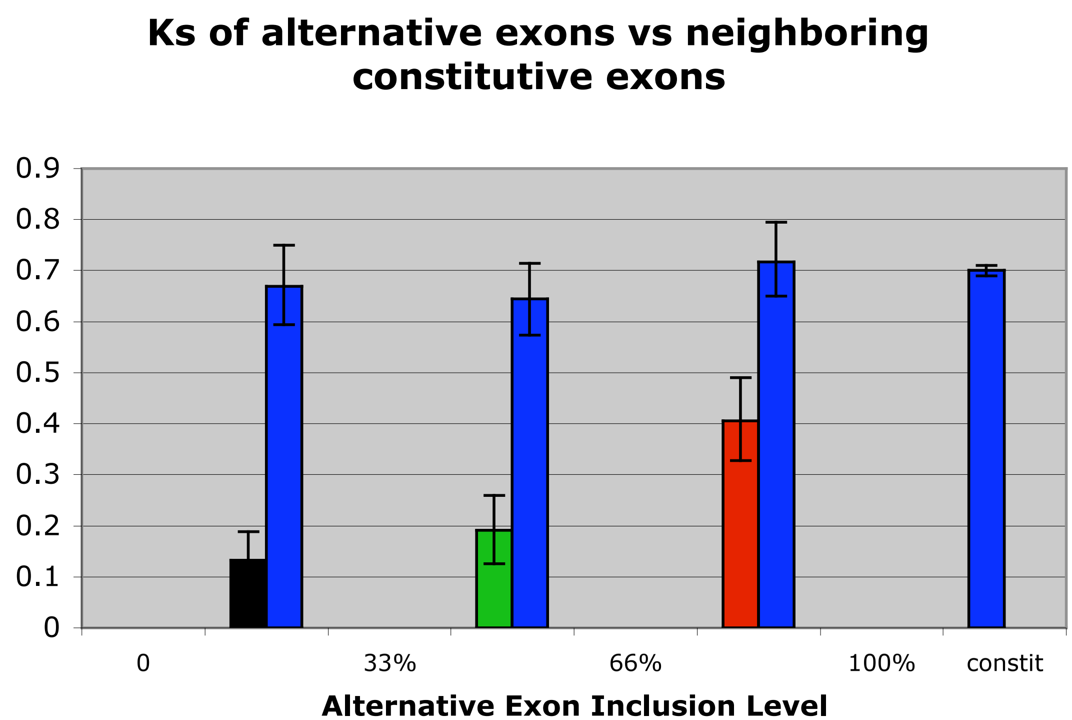

Ks of alternative exons vs neighboring constitutive exons
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
33%
66%
100%
constit
Alternative Exon Inclusion Level

Fig 1C

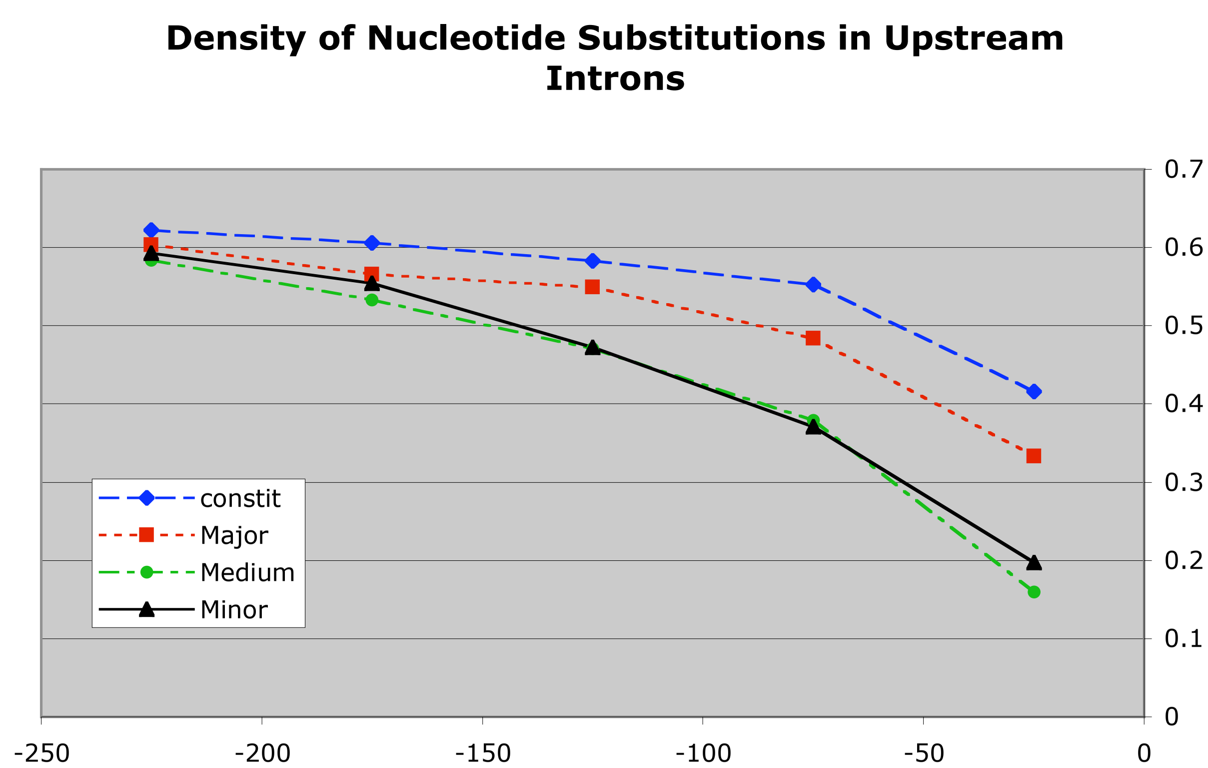

Density of Nucleotide Substitutions in Upstream Introns
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-250
-200
-150
-100
-50
0
constit
Major
Medium
Minor

Fig 2A

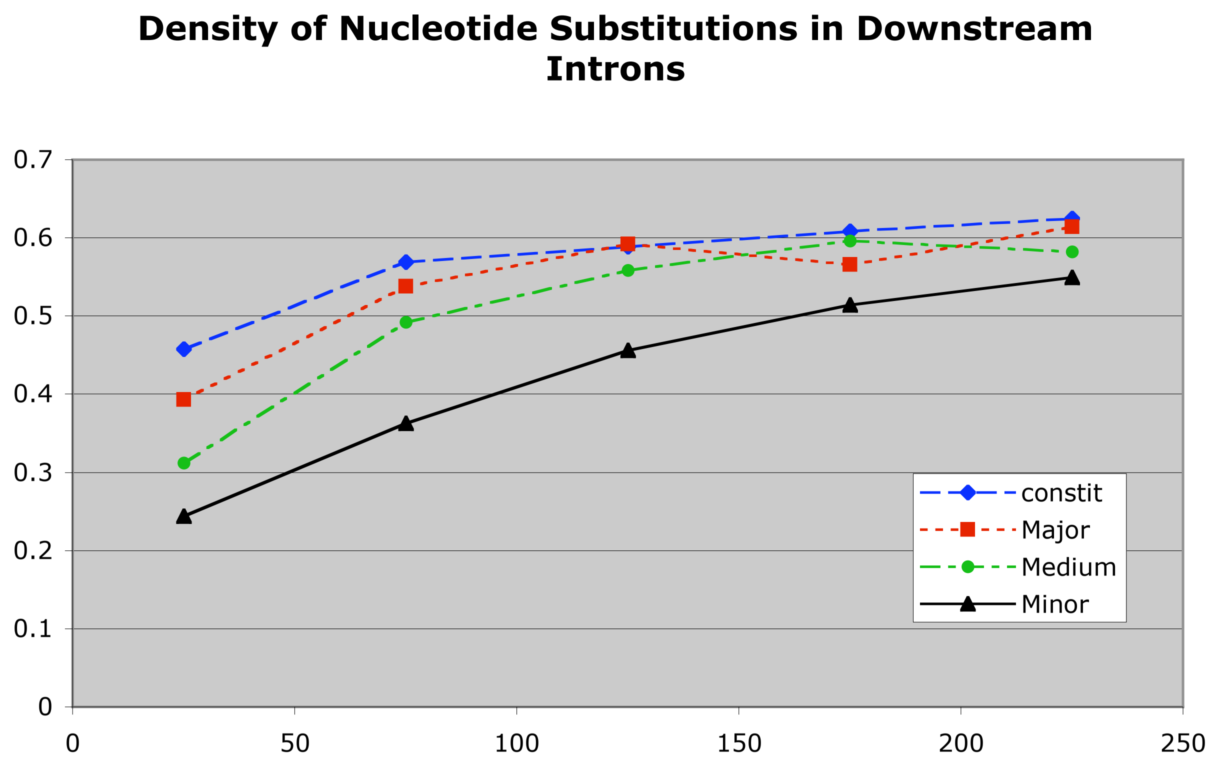

Density of Nucleotide Substitutions in Downstream Introns
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
50
100
150
200
250
constit
Major
Medium
Minor

Fig 2B

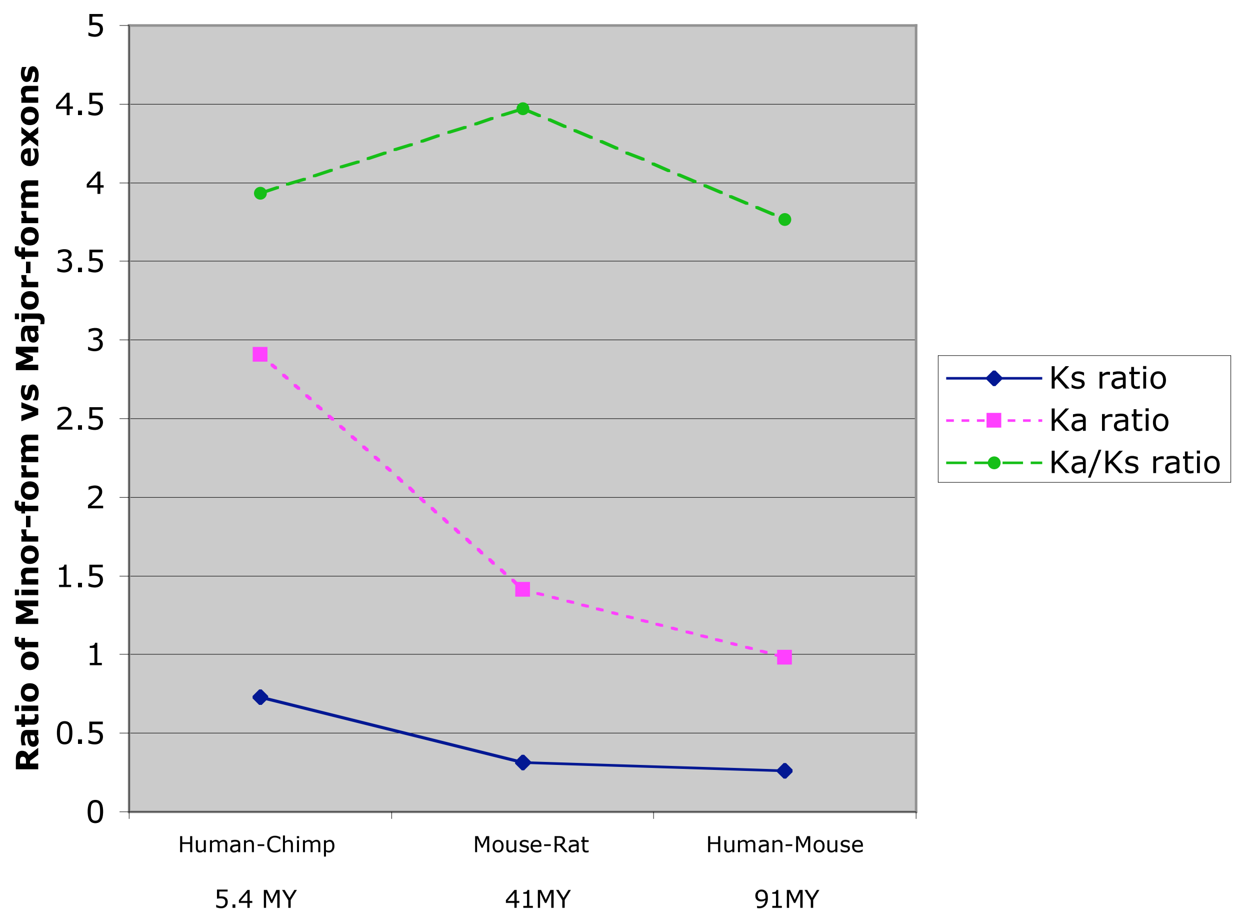
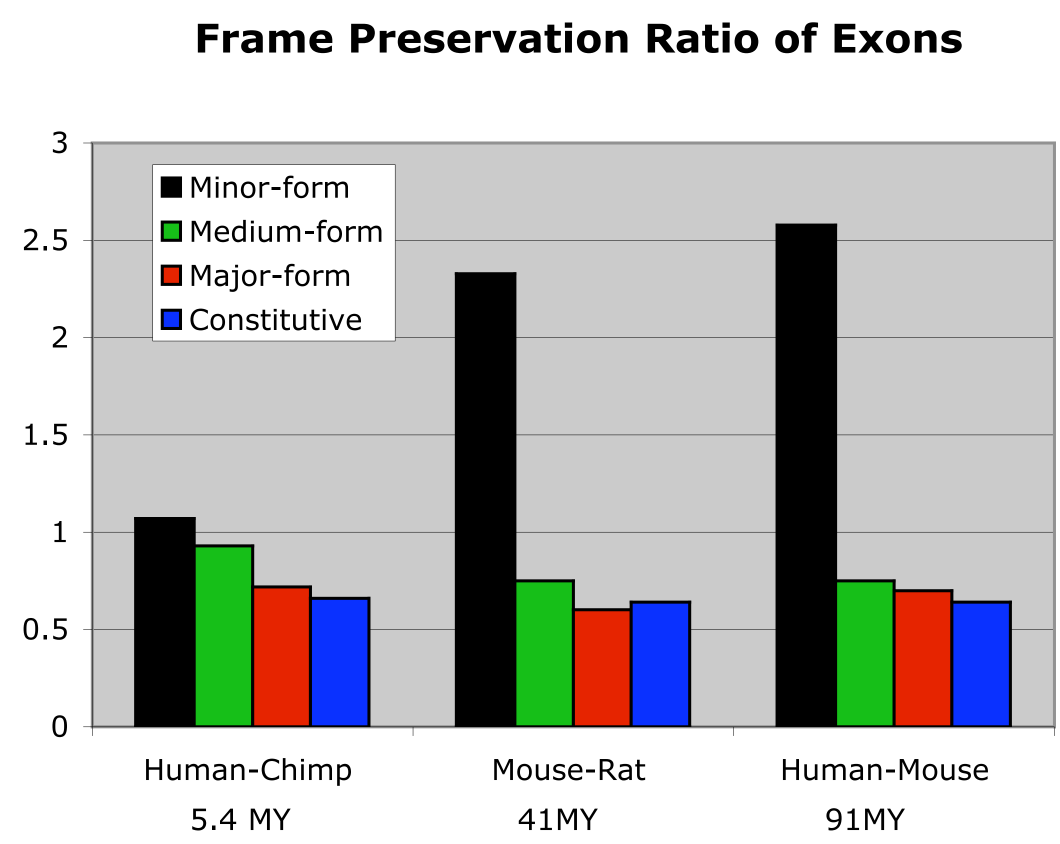

Ratio of Minor-form vs Major-form exons
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Human-Chimp
5.4 MY
Mouse-Rat
41MY
Human-Mouse
91MY
Ks ratio
Ka ratio
Ka/Ks ratio

Fig 3



Frame Preservation Ratio of Exons
3
2.5
2
1.5
1
0.5
0
Minor-form
Medium-form
Major-form
Constitutive
Human-Chimp
5.4 MY
Mouse-Rat
41MY
Human-Mouse
91MY

Fig 4