



## Journal Article

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# Estimates of Positive Darwinian Selection Are Inflated by Errors in Sequencing, Annotation, and Alignment

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Published estimates of the proportion of positively selected genes (PSGs) in human vary over three orders of magnitude. In mammals, estimates of the proportion of PSGs cover an even wider range of values. We used 2,980 orthologous protein-coding genes from human, chimpanzee, macaque, dog, cow, rat, and mouse as well as an established phylogenetic topology to infer the fraction of PSGs in all seven terminal branches. The inferred fraction of PSGs ranged from 0.9% in human through 17.5% in macaque to 23.3% in dog. We found three factors that influence the fraction of genes that exhibit telltale signs of positive selection: the quality of the sequence, the degree of misannotation, and ambiguities in the multiple sequence alignment. The inferred fraction of PSGs in sequences that are deficient in all three criteria of coverage, annotation, and alignment is 7.2 times higher than that in genes with high trace sequencing coverage, “known” annotation status, and perfect alignment scores. We conclude that some estimates on the prevalence of positive Darwinian selection in the literature may be inflated and should be treated with caution.

## Introduction

A general consensus in molecular evolution is that the vast majority of protein-coding genes evolve by random genetic drift. It is also a common lore that genes exhibiting signs of positive Darwinian selection are the “interesting” ones. Much of the recent molecular evolution literature deals with detecting positive selection on a genome-wide scale, sometimes as a tool for gaining insight into function. A wide variety of methods for identifying positively selected genes (PSGs) have been introduced. One of the most commonly used methods is the nonsynonymous to synonymous ratio test pioneered by Li and Gojobori (1983) and Hill and Hastie (1987) and explicitly put forward by Hughes and Nei (1988).

Nonsynonymous changes are far more likely than synonymous changes to improve the function of a protein, that is, to be advantageous. Because advantageous mutations undergo fixation in a population much more rapidly than neutral mutations and because most synonymous substitutions may be assumed to be neutral, the rate of nonsynonymous substitution should exceed that of synonymous substitution if positive Darwinian selection plays a major role in the evolution of a protein-coding gene. Therefore, an excess of nonsynonymous substitutions over synonymous ones is frequently used as an indicator of positive selection at the molecular level.

By using the ratio of nonsynonymous to synonymous substitution ( $dN/dS$ ), it has been shown that the proportion of PSGs varies widely among and within taxonomic lineages. However, the results vary widely depending on taxonomic sampling, sequence sampling, quality filtering, and estimation model. For example, the proportion of PSGs in human was estimated to be 0.02% (Gibbs et al. 2007), 0.07% (Kosiol et al. 2008), 1.1% (Bakewell et al. 2007), 1.5% (Arbiza et al. 2006), 5.2% (Jorgensen et al. 2005), and 8.7% (Clark et al. 2003): a 435-fold range. Some such results were extremely surprising in other respects. In

one such study, positive selection was detected in 77% of vertebrate genes (Studer et al. 2008). In another, the claim was made that the fraction of PSGs in chimpanzees is almost twice that in humans (Bakewell et al. 2007).

Because errors in sequencing, annotation, and alignment are unaffected by codon position and subsequent effect on the amino acid sequence, they are equally likely to affect synonymous as nonsynonymous sites. Because most nonsynonymous mutations are selected against, the vast majority of genes exhibit ratios of nonsynonymous to synonymous substitution that are lower than 1. On the other hand, errors that equally affect synonymous and nonsynonymous sites add a noise component to  $dN/dS = 1$ . Although low levels of such “neutral” noise will not generally raise the overall ratio above 1, estimation models that allow for several categories of sites may detect the noise component as a separate category. Consequently, such models will inflate the fraction of inferred PSGs within a genome.

Here, we investigate quantitatively the effects of errors in sequencing, annotation, and alignment on inferences of positive selection in genomic studies.

## Data and Methods

### Sequence Data

Genomic data from seven eutherian mammalian species were downloaded from Ensembl (Hubbard et al. 2007) and converted to the Darwin database format (Gonnet, Hallett, et al. 2000). The National Center for Biotechnology Information (NCBI) version numbers were 36 for *Homo sapiens*, NCBI m36 for *Mus musculus*, PanTro2.1 for *Pan troglodytes*, Mmul 1.0 for *Macaca mulatta*, RGSC 3.4 for *Rattus norvegicus*, CanFam 2.0 for *Canis familiaris*, and Btau 3.0 for *Bos taurus*. The reason for restricting the data to these species is that their relative phylogenetic position in the eutherian tree is unambiguous (Murphy et al. 2001). Adding more eutherian or noneutherian mammals would have been phylogenetically contentious (e.g., Cannarozzi et al. 2007).

### Orthologous Protein-Coding Sequences

Orthologous sequences were taken from the mammalian data set in Orthologs Matrix project (OMA; Dessimoz

Key words: multiple sequence alignment, sequencing quality, gene annotation, positive Darwinian selection.

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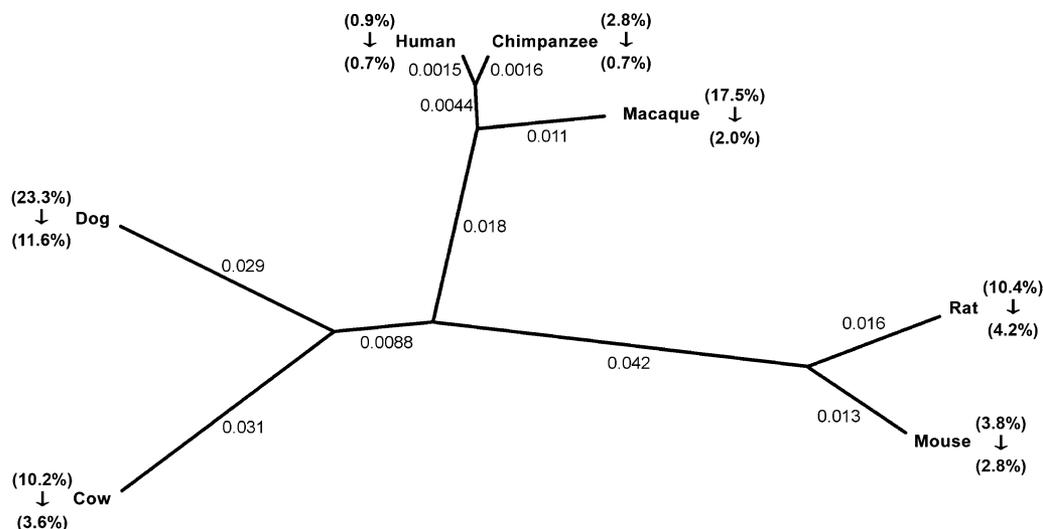


FIG. 1.—Unrooted scaled phylogenetic tree for seven eutherian species. The numbers on the branches are the mean numbers of nonsynonymous substitutions per nonsynonymous sites as inferred from the CODEML analysis. The percentages in parentheses above the downward pointing arrow next to the species name indicate inferred percentages of PSGs in the terminal branch leading from the immediate ancestor to the species when all genes are used. The percentages in parentheses below the downward pointing arrow indicate inferred percentages of PSGs when only all good genes are used. In human, in which a record of trace data has not been kept, all good indicates known annotation status and 100% HoT scores.

et al. 2005; Schneider et al. 2007). Putative orthologous proteins for the seven species under consideration were found in 9,942 OMA groups. Of these, 1,227 contained a coding sequence (CDS) of less than 200 codons and in 5,089 groups, the longest sequence was more than 5% longer than the shortest one. Given the stochastic errors associated with short sequences and the uncertainties of alignment due to indels, we decided not to use these sequences. (These groups may also possibly contain fragments of actual proteins and were, therefore, excluded from further analysis.) Additionally, six groups were excluded because at least one of the CDSs had more than 1% of its bases listed as missing or unknown. The proteins in each group were aligned using the Darwin multiple sequence alignment package (Gonnet, Hallett, et al. 2000; Gonnet, Korostensky, and Benner 2000). The coding DNA was, then, mapped to the aligned amino acids in order to construct codon-wise alignments of the CDSs.

#### Identification of Positive Selection

The CODEML program from version 3.14b of PAML (Yang 1997) was used to estimate branch lengths and nonsynonymous ( $dN$ ) and synonymous ( $dS$ ) rates for each branch of the tree. All analysis was performed on the tree shown in figure 1. The codon frequencies were derived from the average nucleotide frequencies at the three-codon position ( $F3 \times 4$  model), and the model was chosen to compute one ratio of nonsynonymous over synonymous substitution ( $dN/dS$ ) per branch but fixed for all sites (model = 1 and NSsites = 0). In 758 cases (orthologous groups), at least one branch had a length of 0. Such sequences are not suitable for ratio calculations and, hence, were excluded, leaving us with a final data set of 2,980 orthologous trees on which the subsequent analyses were performed. The multiply aligned data are available as Sup-

plementary Material (see Supplementary Material online for alignments).

PSGs were then identified using the improved branch-site model as implemented in CODEML. We performed “test 2” from Zhang et al. (2005) under “model A,” where for the null hypothesis it is assumed that some sites are selected ( $\omega < 1$ ) and some are neutral ( $\omega = 1$ ), whereas for the alternative hypothesis, some sites are allowed to experience positive selection ( $\omega > 1$ ) on one or more a priori-specified “foreground branches.” Each of the seven terminal branches was set up as foreground separately, whereas all other branches were designated as background at that time. Following the recommendations from PAML, CODEML was run three times for the null hypothesis and three times for each alternative with the initial branches set to a random value between 0.8 and 1.2 times the previously estimated branch lengths, allowing for three randomized runs of the optimization process, which increases the chances of reaching the global optimum. The highest likelihood for each alternative was saved for the likelihood ratio test (LRT). Finally, PSGs were identified by comparing the LRT values to the distribution. Rom (1990) correction for multiple testing and a false discovery rate of 5% were applied. Rom (1990) correction has been recently shown to be the very accurate (Anisimova and Yang 2007), but other corrections yielded essentially the same results (data not shown).

#### Quality of Sequences

The quality of each CDS was assessed by the degree of coverage in the trace database (Trace Archive, <http://www.ncbi.nlm.nih.gov/Traces/home/>). Trace data for the human genome have unfortunately not been kept. Each CDS was Blasted against its appropriate trace archive. Only hits with identities of 95% or higher were considered. We experimented with thresholds other than 95%, but the

**Table 1**  
Inferred Percentage of PSGs as a Function of Sequencing Coverage

	Coverage $\geq 3\times$			Coverage $< 3\times$			$P(\chi^2)$
	Total	PSG	% PSG	Total	PSG	% PSG	
Human	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Chimp	1,144	9	0.8	1,836	74	4.0	$9.6 \times 10^{-8}$
Macaque	896	32	3.6	2,084	488	23.4	$8.1 \times 10^{-46}$
Mouse	2,493	77	3.1	487	37	7.6	$1.3 \times 10^{-6}$
Rat	1,841	93	5.1	1,139	217	19.1	$3.2 \times 10^{-38}$
Dog	1,568	212	13.5	1,412	481	34.1	$1.7 \times 10^{-49}$
Cow	1,086	54	5.0	1,894	249	13.1	$4.8 \times 10^{-14}$
Total	9,028	477	5.3	8,852	1,546	17.5	$2.3 \times 10^{-166}$

NOTE.—N/A, Not available.

results (not shown) did not affect the conclusions. For each position in the CDS, the number of hits overlapping this position were counted and used as a measure of sequencing coverage. We divided the sequences into two categories: those in which parts were covered by less than three trace sequences and those in which all the sequence was covered at least three times.

#### Quality of the Alignment

The quality of the multiple sequence alignment was determined with the Heads or Tails (HoT) algorithm (Landan and Graur 2007). Briefly, this methodology is based upon the a priori expectation that sequence alignment results should be independent of the orientation of the input sequences. Thus, for totally unambiguous cases, reversing residue order prior to alignment should yield an exact reversed alignment of that obtained by using the unreversed sequences. The degree of agreement between these two alignments may be used to assess the reliability of the sequence alignment. In this study, we divided the sequences into unambiguous (100% agreement between the head and tail alignments) and ambiguous alignments.

#### Degree of Certainty in Gene Annotation

We used the gene annotation status in Ensembl to divide the genes into two categories: known genes and novel genes. “Ensembl known genes” are predicted on the basis of species-specific database entries from manually curated UniProt/Swiss-Prot, partially manually curated RefSeq, and UniProt/TrEMBL databases. Predictions of “Ensembl novel genes” are based on other experimental evidence such as protein and cDNA sequence information from related species ([http://www.ensembl.org/Homo\\_sapiens/glossaryview](http://www.ensembl.org/Homo_sapiens/glossaryview)). We, therefore, have more confidence in the veracity of the annotation of the “known” category than that in the “novel” (or “inferred”) category.

#### Results

We, first, used the trace database to divide the 17,880 genes from six species (trace data for the human genome are not available) into ones that have poorly sequenced regions (less than three times coverage) and genes, in which all parts have been sequenced at least three times. The results

**Table 2**  
Inferred Percentage of PSGs as a Function of Annotation Status

	Known Genes			Inferred Genes			$P(\chi^2)$
	Total <sup>a</sup>	PSG	% PSG	Total <sup>a</sup>	PSG	% PSG	
Human	2,833	21	0.7	95	5	5.3	$3.5 \times 10^{-6}$
Chimp	318	17	5.3	2,653	66	2.5	0.0031 <sup>b</sup>
Macaque	139	13	9.4	2,840	507	17.9	0.0082
Mouse	2,924	108	3.7	56	6	10.7	0.0063
Rat	2,696	224	8.3	284	86	30.3	$8.9 \times 10^{-33}$
Dog	2,568	491	19.1	412	202	49.0	$7.6 \times 10^{-46}$
Cow	2,670	206	7.7	309	97	31.4	$1.8 \times 10^{-41}$
Total	14,148	1,080	7.6	6,649	969	14.6	$1.4 \times 10^{-61}$

<sup>a</sup> The sums of the two total numbers of genes in each species do not always add up to 2,980 because some genes lack annotation status in the databank.

<sup>b</sup> The difference is in the opposite direction.

are shown in table 1. In all cases, the fraction of genes that have been inferred to evolve by positive selection is significantly higher in poorly covered genes. For the pooled results, the difference is striking with the proportion of PSGs in lowly covered sequences being 3.3 times higher than that in highly covered sequences.

We, then, divided the sequences by annotation status into known and inferred (see Data and Methods). The results are shown in table 2. The known category had a significantly smaller fraction of PSGs than the inferred category. In the pooled data, the proportion of PSGs in putative genes was 1.9 times higher than that in known ones.

In the third step (table 3), we divided the sequences by the quality of alignment into those for which the alignment was judged to be unambiguous by the HoT methodology (Landan and Graur 2007) and those that contained alignment ambiguities. PSGs were less likely to appear in the perfectly aligned category. In the pooled data, the proportion of PSGs in ambiguously aligned genes was 1.6 times higher than that in perfectly aligned ones.

Finally (table 4), we compared the inferred percentages of PSGs between genes with high trace sequencing coverage, known annotation status, and 100% alignment HoT scores (henceforth “all good” genes) and genes that are deficient in all three criteria of coverage, annotation, and alignment (henceforth “all bad” genes). In all cases,

**Table 3**  
Inferred Percentage of PSGs as a Function of Alignment Quality (HoT)

	HoT Score = 100%			HoT Score < 100%			$P(\chi^2)$
	Total	PSG	% PSG	Total	PSG	% PSG	
Human	2,805	23	0.8	175	3	1.7	0.22
Chimp	2,805	78	2.8	175	5	2.9	0.95
Macaque	2,805	468	16.7	175	52	29.7	$5.6 \times 10^{-6}$
Mouse	2,805	99	3.5	175	15	8.6	$6.3 \times 10^{-4}$
Rat	2,805	284	10.1	175	26	14.9	0.042
Dog	2,805	630	22.5	175	63	36.0	$2.1 \times 10^{-5}$
Cow	2,805	276	9.8	175	27	15.4	0.015
Total	19,635	1,858	9.5	1,225	191	15.6	$8.2 \times 10^{-13}$

NOTE.—The quality is determined for the whole multiple sequence alignment, so the numbers of unambiguous and ambiguous alignments is the same for all species.

**Table 4**  
**Differences in Inferred Percentage of PSGs between Genes with High Trace Sequencing Coverage, Known Annotation Status, and 100% Alignment HoT Scores (all good genes) and Genes that Are Deficient in Coverage, Annotation, and Alignment (all bad genes)**

	All Good Genes			All Bad Genes			$P(\chi^2)$
	Total	PSG	% PSG	Total	PSG	% PSG	
Human <sup>a</sup>	2,717	19	0.7	10	1	10.0	$5.7 \times 10^{-4}$
Chimp	137	1	0.7	107	3	2.8	0.2
Macaque	49	1	2.0	134	49	36.6	$2.9 \times 10^{-7}$
Mouse	2,315	65	2.8	0	0	—	—
Rat	1,636	68	4.2	19	8	42.1	$2.8 \times 10^{-15}$
Dog	1,367	159	11.6	29	20	69.0	$2.4 \times 10^{-20}$
Cow	965	35	3.6	21	7	33.3	$1.8 \times 10^{-11}$
Total	9,186	348	3.8	320	88	27.5	$2.4 \times 10^{-90}$

<sup>a</sup> No record of trace data has been kept for human, thus all good indicates known annotation status and 100% HoT scores, and all bad indicates inferred annotation status and less than perfect HoT scores.

except mouse, whose genome does not have all bad genes, the differences in inferred PSG were highly significant. In the pooled data, the proportion of PSGs in all bad genes was 7.3 times higher than that in all good genes.

## Discussion

Our results indicate that the worse the quality of the sequence is, the greater the proportion of PSGs will appear to be. We note that recent developments in technology allow for genomes to be cheaply and rapidly sequenced and that assembly programs are now able to infer contigs from partial trace sequences with little overlap between neighboring ones. As a result, although the quantity of genomic sequences accumulating in databanks is growing exponentially, the quality of the sequences may be concomitantly deteriorating.

We have also shown that uncertainties in alignment and annotation contribute to overestimating the ratio of nonsynonymous to synonymous substitution rates and, consequently, to overestimating the fraction of PSGs. Problems in sequence quality, annotation, and alignment are not independent factors as shown by the fact that their effects are not cumulative. However, they all work in the same direction, inflating estimates of positive selection. Other factors, such as purifying selection on synonymous sites or failure to identify orthology correctly, may also contribute to the overestimation of ratios of nonsynonymous to synonymous substitutions. In figure 1, we see that the estimates of the fraction of PSGs drop precipitously when only good quality sequences that are unambiguously annotated and aligned are considered. In macaque, for instance, the inferred proportion of PSGs drops from 17.5% to almost an order of magnitude smaller (2.0%). Interestingly, when using all good genes, the fraction of PSGs in chimpanzees becomes about equal that in humans, as opposed to the claim that it is twice that in humans (Bakewell et al. 2007).

Finally, we note that there is a strong positive correlation between the inferred fraction of PSGs and branch lengths. If all genes are used, the correlation coefficient is  $r = 0.67$  ( $P = 0.098$ ); if only all good genes are used,

the correlation coefficient is  $r = 0.79$  ( $P = 0.053$ ). These findings indicate that despite the use of sophisticated methods to compensate for saturation in synonymous substitution, we may still have serious underestimations of synonymous rates, resulting in overestimates of the ratio of nonsynonymous to synonymous substitution. For example, in the dog–cow pairwise comparisons, 7.3% of the genes had  $dS > 0.7$ , 13.7% had  $dS > 0.6$ , and 26.0% had  $dS > 0.5$ .

We conclude that estimates on the prevalence of positive Darwinian selection may be inflated and should be treated with caution.

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## Supplementary Material

Supplementary Alignments are available at *Genome Biology and Evolution* online ([http://www.oxfordjournals.org/our\\_journals/gbe/](http://www.oxfordjournals.org/our_journals/gbe/)).

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