Methods

Thin sections

We cut sections 0.7–1.1 mm thick from fragments of the fossil bones with a Leitz Saw Microtome and ground them to 70 μm or less. Sections of Recent bones were cut 1 mm thick with a scroll saw and ground to thicknesses ranging from 20 to 100 μm.

We examined, with transmitted light microscopy and PLM, more than 500 optical thin sections of Recent taxa, taken from several recorded positions along the diaphyses of limb bones in each species. In birds, we sampled the humerus, ulna, femur, tibiotarsus, tarsometatarsus and phalanges in adults of the following orders and species: Struthioniformes: *Dromaius novaehollandiae* (emu); Galliformes: *Meleagris gallopavo* (turkey) and *Phasianus colchicus* (ring-necked pheasant); Anseriformes: *Cygnus columbianus* (tundra swan), *Branta canadensis* (Canada goose) and *Anas strepera* (gadwall); Gaviiformes: *Gavia pacifica* (Pacific loon); Podicipediformes: *Podiceps griseigena* (red-necked grebe); Pelecaniformes: *Pelecanus erythrorhynchos* (white pelican); Ciconiiformes: *Ardea herodias* (great blue heron); Charadriiformes: *Larus delawarensis* (ring-billed gull); Falconiformes: *Haliaeetus leucocephalus* (bald eagle) and *Buteo jamaicensis* (red-tailed hawk); Strigiformes: *Tyto alba* (barn owl) and *Bubo virginianus* (great horned owl); Passeriformes: *Corvus corax* (common raven) and *Carduelis pinus* (pine siskin). We also sampled bones from a nestling *Tyto alba* and a fledgling *Accipiter striatus* (sharp-shinned hawk). We examined approximately 220 thin sections from the major limb bones in adults of the following orders and species of mammals: Marsupialia: *Didelphis marsupialis* (opossum) and *Macropus rufus* (red kangaroo); Insectivoura: *Scapanus townsendii* (Townsend mole); Chiroptera: *Eidolon helvum* (straw-coloured fruit bat); Rodentia: *Sciurus griseus* (grey squirrel) and *Capromys pilorides* (hutia); Carnivoura: *Procyon lotor* (raccoon) and *Puma concolour* (puma); Artiodactyla: *Antilocapra americana* (pronghorn) and *Ammotragus lervia* (Barbary sheep).

Measurements of canalicular directions

The measurements of canalicular directions are based on examples with typical as well as more extreme variations in structure where the canaliculi show good contrast over an extended area. We scanned film images made at a microscope magnification of 400× and locally enhanced the image contrast of the canaliculi, then converted the images to black and white bitmaps. The directions of canalicular boundaries in the bitmaps were calculated using software that measures a direction from each pixel along the boundary to a position six pixels ahead on the same boundary, repeats this for all canalicular edges and prepares a frequency distribution of the directions throughout the image.

Measurements of lamellar thicknesses

Using PLM film images, we measured the degree of uniformity of lamellar thickness in areas with best lamellar contrast. Although we measured lamellae in endosteal, osteonal and periosteal bone tissue, the data shown in Fig. 5 exclude osteonal lamellae because relatively straight lamellae could be measured more exhaustively and accurately by software. We high-pass filtered each digital sample identically to eliminate low-frequency contrast across the image, then increased the contrast of the resulting greyscale image and converted it to a bitmap. The software calculated the distance from one lamellar boundary to the next boundary in the direction perpendicular to the general direction of the lamellar planes, and repeated the process for each pixel along each lamellar boundary.

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Correspondence and requests for materials should be addressed to J.M.R. (e-mail: rensb@u.washington.edu).

Unexpectedly similar rates of nucleotide substitution found in male and female hominids

Hacho B. Bohossian, Helen Skaletsky & David C. Page

Howard Hughes Medical Institute, Whitehead Institute, and Department of Biology, Massachusetts Institute of Technology, 9 Cambridge Center, Cambridge, Massachusetts 02142, USA

In 1947, it was suggested that, in humans, the mutation rate is dramatically higher in the male germ line than in the female germ line[1]. This hypothesis has been supported by the observation that, among primates, Y-linked genes evolved more rapidly than homologous X-linked genes[2–6]. Based on these evolutionary studies, the ratio (α_m) of male to female mutation rates in primates was estimated to be about 5. However, selection could have skewed sequence evolution in introns and exons[7–10]. In addition, some of the X–Y gene pairs studied lie within chromosomal regions with substantially divergent nucleotide sequences[7,11,12]. Here we directly compare human X and Y sequences within a large

Table 1 Numbers of nucleotide substitutions and α_m: direct estimates

	Numbers of substitutions				
	Total	On Y chromosome	On X chromosome	Unresolved	α_m (95% confidence interval)
All substitutions	441	238	175	28	1.66 (1.19–2.45)
Transitions	305	164	121	21	1.65 (1.11–2.66)
Transversions	136	74	54	7	1.68 (0.95–3.68)

region with no known genes. Here the two chromosomes are 99% identical, and X–Y divergence began only three or four million years ago, during hominid evolution[13–15]. In apes, homologous sequences exist only on the X chromosome. We sequenced and compared 38.6 kb of this region from human X, human Y, chimpanzee X and gorilla X chromosomes. We calculated α_m to be 1.7 (95% confidence interval 1.15–2.87), significantly lower than previous estimates in primates. We infer that, in humans and their immediate ancestors, male and female mutation rates were far more similar than previously supposed.

Li *et al.* have suggested that the most accurate and precise estimates of α_m should emerge from comparison of lengthy DNA segments that are non-functional but highly similar in sequence[7]. We therefore focused upon a large region of 99% nucleotide identity between the long arm of the human X chromosome (Xq) and the short arm of the human Y chromosome (Yp). These sequences are present on human Yp because of a massive X-to-Y transposition that occurred about three or four million years ago, after divergence of the human and chimpanzee lineages[13–15]. This Xq–Yp region is poor in genes (T. Kawaguchi *et al.*, unpublished results). The 1% divergence between the human X- and Y-linked sequences presumably reflects the random accumulation of new mutations on both the X and Y chromosomes during the last three to four million years of hominid evolution. Given this evolutionary history and the paucity of genes, the Xq–Yp region offers a nearly ideal substrate for estimation of α_m in hominids.

From within this Xq–Yp region we selected a 38.6-kb segment for detailed study. Human X and Y-chromosomal bacterial artificial chromosomes (BACs) containing this segment had been isolated in our laboratory and sequenced at the Whitehead Institute/MIT Center for Genome Research. This segment has a total content of guanine and cytosine (G+C content) of 35%; Alus, LINES, and other interspersed repeat elements account for 61% of the total sequence on both X and Y. We found no known or electronically predicted genes within this segment or within 100 kb to either side of the segment.

We compared the sequences of this 38.6-kb segment as found on the human X, human Y, chimpanzee X and gorilla X chromosomes. For the chimpanzee and gorilla X chromosomes, we generated sequencing templates by polymerase chain reaction (PCR) amplification using female genomic DNAs as starting material. As controls, we re-sequenced the corresponding portions of the human X and Y BACs, again using PCR-generated templates. In this manner, we were able to assemble 38.6 kb of virtually continuous sequence from all four chromosomes. Assembly and sequencing of genomic PCR products across the entire 38.6-kb segment were straightforward because the region's interspersed repeat elements, though numerous, were ancient and thus did not impede selection of locus-specific oligonucleotide primers. This characteristic of the region, together with the absence of genes, had led us to select it for study.

We discovered that, within this segment, the human X and Y chromosome differed at 441 nucleotides (Table 1); the two human chromosomes were 98.86% identical, in good agreement with previous estimates for the larger Xq–Yp region[15]. Of these 441 nucleotide substitutions, we were able to infer in 413 cases whether the mutation had occurred on the hominid X chromosome (175 cases) or on the hominid Y chromosome (238 cases). This was done by examining, for each substitution, the corresponding nucleotide position on the chimpanzee and gorilla X chromosomes. For example, if a T nucleotide was present on the human Y chromosome, but an A was present at the corresponding site on human, chimpanzee, and gorilla X chromosomes, we inferred that the primitive or ancestral state was A, and that an A-to-T substitution had occurred on the hominid Y chromosome. Conversely, if the human Y, gorilla X, and chimpanzee X chromosomes were identical to each other but differed from the human X chromosome at a particular nucleotide, we inferred that a mutation had arisen on the hominid X chromosome. We were unable to infer the chromosomal origin of the substitution at only 28 nucleotide sites; in most such cases, we observed nucleotide differences between chimpanzee and gorilla. (We also traced the chromosomal origins of small insertions and deletions—15 on the hominid X chromosome, 23 on the hominid Y chromosome—but these events were too few to merit detailed analysis.)

Using the inferred numbers of nucleotide substitutions on the hominid X and Y chromosomes, and ignoring the 28 unresolved differences, we estimated α_m by Miyata's formula:

$$Y/X = 3\alpha_m/(2 + \alpha_m)$$

where Y/X is the ratio of mutation rates on the two sex chromosomes[2]. This formula is based on the expectation that, in any generation, two-thirds of X chromosomes are transmitted through the female germ line, the remaining one-third being transmitted through the male germ line. By contrast, all Y chromosomes are transmitted through the male germ line. Our observed Y/X ratio of (238/175) = 1.36 (95% confidence interval 1.12–1.65) implies that α_m = 1.66 (95% confidence interval 1.19–2.45). Restricting the analysis to either transitions or transversions does not alter the estimate of α_m (Table 1).

Our calculations of Y/X and α_m are based on direct comparison of human X and human Y chromosomal sequences with a readily inferred ancestral sequence. All previous estimates of Y/X (and thus of α_m) in primates involved X–Y gene pairs that were too diverged to allow accurate reconstruction of ancestral sequences. Previous estimates of Y/X required construction and comparison of two trees of evolutionary distances: one tree for orthologous Y-linked

Table 2 Evolutionary distances (substitutions per 100 sites)

	Human Y	Human X	Chimpanzee X
Human X	1.18 ± 0.06		
Chimpanzee X	1.71 ± 0.07	1.56 ± 0.07	
Gorilla X	1.82 ± 0.07	1.66 ± 0.07	1.86 ± 0.07

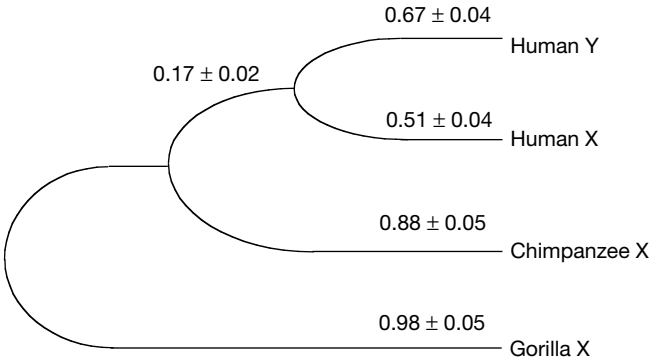

Figure 1 Phylogenetic tree of nucleotide sequences. Branch lengths were estimated from the pairwise evolutionary distances (substitutions per 100 sites) in Table 2.

sequences in several species and a second tree for orthologous X-linked sequences in the same species[3–6]. To ensure that discrepancies between past and present findings were not attributable to different methods of calculation, we re-analysed our sequence data using the traditional method. We first estimated evolutionary distances between the human Y, human X, chimpanzee X and gorilla X sequences using the method of ref. 16 (Table 2). As expected, the resulting phylogenetic tree (Fig. 1) casts chimpanzee and gorilla as outgroups to the human X and Y sequences. The tree yields a Y/X value of (0.67/0.51) = 1.31 (95% confidence interval 1.05–1.55), implying that $\alpha_m = 1.55$ (95% confidence interval 1.08–2.14). Thus, recalculating Y/X and α_m by the traditional phylogenetic method yields essentially the same results as our direct calculation. The phylogenetic tree also suggests that X–Y gene conversion was not a major factor in the evolution of these sequences in hominids. (Even if some X–Y gene conversion had occurred, it would not affect our estimates of α_m.) Finally, the phylogenetic tree corroborates the conclusion[15] that the X-to-Y transposition occurred within one to two million years after the divergence (roughly four to six million years ago) of the human and chimpanzee lineages.

Although our direct calculation indicated that $\alpha_m \approx 1.66$, this may be a slight underestimate. Our calculation assumed that the 1.1% divergence observed between the human X- and Y-linked sequences was entirely attributable to mutations that arose after X-to-Y transposition. However, the 38.6-kb sequence analysed may have been polymorphic (on the X chromosome) at the time of transposition, three to four million years ago. Such ancient polymorphism could account for part of the observed X–Y divergence and could result in our underestimating both Y/X and α_m. Nonetheless, recent studies of sequence diversity on X chromosomes in modern humans and chimpanzees suggest that the correction for ancient polymorphism should be modest. Assuming that X-linked sequence diversity in ancient hominids approximated that in modern human populations (4×10^{-4} per base pair, bp)[17], our estimate of α_m is essentially unchanged. Alternatively, if X-linked sequence diversity in ancient hominids was as high as that in modern chimpanzee populations (1.3×10^{-3} per bp)[18], then our estimate of α_m should be corrected to about 1.8 (95% confidence interval 1.15–2.87).

In any case, our estimate of α_m in hominids is much lower than previous estimates in primates (Table 3). Several factors may account for this discrepancy. Our study addressed the last three to four million years of hominid evolution. By contrast, previous experimental designs required comparisons among divergent primate lineages and thus yielded estimates of α_m averaged across broad swaths of primate evolution[3–6]. These studies could not have detected whether α_m was lower in hominids than in other primates. Second, our study examined a much larger DNA segment and yielded more precise estimates of α_m. Large standard errors in previous estimates (Table 3) may explain, in part, the apparent disparity with present findings. Third, as has been pointed out, nucleotide sequence context may influence or bias substitution rates[7,11,12]. This may have affected previous estimates of α_m, as these were based on relative substitution rates in substantially diverged portions of the X and Y chromosomes[3–6]. Such contextual bias should be negligible in our present examination of X and Y-chromosomal regions whose DNA sequences are 99% identical. Finally, and perhaps most importantly, the present analysis involved sequences which appear to be physically distant from any gene; selective neutrality can reasonably be assumed. By contrast, previous estimates of α_m were based on analyses of introns or exons. The higher estimates of α_m in past studies could reflect: (1) relaxed selective constraints on Y-linked introns and exons as compared with their X-linked homologues[7–9]; (2) diminished mutation rates in X-linked genes[10,19], or both. Future studies of genes within the region of 99% X–Y identity may allow investigators to document the effects, if any, of mutation and selection bias on estimates of α_m.

Our findings suggest that substitution rates were only modestly higher in males than females during the last three to four million years of hominid evolution. If these inferences extend to modern humans, as seems likely, then they have ramifications for medical genetics. Many individuals are afflicted by autosomal dominant or X-linked recessive disorders because of new mutations that appeared in their parents' or grandparents' germlines[1,20,21]. In several such disorders, and especially those caused by recurrence of specific substitutions at particular nucleotide positions in one gene (such as achondroplasia[22]), nearly all new mutations arise in the male germline. Bolstered by studies of X–Y gene evolution in primates[2–6], this dramatic sex bias at a small number of extraordinarily mutable nucleotides has been taken as evidence that substitution rates across the human genome are much higher in males than in females[20,21]. Our results suggest a re-interpretation of these medically ascertained hot spots for mutation. Our estimate of $\alpha_m \approx 1.7$ is based on the complete, diverse set of germline substitutions that accumulated within a large, selectively neutral region. Our data may provide the best global estimate to date of substitutional sex ratios in the human genome. From this point of reference, the large sex biases at some medically important hot spots appear as marked departures from global norms, underscoring the importance of unexplored interactions between sequence context and sex at these unusual sites.

Our findings also challenge the model that human mutation rates are directly proportional to the number of cell divisions, regardless of sex. Beginning with Haldane[1], high α_m values have been attributed to the much greater number of germline cell divisions in males (with mitotically active spermatogonial stem cells) than in females (where germ cells cease dividing during foetal development)[2,3]. Our results, however, suggest that sexual asymmetry in substitution rates is far less striking than sexual asymmetry in numbers of cell divisions, at least in hominids. We suggest two possibilities for further investigation. Perhaps errors in mitotic DNA replication and repair account for a minority of germline substitutions in human genes. Alternatively, perhaps DNA replication and repair are unusually accurate in spermatogonial stem cells and their prospermatogonial precursors, which account for most of the excess cell divisions in the male germ line. □

Table 3 Comparison of Y/X-based estimates of α_m in primates

Genes studied	Exon or intron	DNA segment length (kb)	Y/X ratio	α_m (95% confidence interval)	Reference
ZFX/ZFY	intron	0.9	2.27	6.3 (2.6–32)	3,5
ZFX/ZFY	exon	1.2	2.0	4.2 (not reported)	4
SMCX/SMCY	intron	1.4	2.03	4.2 (2.2–10)	5
AMELX/AMELY	intron	1.1	2.16	5.14 (2.4–17)	6
Pooled data for *ZFX/ZFY*, *SMCX/SMCY* & *AMELX/AMELY*	introns	see above	2.15	5.06 (3.24–8.8)	6
Gene-free Xq–Yp region	neither	38.6	1.36	1.7 (1.15–2.87)	present study

Methods

Reference DNA sequences

We studied a 38.6-kb X–Y homologous segment which corresponds both to nucleotides 28,842–67,422 of a human X-chromosomal BAC (GenBank AC002488) and to nucleotides 88,369–127,049 of a human Y-chromosomal BAC (GenBank AC002509). These BACs, isolated in our laboratory from the California Institute of Technology A (CTA) library[23], had been sequenced at the Whitehead Institute/MIT Center for Genome Research. Interspersed repetitive elements within the 38.6-kb segment were identified electronically using RepeatMasker (http://ftp.genome.washington.edu/RM/RepeatMasker.html); all repeats were included in the analysis of mutations. Electronic searches employing GenScan[24], Grail[25] and BLAST[26] failed to identify any genes or exons within the 38.6-kb segment. Electronic searches also failed to identify any genes or exons in three adjoining Y-chromosomal BACs (GenBank AC012078, AC010094 and AC010737), all sequenced at the Washington University Genome Sequencing Centre.

Resequencing

A series of overlapping fragments, each about 1 kb in length, that collectively spanned the 38.6-kb region was generated by PCR using each of four DNAs as starting material: the human X-chromosomal BAC, the human Y-chromosomal BAC, chimpanzee female genomic DNA, and gorilla female genomic DNA. PCR primers were selected using Primer3 (ref. 27) and are available upon request. PCR products were purified on Sephacryl-S300 columns and sequenced using fluorescent-dye-terminator cycle sequencing protocols (ThermoSequenase kit; Amersham). Primers used in PCR generation of sequencing templates were also used as sequencing primers; additional sequencing primers were selected at sites internal to the PCR-generated templates.

The sequences of each of the four chromosomes (human X, human Y, chimpanzee X, gorilla X) were assembled using Sequencher 3.1 (Gene Codes Corp.). The four chromosomes were aligned using MegAlign (DNASTAR, Inc.), and the alignment was edited manually (alignment available upon request). There was only one discrepancy (T→C at nucleotide 56,776 in X-chromosomal sequence) between our PCR-generated human X and Y-chromosomal sequences and the corresponding, GenBank-deposited reference sequences.

Statistical calculations

In this direct method, α_m was calculated directly from the inferred numbers of Y and X substitutions via the formula $Y/X = 3\alpha_m/(2 + \alpha_m)$ (ref. 2). We calculated confidence intervals for ratios of substitution rates using the formula for relative risk[28].

We also analysed our sequence data using the method of ref. 3. We began by calculating, for each pairwise sequence comparison, the number of substitutions per 100 nucleotides[16]. From there we estimated branch lengths and their variances[29], and these values in turn enabled us to estimate Y/X and α_m (ref. 3).

When correcting estimates of α_m for ancient polymorphism, we calculated means and variances for the numbers of substitutions after X-to-Y transposition, and then calculated means and standard deviations for Y/X using the delta method[30].

GenBank accession numbers

Gorilla: AF190869, AF190870 and AF190871. Chimpanzee: AF190865, AF190866, AF190867 and AF190868.

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Correspondence and requests for materials should be addressed to D.C.P. (e-mail: dcpage@wi.mit.edu).

Evolvability of an RNA virus is determined by its mutational neighbourhood

Christina L. Burch & Lin Chao

Department of Biology, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0116, USA

The ubiquity of mechanisms that generate genetic variation has spurred arguments that evolvability, the ability to generate adaptive variation, has itself evolved in response to natural selection[1,2]. The high mutation rate of RNA viruses is postulated to be an adaptation for evolvability[3,4], but the paradox is that whereas some RNA viruses evolve at high rates[4,5], others are highly stable[5,6]. Here we show that evolvability in the RNA bacteriophage φ6 is also determined by the accessibility of advantageous genotypes within the mutational neighbourhood (the set of mutants one or a few mutational steps away). We found that two φ6 populations that were derived from a single ancestral phage repeatedly evolved at different rates and toward different fitness maxima. Fitness measurements of individual phages showed that the fitness distribution of mutants differed between the two populations. Whereas population A, which evolved toward a higher maximum, had a distribution that contained many advantageous mutants, population B, which evolved toward a lower maximum, had a distribution that contained only deleterious mutants. We interpret these distributions to measure the fitness effects of genotypes that are mutationally available to the two populations. Thus, the / evolvability of φ6 is constrained by the distribution of its mutational neighbours, despite the fact that this phage has the characteristic high mutation rate of RNA viruses.

Populations of φ6 carrying a deleterious mutation have been shown to recover fitness more often by different compensatory mutations than by a back mutation that reverted the deleterious mutation[7]. The mutations were compensatory because their benefit was conditional (epistatic) on the presence of the deleterious mutation in the same viral genome. Thus, the recovered populations could have evolved new co-adapted gene complexes[8] whose subcomponents, the epistatic and the deleterious mutations, were potentially harmful when alone. Such complexes are effectively different evolutionary solutions to maximizing fitness in our culture