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Male-to-Female Ratios of Mutation Rate in Higher Primates Estimated from Intron Sequences

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Benny Hung-Junn Chang, David Hewett-Emmett and Wen-Hsiung Li (1996) Male-to-female ratios of mutation rate in higher primates estimated from intron sequences. *Zoological Studies* **35**(1): 36-48. The male-to-female ratio of mutation rate, α, has been estimated to be 6 using the last intron sequences of the ZFX and ZFY genes in 4 species of higher primates (Shimmin 1993b). This estimate has a very large 95% confidence interval (from 2 to 84) and cannot exclude the possibility that α is very large as suggested by Miyata et al. (1987). To obtain a more reliable estimate, Polymerase Chain Reaction (PCR) was used to amplify and sequence the last introns of the ZFX/ZFY genes in two additional primate species (the colobus monkey and tamarin). We analyzed these and the homologous sequences for 4 other primate species - a total of 12 introns across 6 primate species. Again, an estimate of α = 6 was obtained, but the 95% confidence interval was reduced to (3, 33).

Two introns (A and B) of another pair of genes, SMCX and SMCY, in 4 species of primates were also amplified and sequenced. From intron A sequences, α was estimated to be 4.2 with a 95% confidence interval from 2.2 to 10.0. From intron B sequences, α was estimated to be 12.3 with a large 95% confidence interval (2.8, ∞); this estimate is not reliable because of the small size of the Y-linked intron B ($\sim$418 base pairs). Because the α estimates derived independently from different regions of the genome (ZFX/ZFY and SMCX/SMCY) are close to each other and are also close to the sex ratio of the number of germ-cell divisions, c, in humans ($\sim$6), we conclude that substitution mutation in primate germ cells is largely replication dependent. Other possible factors that may contribute to a higher mutation rate in males and the implication of this study on the molecular clock hypothesis are discussed.

Key words: Substitution mutation, Mutagenesis, SMCX/SMCY, ZFX/ZFY, Primates.

In mammals, the number of germ cell divisions is higher for the production of sperm than for the production of eggs. For this reason, males should have a higher mutation rate if mutation arises mainly from DNA replication during germ cell division. Estimation of sex ratios of mutation rate (α) is therefore important for understanding the mechanism of spontaneous mutation.

Haldane (1935) developed an indirect method to estimate the sex ratio of mutation rate from data on X-linked recessive diseases. Later he estimated a ratio of 10 by using hemophilia data (Haldane 1947). Studies on Lesch-Nyhan syndrome (Francke et al. 1976) and on hemophilia A (Bernardi et al. 1987, Rosendaal et al. 1990) also supported a higher mutation rate in males, whereas studies on Duchenne Muscular Dystrophy (DMD; Moser 1984, Bakker et al. 1989) favored an equal ratio. The ratios estimated in 6 different studies using hemophilia A data varied from 1.2 to 29.3 (see references in Ketterling et al. 1993). The reasons for such a wide range of variation may be ascertainment biases, uncertainties about the reproductive fitness of the carriers and the patients, small sample sizes, and statistical fluctuations (Rosendaal et al. 1990, Ketterling et al. 1993). Moreover, these studies did not distinguish between substitution and insertion/deletion (indel) mutations because defects were not examined at the DNA level. Distinguishing between these two

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kinds of mutation is important because they arise from different mutation mechanisms and thus may have different sex ratios. In fact, it now seems clear that the DMD data supported an equal sex ratio because most of the disease-causing mutations were indels in this huge dystrophin gene, which spans over 2×10^6 base pairs (bp) on the human X chromosome.

With modern molecular techniques, one can identify the type of mutation and can trace its origin. Indeed, Ketterling et al. (1993) have used direct genomic sequencing to study hemophilia B families. However, this approach also has several difficulties. First, it may not be applicable when members of a pedigree are not available for study. Second, ascertainment biases may also be involved if the germ-line origin of mutation cannot be determined unambiguously, which may distort the estimate of α. Third, it is laborious. Ketterling et al. (1993) characterized a total of 570 kilobases (Kb) of sequences in 260 families with hemophilia B but in only 25 of these families could the germ-line origin of mutation be determined. Fourth, it may not be readily applicable to non-human organisms for which no pedigrees have been maintained.

In previous studies, we (Shimmin et al. 1993b, Chang et al. 1994) used the simple method first proposed by Miyata et al. (1987). This approach takes advantage of the fact that genes on different chromosomes segregate differently in the two sexes. However, as was emphasized (Shimmin et al. 1993a), using this method requires one to select DNA regions such as introns that are subject to only weak or no functional constraints. Shimmin et al. (1993b) and Chang et al. (1994) therefore sequenced the last introns of the ZFX and ZFY genes in 4 primate species and in rats and mice, and estimated $\alpha = 6$ in primates and $\alpha = 1.8$ in rodents. The latter estimate had a 95% confidence interval of (1, 3). Moreover, a second estimate from Ube1x/Ube1y introns gave an almost identical estimate, i.e., $\alpha \approx 2$. Therefore, it is fairly certain that $\alpha \approx 2$ in mice and rats. However, the α estimate for primates had a 95% confidence interval ranging from 2 to 84, so the possibility that α is very large in primates as suggested by Miyata et al. (1987) could not be rejected.

In this study, the last introns of the ZFX and ZFY genes in the colobus monkey (an Old World monkey) and in the tamarin (a New World monkey) were sequenced and the sequences were analyzed together with those obtained in our earlier study from 4 species of primates (Shimmin et al. 1993b). It was hoped that the 95% confidence interval of the α estimate would be narrowed by using these extra data. Two intronic regions of another pair of genes, the SMCX and SMCY genes in humans, orangutans, baboons, and squirrel monkeys were also sequenced in order to see whether the α estimates are similar for different genomic regions.

Before describing the present results it is necessary to comment on the 2 gene pairs used in this study. ZFX and ZFY genes are zinc finger protein genes on the X and Y chromosomes, respectively, in all eutherian mammals studied (Page et al. 1987). They are located very close to the pseudoautosomal region of the human sex chromosomes - the only region in which recombination can occur between the X and Y chromosomes. The X-linked SMC cDNA (SMCX) was first cloned in humans (XE169, Wu et al. 1994) and a homologous cDNA was cloned independently in mice (SMC stands for "selected mouse cDNA"; Agulnik et al. 1994a). A partial Y-linked cDNA (SMCY) was also cloned. Both genes were found to be present in the X and Y chromosomes of other eutherian mammals as well as in marsupials (Agulnik et al. 1994b). SMCX is located on Xp11.1-11.2 of the human X chromosome and SMCY is located on Yq11.22 of the human Y chromosome (Agulnik et al. 1994b). The SMCX gene is the only gene outside of the pseudoautosomal region that is known to escape X-inactivation in both humans and mice. The functions of these gene products, however, are yet to be characterized.

MATERIALS AND METHODS

Genomic DNA sources and isolation

Genomic DNAs were isolated from white blood cells of humans and from liver tissues of orangutans (*Pongo pygmaeus*; purchased from Yerkes Primate Center), baboons (*Papio cynocephalus*; gifts from the Southwest Foundation), and squirrel monkeys (*Saimiri boliviensis*). DNAs were purified from one male and one female individual of each species according to the method described in Chang (1995). Male colobus monkey (*Colobus angolensis*) DNA was a gift from Dr. Carol-Beth Stewart, and male tamarin (*Saguinus mystax*) DNA was a gift from Dr. Iracilda Sampaio.

PCR amplification

For the amplification of the last intron of the

ZFX/ZFY genes in the colobus monkey and tamarin, two primers (oLW97 and oLW98; Table 1) were used as described in Shimmin et al. (1993b). Briefly, the PCR reaction contained 200 to 500 ng of genomic DNA as a template and a reaction mixture containing 50 mM KCl, 10 mM Tris (pH 9.0), 0.1 % Triton X-100, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 40 pmole of each primer, and 1 unit of Taq DNA polymerase (Promega). PCR was done in a Omnigene thermal cycler (Hybaid) under a regime of denaturation, 94 °C for 30 sec, annealing, 65 °C for 30 sec, and extension, 72 °C for 2 min + 2 sec auto-extension for 30 cycles and a single final extension of 5 min at 72 °C. All reactions were done in a volume of 40 μl.

The genomic structure of the SMCX and SMCY genes are not known, but positions of two potential introns were determined by Agulnik et al. (1994a) using the PCR approach. Based on this information the cDNA sequences of human SMCX (Wu et al. 1994) and mouse SMCX and SMCY genes (Agulnik et al. 1994a, b) were aligned, and several primers were designed (Table 2) to amplify two introns of the SMCX and SMCY genes in humans, orangutans, baboons, and squirrel monkeys. These two introns are separated by a single exon. Because the genomic organization of the SMCX and SMCY genes are unknown, introns A and B were used to denote these two introns; intron A is 5' to intron B. The PCR conditions were similar to the amplification of the last introns of the ZFX and ZFY genes except that the annealing temperatures were between 55 and 60 °C.

Cloning

Some of the PCR products of SMC introns (Y-linked A intron of the baboon and squirrel monkey; X-linked B intron of all 4 primates studied) were cloned into dephosphorylated blunt-ended plasmid vector or T vector. The former vector was prepared as follows: 1 μg of pBluescript SK/+ plasmid (Stratagene) was digested to completion with SmaI restriction enzyme (New England BioLab), and dephosphorylated by 1 unit of shrimp alkaline phosphatase (United States Biochemical), the enzyme was heat inactivated, and the DNA ethanol precipitated. Twenty nanograms of dephosphory-

Table 1. PCR and sequencing primers used to amplify and sequence the last introns of the primate ZFX/ZFY genes

Primers	Sequence (5' to 3')	Position[a] (genes)	Strand[b]
oLW97	TT(GA)CAC(CA)(AT)AGATGAGTCTG(GC)TGGCCT	1161-1186[c] (ALL)	S
oLW98	TGGT(CT)AGCTTGTGGCTCTCCA(TG)GTG	1441-1417[c] (ALL)	A
oLW100	TAACATTCCTTTTACTGC	~685-665 (ZFY)	A
oLW101	GTACTTCTTCCACTTCTC	~365-345 (ZFY)	S
oLW102	CCTCAAATATGTTA(TC)AAGC	~330-350 (ZFX)	S
oLW103	GCTT(GA)TAACATATTTGAGG	~350-330 (ZFX)	A
oLW104	GAGAAGTGGAAGAAGTAC	~345-365 (ZFY)	S
oLW105	AGGGAGTGAAGG(TC)GGTAC	~990-1010 (ZFX)	S
oLW106	GTACC(AG)CCTTCACTCCCT	~1010-990 (ZFX)	A
oLW107	GCAGTAAAAGGAATGTTA	~665-685 (ZFY)	S
oLW112	C(TA)CACTGCAACCTC(TC)GCC	~670-690 (ZFX)	S
oLW113	GGC(AG)GAGGTTGCAGTG(TA)G	~690-670 (ZFX)	A
oLW118	GTAAAAGGAGTGTAAATAAC	~721-740 (ZFY)	S
oLW119	GTTATTTACACTCCTTTTAC	~740-721 (ZFY)	A
oLW120	AAGTGAACAAAATTACATG	~355-373 (ZFY)	S
oLW121	CATGTAATTTTGTTCACTT	~373-355 (ZFY)	A
ZFX51	GAATAAT(AT)TTCCACCAGT	~453-470 (ZFX)	S
ZFX31	(GC)AGAGCAAGATTCTGCC	~502-485 (ZFX)	A
ZFX52	AATTTTCCACCAATTTC	~774-790 (ZFX)	S

[a]Numbers are the positions of the primers (except for the oLW97 and oLW98) starting from the 5' end of the amplified PCR products.
[b]Primers were used for generating antisense sequences, A, or sense sequences, S.
[c]Position corresponding to published human ZFX cDNA sequence (Schneider-Gadicke et al. 1989)

lated blunt-ended vector was ligated to 40 ng of PCR product. To facilitate ligation reaction, the PCR products were treated with 1 unit of Klenow to remove the Taq-generated template-independent dATP overhang at the 3' ends and with 10 units of kinase to add a phosphate group at the 5' ends. The T-vector was prepared by restricting the vector DNA with EcoRV to completion, purifying the DNA by ethanol precipitation, and then, incubating the DNA in a solution containing 1X PCR buffer with the supplement of 2 mM dTTP and 1 unit of Taq DNA polymerase at 72 °C for 2 h. PCR products were ligated to T-vector without any pre-treatment. One-fifth of the ligated DNA was used to transform

Table 2. PCR and sequencing primers used to amplify and sequence introns A and B of the SMCX and SMCY genes

Primers	Sequence (5' to 3')	Position[a] (Intron; Linkage)	Strand[b]
smc1	GGATGTGTTCTCG(AT)GGGGATGA	1535 (Intron B; X and Y)	S
smc2	GCCATCTC(AT)CCAAAGCTCTGCA	1713 (Intron B; X and Y)	A
smc3	GTGGTGAAGG(AG)GGAG(TG)T(AC)GGTG	1366 (Intron A; X)	S
smc4	GTAGTTGTCAT(TC)(AG)CAGCCATCACA	1555 (Intron A; X)	A
smc5	TTTGGGCACCTCCAGATGCCTC	1619 (Intron A; Y)	A
smc6	GGAG(GC)AATCACAGCAGTGCCCAG	1499 (Intron A; Y)	S
smc12	TGCCCAGTTTGTAAGGAC(AT)CATG	12 (Intron A; Y)	S
smc11s	TCAGCCATAGTCTTAG	99 (Intron B; X)	S
smc12s	GGGAAAATGTCAGAAATG	343 (Intron B; X)	S
smc13s	TGAGGCAGTG(CA)TACAGG	1849 (Intron B; X)	S
smc14s	TGCATATTG(GA)CCTTGGTA	2219 (Intron B; X)	S
smc15s	GCCAGTG(TC)CTTCATTTT	2812 (Intron B; X)	S
smc151s	CTCCTGACCTCA(GA)GTGA	3075 (Intron B; X)	S
smc16s	AAAAA(TC)(GA)GTGGCAGAAG	3586 (Intron B; X)	S
smc21s	TCCTCCCTACTGAAATG	3723 (Intron B; X)	A
smc23s	GTCTG(GA)TCCACTGACTTA	2308 (Intron B; X)	A
smc24s	GCCCTTACTGCTGCTTC	1966 (Intron B; X)	A
smc25s	GCACTCCAGCCTGG(GT)T	2871 (Intron B: X)	A
smc26s	ATGGTTATAAAG(TG)AACA	1518 (Intron B; X)	A
smc27s	CTCAGAA(TA)TTGAAAG	685 (Intron B; X)	A
smc31s	GCAAGAAAGGGTGATCA	277 (Intron A; X)	S
smc32s	TTGTTTTGCC(TC)TCAGGT	705 (Intron A; X)	S
smc41s	(TC)ATCATCCAGTCTTGAA	894 (Intron A; X)	A
smc42s	G(GA)ACTT(CA)ATTGAAAAGGT	636 (Intron A; X)	A
smc51	ACTCC(TC)A(GC)TTCAATCTTGATG	780 (Intron A; Y)	A
smc52	CACAGAAGAGAAACATGAAGCAAAG	2183 (Intron A; Y)	A
smc53	TACTCA(CT)CTCCCAGAATGGCTA	1357 (Intron A; Y)	A
smc54	ATCCAGATATTTTTGTG	887 (Intron A; Y)	A
smc61	CTTGTAGAAAAGC(CT)CATCCTACT	2056 (Intron A; Y)	S
smc62	GATTGGGAGATCAT(GC)TTTAC(TC)A	808 (Intron A; Y)	S
smc63	TATTGGAT(GA)CCAGTTCT	1303 (Intron A; Y)	S
smc64	CTTTTACAGCTTAATATAGGAGA	293 (Intron A; Y)	S
smc523	TAACTCAA(GT)AGCAGAAG	1375 (Intron A; Y)	A
smc611	G(GC)CAG(AT)TAAATTTTTAAC	2377 (Intron A; Y)	S
smc623	ATGTTACCTAGAAACATA	1163 (Intron A; Y)	A
smc625	TTTTGTATTG(GA)CTTCTT	962 (Intron A; Y)	S

[a]Underlined numbers are the positions of the 3' end of the primers corresponding to the human SMCX cDNA sequence (Wu et al. 1994; GenBank accession no. L25270); the other numbers are the positions of the 3' end of the primers corresponding to the aligned sequences (available upon request).
[b]Primers were used for generating antisense sequences, A, or sense sequences, S.

competent *E. coli* cells prepared as described in Inoue et al. (1990), and the recombinant colonies were screened using PCR (Chang 1995).

Sequencing

The sequences of the cloned SMC introns were determined in one strand by sequencing the serially deleted subclones generated by the Erase-a-Base System (Promega) according to the protocol provided by the vendor. Internal primers were then designed to "walk" the PCR products by direct cycle sequencing.

All the sequences were determined from both strands using fmol Cycle Sequencing (Promega) on purified PCR products or unidirectionally deleted subclones. PCR and sequencing primers (Tables 1 and 2) were end labeled, and a typical end-labeling reaction contained 1X kinase buffer (Promega), 4 pmole of primer, and 2.5 μl of 32P ATP (3000 Ci/mmol) in a volume of 7.5 μl, which was enough for sequencing 5 templates. For each sequencing reaction 10 to 100 fmole of template in a volume of 5 μl was used. The template was mixed with 12 μl of reaction mix containing 1.5 μl of labeled primer, 6.05 μl H_2O, 4.25 μl of 5X reaction buffer (200 mM Tris pH 9.0, 10 mM $MgCl_2$), and 1 unit (0.2 μl) of Sequencing Grade Taq DNA polymerase (Promega). Four microliters of the above mixture was added to 1 μl of each of the 4 dideoxynucleotide mixes provided in the kit, and the total 5 μl solution was overlaid with ~50 μl of mineral oil. The cycle sequencing reaction was done in a Omnigene thermal cycler (Hybaid) by first heating the mix at 92 °C for 1 min, then 94 °C for 30 sec, 50 to 70 °C for 30 sec, and 72 °C for 30 sec through a total of 20 cycles, and finally 94 °C for 30 sec, 72 °C for 30 seconds through 10 cycles. Four microliters of stop buffer (0.025% Bromophenol Blue and 0.025% Xylene cyanol in 95% formamide) was added to the reaction, and 1 μl of the mixture was used to run 6% Long Ranger gel (AT Biochemistry) at a constant wattage of 50 W for 3 to 4 h. The gel was lifted, dried, and exposed to Kodak XAR5 X-ray film for 12 to 24 hours.

Sequence analysis

Sequences were aligned using the GCG package (Devereux et al. 1984) installed at the M.D. Anderson Cancer Center and refined using the VOSTORG software (Zharkikh et al. 1991). The sequence divergences were calculated using Tajima and Nei's (1984) method in the MEGA package (Kumar et al. 1993). Gaps were excluded from comparison. Phylogenetic trees were constructed using the neighbor-joining method (Saito and Nei 1987). Estimation of α was as described previously (Shimmin et al. 1993b).

RESULTS

Last intron sequences of ZFX/ZFY

The consensus primers oLW97 and oLW98 amplified the targeted region of the ZFX and ZFY genes. The amplified sequences contained 65 base pairs (bp) of the upstream exon, 185 bp of the downstream exon, and the last intron of variable length in different species and genes. The sizes of the last ZFX introns are 1 140 and 1 146 bp for the colobus monkey and tamarin, respectively, and those of the last ZFY introns are 777 and 779 bp for the colobus monkey and tamarin, respectively. The ZFX introns are larger than the ZFY introns because of the presence of an Alu repeat.

SMCX/SMCY intron A sequences

Primers smc3 and smc4 amplified the regions of the SMCX genes containing 88 and 33 bp of the upstream and downstream exons, respectively, and intron A of various lengths in different species. The sizes of intron A are 946, 947, 939, and 941 bp, respectively, in the human, orangutan, baboon, and squirrel monkey.

Primers smc5 and smc6 amplified the regions of the SMCY gene containing 3 and 124 bp of the upstream and downstream exons, respectively, and intron A of 2 604 bp in the baboon and of 2 247 bp in the squirrel monkey. They failed to produce any PCR product from the human or orangutan. Baboon and squirrel monkey PCR products were cloned and their sequences determined in one strand using the Erase-a-Base System (Promega). Consensus primers (Table 2) between baboon and squirrel monkey sequences were designed to amplify short and overlapping fragments in the human and orangutan. This succeeded in the human and yielded 2 550 bp of intron A sequence with perhaps a few bp at the 5' end of intron A undetermined because one of the primers (smc12; Table 2) was 13 bp 3' toward the splicing junction of the aligned baboon and squirrel monkey sequences. This strategy succeeded only partially in the orangutan and yielded

a 1 284 bp partial sequence belonging to the 3' half of intron A. An Alu repeat was found inserted in a conserved position of human, orangutan, and baboon intron A of the SMCY gene but was absent from the squirrel monkey.

SMCX/SMCY intron B sequences

Smc1 and smc2 primers amplified regions of the SMCX and SMCY genes containing 118 bp of the 5' exon, 58 bp of the 3' exon, and intron B sequences of various lengths in different species. The sizes of SMCX intron B, in the order of human, orangutan, baboon, and squirrel monkey, are 2 776, 2 774, 3 081, and 3 253 bp, respectively; and those of the SMCY, in the human, orangutan, and baboon, are 418, 419, and 418 bp, respectively. These two primers and two other consensus primers (data not shown) designed from aligned human, orangutan, and baboon SMCY sequences failed to amplify the homologous squirrel monkey sequence. Because the size of this Y-linked intron was small we did not pursue it further.

Many Alu repeats were found in intron B of the SMCX gene. Four Alu repeats were present in all 4 species studied, 2 of which were inserted back to back into intron B and were partially deleted from the human, orangutan, and baboon. Another was present in the human, orangutan, and baboon, but was absent from the squirrel monkey. The baboon and squirrel monkey each have an additional unique Alu insertion.

Sequence divergence

ZFX/ZFY introns

The last intron sequences of the colobus monkey and tamarin ZFX/ZFY genes were aligned (the alignment not shown but available upon request) with the homologous sequences generated in an earlier study of ours (Shimmin et al. 1993b); the Alu repeats were not included. These sequences were used to calculate sequence divergences using Tajima and Nei's (1984) method, and the results are shown in Tables 3 and 4. The divergences of the ZFX introns are in the range of 2.10% - 8.31%, and those of the ZFY introns are in the range of 3.44% - 17.63%. The divergences between the same species pair are larger in the ZFY introns than in the ZFX introns. For example, between the human and orangutan the divergence in the ZFY introns is 4.35% (Table 4), while that in the ZFX introns is 2.10% (Table 3).

The degrees of divergence fit well with the evolutionary scheme of these primates. As shown in Figure 1, the ZFX and ZFY introns form 2 separate clusters, and in each cluster, introns of the 2 New World monkeys (the squirrel monkey and tamarin) form 1 clade. This clade is then joined to introns of the other 2 clades, the human-orangutan

Table 3. The mean (below diagonal) and standard error (above diagonal) of the number of nucleotide substitutions per site between primate ZFX intron sequences

	Human	Orangutan	Baboon	Colobus monkey	Squirrel monkey	Tamarin
Human		0.0053	0.0060	0.0071	0.0102	0.0097
Orangutan	0.0210		0.0063	0.0071	0.0101	0.0094
Baboon	0.0265	0.0291		0.0055	0.0101	0.0106
Colobus monkey	0.0360	0.0373	0.0224		0.0111	0.0106
Squirrel monkey	0.0716	0.0711	0.0699	0.0831		0.0074
Tamarin	0.0657	0.0625	0.0641	0.0771	0.0401	

Table 4. The mean (below diagonal) and standard error (above diagonal) of the number of nucleotide substitutions per site between primate ZFY intron sequences

	Human	Orangutan	Baboon	Colobus monkey	Squirrel monkey	Tamarin
Human		0.0082	0.0104	0.0103	0.0183	0.0179
Orangutan	0.0435		0.0103	0.0100	0.0179	0.0176
Baboon	0.0675	0.0659		0.0073	0.0183	0.0178
Colobus monkey	0.0659	0.0626	0.0344		0.0182	0.0174
Squirrel monkey	0.1781	0.1707	0.1768	0.1763		0.0129
Tamarin	0.1736	0.1681	0.1717	0.1675	0.0989	

clade and the Old World monkey clade (the baboon and colobus monkey). Since the branch length of a neighbor-joining tree is a measure of degree of divergence, it is noteworthy that, with the exception of the branch leading to the colobus lineage, every branch in the ZFY cluster is longer than the corresponding branch of the ZFX cluster - a manifestation of ZFY introns evolving faster than ZFX introns.

The degrees of divergence between ZFX introns and ZFY introns are quite uniform across all pairwise comparisons ranging from 30.76% to 36.41% (Table 5).

SMCX/SMCY introns

Similar trends in divergence can be observed in the introns of the SMCX and SMCY genes (Tables 6 - 9) in that all the Y-linked introns are evolving faster than the X-linked introns. However, because of the large size differences between homologous introns of the SMCX and SMCY genes, it is difficult to align the X-linked sequences to the homologous Y-linked sequences for phylogenetic tree construction.

Male-to-female ratio of mutation rate

ZFX/ZFY introns

Figure 2 shows a simplified tree in which the branch lengths of the ZFY intron sequences (Y) and those of ZFX intron sequences (X) are indicated as Y/X above each branch, and the ratio of these two numbers is indicated below each branch. Although, as stated earlier, almost every

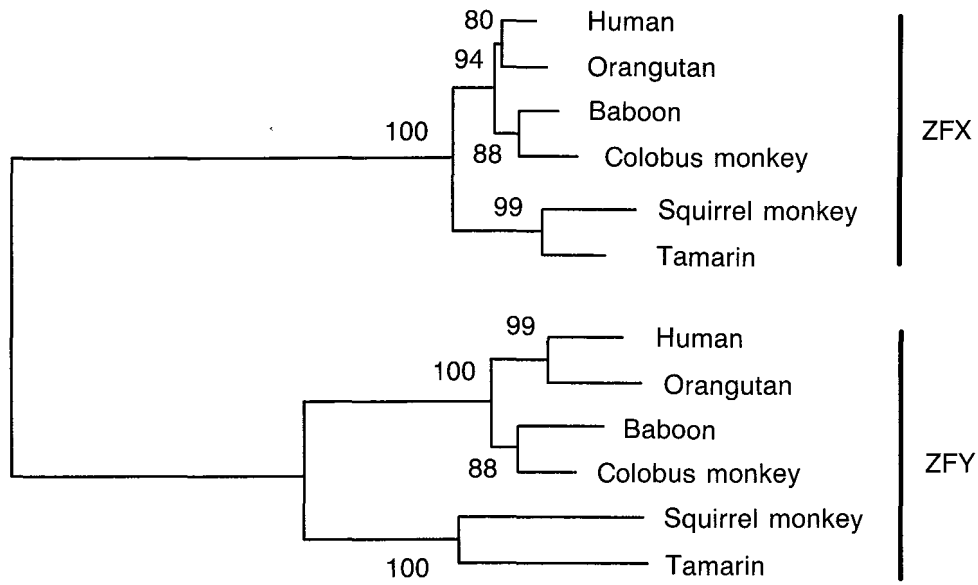

Fig. 1. Phylogenetic tree of the last introns of the ZFX and ZFY genes in primates constructed by the neighbor-joining method (Saito and Nei 1984). Numbers represent bootstrap proportions (100 replicates).

Table 5. The mean and standard error of the number of nucleotide substitutions per site in the last introns of the primate ZFX and ZFY genes. Species: Hum, humans; Ora, orangutans; Bab, baboons; Col, colobus monkeys; Squ, squirrel monkeys; Tam, tamarins. Genes: X, ZFX; Y, ZFY

	HumX	OraX	BabX	ColX	SquX	TamX
HumY	0.3196 ± 0.0291	0.3251 ± 0.0296	0.3171 ± 0.0291	0.3381 ± 0.0285	0.3490 ± 0.0290	0.3415 ± 0.0290
OraY	0.3225 ± 0.0295	0.3362 ± 0.0303	0.3228 ± 0.0298	0.3441 ± 0.0289	0.3641 ± 0.0300	0.3509 ± 0.0295
BabY	0.3158 ± 0.0290	0.3264 ± 0.0294	0.3076 ± 0.0285	0.3224 ± 0.0280	0.3452 ± 0.0293	0.3376 ± 0.0294
ColY	0.3095 ± 0.0307	0.3146 ± 0.0311	0.3015 ± 0.0297	0.3219 ± 0.0296	0.3388 ± 0.0306	0.3260 ± 0.0310
SquY	0.3244 ± 0.0310	0.3383 ± 0.0320	0.3276 ± 0.0310	0.3453 ± 0.0305	0.3467 ± 0.0306	0.3380 ± 0.0309
TamY	0.3278 ± 0.0306	0.3359 ± 0.0314	0.3319 ± 0.0306	0.3528 ± 0.0297	0.3559 ± 0.0299	0.3446 ± 0.0302

branch of the ZFY intron sequence is longer (evolving faster) than the ZFX intron sequence, the ratio Y/X varies considerably among branches; for example, in the branch leading to the colobus monkey the branch length ratio Y/X is 0.99, while in the branch leading to the baboon Y/X is 3.03. This is perhaps due to statistical fluctuations resulting from the relatively small number of substitutions in each branch. To reduce such statistical fluctuations all the branches are considered together, and the Y/X ratio becomes (0.023 96 + 0.019 54 + 0.010 78 + 0.015 78 + 0.018 39 + 0.015 97 + 0.054 01 + 0.036 65 + 0.052 10 + 0.046 83) / (0.010 48 + 0.010 54 + 0.002 68 + 0.007 84 + 0.006 06 + 0.016 33 + 0.016 82 + 0.017 68 + 0.023 31 + 0.016 74) = 0.2940 / 0.1285 = 2.288. As noted in Shimmin et al. (1993b), this quantity is actually E(Y/X), where E means taking expectation. What is needed is E(Y)/E(X), which is given by $E(Y)/E(X) \approx E(Y/X) - E(X)V(Y)/E(Y)^3$, where V means variance. E(Y)/E(X) is approximately equal to 2.265. Using Miyata et al's (1987) formula, $Y/X = 3\alpha/(\alpha+2)$, α is estimated to be 6.2, which is an average estimate for these primates. The variance is approximately 0.0815. Therefore, the estimate of E(X)/E(Y) is 2.265 ± 0.286, from which the 95 % confidence interval of α can be calculated to be roughly between 2.6 and 33.3.

SMCX/SMCY introns

Using the same method as above, the α values from SMCX and SMCY intron sequences were calculated. Because there is a stretch of sequence missing from the orangutan Y-linked intron A as compared to those from other species, the branch lengths of this Y-linked intron and the α estimates

Table 6. Mean and standard error of the number of nucleotide substitutions per site between intron A sequences of the primate SMCX genes

	Human	Orangutan	Baboon
Human			
Orangutan	0.0153 ± 0.0041		
Baboon	0.0356 ± 0.0064	0.0344 ± 0.0063	
Squirrel monkey	0.0874 ± 0.0105	0.0886 ± 0.0106	0.0936 ± 0.0109

Table 7. Mean and standard error of the number of nucleotide substitutions per site between intron A sequences of the primate SMCY genes

	Human	Orangutan	Baboon
Human			
Orangutan	0.0530 ± 0.0081		
Baboon	0.0888 ± 0.0108	0.0837 ± 0.0105	
Squirrel monkey	0.1832 ± 0.0169	0.1751 ± 0.0164	0.1708 ± 0.0159

Table 8. Mean and standard error of the number of nucleotide substitutions per site between intron B sequences of the primate SMCX genes

	Human	Orangutan	Baboon
Human			
Orangutan	0.0212 ± 0.0036		
Baboon	0.0419 ± 0.0051	0.0475 ± 0.0054	
Squirrel monkey	0.0843 ± 0.0075	0.0897 ± 0.0077	0.0977 ± 0.0081

Table 9. Mean and standard error of the number of nucleotide substitutions per site between intron B sequences of the primate SMCY genes

	Human	Orangutan
Human		
Orangutan	0.0755 ± 0.0145	
Baboon	0.1146 ± 0.0182	0.1028 ± 0.0171

were estimated in two ways. First, the orangutan Y-linked intron A sequence was excluded, and a phylogenetic tree (not shown) was reconstructed using only human, baboon, and squirrel monkey Y-linked intron A sequences. These branch lengths were then compared to the corresponding branch lengths of the X-linked intron A sequences in a separate phylogenetic tree reconstruction (orangutan SMCX intron A sequence was excluded; data not shown). The estimate of α is 4.2 with a 95% confidence interval from 2.2 to 10.0 for the three primate species used. Second, the part of the sequences in the human, baboon, and squirrel monkey which is missing from the orangutan was excluded and a phylogenetic tree was constructed. The branch lengths of this tree were compared to those of the X-linked intron A phylogenetic tree, and an α estimate of 3.5 was obtained with a 95% confidence interval of 1.7 to 8.5. The average of the two estimates is 3.9.

The sequence of the squirrel monkey Y-linked intron B was not obtained so the comparison was made between the Y-linked and the X-linked intron B for the human, orangutan, and baboon only. The estimate of α from these data is 12.3 with a 95% confidence interval from 2.8 to infinity. This large interval is due to the small size of the Y-linked introns.

DISCUSSION

In this study the last introns of the ZFX/ZFY genes and 2 introns of the SMCX/SMCY genes were used to estimate the sex ratio of mutation rate, α, in higher primates. It was assumed that introns are selectively neutral because most of the intron sequences are non-functional with the exception of some sites such as splicing junctions and lariate-forming sites, which are very short and may not have affected our analysis. Certain intronic regions may have regulatory functions and may be conserved across species, but no conserved regions seem to exist in the introns that were analyzed.

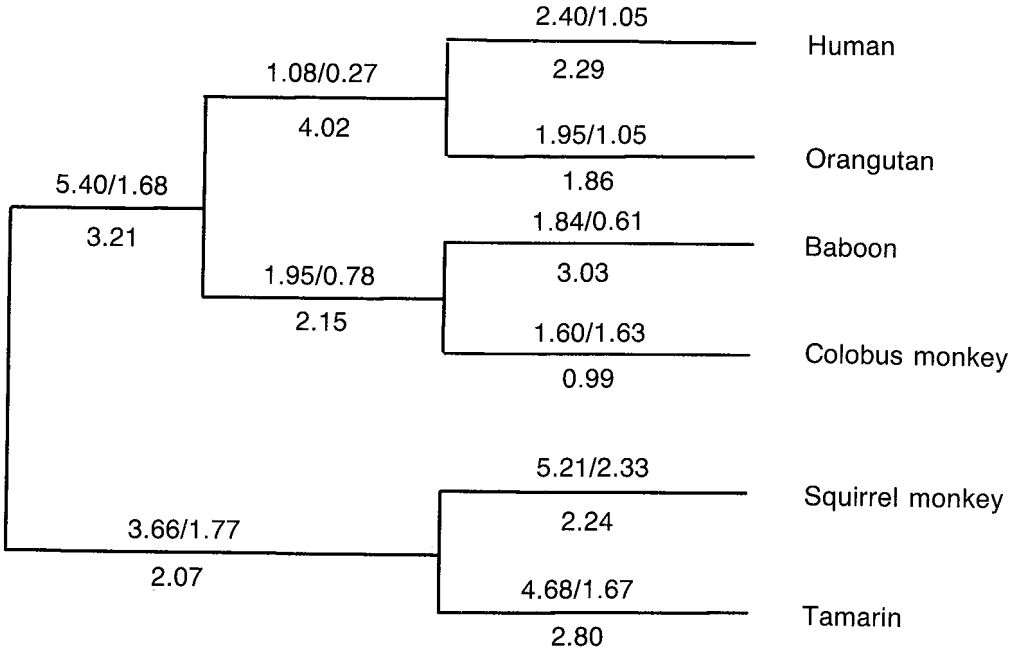

Fig. 2. Schematic phylogeny of the primate ZFX/ZFY last intron sequences. The numbers above each branch are branch lengths estimated from the Y-linked and X-linked introns (Table 2), respectively, and the number below each branch is the ratio of the preceding two numbers.

The chromosomal locations of ZFX/ZFY and SMCX/SMCY are quite different. The ZFX/ZFY genes are known to be located close to the pseudoautosomal boundary at the p-ter of the human sex chromosomes (Coopman et al. 1991). Human SMCX is located at Xp11.1-Xp11.2 near the centromere and human SMCY is located at Yq11.22 (Agulnik et al. 1994b). The mutation rates in ZFX/ZFY and SMCX/SMCY introns are somewhat different (Tables 3 - 4; 6 - 9). For example, the divergence between the human and orangutan in the ZFY introns is 0.0435 (Table 4), while it is 0.0530 in intron A of the SMCY genes (Table 7). The estimates of α from introns of these two sets of genes (6 and 4.2, respectively), however, are not significantly different from each other or from the estimate using direct genomic sequencing of the factor IX gene (α = 3.5; Ketterling et al. 1993). Therefore, the α value in higher primates is probably between 3 and 6.

For the ZFX/ZFY introns, compared with the smaller data set in Shimmin et al. (1993b), the significance of the current estimate is the reduction of the 95% confidence interval from (2, 84) to (3, 33). Thus, by increasing the data by about 50% the variability of the estimate has been narrowed more than 2-fold, while the mean estimate stays the same ($\sim$6). Using SMCX/SMCY introns, which are longer than the last intron of the ZFX/ZFY genes, a smaller 95% confidence interval (from 2.2 to 10.0) was obtained. So, it seems that these estimates are quite accurate for higher primates.

There are several possible factors that may contribute to sex differences in germ-line substitution mutations: (a) differential protection in the male and female germ-lines against insults from environmental and biological mutagens, (b) differential methylation in male and female germ-lines, and (c) sex differences in the number of germ cell divisions that lead to more chances for misincorporation during DNA replication in the male germ-line.

Studies on people who survived the atomic bombs in Nagasaki and Hiroshima have revealed that germ-lines are more protected from gamma radiation than somatic tissues; however, relatively little is known as to whether there are sexual differences in terms of physical and biochemical protection mechanisms against mutagens in germ-lines. So it is difficult to correlate the observed sexual difference in mutation rate to this factor.

Studies on germ-line DNA methylation have shown that female germ-line DNA is markedly unmethylated, while male germ-line DNA is heavily methylated (Monk et al. 1987, Driscoll and Migeon 1990). DNA methylation occurs mostly at CpG dinucleotides (Singer and Berg 1991) and these dinucleotides are prone to transition mutation. On the sense strand a methylated C residue at the CpG dinucleotide is easily transformed to a T residue through deamination reaction and this creates a C to T transition. On the antisense strand a methylated C can be transformed to a T, and then by chance the complementary G is mistakenly repaired to an A, which creates a G to A transition. Studies on the human low-density lipoprotein receptor (LDLR) and p53 genes suggested that certain cytosine residues in these two genes that are known to have gone through germ-line mutations are indeed methylated in the germ-line (Rideout 1990). Ketterling et al. (1993) studied the sex ratio of mutation rate in the factor IX gene and found an 11-fold excess of point mutation in males over females at CpG dinucleotides. They postulated that the hypo-methylation in oocyte DNA is the major reason for this huge difference. This may indeed be the case for the factor IX gene, as 35% of the point mutations that have been identified so far in this gene were transitions occurring at CpG dinucleotides (Koeberl et al. 1990). However, the CpG frequency is variable among genes, and not all CpG's are highly mutable; for example, mutation in CpG accounts for only $\sim$10% of all known mutations in the β globin and HPRT genes, though more than 60% in the ADA gene (reviewed in Cooper et al. 1995). Moreover, mutability is also variable within a gene; for example, in exon 7 of the Protein C gene, 9 out of the 12 known mutations occurred at CpG, while, in exons 5 and 6 of this gene, none of the 13 known mutations was at CpG, even though these 2 exons have many CpG sites (Cooper et al. 1995). So, high mutability at CpG does not seem to affect all genes and/or DNA regions. In the introns we examined, only 5 CpG's were found in the primate ZFX and only 2 of these were mutated in at least 1 of the lineages, which represent only about 2% of the total number of variable sites (97) in the alignment. No CpG was found in the last intron of primate ZFY genes. In intron A of the SMC genes, there were 4 CpG's in the SMCX genes, 3 of which have mutated in at least 1 of the lineages ($\sim$3% of the total mutations). One CpG was found in the SMCY genes and it was mutated, which represents less than 0.5% of the total number of variable sites (208). In intron B of the SMC genes, 5 CpG's were found in the SMCX genes and 3 of them were mutated ($\sim$1.5% of the total mutations), while,

no CpG was found in the SMCY genes. We have also found low CpG frequencies in the Zfx/Zfy and Ube1x/Ube1y introns of rodents (Chang 1995). Hence, even though the C residue at CpG dinucleotides may be highly mutable, the frequencies of CpG are very low in the intron sequences analyzed. So, it is very unlikely that the higher mutation rate in males observed in this study was a result of the methylation-mediated deamination at CpG dinucleotides.

The accuracy of DNA polymerization is determined by a combination of different factors such as the frequency of misincorporation and the efficiency of proof-reading, and these depend on the properties of different DNA polymerases and the microenvironment at the time of replication. Cooper and Krawczak (1993) collected known substitution mutations that caused human diseases and compared the mutation spectrum with that observed in vitro for 3 different eukaryote DNA polymerases (α, β, and δ). They found that the spectrum observed in human diseases caused by mutation fits well with the mutation spectrum generated by DNA polymerase β. Since the in vitro studies used purified polymerases, which lack proofreading and repairing abilities, they concluded that most of the human disease-causing substitution mutations are perhaps generated through the misincorporation by DNA polymerase β. If this is indeed the case, then it follows that the more rounds of replication the DNA undergoes, the higher the substitution rate, and thus the more times the cell divides, the higher the mutation rate. The results obtained in our studies support the above explanation (Shimmin et al. 1993b, Chang et al. 1994, Shimmin et al. 1994, Chang and Li 1995). We have shown, not only that mammalian males have a higher substitution mutation rate than do females, but also that the male-to-female ratios of substitution rate (α) are close to the male-to-female ratios of germ cell divisions (c; Chang et al. 1994). So, it is quite convincing that replication errors are a major factor (in addition to methylation-mediated deamination at CpG dinucleotides) in accounting for the higher substitution rate in males.

There has been a long controversy over the existence of a molecular clock, that is, all protein and DNA sequences evolve at constant rates among lineages (Zukerkandl and Pauling 1965). According to this hypothesis, the number of changes between two sequences can be translated into the number of years since the two sequences diverged. Replication-dependent mutagenesis as suggested in our studies argues against the existence of a global clock, and predicts a generation-time effect on the rate of DNA sequence evolution, that is, an inverse correlation between generation time and substitution rate. This generation-time effect is consistent with many studies on DNA sequence divergences in different lineages of mammals (reviewed in Li and Graur 1991).

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利用intron序列估計高等靈長類之雌雄突變速率比例

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人類遺傳學家Haldane認爲男性的突變速率比女性的高出許多，因爲在一個世代中，爲製造精子所必須經過的生殖細胞分裂數目要遠比製造卵子所必須經過的細胞分裂數目大得多。如果絕大部分的突變都發生在細胞分裂週期中的染色體複製過程，則Haldane的推測可能是正確的，但是，究竟男性的突變速率比女性高多少？又雌雄突變速率比是否與雄雌生殖細胞分裂數目比相同？爲解決數量上的問題，Miyata et al. 提出了一套方法，此方法利用不同染色體在不同性別個體上有不同分離率之性質—亦即，體染色體半數在雄性，半數在雌性；X染色體1/3在雄性，2/3在雌性；Y染色體完全在雄性。所以比較同源基因在不同染色體上的突變速率可以讓我們估計出雄雌突變速率之比值。

我們先前用了四種高等靈長類的ZFX(在X染色體)及ZFY(在Y染色體)兩個基因的最後一段intron的DNA序列估算出雄雌突變速率比是6，但是由於此估值的百分之九十五的信賴區間很大(從2到84)，所以我們希望能用更多的DNA序列來做更精確的估計。我們用聚合酶連鎖反應(PCR)的方法擴大合成了兩種猴子(colobus monkey及tamarin)的ZFX/ZFY基因的最後一段intron，我們決定了這些introns的DNA序列，然後將這些資料與先前四種靈長類同源introns的DNA序列合併起來做分析，我們估算出雄雌突變速率比值仍然是6，但百分之九十五信賴區間已經縮小到(3, 33)。

利用同樣的方法，我們也決定了四種高等靈長類的SMCX/SMCY基因的兩段introns（A和B)的DNA序列。用intron A的DNA序列，我們得到的估值是4.2，其百分之九十五信賴區間是(2.2, 10.0)；用intron B所得的估值是12.3，其百分之九十五信賴區間是(2.8, ∞)；後者的估值有較大的誤差，可能是因爲在Y染色體上的intron B非常短的緣故。

由於從不同基因組所得到的高等靈長類雄雌突變速率比值相接近(6及4.2)，而且這些估值也很接近人類雄雌細胞分裂數目的比值(3～6)，這顯示大部份的突變是源自細胞分裂週期中的染色體複製過程。我們同時也討論了其他可能造成這種現象的因素，以及本研究結果提供「分子時鐘」假說的反證據。

關鍵詞：置換突變，突變機制，性別差異，靈長類。

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