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Analyses of human–chimpanzee orthologous gene pairs to explore evolutionary hypotheses of aging

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Abstract

Compared to chimpanzees (*Pan troglodytes*), the onset of aging appears to be delayed in the human species. Herein, we studied human–chimpanzee orthologous gene pairs to investigate the selective forces acting on genes associated with aging in different model systems, which allowed us to explore evolutionary hypotheses of aging. Our results show that aging-associated genes tend to be under purifying selection and stronger-than-average functional constraints. We found little evidence of accelerated evolution in aging-associated genes in the hominid or human lineages, and pathways previously related to aging were largely conserved between humans and chimpanzees. In particular, genes associated with aging in non-mammalian model organisms and cellular systems appear to be under stronger functional constraints than those associated with aging in mammals. One gene that might have undergone rapid evolution in hominids is the Werner syndrome gene. Overall, our findings offer novel insights regarding the evolutionary forces acting on genes associated with aging in model systems. We propose that genes associated with aging in model organisms may be part of conserved pathways related to pleiotropic effects on aging that might not regulate species differences in aging.

Keywords: Comparative genomics; Life history; Longevity; Natural selection

1. Introduction

Genome comparisons between humans and our closest living evolutionary relatives, chimpanzees (*Pan troglodytes*), may shed light on the genetic changes involved in the evolution of the unique human traits (Watanabe et al., 2004; Chimpanzee Sequencing and Analysis Consortium, 2005; Varki and Altheide, 2005). A first step to understand the evolution of human features is to study the selective forces that originated them. Typically, natural selection shapes the evolution of species through one of two mechanisms: purifying selection, which promotes the conservation of existing phenotypes, and positive selection, which favors the emergence of new phenotypes (Vallender and Lahn, 2004). Despite the low sequence divergence between human and chimpanzee genes, it is possible to study these selective forces throughout the genome and identify signatures of evolutionary changes that can provide insights about the evolution of human-specific traits (Chimpanzee Sequencing and Analysis Consortium, 2005; Nielsen et al., 2005).

The most commonly used method to study selective forces is the K_a/K_s test, where K_a and K_s represent the number of substitutions per, respectively, nonsynonymous and synonymous sites between two protein-coding sequences. Nonsynonymous mutations result in changes in the amino acid sequence of the protein while synonymous mutations do not. Although K_a gives an estimate of a gene's rate of evolution, because mutation rates vary across the genome, K_s is used to estimate the background mutation rate and normalize K_a for gene comparisons. In the classical test, if nucleotide substitutions occur through a purely random process $K_a/K_s = 1$, which indicates neutral selection, $K_a/K_s < 1$ indicates purifying selection or structural constraints and $K_a/K_s > 1$ indicates, though it does not necessarily prove or disprove, positive selection. Even though post-translational and regulatory differences are impossible to detect using this method, the K_a/K_s test has proven to be a powerful and accurate tool in detecting selective pressures across groups of genes and even to provide clues about the selective pressures acting on individual

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genes (Clark et al., 2003; Dorus et al., 2004; Vallender and Lahn, 2004; Chimpanzee Sequencing and Analysis Consortium, 2005; Nielsen et al., 2005).

In this work, we studied the selective forces operating since the divergence of humans and chimpanzees 5–7 million years ago (Varki and Altheide, 2005). While differences in cognitive functions, speech, or bipedalism have so far received the bulk of attention in such studies (Clark et al., 2003; Dorus et al., 2004; Watanabe et al., 2004; Chimpanzee Sequencing and Analysis Consortium, 2005; Nielsen et al., 2005), one major difference between chimpanzees and humans concerns life history traits: development, all growth phases, and lifespan are considerably extended in humans when compared to chimpanzees (McKinney and McNamara, 1991). Life history theory predicts that under lower adult mortality rates selection will favor a later maturity and a longer lifespan (reviewed in Charnov, 1993). Indeed, it has been estimated that the adult life expectancy of chimpanzees in the wild is about 60% less than it was for human hunter-gatherers (Hill et al., 2001). Nonetheless, little is known concerning the genetic mechanisms responsible for the evolution of human life history.

Of great interest to biomedical research is the fact that the onset of aging appears to occur at later ages in humans, even when compared to chimpanzees in captivity. Humans are longer-lived than chimpanzees, with a record longevity of 122 years compared to 74 years in chimpanzees, albeit the vastly larger sample size for humans may overestimate the difference between the two species. Importantly, humans appear to feature a delayed onset of several age-related diseases and a later acceleration of mortality, a common characteristic of aging (Finch, 1990; Hill et al., 2001; Erwin et al., 2002; Morbeck et al., 2002; Finch and Stanford, 2004; de Magalhaes, 2006). Such differences in aging might be explained by changes in extrinsic mortality rates during the evolution of hominins (herein used to refer to the lineage leading to modern humans whereas hominids refers to humans and the great apes) that possibly affect selection on the pathways that govern aging (Kirkwood and Austad, 2000). Even if such hypothesis is correct, however, and even though it is clear that genetic alterations must be responsible for the delayed onset of aging in humans (Miller, 1999; de Magalhaes, 2003; Partridge and Gems, 2006), the essence of those genetic changes remains largely unknown.

Because natural selection is the ultimate cause of aging, evolutionary biology is crucial to understand the aging phenotype (Rose, 1991). From an evolutionary perspective, aging can be seen as a polygenic and highly complex process whose phenotype changed markedly since chimpanzees and humans shared their last common ancestor. Herein, we took advantage of the recent sequencing of the chimpanzee and human genomes to investigate – using K_a/K_s ratios – the selective forces acting on genes previously related to aging in model systems, which in turn allowed us to explore evolutionary hypotheses of aging. One controversial topic in evolutionary models of aging concerns the extent to which mechanisms of aging are conserved (''public'') across diverse organisms (Martin et al., 1996; Partridge and Gems, 2002). Here, we explored whether mechanisms of aging identified in different model organisms may also specify species differences in aging (Partridge and Gems, 2006). We also investigated whether the evolution of longevity in hominins may be due to multiple changes in a wide range of defensive and repair mechanisms (Partridge and Gems, 2002; Kirkwood, 2005). Lastly, mutations in the *ASPM* and *MCPH1* genes result in microcephaly, and evidence of positive selection in these genes in the primate lineage leading to humans has been used to hypothesize that they were involved in the evolution of brain size (reviewed in Vallender and Lahn, 2004). Following this rationale, we explored whether genes associated with premature aging phenotypes in humans may also be potential targets of positive selection.

2. Materials and methods

2.1. Sources and types of data

We employed the datasets assembled by the Chimpanzee Sequencing and Analysis Consortium (2005), consisting of 13,454 human–chimpanzee orthologous gene pairs plus 7043 four-way gene alignments of human, chimpanzee, mouse, and rat orthologs. The orthology of these gene alignments is considered unambiguous. K_a/K_s ratios for each alignment were previously calculated by the Chimpanzee Sequencing and Analysis Consortium (2005) as well as estimates of lineage-specific protein changes. Given the similarity between human and chimpanzee genes, however, there will be few, and not infrequently zero, synonymous substitutions. To obviate this problem, another estimate of background mutation rates used was K_i, which is calculated based on the local intergenic/intronic substitution rate. K_a/K_i ratios have the same interpretation as K_a/K_s ratios, and K_a/K_i ratios provide an additional control for false positives when looking at individual genes. The average K_a/K_s ratio for human–chimpanzee orthologous gene pairs was 0.23. The average K_a/K_i ratio was also 0.23 with 4.4% of the genes with $K_a/K_i > 1$ and 29% of human and chimpanzee orthologs encoding identical proteins. For four-way comparisons, the average K_a/K_s ratio in hominids was 0.20 while in murids it was 0.13 (Chimpanzee Sequencing and Analysis Consortium, 2005). We also used the dataset of Clark et al. (2003), which consists of 7645 human–chimpanzee–mouse alignments, to discriminate between selection in the chimpanzee lineage and selection in the human lineage and attempt to infer accelerated evolution in the human lineage. *p*-Values from Model 2 in the calculations by Clark et al. (2003), which give a likelihood ratio test of rejecting the null hypothesis of no positive selection in the human lineage, were used in conjunction with the dataset of the Chimpanzee Sequencing and Analysis Consortium (2005).

Rhesus monkeys (*Macaca mulatta*), a species of Old World monkeys that diverged from apes about 25 million years ago, live up to 40 years and their onset of aging occurs at considerably earlier ages than in humans or chimpanzees; mice (*Mus musculus*) and rats (*Rattus norvegicus*), rodents that diverged from primates roughly 80 million years ago, live less than 5 years and age considerably faster than primates (Finch, 1990; de Magalhaes, 2006). To serve as an additional test, human–rhesus, human–mouse, and human–rat homologous gene pairs were downloaded from ENSEMBL v.37 (Birney et al., 2006). K_a/K_s ratios were obtained only for genes for which we had the highest confidence, and we excluded pairs such as pseudogenes and poorly annotated genes. (Nonetheless, not eliminating gene pairs at all for any of these datasets did not significantly affect our general results and conclusions.) Overall, for 9857 human–rhesus genes the average K_a/K_s ratio was 0.20, for 12,063 human–mouse genes the average K_a/K_s ratio was 0.13, and for 11,594 human–rat genes the average K_a/K_s ratio was 0.13. Where applicable, we also employed orangutan (*Pongo pygmaeus*) genes to infer whether differences between human and chimpanzee proteins were due to human- or chimpanzee-lineage specific changes.

2.2. Selection of putative aging-related genes

To study genes previously related to aging, we obtained a list of 243 curated human genes, of which 242 encode proteins, associated with aging in different

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model systems from the GenAge dataset (de Magalhaes et al., 2005). These genes are candidates for determinants of human longevity and aging, though none of them have been proven to play a causal role in human aging and the degree of evolutionary conservation in function of genes and pathways that control aging is unclear. We also investigated a group of 14 genes that have been associated with human longevity or survival in old age. Both lists of genes are available online (http://genomics.senescence.info/evolution/chimp.html).

In addition, we used Gene Ontology (GO) annotation, which describes how gene products behave in a cellular context (Ashburner et al., 2000), to select GO categories encompassing mechanisms or functions previously associated with aging in model systems or hypothesized to be related to aging (reviewed in Martin et al., 1996; Partridge and Gems, 2002; de Magalhaes, 2005a). Briefly, we selected GO categories related to DNA repair and processing based on empirical evidence of an association between these pathways and aging in model systems (e.g., Fraga et al., 1990), including DNA repair (reviewed in Hasty et al., 2003; Lombard et al., 2005; Martin, 2005), DNA methylation (reviewed in Richardson, 2003), and protein amino acid ADP-ribosylation (reviewed in Burkle et al., 2004). Stress, oxidative stress, the electron transport chain, and hypoxia have also been hypothesized to play a role in aging (reviewed in Sohal and Weindruch, 1996; Cadenas and Davies, 2000; Finkel and Holbrook, 2000; Katschinski, 2006), as supported by experimental results from model systems (e.g., Lin et al., 1998; Migliaccio et al., 1999; Moskovitz et al., 2001). There is also abundant empirical evidence associating hormones, and neuropeptide hormones in particular, with aging in a number of model organisms (Brown-Borg et al., 1996; Flurkey et al., 2001; Tatar et al., 2003). Other aging-associated pathways in model organisms that we selected include ubiquitin (reviewed in Gray et al., 2003), heat shock proteins (Yokoyama et al., 2002), JNK (Wang et al., 2003), glutathione transferase enzymes (Ayyadevara et al., 2005), and histone deacetylase genes (Rogina et al., 2002).

2.3. Calculating average K_a/K_s ratios for gene categories

The K_a and K_s values of GO categories were taken from the Chimpanzee Sequencing and Analysis Consortium (2005). To calculate K_a and K_s values for other gene categories or groups, such as GenAge or a GenAge subset, we employed the method used by the Chimpanzee Sequencing and Analysis Consortium (2005) to calculate the K_a/K_s ratio for each GO category. Values of K_a and K_s concatenated for all genes in a given category C were calculated using:

$$K_a = \frac{\sum_{i \in C} a_i}{\sum_{i \in C} A_i} \quad \text{and} \quad K_s = \frac{\sum_{i \in C} s_i}{\sum_{i \in C} S_i}$$

being a_i and A_i the number of nonsynonymous substitutions and sites and being s_i and S_i the number of synonymous substitutions and sites per gene i in category C.

2.4. Detecting rapidly and slowly evolving gene categories

We employed the binomial probability method used by the Chimpanzee Sequencing and Analysis Consortium (2005) to identify rapidly (i.e., with higher-than-average K_a/K_s ratios) and slowly (i.e., with lower-than-average K_a/K_s ratios) evolving GO categories. To identify other gene categories potentially undergoing rapid or slow evolution, we also employed the method used by these authors. The binomial probability is typically used to calculate the probability of several successive events, each with two possible outcomes. Herein, the events are substitutions which can be synonymous or nonsynonymous. Assuming a binominal distribution, we calculated the binomial probability as follows (Chimpanzee Sequencing and Analysis Consortium, 2005).

The probability p_A of a nonsynonymous substitution event occurring in a given category C was estimated from the expected proportion of nonsynonymous substitutions using:

$$p_A = \frac{k_a \sum_{i \in C} A_i}{k_a \sum_{i \in C} A_i + k_s \sum_{i \in C} S_i}$$

where k_a and k_s represent average K_a and K_s values for all human–chimpanzee gene pairs. Consequently, for the total number of substitution events in category C, the probability p_C that we observe an equal or higher number of nonsynonymous substitutions was calculated as:

$$p_C = \sum_{j=a_C}^{a_C+s_C} \binom{a_C + s_C}{j} {p_A}^j (1 - p_A)^{a_C+s_C-j}$$

being a_C and s_C the total number of nonsynonymous and synonymous substitutions in category C. Likewise, we identified slowly evolving categories by calculating the probability of observing an equal or smaller number of nonsynonymous substitutions.

This method allowed us to identify categories that have a K_a/K_s ratio significantly above or below the average. The binomial probability does not allow a direct rejection of the null hypothesis of no acceleration or no constraint in a particular category. Instead, it is a metric designed to detect the most extreme outliers that are potentially undergoing rapid or slow evolution. Based on simulations conducted by the Chimpanzee Sequencing and Analysis Consortium (2005), its cut-off value was set at 0.001. With test statistic <0.001, 98 GO categories were found to feature elevated K_a/K_s ratios but only 30 would be expected by chance. This means that most, but not all, of these categories have undergone rapid evolution when compared to the genome-wide average. Similarly, 251 GO categories were found to feature lower K_a/K_s ratios when only 32 would be expected by chance (Chimpanzee Sequencing and Analysis Consortium, 2005).

2.5. Genome coverage

Because the dataset of the Chimpanzee Sequencing and Analysis Consortium (2005) is derived from a draft sequence of the chimpanzee genome, not all human genes have been matched to orthologs in the chimpanzee genome and so our results should not be considered as comprehensive. For instance, of GenAge's 242 protein-coding genes, 211 could be correctly analyzed. Similarly, of GenAge's 242 protein-coding genes, we obtained 169 human–rhesus, 216 human–mouse, and 205 human–rat gene pairs that could be correctly analyzed. Therefore, genes of potential interest may have been left out of our analysis, but trends in groups – such as GO or GenAge categories – with a large number of genes are unlikely to change even when complete results are available.

2.6. Computational and statistical analyses

To retrieve the genes of interest and analyze our results, we designed and implemented an automated system of modules written in Perl. The system is part of the Ageing Research Computational Tools (ARCT, described in de Magalhaes et al., 2005) and is used to retrieve, parse, and data-mine the different datasets used in this work: the datasets of the Chimpanzee Sequencing and Analysis Consortium (2005), the GenAge dataset, and the dataset of Clark et al. (2003). The system then generates a new set of results, such as K_a/K_s ratios for each GenAge category, which can be statistically analyzed.

As mentioned above, we employed the statistical tests used by the Chimpanzee Sequencing and Analysis Consortium (2005), which were also the ones used in similar works (e.g., Nielsen et al., 2005). The Mann–Whitney U-test (MWU) was used to compare different groups of genes. When comparing a subset of genes with the whole dataset we excluded the subset of genes from the dataset to avoid non-independence problems. Where necessary, computer simulations were conducted with randomly selected gene pairs to infer the probability of, by chance, observing our results. Each simulation involved 100,000 randomly permuted data sets. Statistical analyses were performed using the SPSS package version 11.5 (SPSS Inc., Chicago, IL).

3. Results

3.1. Selective forces acting on putative aging-related genes

To investigate the overall selective forces acting on genes associated with aging in model systems, we first compared the K_a/K_s ratios of these genes and pathways with the K_a/K_s ratio of all human–chimpanzee orthologous gene pairs. Typically,

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purifying selection is predominant in human genes and the average K_a/K_s ratio for human–chimpanzee orthologs is 0.23 (Chimpanzee Sequencing and Analysis Consortium, 2005). Groups of genes with significantly higher K_a/K_s ratios are putative cases of rapid evolution, and thus more likely to be associated with phenotypic changes, and vice versa. Consequently, we tried to find evidence of positive selection or, at least, evidence of rapid or accelerated evolution in pathways and genes previously related to aging, based on the assumption that such genes and pathways are more likely to specify the greater longevity of humans relative to chimpanzees. As detailed in Section 2, we used the datasets and statistical methods developed by the Chimpanzee Sequencing and Analysis Consortium (2005), which have been previously shown to be capable of detecting meaningful biological findings.

3.1.1. Purifying selection is predominant in aging-associated genes

We obtained 242 protein-coding human genes associated with aging, primarily in model organisms, from build 12 of the GenAge database (de Magalhaes et al., 2005), of which 211 genes could be correctly analyzed. While it is unclear what proportion, if any, of these 211 genes control human aging, it is reasonable to hypothesize that this list is enriched for such genes, and it allowed us to estimate the evolutionary pressures affecting putative aging-related genes. We were surprised to find that the GenAge dataset as a whole – i.e., using concatenated K_a and K_s values (see Section 2) – had a statistically significant lower K_a/K_s ratio (0.16) than the average (0.23) for human–chimpanzee orthologs ($p < 0.001$; MWU). This suggests that aging-associated genes tend to be under stronger functional constraints than what would be expected by chance. Likewise, functional categories present in GenAge appear to have undergone purifying selection since chimpanzees and humans diverged (Table 1), and we found little evidence of rapid evolution (but see below).

It is possible for functionally constrained genes to be linked to lineage-specific phenotypic changes, which can be detected as lineage-specific accelerated evolution in, for instance, hominids when compared to murids (Dorus et al., 2004). For all 7043 four-way gene alignments using chimpanzee, human, mouse, and rat orthologs computed by the Chimpanzee Sequencing and Analysis Consortium (2005), the average K_a/K_s ratio was 0.20 in hominids and 0.13 in murids. The average K_a/K_s ratio for genes in GenAge was 0.13 in hominids and 0.12 in murids, which does not suggest a general pattern of accelerated evolution of aging-associated genes in hominids. We also failed to detect accelerated evolution in GenAge's functional categories (Table 1). Categories like calcium metabolism ($n = 3$) and DNA condensation ($n = 8$) had considerably higher K_a/K_s ratios in the hominid lineage but given the small number of genes in each of these categories we cannot exclude these are false positives.

We also tried to find evidence of human lineage-specific protein changes in GenAge. Overall, there were 66 human lineage-specific changes compared to 55 chimpanzee lineage-specific changes. Based on simulations, the probability of observing such a higher proportion of human lineage-specific changes over chimpanzee lineage-specific changes in a group of genes with this many amino acid changes is 32%, which does not suggest that the proportion of human lineage-specific protein changes in GenAge is higher than what would be expected by chance. In GenAge categories the strongest outlier was growth and development proteins in which more than twice as many changes occurred in the human lineage than in the chimpanzee lineage (Table 1). This could suggest lineage-specific selection since the probability of observing such a high proportion of human lineage-specific changes is 1.2%. Lastly, we employed the dataset of Clark et al. (2003) to determine whether human lineage-specific evolution occurred in GenAge

Table 1
Selective forces operating on GenAge functional categories

GenAge category	n	K_a/K_s	p-Value high[a]	p-Value low[a]	n in four-way comparisons[b]	K_a/K_s in hominids	K_a/K_s in murids	*Homo* AA diff.[c]	*Pan* AA diff.[c]
Apoptosis	65	0.13	1	2.8E−9	43	0.082	0.10	14	15
Calcium metabolism	6	0.18	0.75	0.25	3	0.29	0.17	2	0
Cell cycle control	42	0.13	1	7.5E−7	26	0.057	0.11	10	9
DNA condensation	28	0.22	0.67	0.33	8	0.32	0.16	6	9
DNA repair	38	0.25	0.24	0.79	16	0.17	0.17	9	11
DNA replication	15	0.24	0.42	0.58	7	0.079	0.14	4	2
Energy apparatus	19	0.12	1	5.1E−5	12	0.13	0.082	7	6
Growth and development	66	0.14	1	5.9E−10	44	0.13	0.13	37	17
Other	47	0.17	1	2.9E−3	27	0.17	0.14	12	16
Redox and oxidative regulation	23	0.18	0.92	0.11	13	0.10	0.071	4	3
Signaling	52	0.13	1	1.9E−8	27	0.13	0.097	15	16
Stress response	31	0.14	1	2.4E−4	14	0.10	0.087	4	5
Transcriptional regulation	39	0.15	1	2.6E−3	22	0.062	0.084	3	7

[a] This binomial probability is not used to directly reject the null hypothesis of a given GenAge category not undergoing rapid (p-Value high) or slow (p-Value low) evolution but provides a metric to identify categories potentially undergoing rapid or slow evolution. The test statistic threshold was set at 0.001 (see Section 2).
[b] K_a/K_s ratios in hominids are based on four-way alignments and hence are expected to be different from K_a/K_s ratios based on only chimpanzee and human alignments, as described (Chimpanzee Sequencing and Analysis Consortium, 2005).
[c] Amino acid differences (AA diff.) represent amino acid changes estimated to have occurred specifically in the human (*Homo*) or chimpanzee (*Pan*) lineage.

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or its categories (see Section 2), but we found no evidence of accelerated evolution in the human lineage on any of these pathways (not shown).

Due to the close similarity between humans and chimpanzees, analyses of K_a/K_s ratios are more accurate when focusing on groups of genes. Nonetheless, results from individual genes in GenAge confirm our assessment that these putative aging-related genes tend to undergo purifying selection. Barring sequencing errors, and already excluding proteins with less than 95% of the human sequence aligned with the chimpanzee sequence, 56 proteins are perfectly conserved between humans and chimpanzees. By chance one would expect to find 43 such proteins—i.e., 29% of GenAge proteins aligned at least 95% with the chimpanzee orthologous sequence. Simulations indicate that the probability of observing such a high proportion of proteins perfectly conserved between humans and chimpanzees is 8.1%. Similarly, even though 4.4% of all genes have a K_a/K_i ratio above 1, only 2 of 211 genes in GenAge (*APOC3* and *PMCH*) fulfilled this criterion. Based on simulations, the probability of observing such a low number of genes with a K_a/K_i ratio above 1 is 0.45%. In addition to *BRCA1*, which previous results including linkage disequilibrium analysis have suggested to be under positive selection (Huttley et al., 2000), and *WRN* (see below), other genes in GenAge with potential for positive selection include *CDKN2A* ($K_a/K_i = 0.90$) and *EMD* ($K_a/K_i = 0.84$ and $K_a/K_s = 1.22$).

We also studied the K_a/K_s ratios of GenAge using human–rhesus, human–mouse, and human–rat gene pairs. Although shorter-lived and evolutionary and biologically more distant organisms from humans (see Section 2), these additional species provide a complementary model to human–chimpanzee gene pairs. Using human–rhesus gene pairs, the average K_a/K_s ratio for genes in GenAge was 0.16, significantly lower than the average (0.20) for all human–rhesus gene pairs ($p < 0.001$; MWU). The results from human–mouse and human–rat gene pairs did not confirm this idea, though, and the average K_a/K_s ratio of genes in GenAge was not lower than expected by chance (not shown). Therefore, it appears that the stronger-than-average functional constraints observed in GenAge genes in primates are not observed in the rodent lineage. We have no satisfactory explanation for this observation.

3.1.2. Selection in aging-associated GO categories

Of course, it can be argued that GenAge contains only a fraction, if any, of genes involved in human aging because most remain to be identified. To further investigate the selective forces acting on putative aging-related pathways and explore whether positive selection or rapid evolution may have acted on

Table 2
List of GO categories hypothesized to be related to aging[a]

GO[b]	Name	*n*	K_a/K_s	*p*-Value high[c]	*p*-Value low[c]
Biological process GO categories related to DNA repair or processing					
0006974	Response to DNA damage stimulus	148	0.27	9.0E−7	1
0006281	DNA repair	121	0.26	1.1E−4	1
0042770	DNA damage response, signal transduction	15	0.38	3.2E−4	1
0006306	DNA methylation	11	0.087	1	1.8E−4
0006471	Protein amino acid ADP-ribosylation	10	0.36	3.3E−3	1
0006298	Mismatch repair	9	0.32	5.8E−3	1
0006282	Regulation of DNA repair	5	1.1	4.6E−8	1
0030330	DNA damage response, signal transduction by p53 class mediator	3	1.4	1.3E−13	1
Other biological process GO categories					
0006950	Response to stress	594	0.27	1.1E−17	1
0006118	Electron transport	216	0.23	0.039	0.96
0006511	Ubiquitin-dependent protein catabolism	82	0.20	0.65	0.38
0006512	Ubiquitin cycle	70	0.10	1	4.6E−11
0006979	Response to oxidative stress	27	0.16	0.95	0.066
0007254	JNK cascade	23	0.12	1	2.6E−4
0006120	Mitochondrial electron transport, NADH to ubiquinone	12	0.12	0.97	0.054
0001666	Response to hypoxia	5	0.043	1	0.014
Molecular function GO categories					
0005179	Hormone activity	77	0.37	1.2E−8	1
0004840	Ubiquitin conjugating enzyme activity	44	0.094	1	5.1E−6
0003773	Heat shock protein activity	29	0.080	1	4.4E−8
0003684	Damaged DNA binding	24	0.37	8.5E−5	1
0005184	Neuropeptide hormone activity	15	0.39	4.8E−3	1
0004364	Glutathione transferase activity	14	0.50	4.5E−4	1
0004407	Histone deacetylase activity	8	0.14	0.95	0.082

[a] Although the topic remains highly contentious, and the mechanisms of aging in primates are largely unknown, we selected GO categories encompassing mechanisms or functions typically associated with aging in model systems or hypothesized to be related to aging (see Section 2).
[b] Subcategories without evidence of positive selection or rapid evolution were omitted unless they were deemed as potentially relevant to aging.
[c] This binomial probability is not used to directly reject the null hypothesis of a given GenAge category not undergoing rapid (*p*-Value high) or slow (*p*-Value low) evolution but provides a metric to identify categories potentially undergoing rapid or slow evolution. The test statistic threshold was set at 0.001 (see Section 2).

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repair and defense mechanisms since humans and chimpanzees diverged, we examined K_a/K_s ratios, obtained from the Chimpanzee Sequencing and Analysis Consortium (2005), in GO categories that could potentially be related to aging (Table 2). Most pathways hypothesized to play a role in aging and somatic maintenance do not appear to have undergone rapid evolution, but there are a few exceptions. For example, there is some evidence that DNA repair (GO:0006281; $K_a/K_s = 0.26$; $n = 121$) and GO categories associated with DNA repair may have undergone rapid evolution after humans and chimpanzees diverged (Table 2). It was not clear, however, whether accelerated evolution in these GO categories occurred in the human rather than the chimpanzee lineage as lineage-specific protein changes occurred in both lineages (not shown but see Section 4).

We found no evidence that GO categories related to oxidative stress, such as response to oxidative stress (GO:0006979; $K_a/K_s = 0.16$; $n = 27$), have undergone rapid evolution (Table 2). The response to stress category (GO:0006950) could have undergone rapid evolution, but this is misleading because of its subcategories, namely, DNA repair and response to pest/pathogen/parasite (GO:0009613) that appear to have undergone rapid evolution. When the influence of these subcategories that could represent sources of bias was eliminated, response to stress was no longer considered under rapid selection (not shown). Similarly, there was evidence of rapid evolution ($K_a/K_s = 0.50$; $n = 14$) in the glutathione transferase activity (GO:0004364). Because glutathione transferases typically associated with aging, such as *GSTA4* and *GSTP1*, were not found to be undergoing positive selection, and because some proteins from this GO category play a role in response to environmental stressors, which typically tend to be associated with rapid evolution, these findings are not necessarily related to aging, even if intriguing. Similarly, hormone activity (GO:0005179) appeared to have undergone rapid evolution ($K_a/K_s = 0.37$ with $n = 77$), yet changes in hormones may be related to adaptations in processes other than aging. Indeed, genes from this GO category with $K_a/K_s > 1$ have been related to different functions, including processes typically associated with rapid evolution such as immune response, reproduction, and development, so it is not clear that rapid evolution in this GO category is associated with aging.

Overall, pathways and mechanisms previously related to aging found highly conserved between humans and chimpanzees include topoisomerases such as TOP3B, the JNK pathway including MAPK8 and MAPK9, IGF1, its receptor IGF1R, and IGFBP3, the Sir2 homologue SIRT1, the TOR homologue FRAP1, TP53, heat shock proteins (GO:0003773) such as HSPA1B, HSPA8, HSPA9B, HSP90AA1, and HSPD1, histone deacetylases (GO:0004407) like HDAC1 and HDAC3, and ubiquitin cycle (GO:0006512) such as SUMO, UBB, UBE2I, and UCHL1. The complete results are available online (http://genomics.senescence.info/evolution/chimp.html).

3.2. Genes linked to aging in different models are under different selection forces

As mentioned before, GenAge features genes associated with aging in different model systems, including genes derived from organisms ranging from yeast to rodents, cellular models, and progeroid syndromes—though some overlap is possible. Therefore, we investigated whether different evolutionary pressures acted on genes associated with aging in different model systems (Table 3). Interestingly, genes directly related to aging in non-human mammals ($n = 21$) and humans ($n = 3$) had the highest average K_a/K_s ratios (0.33 and 0.47, respectively) while genes associated with aging in non-mammalian models – typically invertebrates – had the lowest average K_a/K_s ratio (0.083). The higher average K_a/K_s ratio of genes linked to aging in non-human mammals – typically rodents – could be due to a

Table 3
Selective forces operating on GenAge categories by selection process

GenAge category[a]	*n*	K_a/K_s	*p*-Value high[b]	*p*-Value low[b]	*n* in four-way comparisons[c]	K_a/K_s in hominids	K_a/K_s in murids	*Homo* AA diff.[d]	*Pan* AA diff.[d]
Human	3	0.472	0.017	0.99	2	0.46	0.22	4	8
Mammal	21	0.331	0.0026	1	9	0.32	0.20	9	10
Model	22	0.083	1	4.6E−7	14	0.069	0.10	2	3
Cell	14	0.109	1	5.7E−4	6	0.068	0.19	2	1
Functional	55	0.184	0.99	0.017	22	0.094	0.11	8	4
Upstream	25	0.138	1	1.1E−3	15	0.18	0.21	9	2
Downstream	46	0.097	1	7.6E−16	28	0.085	0.080	22	17
Putative	56	0.159	1	2.3E−4	38	0.15	0.13	16	19

[a] Genes linked to aging based on: human = evidence directly linking the gene product to aging in humans; mammal = evidence directly linking the gene product to aging in a non-human mammalian model organism; model = evidence directly linking the gene product to aging in a non-mammalian animal model organism; cell = evidence directly linking the gene product to aging in a cellular model system; functional = evidence linking the gene product to a pathway or mechanism linked to aging; upstream = evidence directly linking the gene product to the regulation or control of genes previously linked to aging; downstream = evidence showing the gene product to act downstream of a pathway, mechanism, or other gene product linked with aging; putative = indirect or inconclusive evidence linking the gene product to aging.

[b] This binomial probability is not used to directly reject the null hypothesis of a given GenAge category not undergoing rapid (*p*-Value high) or slow (*p*-Value low) evolution but provides a metric to identify categories potentially undergoing rapid or slow evolution. The test statistic threshold was set at 0.001 (see Section 2).

[c] K_a/K_s ratios in hominids are based on four-way alignments and hence are expected to be different from K_a/K_s ratios based on only chimpanzee and human alignments, as described (Chimpanzee Sequencing and Analysis Consortium, 2005).

[d] Amino acid differences (AA diff.) represent amino acid changes estimated to have occurred specifically in the human (*Homo*) or chimpanzee (*Pan*) lineage.

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few genes, such as *WRN* and *BRCA1*, because if these two genes were excluded the average K_a/K_s ratio of this category dropped to 0.19. Also in non-human mammals, genes associated with premature aging had a slightly higher K_a/K_s ratio (0.34; $n = 16$) than genes associated with life-extension (0.27; $n = 6$), though again this appears to be due to the presence of *BRCA1* and *WRN* in the former. Genes associated with cellular models of aging also had a very low K_a/K_s ratio (0.11). Lastly, we found no significant indication of accelerated evolution in hominids when compared to murids, except in genes related to aging in mammals which may again be due to the influence of a few genes such as *BRCA1* and *WRN*.

We also looked at the K_a/K_s ratios of genes ($n = 14$) associated with human longevity or survival in the elderly that might thus be associated with healthy aging. The average K_a/K_s ratio was 0.16 with no significant evidence of a higher or lower than average K_a/K_s ratio. None of these genes had a K_a/K_s ratio > 1. The strongest outliers were *SIRT3* with a $K_a/K_s = 0.85$ (but $K_a/K_i = 0.42$) and *APOC3* with a $K_a/K_i = 1.19$ (but $K_a/K_s = 0.35$). The complete results are available online (http://genomics.senescence.info/evolution/chimp.html).

Analyses of the GenAge dataset according to selection clusters using human–rhesus, human–mouse, and human–rat gene pairs were largely in accordance with the abovementioned results obtained using human–chimpanzee gene pairs. For example, genes associated with aging in lower organisms and cellular models always had a lower K_a/K_s ratio than genes associated with aging in mammals.

3.3. Evolution of genes associated with segmental progeroid syndromes

As mentioned above, we found limited evidence of positive selection or rapid or accelerated evolution in GenAge genes. Apart from *BRCA1*, it is unclear whether any of the genes in GenAge have undergone positive selection since humans and chimpanzees diverged. Nonetheless, we wanted to evaluate whether genes associated with premature aging in humans could also be related to the evolution of aging in the human lineage.

The three genes in which mutations cause what most dramatically resembles premature aging in humans are *WRN*, *ERCC8*, and *LMNA*, of which *WRN*, the gene responsible for Werner's syndrome, is the most striking case. Other genes that might also be considered segmental progeroid syndromes include *BSCL2*, *ERCC6*, *NBN*, and *AGPAT2* (Martin, 1978, 2005; de Magalhaes, 2005a). We failed to find significant evidence of positive selection or rapid evolution in either hominins or hominids in any of these genes, except for *WRN*.

In the case of *WRN*, both the K_a/K_s (0.91) and K_a/K_i (0.59) ratios indicate the gene could have undergone rapid evolution since humans and chimpanzees diverged. *WRN* also had a higher K_a/K_s ratio (0.75) in hominids than in murids (0.31), which could suggest accelerated evolution in the former, and higher-than-average K_a/K_s ratios in the human–rhesus gene pair (0.53), human–mouse gene pair (0.32), and human–rat gene pair (0.29). Although these results could be false positives (see Section 4), they suggest possible adaptive changes in the *WRN* gene in the hominid lineage. Previously, Clark et al. (2003) reported that *WRN* could have undergone accelerated evolution in the human lineage ($p = 0.028$). Using a human–chimpanzee–orangutan protein alignment, however, we found 11 chimpanzee-specific protein changes but only 4 human-specific protein changes in WRN plus 2 changes that could not be attributed to either lineage. Because the chimpanzee genome sequence is still considered a draft, however, errors in it could artificially increase the number of chimpanzee lineage-specific protein changes.

Our results and K_a/K_s ratios have been incorporated into the GenAge database (http://genomics.senescence.info/genes/human.html) and could be useful for researchers working on aging to study the evolution of aging-associated genes across primates. They are further available online (http://genomics.senescence.info/evolution/chimp.html).

4. Discussion

Multiple primate genomes will be necessary to understand the genetic changes that contributed to the distinguishing features of the human aging process and thus our work should be considered preliminary or explorative. Our approach for identifying genes responsible for the interspecies differences in longevity is novel and promising but its limit of detection is still confined to relatively strong signals (Fig. 1). Moreover, such large-scale, genome-wide analyses are so far only feasible in protein-coding regions, yet it is likely that differences in gene regulation also specify species differences in aging and there could be important changes in regulatory regions, RNA genes, or even some unknown gene product that are not detectable by our method. Despite these limitations, our work offers new insights regarding the evolutionary forces acting on genes associated with aging in model systems.

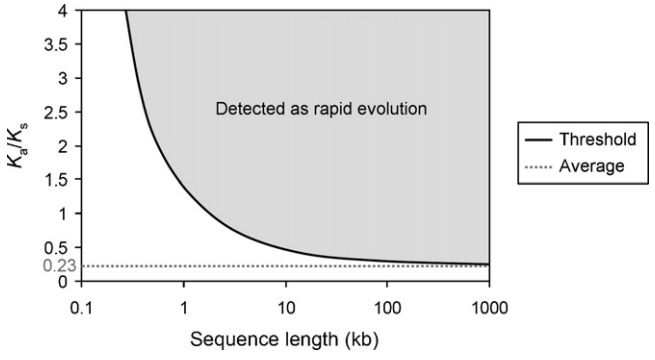

Fig. 1. Approximate limit of detection of rapid evolution using our test statistic as a function of sequence length and K_a/K_s ratio. The straight black line represents the K_a/K_s ratio threshold above which a given protein-coding sequence will be detected as undergoing rapid evolution. Sequence length, which may be a single gene or a concatenated sequence of a large group of genes (the average human–chimpanzee gene pair, only including the protein-coding sequence, is approximately 1.4 kb), is plotted on a logarithmic scale. The dotted gray line is the average K_a/K_s ratio for all human–chimpanzee gene pairs (0.23). K_a/K_s ratios assume a constant K_s and a constant proportion of synonymous and nonsynonymous sites in relation to sequence length, both of which were estimated from the average of all human–chimpanzee gene pairs.

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Although we found evidence of selection in a few genes and pathways previously associated with aging, we urge caution in interpreting these results. For instance, genes in GO categories may be involved in functions unrelated to aging and thus any evidence of rapid evolution may reflect selection on another trait (but see below). One potentially interesting finding was the putative rapid evolution of DNA repair proteins. Other works have reported evidence of positive selection in DNA metabolism genes, including *ERCC8*, in recent human evolution (Wang et al., 2006). These results could be associated with hypotheses of aging predicting that DNA repair/metabolism genes play a role in species differences in aging (reviewed in de Magalhaes, 2005a; Martin, 2005). On the other hand, rapid evolution of DNA repair genes is not unique of long-lived humans or hominids and, in fact, some results suggest accelerated evolution in DNA repair genes in murids when compared to hominids (Chimpanzee Sequencing and Analysis Consortium, 2005). More detailed studies encompassing several mammalian genomes are warranted to examine whether changes to DNA repair/metabolism genes are involved in the evolution of aging.

Overall, our results using the GenAge dataset show that, at least in the primate lineage, genes associated with aging in model systems tend to be under purifying selection and stronger-than-average functional constraints. Even though it is possible for genes under strong functional constraints to play a role in the evolution of lineage-specific traits, we found little evidence of accelerated evolution in putative aging-related genes in the hominid or human lineages. Genes with such patterns of selection tend to have key biological functions conserved among mammals and these evolutionary forces typically preserve existing phenotypes. Because our work is the first to explore the evolutionary pressures acting on aging-associated genes and pathways, however, different explanations can be conceptualized, and our results do not disprove any potential involvement these genes may have on human aging. Certainly, phenotypically important changes in one or a few genes – maybe even changes in regulatory regions – are likely to be undetected by our analyses. For instance, although we found no evidence of rapid evolution in the mitochondrial electron transport chain (GO:0006118 and GO:0006120), rapid evolution in a few of these proteins has been reported in anthropoid primates (Grossman et al., 2004), and it has been hypothesized that this represents a life-extending adaptation (de Magalhaes, 2005b). Though speculative, an alternative hypothesis is that genes involved in aging require extreme precision, because they are likely to have other functions, and subtle, so far impossible to detect changes led to the life-extension of humans.

Apart from the GO categories results, which can be subject to different interpretations, we found little evidence of a widespread optimization of multiple maintenance and repair mechanisms, even though this may be due to limitations in our analyses. The differences we found in selection pressures acting on genes associated with aging in different models systems, such as the observation that genes associated with aging in non-mammalian model organisms are under stronger functional constraints than genes associated with aging in mammals, are novel. These results might reflect, in human–chimpanzee gene pairs, a higher degree of conservation throughout evolution of genes whose homologs are involved in aging in non-mammalian model organisms. In fact, the proportion of human homologs of aging-related genes in model organisms has been reported to be higher than expected by chance (Budovsky et al., 2007). This high conservation of aging-related genes might be related to their reported higher-than-average interactions (Promislow, 2004; Budovsky et al., 2007). Our results, albeit only explorative, do not appear to support the hypothesis that most genes and mechanisms that have been the focus of research in model organisms are likely to regulate species differences in aging, at least in primates. Even if the mechanisms of aging identified so far in model systems do not explain differences in aging between hominids, it is plausible that these mechanisms are conserved between organisms, for instance, as part of a conserved mechanism linking reproduction and food availability to aging (Partridge and Gems, 2002). In other words, perhaps the biological functions of these genes are part of conserved pathways related to pleiotropic effects on aging that impact on intra-species differences in aging but do not regulate inter-species differences in aging.

As mentioned in the introduction, our rationale in this work is based on the concept, supported by modern evolutionary theory (e.g., Charnov, 1993; Kirkwood and Austad, 2000), that the extended human lifespan and delayed onset of aging are a product of direct selective pressures. With the little that we know about the genetics of aging in primates, however, we cannot exclude alternatives. Maybe the delayed onset of aging in humans is a by-product of selection acting on other traits, such as development, growth, or reproduction. For instance, one hypothesis is that, after humans and chimpanzees diverged, natural selection may have acted primarily on developmental pathways to extend development, growth, and lifespan (de Magalhaes and Church, 2005). In fact, one mechanism shown to act on development, growth, and aging is the endocrine system (reviewed in McKinney and McNamara, 1991; Partridge and Gems, 2002; de Magalhaes, 2005a; de Magalhaes and Church, 2005). Hormonal adaptations, even those related to development and reproduction, may thus have some impact on aging. The higher proportion of human-specific protein changes in GenAge proteins associated with growth and development and the putative rapid evolution in hormone activity GO categories support this view. If the delayed onset of human aging is a by-product of the delayed human development then genes related to aging in model systems may be expected to be under purifying selection.

Lastly, our results indicate a putative rapid evolution of *WRN*, a gene in which mutations result in a phenotype that resembles accelerated aging in human patients. Our results do not prove that *WRN* has undergone adaptive changes, and admittedly may be a result of chance, though because *WRN* encodes a relatively large protein with 1432 amino acids, K_a/K_s ratios are less likely to be exaggerated than those of genes encoding smaller proteins. Moreover, it is an intriguing

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coincidence that the gene associated with the most impressive human progeroid syndrome is one of the genes in GenAge with the highest K_a/K_s ratio. It is tempting to speculate that changes to *WRN* are also involved in the evolution of aging in primates and hominids. Since humans and chimpanzees diverged, most protein changes to WRN were chimpanzee lineage-specific, which makes it unlikely that protein changes to WRN contributed to the evolution of human longevity. This could suggest a pattern of selection on *WRN* similar to that observed in the *MCPH1* gene where selection is most pronounced in earlier portions of the lineage (Vallender and Lahn, 2004). Analyses of changes to the WRN protein during primate and mammalian evolution merit further attention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mad.2007.03.004.

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