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Comparison of Substitution Rates in *ZFX* and *ZFY* Introns of Sheep and Goat Related Species Supports the Hypothesis of Male-Biased Mutation Rates

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Abstract. There is a growing body of evidence that males serve as the major generators of mutations, due to the larger number of cell divisions involved in sperm compared to egg production. In mammals, this hypothesis (referred to as "male-driven evolution") has been tested by comparison of nucleotide substitution rates on the X and Y sex chromosomes in a limited number of taxa, predominantly primates and rodents. This study asks whether male-driven evolution is a more general phenomenon among mammals, by comparison of paralogous *ZFX* and *ZFY* intron sequences in sheep and goat species (the tribe Caprini). The male-to-female mutation ratio, α_m, was estimated to be between 2.93 (95% CI, 1.51–8.61) and 3.94 (95% CI, 1.25–32.29) when calculated using pairwise distance and branch length, respectively, suggesting that the Caprini are subject to weak, male-driven evolution. Comparison to published values for primates, felids, and rodents implies that there may be some correlation with reproductive life span. However, this is difficult to test with current data because confidence intervals are large and overlapping. Nonindependent evolution of paralogous sequences and/or the presence of selective constraints could lead to inaccurate estimates of α_m. No evidence for gene conversion between the *ZFX* and the *ZFY* introns was found, and this suggests that they have evolved independently during the radiation of the Caprini. Finally, there was no apparent evidence that these introns are subject to selective constraints, although low levels of intraspecific polymorphism reduce the power of neutrality tests.

Key words: *ZFX* — *ZFY* — Male-driven evolution — Sex chromosomes — Generation time — Caprini — Sheep — Goat

Introduction

From observations during the 1930s and 1940s, J.B.S. Haldane deduced that the mutations responsible for inducing hemophilia in humans were generated predominantly in the male germ line (Haldane 1947). Since then it has been broadly assumed that the mutation rate is higher in males than in females because of the higher number of germ-cell divisions in spermatogenesis compared to oogenesis. For example, in humans, female germ cells undergo approximately 22 cell divisions by the fifth month of gestation, and another 2 meiotic divisions at sexual maturity, therefore 24 divisions in total (Strachan and Read 1997; Vogel and Motulsky 1997). In contrast, male stem spermatagonia undergo approximately 30 cell divisions by the onset of puberty, but these stem cells then divide 23 times per year throughout adulthood. Hence the number of cell divisions at age 20 will be 150, but that at age 40 will be 610 (for review see Hurst and Ellegren 1998).

If errors in DNA replication during cell division are the major source of mutations that contribute to molecu-

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lar evolution, then we would expect males to serve as the major generators of mutations given the assumption of a bias in germ cell division. The male-to-female ratio in the number of germ cell divisions per generations is commonly denoted α, and Miyata et al. (1987) describe a model for estimating the relative ratio of mutation rate in males compared to that in females (α_m) based on these assumptions. This model predicts that the asymmetry in the number of germ cell divisions between the male and the female will result in differences in mutation frequency between the autosomes and the sex chromosomes, a phenomenon commonly referred to as "male-driven evolution." In species with heterogametic sex chromosomes, the sex chromosomes and the autosomes spend different times in the two sexes. Therefore if there is a sex bias in the mutation rate, this should be reflected in the rate of nucleotide substitutions between the sex chromosomes themselves and between the sex chromosomes and the autosomes. In the XX/XY system of eutherian mammals, where the male is the heterogametic sex, the expected mutation frequency per generation for the Y chromosome will be proportional to α because Y is always carried by the male. The expected mutation frequency per generation for the X chromosome will be $(\alpha + 2)/3$ because the female carries two X chromosomes, whereas the male carries one (Miyata et al. 1987). Miyata et al.'s study implies that the female contribution to mutation is negligibly small and that males serve as the major generator of mutations that contribute to molecular evolution. In the same study, calculations of α_m based on nonhomologous sex chromosome and autosomal sequences were found tentatively to support these predictions.

This hypothesis of male-driven evolution has since been tested by comparison of paralogous sequences on the X and Y chromosomes of primates and rodents (Lanfear and Holland 1991; Hayashida et al. 1992; Pamilo and Bianchi 1993; Shimmin et al. 1993a, b, 1994; Chang et al. 1994, 1996; Chang and Li 1995; Agulnik et al. 1997; Huang et al. 1997). Very few data exist for other mammalian taxa, but α_m has been estimated in the Felidae through the comparative substitution rate of the final intron of the sex chromosome zinc finger genes, *ZFX* and *ZFY* (Slattery and O'Brien 1998). Results generated from these introns lead to estimates of α_m of 1.8 (95% CI, 1–3) for rodents (Change et al. 1994), 4.38 (95% CI, 3.76–5.14) for felids (Slattery and O'Brien 1998), and 6 (95% CI, 2–84) for humans (Shimmin et al. 1993a). The relative size of these estimates implies that the male-to-female mutation ratio may be correlated with the generation time, with species with a faster reproductive rate and short generation time having a lower α_m compared to species with a longer generation time and long reproductive life. At present this correlation is ambiguous because of the limited data and large confidence intervals associated with α_m.

The present study asks whether male-driven evolution is a more general phenomenon and whether different breeding systems and reproductive life span affect estimates of the male-to-female mutation ratio, α_m. We calculate α_m from the final introns of *ZFX* and *ZFY* in sheep and goats and their wild relatives from the tribe Caprini (family Bovidae, subfamily Caprinae), so that results will be comparable to published estimates for rodents, felids, and primates. The breeding systems and reproductive life span of wild sheep and goats are different from those of organisms studied previously. The Caprini are generally polygynous, with only a small number of males contributing to the next generation. The average reproductive life span of female caprids is approximately 6–8 years (Geist 1971). Males, on the other hand, may not actually participate in mating until several years later than females born in the same year, and their reproductive life span is much shorter (Shackleton 1997). The older the age at first reproduction, the more time has passed to accumulate germ-line mutations, which could have consequences for α_m.

To calculate an unbiased estimate of α_m it is important to address factors that may influence the mutation rate in sex chromosome-linked sequences. One major assumption in this study is that evolutionary changes in the X- and Y-linked sequences are independent of one another. If they are not, then calculation of the substitution ratio in Y/X will lead to confounded estimates of α_m. This can be tested relatively simply by comparison of phylogenetic divergence between the two paralogous introns and by detection of gene conversion tracts between the sequences. A gene conversion, or nonreciprocal recombination event, will result in increased homogeneity of the sequences and therefore reduce estimates of divergence between the X and the Y sequences.

Finally it is assumed that the sequences are not influenced by selection. If a gene is evolving in accordance with the neutral theory of molecular evolution (Kimura 1983), then the number of nucleotide substitutions can be used as a measure of the mutation rate. In this study we test the two assumptions that the final introns of the *ZFX* and *ZFY* genes are evolving under the neutral mutation hypothesis (Kimura 1983) and that all mutations in the DNA sequence are selectively neutral (Tajima 1989; Fu and Li 1993). The former assumption is slightly weaker than the latter because it considers that the *majority* of mutations that contribute to genetic variation are neutral. Introns were chosen in this study because they are under less selective constraint than are protein coding regions. However, there is some debate as to whether reduced levels of polymorphism in the final intron of *ZFY* in humans and other primates could be the result of selection (Shimmin et al. 1993a; Dorit et al. 1995; Jaruzelska et al. 1999). Since the Y chromosome is nonrecombining along most of its length, a favorable mutation could lead to a selective sweep, so the absence of selection is un-

likely (Charlesworth et al. 1993). It has been suggested that selection may be very important on the Y chromosome because it carries several loci affecting male fertility (Jobling et al. 1998). If selection is indeed responsible for observed levels of polymorphism at X- and Y-linked loci, this will bias estimates of α_m.

Materials and Methods

Samples and DNA Extraction. Samples from 21 species and subspecies within the tribe Caprini and 1 outgroup (*Bos taurus*) were analyzed for sequence variation at the final intron of *ZFX* and *ZFY*. Of these, only eight taxa exhibited unique sequences at both *ZFX* and *ZFY*. Therefore only the following taxa were included in the calculation of α_m: *Ammotragus lervia* (3), *Hemitragus jayakari* (3), *Ovis aries* (16), *O. ammon* (3), *O. dalli,* (6), *Pseudois nayaur* (2), *Capra cylindricornis* (1), and *Bos taurus* (1), where numbers in parentheses are the numbers of animals sequenced. *Capra hircus* (22) was included in the neutrality tests, but omitted from α_m calculations because it was identical to *C. cylindricornis* at *ZFX*. DNA was extracted from dried skin and ethanol-stored muscle from museum collections and from fresh hair, by a standard proteinase K digestion and chloroform purification, adapted from Hoelzel and Green (1998).

Locus Information and PCR Amplification. ZFX is located on the long arm of the X chromosome (Xq34 in cow, Xq13 in sheep and goat), while *ZFY* is situated on the short arm at Yp12-13 in all three species (Xiao et al. 1998). Both genes are located just outside the pseudoautosomal region (PAR) and are thought to have evolved independently without recombination since the radiation of eutherian mammals. Conserved oligonucleotide primers were designed from published sequences from a diverse range of mammalian species in the exons that flank the final introns of the *ZFX* and *ZFY* genes. The primers UEA-ZF1F (5′ AAG TGC CTT CTT GCA CAT AGA TGA) and UEA-ZF2R (5′ TTG TGG CTC TCC AGG TGG TTG T) amplify the final introns of *ZFX* and *ZFY* simultaneously. PCR reactions were carried out in 25-μl reaction volumes, containing approximately 100 ng of DNA template, a 2 μ*M* concentration of each primer 1 × NH_4 buffer, 2 m*M* $MgCl_2$ (both Bioline), 0.175 m*M* dNTPs (Pharmacia), and 1 U of Prozyme DNA polymerase (Bioline). Reactions were performed in a PE9700 thermocycler (PE-Applied Biosystems) using the following parameters: initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 30 s, 55°C annealing for 40 s, then extension at 72°C for 2 min.

Cloning and Sequencing. Products of the first-round amplification of *ZFX* and *ZFY* in *Ovis aries* and *Bos taurus* (two samples from each species) were cloned and sequenced to design X- and Y-specific primers. PCR product was purified prior to cloning using the Wizard PCR Preps Kit (Promega) and ligated into pGEM-T easy vector (Promega) according to the manufacturer's protocols. Ligation products were transformed into competent *Epicurian coli* XL-1 blue MRF cells (Stratagene), prepared according to Chung et al. (1989). Inserts from positive clones were amplified using the vector-specific primers OMNI (5′-ACA GGA AAC AGC TAT GAC CAT GAT) and M13F (5′-CGA CGT TGT AAA ACG ACG GCC, Promega). PCR conditions were identical to those described above, except that a 65°C anneal step was used. Purified PCR products were sequenced in forward and reverse directions with OMNI or M13F primers, using the Big Dye Terminator kit (PE-Applied Biosystems). Samples were electrophoresed on an ABI377 automated DNA sequencer and raw sequence data analyzed using ABI Prism DNA Sequencing Analysis software (both PE-Applied Biosystems). *ZFX* and *ZFY* sequences from these clones have been submitted to the GenBank database (accession numbers AF241271–241274). Internal primers were then designed to allow specific nested amplification of *ZFX* or *ZFY*. The same internal primer was used in both cases (ZF2F, 5′ AAG ACC TGA TTC CAG GCA GTA). The reverse internal primers are located in the same position in alignments of *ZFX* (UEA-ZFXR 5′ CTT CTT GTT GGT AGT GTA ATC ACA G) and *ZFY* (UEA-ZFYR 5′ CTT CTT ATT GGT AGT GTA ATC ACA A), but they differ at two nucleotide positions (boldfaced). Conditions for PCR amplification were as before, however the annealing temperature for *ZFX* was 67°C, and that for *ZFY* was 64°C. Nested PCR products were then sequenced directly to screen individuals for substitutional polymorphisms. Internal sequencing primers were designed approximately 400 bp from the external primers (*ZFX:* forward, FINX 5′ TTC TTA CAT GCT GAT TTG C; reverse, RINX 5′ AAG AGA TTA GCA GCC TCA; and *ZFY:* forward, FINY 5′ GAG GAG CAG GAC AAA TAC TAC; reverse, RINY 5′ CAT TCA TAA AGC TCA AGG).

Analysis of Sequence Data. Pairwise distances were calculated from sequence data with gaps excluded, using the Tajima–Nei (1984) distance measure in PAUP* (Swofford 1996). The Tajima–Nei method, which does not assume equal frequencies of the four nucleotides, was used so that results would be directly comparable to previous studies. Calculation of the male-to-female mutation rate is detailed under Results.

Estimating Divergence Between ZFX *and* ZFY. The average divergence between the *ZFX* and the *ZFY* final introns was estimated from pairwise comparisons using the Tajima–Nei distance measure. A neighbor-joining tree was constructed for *ZFX* and *ZFY* combined, to estimate the branch length between the paralogues. To test whether gene conversion has been influential in the evolution of these two introns, we searched for shared substitutions between X and Y in a given species using the algorithm ψ, as defined by Betrán et al. (1997), implemented in DNAsp 3.0 (Rozas and Rozas 1995). ψ is a measure of the probability of detecting a gene conversion event between two paralogues.

Testing for Deviations from Neutral Sequence Evolution. The hypothesis that all mutations in *ZFX* and *ZFY* are selectively neutral was tested using Fu and Li's (1993) test with an outgroup (*D* and *F*) and Tajima's (1989) *D* statistic. In addition, the HKA test (Hudson, Kreitman, and Agaude 1987) was employed to test Kimura's (1983) neutral mutation hypothesis. The latter test assumes that the majority of mutations that contribute significantly to genetic variation at a locus are neutral. This is a weaker assumption than the one that all mutations at a locus are neutral (Fu and Li 1993), but the HKA test has advantages in that two genomic regions (in this case X and Y) can be compared. Tests were performed using population samples of *Ovis aries* and *Capra hircus.* For *O. aries* two haplotypes at both *ZFX* and *ZFY* were found in 16 individuals, whereas in *C. hircus* three *ZFY* haplotypes were found in 22 individuals, but no polymorphisms were found at *ZFX*. Therefore tests could not be performed for *C. hircus ZFX. Ovis dalli* was used as an outgroup to *O. aries,* while *Capra cylindricornis* was used as an outgroup to *C. hircus.* Neutrality tests were performed using DNAsp 3.0 (Rozas and Rozas 1995).

Results

Calculation of the Male-to-Female Mutation Ratio, α_m. Two approaches were adopted to estimate the male-to-female mutation ratio, α_m. In the first approach, the mean pairwise estimate and standard error were calculated separately for *ZFX* and *ZFY,* from the data in Tables 1a and b, using MEGA (Kumar et al. 1993). The mean pairwise distance for *ZFY* is 0.025 ± 0.003, and that for

Table 1. Percentage pairwise distances (a) for *ZFY* and (b) for *ZFX* among taxa calculated using the Tajima–Nei distance measure, with gaps excluded (lower triangles) with standard errors provided in upper triangles[a]

(a) *ZFY*

	AL	HJ	PN	CC	OA	OAm	OD	BT
AL	—	0.315	0.430	0.470	0.431	0.431	0.469	0.841
HJ	0.777	—	0.505	0.540	0.505	0.505	0.539	0.888
PN	1.434	1.962	—	0.410	0.522	0.522	0.555	0.876
CC	1.699	2.230	1.304	—	0.489	0.489	0.489	0.929
OA	1.433	1.962	2.096	1.832	—	0.257	0.257	0.818
OAm	1.557	2.096	2.098	1.965	0.129	—	0.257	0.818
OD	1.696	2.227	2.362	1.831.	0.517	0.648	—	0.855
BT	5.370	5.932	5.797	6.380	5.096	5.234%	5.518	—

(b) *ZFX*

	AL	HJ	PN	CC	OA	OAm	OD	BT
AL	—	0.262	0.294	0.262	0.322	0.348	0.348	0.603
HJ	0.527	—	0.349	0.323	0.373	0.396	0.397	0.636
PN	0.660	0.927	—	0.227	0.397	0.419	0.419	0.651
CC	0.527	0.793	0.395	—	0.373	0.396	0.397	0.635
OA	0.792	1.059	1.194	1.059	—	0.130	0.130	0.633
OAm	0.925	1.192	1.327	1.192	0.131	—	0.185	0.647
OD	0.925	1.193	1.328	1.193	0.131	0.263	—	0.648
BT	2.688	2.965	3.103	2.963	2.958	3.095	3.097	—

[a] Taxa are as follows: AL, *Ammotragus lervia* (3); HJ, *Hemitragus jayakari* (3); PN, *Pseudois nayaur* (2); CC, *Capra cylindricornis* (a); OA, *Ovis aries* (16); OAm, *O. ammon* (3); OD, *O. dalli* (6); and BT, *Bos taurus* (1). Numbers in parentheses are the numbers of samples sequenced.

ZFX is 0.014 ± 0.002. Therefore Y/X = 1.786, however, we are interested in the expected ratio of Y/X, which can be estimated by $E(Y)/E(X) \approx E(Y/X) - E(X)V(Y)/E(Y)^3$, where V is the variance of Y (Shimmin et al. 1993a). $E(Y)/E(X)$ is therefore 1.782. The variance of Y/X is then estimated by $V(Y/X) = V(Y)/E(X)^2 + E(Y)^2V(X)/E(X)^4$, leading to a standard error of 0.333, hence $E(Y)/E(X) = 1.782 \pm 0.333$ (Shimmin et al. 1993a). The 95% confidence intervals (CI) for $E(Y)/E(X)$ can now be calculated as follows. The lower-bound confidence interval is $E(Y)/E(X)^- = E(Y)/E(X) - 1.96s$, where s is the standard error. Similarly the upper-bound CI is $E(Y)/E(X)^+ = E(Y)/E(X) + 1.96s$ (Huang et al. 1997), therefore $E(Y)/E(X) = 1.782$ (95% CI, 1.129–2.435). The male-to-female mutation ratio α_m is then calculated from $E(Y)/E(X) = 3\alpha/(\alpha + 2)$ (Miyata et al. 1987), where α is the male-to-female ratio in the number of germ-cell divisions per generation. Confidence intervals for α_m are estimated as follows: $\alpha_m = 2[E(Y)/E(X)^-]/\{3 - [E(Y)/E(X)^-]\}$, and $\alpha_m{}^+ = 2[E(Y)/E(X)^+]/\{3 - [E(Y)/E(X)^+]\}$, according to Huang et al. (1997). Based on pairwise distances, we therefore estimate α_m to be 2.93 (95% CI, 1.51–8.61). It has been argued, however, that using pairwise distances to compute α_m may produce a biased estimate of the variance because the internal branches of the genealogy can be sampled more than once (Ellegren and Fridolfsson 1997). In other words, the pairwise distances are not necessarily independent of one another. For this reason, a second approach was taken which uses the sum of branch lengths to calculate Y/X and then α_m. The sum of branch lengths from the neighbor-joining trees (Fig. 1) constructed using Tajima–Nei distances is 0.088 for *ZFY* and 0.044 for *ZFX,* therefore Y/X = 2. Following the procedures described above for pairwise estimates, $E(Y)/E(X) = 1.99 \pm 0.426$, so $\alpha_m = 3.94$ (95% CI, 1.25–32.29). Both of these estimates suggest that molecular evolution is male-driven in the Caprini.

Comparison of DNA Sequence Polymorphism in ZFX *and* ZFY. Analysis of sequence data was performed on 777 bp *ZFX* and 811 bp *ZFY* excluding alignment gaps. Table 2 summarizes the basic polymorphism statistics for both loci. Corrected for sequence length, the number of segregating sites (S), total number of mutations (η), nucleotide diversity (π), and parsimony informative sites are consistently higher for *ZFY* than for *ZFX*. Values of S and η are different for *ZFX* and *ZFY* because one site exhibited three nucleotides in *ZFX* and two sites showed similar behavior in *ZFY*. This is in accordance with the finite sites model, which assumes four possible nucleotides per site (Tajima 1996). Estimates of θ per site (where $\theta = 4Ne\mu$) based on the finite sites model are similar for S and η, with the small discrepancy being due to the difference in S and η, mentioned previously. A lower estimate of θ is obtained from π because the average nucleotide diversity between pairs of sequences is low compared to the total number of mutations or segregating sites.

Are ZFX *and* ZFY *Evolving Under Neutrality?* Overall, Fu and Li's tests for both *ZFX* and *ZFY* showed no significant deviation from neutrality in the comparison of

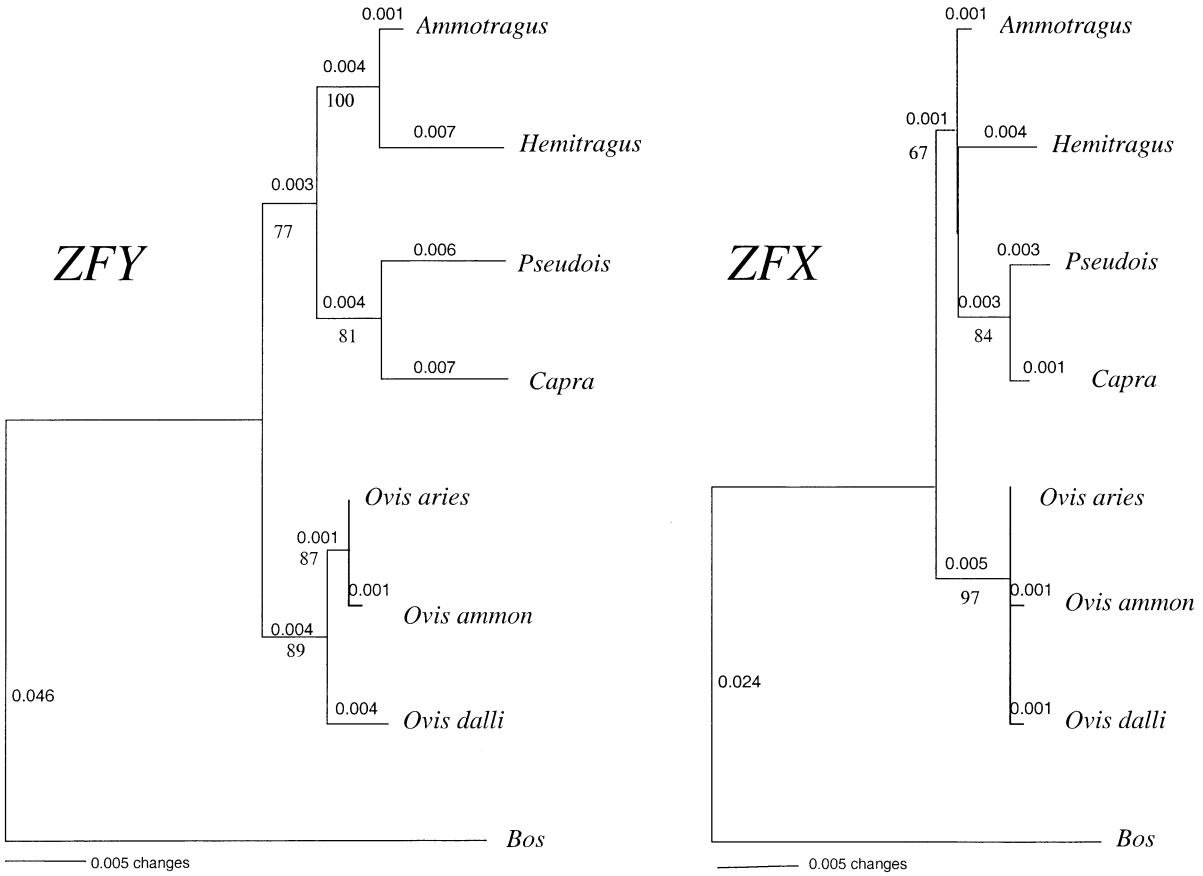

Fig. 1. Rooted neighbor-joining trees of *ZFX* and *ZFY*, constructed using the Tajima–Nei distance measure, with *Bos taurus* as outgroup. Tree lengths are 0.088 for *ZFY* and 0.044 for *ZFX*. Branch lengths are given above branches, and bootstrap values below, after 1000 replicates.

Table 2. DNA polymorphism statistics for *ZFX* and *ZFY*

	ZFX	*ZFY*
Total length (bp)	777	811
A + T	0.69	0.67
G + C	0.31	0.33
Ts:Tv	1.23	1.60
Number of indels[a]	14	35
Number of variable, uninformative characters	28	49
Number of parsimony informative characters	7	23
Segregating sites, S	29	72
Total number of mutations, η	30	74
Nucleotide diversity, π (SD)	0.0054 (0.0008)	0.0124 (0.0021)
Finite sites model		
θ per site from π	0.00541	0.01260
θ per site from S	0.00958	0.02215
θ per site from η	0.00979	0.02211

[a] Total number of indels between *ZFX* and *ZFY* when aligned = 184.

Ovis aries versus *O. dalli* (*ZFX*, $D = 0.763$, $F = 0.729$, $p > 0.10$; *ZFY*, $D = -2.032$, $F = 2.21$, $p > 0.05$) and *Capra hircus* versus *C. cylindricornis* (*ZFY* only: $D = 0.596$, $F = 0.409$, $p > 0.10$). Similarly, intraspecific tests using Tajima's D showed no deviation from neutrality either within *Ovis aries* (*ZFY* and *ZFX*) or within *Capra hircus* (*ZFY* only). Values of Tajima's D were negative except in the *O. aries* test at *ZFY* ($D = 0.156$). This positive value could indicate the influence of overdominant selection at the *ZFY* locus in sheep (Li 1997). However, since the value is not significant, it is more likely an effect of the low levels of intraspecific polymorphism found in this analysis. Results for Tajima's test were comparable when based on S or η, although values from S were slightly lower as expected because of the presence of sites segregating with more than two nucleotides. Finally, in a comparison of *O. aries* versus *O. dalli,* at both loci, the HKA test was not significant ($\chi^2 = 0.174$, $p > 0.10$).

Are ZFX *and* ZFY *Evolving Independently?* The mean Tajima–Nei distance between *ZFX* and *ZFY* was calculated to be 40.3% (95% CI, 0.035) (Table 3). This result is higher than the estimates for the same introns in primates [30% (Shimmin et al. 1993a)] and felids [31% (Slattery and O'Brien 1998)]. The divergence between these two introns is also illustrated by the branch length of 37.2 which separates *ZFX* and *ZFY* (data not shown). The number of fixed differences (Hey 1991) between *ZFX* and *ZFY* was 173, and no shared polymorphisms were found between the two introns. Although these results suggest that there has been no exchange between

Table 3. Percentage pairwise differences (Y–X) with standard errors between *ZFX* and *ZFY,* calculated using the Tajima–Nei distance with gaps excluded[a]

	AL-Y	HJ-Y	PN-Y	CC-Y	OA-Y	OAm-Y	OD-Y	BT-Y
AL-X	39.6 ± 3.27	39.4 ± 3.26	39.1 ± 3.24	39.9 ± 3.30	39.8 ± 3.29	39.8 ± 3.29	39.8 ± 3.29	42.6 ± 3.50
HJ-Y	39.3 ± 3.25	39.1 ± 3.24	38.8 ± 3.21	39.6 ± 3.27	39.5 ± 3.27	39.5 ± 3.27	39.4 ± 3.27	42.2 ± 3.48
PN-X	42.0 ± 3.31	40.0 ± 3.30	39.7 ± 3.28	40.5 ± 3.34	44.0 ± 3.31	40.4 ± 3.33	40.3 ± 3.33	42.9 ± 3.53
CC-X	39.8 ± 3.28	39.6 ± 3.27	39.3 ± 3.25	40.1 ± 3.31	40.0 ± 3.30	40.0 ± 3.30	40.0 ± 3.30	42.5 ± 3.50
OA-X	39.5 ± 3.26	39.3 ± 3.25	39.0 ± 3.23	39.8 ± 3.28	39.7 ± 3.28	39.7 ± 3.28	39.7 ± 3.28	42.5 ± 3.49
OAM-X	39.5 ± 3.25	39.3 ± 3.24	39.0 ± 3.22	39.8 ± 3.27	39.7 ± 3.27	39.7 ± 3.27	39.6 ± 3.27	42.4 ± 3.48
OD-X	42.0 ± 3.30	40.1 ± 3.29	39.8 ± 3.26	40.5 ± 3.32	44.0 ± 3.31	40.4 ± 3.32	40.4 ± 3.32	43.3 ± 3.53
BT-X	41.0 ± 3.28	39.8 ± 3.27	39.6 ± 3.25	40.4 ± 3.31	43.0 ± 3.30	40.3 ± 3.30	40.3 ± 3.31	43.0 ± 3.51

[a] Taxa are as follows: AL, *Ammotragus lervia* (3); HJ, *Hemitragus jayakari* (3); PN, *Pseudois nayaur* (2); CC, *Capra cylindricornis* (a); OA, *Ovis aries* (16); OAm, *O. ammon* (3); OD, *O. dalli* (6); and BT, *Bos taurus* (1). Numbers in parentheses are the numbers of samples sequenced.

ZFX and *ZFY* for a considerable amount of time, it is possible that small regions of these introns have been subject to gene conversion. Using the algorithm of Betrán et al. (1997), five 10-bp regions were identified that gave values of ψ greater than 0.5. These probabilities were, however, not significant ($p > 0.05$), suggesting that gene conversion has not been influential in the evolution of these introns.

Presence of a 50-bp Insertion in ZFX. An insertion 50 bp in length was found near the 3′ end of the *ZFX* intron in all the Caprini samples. However, the sequence was not found in the *Bos taurus ZFX,* which suggests that it either appeared or was lost very early in the radiation of the Bovidae family. The sequence contained a poly (T) tract with 14–17 T nucleotides, which made this area difficult to sequence through, and it was therefore omitted from our analysis. The presence of a poly (T) region at the 5′ end of this sequence suggests that it could be a SINE (short interspersed nucleotide element) since they are characterized by a poly (A) region at their 3′ end (opposite strand). However, there was no homology to published sequences from the BLAST database, and no matching repeats were found with the RepeatMasker program (http://ftp.genome.washington.edu/cgi-bin/RepeatMasker).

Discussion

The Caprini Are Subject to Male-Biased Mutation Rates. In the Caprini, the final intron of the *ZFX* gene appears to be more conserved than its Y chromosomal counterpart (Tables 1a and b and 2). The nucleotide substitution ratio between these two introns is $E(Y)/E(X) = 1.782 \pm 0.333$ to 1.99 ± 0.426 calculated from pairwise distances and branch length, respectively. The corresponding values for α_m are therefore 2.93 (95% CI, 1.51–8.61) and 3.94 (95% CI, 1.25–32.29), where α_m is the ratio of male-to-female mutation rate resulting from the difference in the number of germ-cell divisions (Miyata et al. 1987). Our calculations therefore suggest that the Caprini are subject to male-driven molecular evolution. We obtain a slightly more conservative estimate of α_m from pairwise distances rather than from branch lengths, which we found surprising given that the former method involves statistical resampling of branches (Ellegren and Fridolfsson 1997). Results from the present study combined with the previous estimates of α_m in rodents, felids, and primates suggest that male-driven molecular evolution is a general phenomenon, at least among mammals.

The Effect of Generation Time. The "generation time effect" hypothesis postulates higher evolutionary rate in organisms with short generation times because, in 1 unit of time, organisms with short generation times will go through more generations and therefore more rounds of germ-cell division (Laird et al. 1969). As mentioned previously, the fundamental assumption underlying the hypothesis of male-driven evolution is that errors in DNA replication during germ-line cell division are the major source of mutations. Therefore α_m should be directly proportional to the number of germ-cell divisions, $C = n_m/n_f$, where n_m and n_f are the number of germ-cell divisions in males and females, respectively (Li 1997). Preliminary calculations for rodents and humans suggest that estimates of α_m and C are similar (Li 1997), although this is with some degree of ambiguity since C assumes that the mutation rate is unaffected by senescence, which is probably not the case (Hurst and Ellegren 1998). At present there is no information available as to the number of germ-cell divisions per generation in any of the species closely related to the Caprini or in the Felidae. We therefore cannot assume that the sex ratio in mutation rate (α_m) is comparable just to the number of germ-cell divisions (C) in these species. The confidence intervals for our estimates of α_m overlap with published results for the same intron in primates [$\alpha_m = 6$; 95% CI, 2–84 (Shimmin et al. 1993a)], felids [$\alpha_m = 4.38$; 95% CI, 3.76–5.14 (Slattery and O'Brien 1998)], and rodents [$\alpha_m = 1.8$; 95% CI, 1–3 (Chang et al. 1994)]. We are therefore unable to conclude any correlation of α_m with generation time or life history parameters, even though

our values of α_m are between the rodent and the felid estimates. This is unfortunate because it means that the generation time effect hypothesis is difficult to test from existing data. However, even with the ambiguity due to large confidence intervals, these results do tentatively imply that relative breeding system dynamics and generation time may be responsible for different rates among taxa.

Evolution of the ZFX *and* XFY *Final Introns in the Caprini.* In this study intron sequences were chosen to try to overcome the influence of selection, which could also lead to inaccurate estimates of α_m. However, low levels of polymorphism have been found in the *ZFY* intron in humans (Dorit et al. 1995; Jaruzelska et al. 1999), and this coupled with the unusual transmission properties of the Y chromosome suggests that the introns may not actually be evolving under neutrality. Here we used intraspecific polymorphism data from domestic sheep *Ovis aries* and goats *Capra hircus* along with interspecific outgroup comparisons to test for deviations from neutral evolution. The efficacy of these tests was greatly reduced by the low levels of intraspecific polymorphism exhibited in the *ZFX* and *ZFY* final introns. However, since no significant deviations from neutrality were found during any of the tests, the null hypothesis that "polymorphisms are maintained without selection at the *ZFX* and *ZFY* final introns" could not be rejected.

It has been suggested previously that the *ZFX* and *ZFY* genes have been subject to gene conversion events during primate (Schneider-Gädicke et al. 1989; Pamilo and Bianchi 1993; Shimmin et al. 1993b; Hayashida et al. 1992) and felid evolution (Slattery et al. 2000). However, in this study, we found no evidence for gene conversion between the final introns of the *ZFX* and *ZFY* genes. There is a higher mean divergence between *ZFX* and *ZFY* than has been reported for other taxa [40.3%, compared to 30% in primates and 31% in felids (Shimmin et al. 1993a; Slattery and O'Brien 1998], and *ZFX* and *ZFY* clades are well separated. In addition, we found no significant evidence for small gene conversion tracts between X and Y sequences using the parameter ψ (Betrán et al. 1997). We can therefore assume that these paralogous sequences have been evolving independently for a considerable amount of time, and gene conversion is not responsible for reducing our estimate of α_m.

During this study a region of DNA sequence approximately 50 bp in length, characterized by a poly (T) tract at the 5′ end was detected in the *ZFX* intron in all Caprini samples tested but not in the outgroup *Bos taurus*. This suggests that it either appeared or was lost very early during the Bovidae radiation. Transposable elements and simple repeat sequences are expected to accumulate in regions of no recombination and may be one of the factors contributing to degeneration of sex chromosome genes (Charlesworth 1991). In humans both sex chromosomes have been found to harbor significantly more interspersed elements than the autosomes (Shimmin et al. 1993b; Jaruzelska et al. 1999; Erlandsson et al. 2000), while a SINE insert has also been found in the *ZFY* intron of domestic cats (Slattery and O'Brien 1998).

It is possible that fewer mutations due to functional constraints on the X chromosome could be implicated in these results rather than germ-cell division bias (McVean and Hurst 1997). At present there is no way to decide between these two hypotheses in mammals. However, recent studies in birds where the female is the heterogametic sex, suggest that functional constraints on the paired sex chromosome are not responsible for the difference in mutation rates between sex chromosomes. Indeed these results are concordant with a male mutation bias (Ellegren and Fridolfsson 1997; Kahn and Quinn 1999; Carmichael et al. 2000). Finally, it is possible that the relative rates of mutation in *ZFY* and *ZFX* are locus-specific effects and that estimates of α_m may vary substantially between loci (Hurst and Ellegren 1998). For example, a recent study of this type in humans obtained a value of $\alpha_m = 2.5$ (Erlandsson et al. 2000), which is much smaller than that predicted using just *ZFX* and *ZFY* [compare to $\alpha_m = 6$ (Shimmin et al. 1993a)]. In addition, a comprehensive survey of nonfunctional but highly similar X and Y sequence in primates proposed an α_m of 1.7 (95% CI, 1.15–2.87), which is significantly lower than single-locus estimates calculated previously (Bohossian et al. 2000). It is therefore imperative that information from other loci be gathered to test for locus-specific effects and to calculate more accurate estimates of α_m. Calculation of smaller confidence intervals for α_m may in turn enable testing of the generation time effect hypothesis.

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