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Evolution of the X-linked Zinc Finger Gene and the Y-linked Zinc Finger Gene in Primates

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We have sequenced the partial exon of the zinc finger genes (ZFX and ZFY) in 5 hominoids, 2 Old World monkeys, 1 New World monkey, and 1 prosimian. Among these primate species, the percentage similarities of the nucleotide sequence of the ZFX gene were 96–100% and 91.2–99.7% for the ZFY gene. Of 397 sites in the ZFX and ZFY gene sequences, 20 for ZFX gene and 42 for ZFY gene were found to be variable. Substitution causes 1 amino acid change in ZFX, and 5 in ZFY, among 132 amino acids. The numbers of synonymous substitutions per site (Ks) between human and the chimpanzee, gorilla and orangutan for ZFY gene were 0.026, 0.033, and 0.085, respectively. In contrast, the Ks value between human and hominoid primates for the ZFX gene was 0.008 for each comparison. Comparison of the ZFX and ZFY genes revealed that the synonymous substitution levels were higher in hominoids than in other primates. The rates of synonymous substitution per site per year were higher in the ZFY exon than in the SRY exon, and higher in the ZFY exon than in the ZFY intron, in hominoid primates.

Keywords: Evolution Rate; Primates; Synonymous Substitution; ZFX Gene; ZFY Gene.

Introduction

The Y-linked zinc finger gene (ZFY) is located on the Y-chromosome near the pseudoautosomal boundary (Page *et al*., 1987) and a closely related gene, ZFX, is present on the X-chromosome (Schneider-Gädicke *et al*., 1989). The mouse has four zinc finger related genes. These are two related Y-linked genes (Zfy-1 and Zfy-2), one X-linked gene (Zfx), and an autosomal homologue on chromosome 10 (Zfa) (Ashworth *et al*., 1989; 1990; Mardon and Page, 1989; Mardon *et al*., 1990). Lanfear and Holland (1991) identified a Zfx from Chinese hamster, Zfx and Zfy from a crab-eating fox and related Zf genes from two bird species, the great tit and the lesser black-backed gull. The Zfy gene has been also detected in the wood lemming (Lau *et al*., 1992).

The mutation rates of DNA sequences during evolution can be estimated from DNA sequence differences, which are due to phenotypic variation and the selection of biochemical mechanisms such as DNA replication or repair (Britten, 1986). The number of cell divisions differs between sperm and ova (Winter, 1983). The different number of germ-cell divisions between males and females result in different mutation frequencies between autosomes and sex chromosomes (Miyata *et al*., 1987). In estimation of the sex ratio of the mutation rate, direct genomic sequencing provides a good way to trace the origin of mutations (Ketterling *et al*., 1993). Shimmin *et al*. (1993) sequenced the last intron of the Y-linked and X-linked zinc finger protein genes in the human, orangutan, baboon and squirrel monkey. The ratio Y/X of the substitution rate in the Y-linked intron to that in the X-linked intron is 2.3, which is close to that estimated from synonymous rates in the ZFY and ZFX genes (Lanfear and Holland, 1991). In contrast, the last intron of the zinc finger protein genes (Zfy and Zfx) in mice and rats showed weak male-driven molecular evolution (Chang *et al*., 1994). The ratio of the rates of synonymous substitution in the ZFY and ZFX exon genes is estimated to be 2.1 in primates, 1.3 in the rat lineage, and 4.2 in the mouse lineage (Shimmin *et al*., 1994). Therefore, the rate of molecular evolution is not constant in mammals (Britten, 1986; Kikuno *et al*., 1985).

The aim of the present study is: (1) to examine the number of synonymous substitutions per site in the

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Abbreviations: ZFX, X-linked zinc finger gene; ZFY, Y-linked zinc finger gene.

ZFX and ZFY genes of 9 non-human primates, (2) to determine which species has higher synonymous substitution levels by comparing ZFX and ZFY genes, and (3) to compare rates of synonymous substitution per site per year with other genes.

Materials and Methods

Primate samples Heparinized blood samples from the chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), agile gibbon (*Hylobates agilis*), siamang (*Hylobates syndactylus*), Japanese monkey (*Macaca fuscata*), green monkey (*Cercopithecus aethiops*), tamarin (*Saguinus tripartitus*), and the ring-tailed lemur (*Lemur catta*) were collected at the Primate Research Institute, Kyoto University.

DNA isolation DNA for titration experiments was isolated from primate blood samples using a standard lysis solution (5 mg/ml Proteinase K, 10 mM Tris, 100 mM NaCl, 1 mM EDTA, 10% SDS). All samples were dialyzed overnight in TE solution (10 mM Tris and 1 mM EDTA) after being incubated for 2 h at 55°C for phenol extraction. DNA concentration was calculated from the OD_{260} and the DNA then diluted to 100 ng/μl.

PCR amplification The ZFX and ZFY genes were amplified in a 50 μl-volume polymerase chain reaction using 25-mer oligonucleotides primers ZF-a and ZF-b representing nucleotides 253–277 and 675–699 respectively, from a region of the published genome sequence of the human zinc finger domain (Schneider-Gädicke *et al*., 1989). Primer ZF-a is 5′-ATAACCACCTGGAGAGCCACAAGCT-3′ and primer ZF-b is 5′-ACTTTCTCAGATACCAAAGAAGTGC-3′. The PCR consisted of 30 cycles performed as follows: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min, on a Perkin Elmer Cetus DNA thermal cycler. This main PCR amplification cycle was preceded by an initial denaturation step of 94°C for 3 min and followed by an additional extention period of 72°C for 5 min.

Cloning of PCR products PCR products were gel-purified by electro-elution into DEAE 81 paper. When all of the DNA fragments left the gel and were trapped on the DEAE 81 paper, the electric current was turned off. Fragments of DNA were separated by concentration of the low-salt wash buffer and the high-salt elution buffer, extracted with phenol: chloroform, precipitated with EtOH, and quantitated after resuspension in TE buffer. The ZFX and ZFY products were treated with T4 DNA polynucleotide kinase, and ligated into alkaline phosphatase-treated *Sma*I-cut plasmid 118 (Takara). The ligation reaction products were then transformed into competent *Escherichia coli* JM 109 (Takara). Plasmid DNA was extracted by an automatic plasmid isolation system (Pharmacia).

DNA sequencing and data analysis Both strands of the insert of plasmid DNA were sequenced by dideoxy chain termination (Sanger *et al*., 1977) with an autocycle sequencing kit (Pharmacia). At least three of the cloned fragments from each of the PCR-amplified DNAs were sequenced. The determined sequences were aligned with the aid of GENETYX (Ver. 9, SDC, Tokyo) and the numbers of synonymous and nonsynonymous substitutions were estimated by the method of Li (1993). The various analyses of nucleotide sequences were done using the MEGA program (Ver. 1.01, USA).

Results

Nucleotide sequence of ZFX and ZFY genes We analysed the last exon partial sequences of the ZFX and ZFY genes in 5 hominoids, 2 Old World monkeys, 1 New World monkey and 1 prosimian. No insertions or deletions were found in this segment in any individual examined. Of 397 sites in the ZFX and ZFY gene sequences, twenty from ZFX and forty two from ZFY were found to be variable. Substitutions cause 1 amino acid change in ZFX and 5 in ZFY, among 132 amino acids. Comparison of mouse Zfy-1 and human ZFY revealed that thirty three putative amino acids were different. Comparison between mouse Zfx and human ZFX, however, showed only one putative amino acid difference, so the amino acid sequence is highly conserved in the X-chromosome of primates and rodents.

Base composition was calculated from the nucleotide sequence data (Table 1). The G + C content of the ZFX gene ranged from 43.32 to 44.84% and that of the ZFY gene ranged from 43.07 to 44.33% among all primate species. Mouse Zfx gene sequences have a G + C content of 42.82%, while Zfy-1 has a G + C content of 40.30%. The percentage similarity of nucleotide sequences of ZFX and ZFY among primates is shown in Table 2. Identical sequences in the ZFX gene were found in hominoid primates: chimpanzee, gorilla, orangutan, and gibbon. Homology for the ZFY gene ranged from 80.8% (tamarin-mouse) to 99.7% (Japanese monkey – green monkey). The percentage similarity of nucleotide

Table 1. Base composition of 397-bp fragments of the ZFX and ZFY genes in primates.

Species	ZFX/ZFY			
Human	A125/128	T95/95	G97/96	C80/78
Chimpanzee	A124/129	T95/95	G98/94	C80/79
Gorilla	A124/129	T95/94	G98/94	C80/80
Orangutan	A124/128	T95/93	G98/96	C80/80
Agile gibbon	A124/129	T95/92	G98/93	C80/83
Siamang	A124/128	T95/93	G98/94	C80/82
Japanese monkey	A126/127	T93/95	G96/96	C82/79
Green monkey	A126/127	T93/95	G96/96	C82/79
Tamarin	A125/127	T95/96	G96/96	C81/78
Ring-tailed lemur	A129/130	T96/96	G93/91	C79/80
Mouse	A132/140	T95/97	G87/80	C83/80

sequence is higher in the ZFX gene than in the ZFY gene.

The nucleotide sequence data reported in this paper appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB041907 (chimpanzee, ZFX), AB041908 (chimpanzee, ZFY), AB041909 (gorilla, ZFX), AB041910 (gorilla, ZFY), AB041911 (orangutan, ZFX), AB041912 (orangutan, ZFY), AB041913 (agile gibbon, ZFX), AB041914 (agile gibbon, ZFY), AB041915 (siamang, ZFX), AB041916 (siamang, ZFY), AB041917 (Japanese monkey, ZFX), AB041918 (Japanese monkey, ZFY), AB041919 (green monkey, ZFX), AB041920 (green monkey, ZFY), AB041921 (tamarin, ZFX), AB041922 (tamarin, ZFY), AB041923 (ring-tailed lemur, ZFX), and AB041924 (ring-tailed lemur, ZFY).

Synonymous substitutions We have computed the number of synonymous substitution per site (Ks). As shown in Table 3, the Ks values between the human and the chimpanzee, gorilla and orangutan in the ZFY gene were 0.026, 0.033, and 0.085, respectively. But an identical value of 0.008 for the ZFX gene was found in each comparison. The Ks values between the agile gibbon and the siamang and between the Japanese monkey and the green monkey in the ZFY gene were 0.015 and 0.007, respectively. In addition, the Ks value between the human and the gibbon in the ZFY (ZFX) gene was 0.135 (0.008), and the Ks value between the human and the mouse in the ZFY (ZFX) gene was 0.577 (0.426). The number of synonymous substitutions per site was higher in hominoid primates than in other primates in a comparison of the ZFX and ZFY genes (Table 4). The Ks values of the orangutan and Old World monkeys were 0.289 and 0.106, respectively. In gibbons, the Ks value of the agile gibbon was 0.259 and that of the siamang was 0.24. The value for the siamang was similar to that of the human (0.241). The Ks value of the chimpanzee was the lowest (0.222) of the hominoid primates, followed by the gorilla (0.231). The Ks value of mouse was found to be 0.495. Thus, synonymous substitution levels of comparisons within pairs of species were higher in Y-linked sequences than in X-linked sequences, and the synonymous substitution levels of ZFX and ZFY were higher in mouse than in primates.

Rates of synonymous substitutions To estimate the substitution rate between two species, we assumed that the divergence time was considered as shown in Table 5 from the paleontological data (Pilbeam, 1984). Using these divergence times and the number of synonymous substitutions, we estimated the rate of synonymous

Table 2. Percentage similarity of the nucleotide sequence of ZFX (above the diagonal) and ZFY (below the diagonal) between species.

Species	1	2	3	4	5	6	7	8	9	10	11
Human		99.7	99.7	99.7	99.7	99.7	98.7	98.7	98.0	96.7	91.7
Chimpanzee	99.5		100.0	100.0	100.0	100.0	99.0	99.0	97.7	96.5	91.4
Gorilla	99.2	99.7		100.0	100.0	100.0	99.0	99.0	97.7	96.5	91.4
Orangutan	98.0	98.5	98.2		100.0	100.0	99.0	99.0	97.7	96.5	91.4
Agile gibbon	96.7	97.2	97.5	96.7		100.0	99.0	99.0	97.7	96.5	91.4
Siamang	96.7	97.2	97.5	96.7	99.5		99.0	99.0	97.7	96.5	91.4
Japanese monkey	95.2	95.7	96.0	95.2	96.7	97.2		100.0	97.2	96.0	90.9
Green monkey	95.0	95.5	95.7	95.2	96.5	97.0	99.7		97.2	96.0	90.9
Tamarin	93.7	94.2	93.9	93.7	94.7	95.2	98.0	98.2		96.7	91.4
Ring-tailed lemur	91.4	91.9	91.7	91.2	92.7	92.7	94.2	94.5	95.2		91.9
Mouse	81.8	82.3	82.1	81.1	82.1	82.1	82.1	82.1	80.8	82.1	

Table 3. Number of synonymous substitutions per site in ZFX (above the diagonal) and ZFY (below the diagonal) genes.

Species	1	2	3	4	5	6	7	8	9	10	11
Human		0.008	0.008	0.008	0.008	0.008	0.056	0.056	0.099	0.153	0.426
Chimpanzee	0.026		0.000	0.000	0.000	0.000	0.048	0.048	0.108	0.163	0.441
Gorilla	0.033	0.007		0.000	0.000	0.000	0.048	0.048	0.108	0.163	0.441
Orangutan	0.085	0.057	0.065		0.000	0.000	0.048	0.048	0.108	0.163	0.441
Agile gibbon	0.135	0.107	0.099	0.138		0.000	0.048	0.048	0.108	0.163	0.441
Siamang	0.135	0.107	0.099	0.138	0.015		0.048	0.048	0.108	0.163	0.441
Japanese monkey	0.239	0.207	0.199	0.246	0.185	0.168		0.000	0.105	0.199	0.489
Green monkey	0.248	0.216	0.207	0.254	0.194	0.176	0.007		0.105	0.199	0.489
Tamarin	0.313	0.279	0.288	0.321	0.271	0.252	0.072	0.064		0.152	0.439
Ring-tailed lemur	0.435	0.398	0.408	0.465	0.332	0.332	0.229	0.218	0.218		0.337
Mouse	0.577	0.535	0.547	0.665	0.546	0.546	0.463	0.479	0.545	0.450	

substitutions per site per year (Vs). In the ZFY exon, Vs was: (1) 1.9×10^{-9}/site/year between human and chimpanzee, (2) 1.8×10^{-9}/site/year between human and gorilla, (3) 2.8×10^{-9}/site/year between human and orangutan, and (4) 2.6×10^{-9}/site/year between human and baboon. In the ZFY intron, the human-gorilla and human-orangutan comparisons showed the same rate of synonymous substitutions at 0.6×10^{-9}/site/year. This rate was similar to that of the SRY exon in the human-chimpanzee comparison. The rate of synonymous substitutions of human-gorilla and human-orangutan were 1.4×10^{-9}/site/year and 0.8×10^{-9}/site/year respectively in the SRY exon. Thus, in hominoid primates, the rates of synonymous substitutions per site per year were higher in the ZFY exon than in the SRY exon, and higher in the ZFY exon than in the ZFY intron.

Table 4. Number of synonymous substitutions (Ks) per site between ZFX and ZFY genes.

Species	Ks ± SE	Species	Ks ± SE
Human	0.241 ± 0.069	Japanese monkey	0.106 ± 0.037
Chimpanzee	0.222 ± 0.062	Green monkey	0.106 ± 0.037
Gorilla	0.231 ± 0.063	Tamarin	0.107 ± 0.043
Orangutan	0.289 ± 0.078	Ring-tailed lemur	0.123 ± 0.039
Agile gibbon	0.259 ± 0.070	Mouse	0.495 ± 0.103
Siamang	0.240 ± 0.068		

Discussion

It is clear from the results that the ZFX gene has been highly conserved in primates. This suggests that the structural requirement for zinc finger domains is stringent. In contrast, the ZFY gene has evolved rather rapidly in hominoids, but more slowly in other primates (Fig. 1). As for the rate variation within the primates, Li and Tanimura (1987) and Li *et al.* (1987) argued for a slowdown of rate in the hominoids, on the basis of a comparison of both noncoding regions and synonymous sites of DNA sequence data. However, a statistically significant slowdown of evolutionary rate in the hominoid lineage has been observed only in the $\phi\eta$-globin gene region. A study of the immunoglobulin alpha

Table 5. Rates of synonymous substitution per site per year in primates.

Species pair	Divergence time[a] (10^6 yr)	Percent divergence			Rate ($\times 10^{-9}$)		
		SRY[b]	ZFY		SRY	ZFY	
		Exon	Exon	Intron[d]	Exon	Exon	Intron
Human–Chimpanzee	7 (5–10)	0.8	2.6	0.7	0.6 (0.4–0.8)	1.9 (1.3–2.6)	0.5 (0.4–0.7)
Human–Gorilla	9 (7–12)	2.5	3.3	1.1	1.4 (1.0–1.8)	1.8 (1.4–2.4)	0.6 (0.5–0.8)
Human–Orangutan	15 (13–18)	2.5	8.5	1.9	0.8 (0.7–1.0)	2.8 (2.4–3.3)	0.6 (0.5–0.7)
Human–Baboon	25 (20–30)	14.3	12.8[c]	–	2.9 (2.4–3.6)	2.6 (2.1–3.2)	–

[a] From paleontological data (Pilbeam 1984).
[b] From Whitfield *et al.* (1993).
[c] From Shimmin *et al.* (1994).
[d] From Dorit *et al.* (1995).

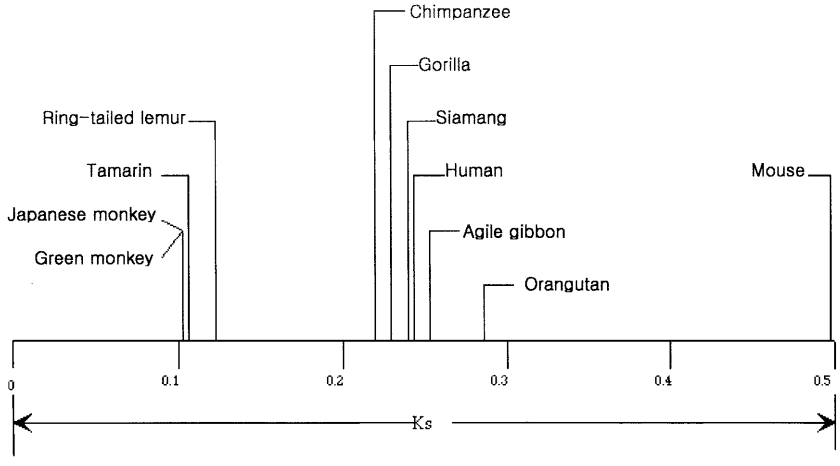

Fig. 1. Comparison of Ks values between ZFX and ZFY genes among species.

noncoding region did not support the hypothesis of a hominoid slowdown (Kawamura *et al*., 1991).

According to the neutral theory of molecular evolution, the long-term rate of nucleotide substitution is determined by the rate of selectively neutral mutations (Kimura, 1987). Calibrations of the molecular clock have generally indicated that the evolutionary distance between two lineages depends approximately linearly on the time of separation in years. It has, however, been proposed that the mutation rate should depend on the number of cell divisions rather than on the absolute time, because new mutations arise during DNA replication (Laird *et al*., 1969). As shown in Table 3, the synonymous positions of the X-linked genes are conserved much more strongly than in the Y-chromosome. According to Miyata *et al*. (1987), a Y-linked sequence would be predicted to have a rate of nucleotide substitution three times that of an X-linked sequence, but they were unable to test this adequately because of insufficient data. Lanfear and Holland (1991) concluded, on the basis of the relative differences between human and murine X- and Y-chromosomal genes, that the rate of synonymous substitutions in the Y-chromosome has been twice that in the X-chromosome. At the protein level, the ZFX gene has been highly conserved in all placental mammals studied, while the ZFY gene has been well conserved in primates and foxes but has evolved rapidly in mice and rats, possibly due to relaxation of functional constraints as a result of the development of X inactivation of the ZFX gene in rodents (Shimmin *et al*., 1994). Miyata *et al*. (1987) also suggested that the limited rate of synonymous substitutions in X-linked genes is due to functional regions but not to different numbers of germ-cell divisions between male and female. Because many vital genes exist on the X-chromosome, mutation frequency occurs to a lesser extent for the X-chromosome than the Y-chromosome. In comparison between human and primates, the extent of functional constraints is higher in the ZFX gene of hominoids than in ZFY as shown in Table 3. This may have arisen from a relaxation of selective constraints on synonymous changes in the ZFY gene of hominoid primates.

From the paleontological data (Pilbeam 1984), we estimated the rate of synonymous substitution per site per year (Vs). The Vs of the ZFY exon gene (1.9×10^{-9}/site/year) was similar to that of the $\alpha 1$ globin gene (2.0×10^{-9}/site/year) in the human-chimpanzee comparison, but the Ig ε and γ globin genes (1.4×10^{-9}/site/year) have low rates of synonymous substitution (Li *et al*., 1987). The Vs of the ZFY exon (2.8×10^{-9}/site/year) and the α ($\alpha 1 + \alpha 2$) globin (2.9×10^{-9}/site/year) genes were similar in the human-orangutan comparison. A comparison between the ZFY exon and the ND4 (ND5) genes (Hayasaka *et al*., 1988) in a human - orangutan comparison revealed that the ND4 (ND5) gene was about 8 times higher than that of ZFY exon gene in the rate of synonymous substitution. As shown in Table 5, the rates of synonymous substitution were higher in the ZFY exon than in the ZFY intron. Similarly, it has been shown that the exon region of the protamine P1 gene was variable, whereas the sequence of the single intron of the protamine P1 gene was highly conserved (Retief *et al*., 1993).

Synonymous codon usage is highly heterogeneous among mammalian genes, with the principal variation being in the base composition (G + C content) at silent sites (Ikemura, 1985). The AMG gene has been subject to strong mutation pressure from G or C to A or T as shown in KA and KA/Ks values. Nevertheless, the G + C content of the AMG gene is 61.45% (X-chromosome) and 61% (Y-chromosome), which is higher than those of other genes (Nakahori *et al*., 1991). For the ZF gene, the G + C contents of the X-chromosome and the Y-chromosome are 44.58% and 43.83%, respectively (Schneider-Gädicke *et al*., 1989). The G + C content of the ZF gene is slightly lower in other organisms. For example, mouse Zfy-1 gene sequences have a G + C content of 40.30% (Ashworth *et al*., 1989), while rat ZFY gene sequences have a G + C content of 39.64% (Shimmin *et al*., 1994). In the final intron of the ZFY gene, squirrel monkeys have a G + C content of 32.78% (Shimmin *et al*., 1993). Conversely, the insulin and IGF2 gene regions are very G + C rich, with approximately 66.3% and 61.4% of G + C, respectively (Ellsworth *et al*., 1993).

We have compared the Ks and KA values among sex-specific genes (RPS4, ZF, AMG, and STS) only in humans (Table 6). When four Y-specific genes are

Table 6. Comparison between male and female sex-specific gene in humans.

Gene	BP	Ks ± SE	KA ± SE	KA/Ks	G + C (%)	Homology (%)
RPS4	789	0.995 ± 0.134	0.045 ± 0.009	0.045	X:48.54 Y:48.42	82.2
ZF	396	0.241 ± 0.069	0.008 ± 0.006	0.033	X:44.58 Y:43.83	94.7
AMG	441	0.043 ± 0.018	0.085 ± 0.017	1.977	X:61.45 Y:61.00	92.7
STS	417	0.115 ± 0.033	0.176 ± 0.027	1.530	X:53.13 Y:49.28	85.3

Data sources: RPS4 (Fisher *et al*., 1990); ZF (Schneider-Gaedicke *et al*., 1989; Page *et al*., 1987); AMG (Nakahori *et al*., 1991); STS (Yen *et al*., 1988).
Ks and KA: mean of synonymous and nonsynonymous substitutions per site, respectively.

compared with X-specific genes in humans, KA/Ks lies between 0.033 (ZF gene) and 1.977 (AMG gene). The extent of synonymous substitution also varies between genes. The highest Ks value is 0.995 for the RPS4 gene, and the lowest is 0.043 for the AMG gene. The lowest KA and KA/Ks values were found in the ZF gene. This means that the ZF genes are subject to stronger functional constraints than other genes. In addition, the SRY gene has much stronger functional constraints than the ZFY gene in apes (Table 5). The Ks value of the SRY gene is slightly higher than that of the ZFY only in the baboon, which is overestimated by the LWL method (Li *et al*., 1985) but not by the method of Li (1993). The KA/Ks value for the SRY gene between human and chimpanzee is 0.018 (Whitfield *et al*., 1993). The AMG and STS genes have undergone functional change, which, in turn, has permitted an accelerated rate of amino acid substitution compared to other genes.

In conclusion, we have sequenced the zinc finger genes (ZFX and ZFY) in 5 hominoids (chimpanzee, gorilla, orangutan, agile gibbon, and siamang), 2 Old World monkeys (Japanese monkey and green monkey), 1 New World monkey (tamarin), and 1 prosimian (ring-tailed lemur). From a comparison of the ZFX and ZFY genes, it was found that the synonymous substitutions per site were higher in hominoids than in other primates. Additionally, in hominoid primates, the rates of synonymous substitution per site per year were higher in the ZFY exon compared to the ZFY intron and the SRY exon, respectively.

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