Patterns of *Y* and *X* Chromosome DNA Sequence Divergence During the Felidae Radiation

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ABSTRACT

The 37 species of modern cats have evolved from approximately eight phylogenetic lineages within the past 10 to 15 million years. The Felidae family has been described with multiple measures of morphologic and molecular evolutionary methods that serve as a framework for tracking gene divergence during brief evolutionary periods. In this report, we compare the mode and tempo of evolution of noncoding sequences of a large intron within Zfy (783 bp) and Zfx (854 bp), homologous genes located on the felid *Y* and *X* chromosomes, respectively. Zfy sequence variation evolves at about twice the rate of Zfx, and both gene intron sequences track feline hierarchical topologies accurately. As homoplasies are infrequent in patterns of nucleotide substitution, the *Y* chromosome sequence displays a remarkable degree of phylogenetic consistency among cat species and provides a highly informative glimpse of divergence of sex chromosome sequences in Felidae.

AN intriguing aspect to mammalian evolution concerns the maintenance and segregation of genetic diversity within sex chromosomes. In eutherian mammals, only a small portion of the *Y* chromosome undergoes recombination with the *X*, fueling speculation about the fate of *Y*-linked genes located outside of this pseudoautosomal region. In particular, the mode and tempo by which *Y*-linked genes change are predicted to differ from those genes located on either autosomes or *X* chromosomes. In this report, we examine differential evolution between sex chromosomes by a comprehensive comparison of substitution rates within a large intron located in homologous genes, Zfy and Zfx, within 34 species of the cat family Felidae.

Mutation rate differences between sex chromosomes are viewed as evidence of the outcome of differential selection pressures, or as merely a consequence of unequal numbers of mutations generated by errors in DNA replication during germ cell division. Phenomena such as dosage compensation, increased frequency of retroposon insertion, and gene duplication events support hypotheses that predict the gradual degeneration of genes located on the *Y* and arguments that favor differential selection pressure between sex chromosomes (Charlesworth 1978, 1991, 1993; Mardon *et al.* 1989; Graves 1995; Rice 1996; McVean and Hurst 1997). In contrast, under the hypothesis of male-driven evolution (Haldane 1947), higher mutation rates are predicted for *Y*-linked genes relative to *X* because of the greater number of germ cell divisions required for spermatogenesis relative to oogenesis. Although not mutually exclusive, these two categories of hypotheses do not necessarily agree on the relative roles of either selection in the maintenance of mutations, or on the inherent rates by which mutations are generated on the *Y* and *X*, in sex chromosome evolution.

Empirical estimates of the male:female mutation rate ratio, α_m, vary considerably among studies based on human and rodent genes. Initial indirect estimations based on several *X*-linked and autosomal genes give a value of $\alpha_m \sim \infty$ (Miyata *et al.* 1987; Wolfe and Sharp 1993). Evidence from studies of both coding and noncoding regions of *Y* and *X* chromosomes, however, consist of much lower estimates, with $\alpha_m \sim 2$ and 5 for rodents and primates, respectively (Shimmin *et al.* 1993a; Chang *et al.* 1994; Chang and Li 1995; Huang *et al.* 1997).

These original studies, useful in delineating the controversy in estimating substitution differences between sex chromosomes, are restricted in sampling design. The taxa are few and limited to either rodents and primates. Consequently, some estimates of α_m have large confidence intervals because of small sample size (Shimmin *et al.* 1993; Chang *et al.* 1994). Furthermore, studies that combine rodents and primates may exhibit bias in mutation rate estimates for sex-linked genes because of the generation time effect (*i.e.,* more germ cell divisions occur over a given time interval in short-lived animals) between these two mammalian orders (Li and Graur 1991; Shimmin *et al.* 1993b).

To ameliorate substitution rate heterogeneity and sample size effects, a well-defined taxonomic group rep-

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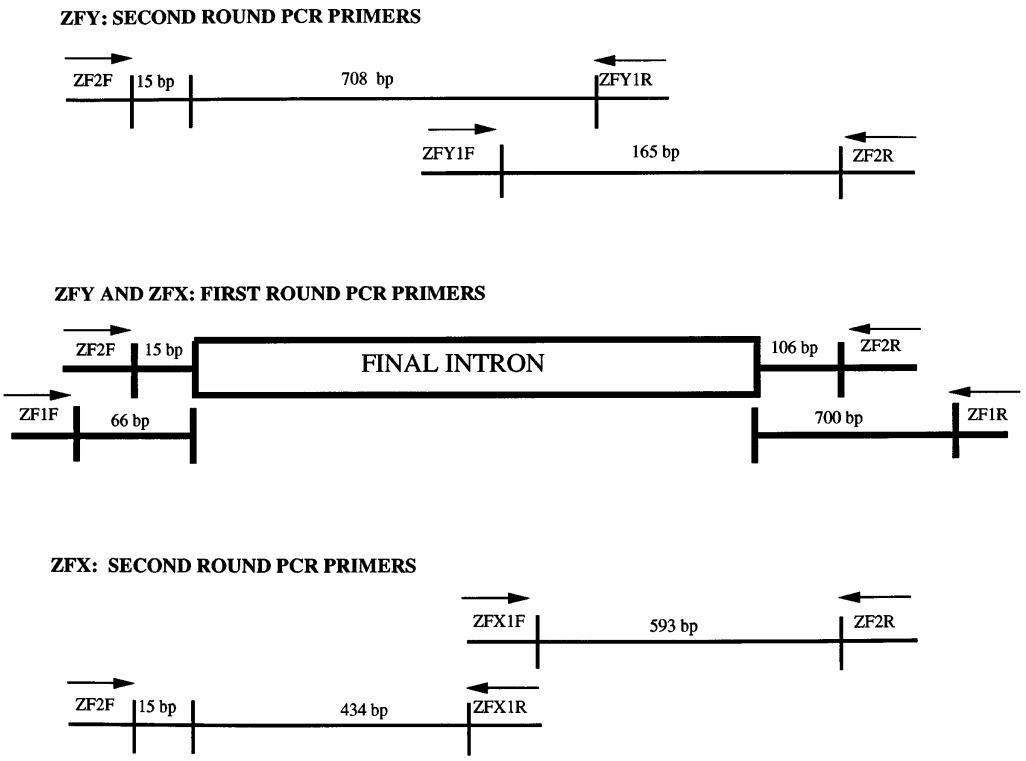

Figure 1.—Schematic diagram of primer pairs used in the amplification of the final intron of Zfy and Zfx in Felidae (see materials and methods). Generation of the gene segment encompassing the final intron for both Zfy and Zfx used conserved primers ZF1F (66 bp upstream): 5′-ATAGATGAGTCTGCTGGC and ZF1R (700 bp downstream): 5′-CGTTTCAAATCACTTGA or ZF2F (15 bp upstream): 5′-GGTGATTCCAGGCAGTAC and ZF2R (106 bp downstream): 5′-TGGTCAGCTTGTGGCTCTCCT. Second-round primers to isolate Zfy were ZF2F paired with ZFY1R: 5′-AAGCATTTGAAGTGTGTG and ZFY1F: 5′-TGGAGTTTGCT GTTACCT paired with ZF2R, as well as ZFY2F: 5′-TGTCAGCATAAGCAGGCT (located ~325 bp inside intron; not shown in figure). Second-round primers to isolate Zfx were ZF2F paired with ZFX1R: 5′-CAGTAGAGCTTAAACCCA and ZFX1F: 5′-TG GGTTTAAGCTCTACTG paired with ZF2R, as well as ZFX2F: 5′-GCTTCTGTGTACTTG (located at ~150 bp inside intron; not shown in figure).

resented by 34 of 37 species of the cat family Felidae is used. We analyze the genetic variation of the final intron of homologous genes, Zfy and Zfx, located outside of the pseudoautosomal region (Mardon and Page 1987; Page *et al.* 1987) of the *Y* and *X* chromosomes, respectively. The events by which Zfy and Zfx became sex-linked occurred early within eutherian mammal evolution, and they predate the emergence of modern day cat species. Autosomal in marsupials (Sinclair *et al.* 1988) and monotremes (Watson *et al.* 1993), Zfy and Zfx have been found in sex chromosomes in Rodentia (Bianchi *et al.* 1992), Primates (Schneider-Gadicke *et al.* 1989a; Palmer *et al.* 1990; Shimmin *et al.* 1994), and Carnivora (Lanfear and Holland 1991). Felid phylogeny, supported by congruent results from multiple nuclear and mitochondrial genetic markers (Collier and O'Brien 1985; O'Brien *et al.* 1987; Modi and O'Brien 1988; Pecon Slattery *et al.* 1994; Janczeweski *et al.* 1995; Johnson *et al.* 1996; Masuda *et al.* 1996; Johnson and O'Brien 1997), exhibits an evolutionary pattern marked by a recent rapid speciation with several recognized monophyletic clades. The evolution of modern felids occurred ~12 to 15 mya and consists of eight major clades and four unaligned species (see Johnson and O'Brien 1997).

Sequence diversity of Zfy and Zfx introns across 34 species of Felidae offers additional perspectives on evolutionary differences between sex chromosomes. Furthermore, our results illustrate the usefulness of phylogenetic methods in assessing not only the relative rates of substitution but in comparing the pattern of nucleotide changes between sex-linked introns accumulated over evolutionary history.

MATERIALS AND METHODS

Isolation and characterization of intron sequence from Felidae: Primers were designed from conserved regions flanking the final intron of Zfy and Zfx based on alignments of published cDNA sequences for humans and mice. (Schneider-Gadicke *et al.* 1989b; Mardon and Page 1989; Palmer *et al.* 1990). Each primer pair was tested in humans, mice, and three felid species representing diverse lineages within the cat family: puma (*Puma concolor*), pampas cat (*Lynchailurus colocolo*), and domestic cat (*Felis catus*). Nested PCR was performed starting with primers ZF1F, located 66 bp upstream of the intron, and ZF1R, situated 700 bp into the adjacent

TABLE 1

List of felid species used in phylogenetic analysis, DNA code, and source of tissue samples

Common name	Scientific name	Code	Source
	Ocelot lineage		
Ocelot	*Leopardus pardalis*	Lpa 14	Summit Zoo, Panama
Tigrina	*Leopardus tigrina*	Lti 13	Canas, Las Pumas, Costa Rica
Margay	*Leopardus weidii*	Lwi 69	Itaipu, Brazil
Pampas cat	*Lynchailurus colocolo*	Lco 6	Mendoza Zoological Park, Argentina
Geoffroy's cat	*Oncifelis geoffroyi*	Oge 38	Pan de Azocos, Uruguay
	Domestic cat lineage		
Domestic cat	*Felis catus*	Fca 14	NIH Animal Center, Bethesda, MD
Jungle cat[a]	*Felis chaus*	Fch 2	Blijdorp Zoo, Rotterdam, The Netherlands
African wild cat	*Felis libyca*	Fli 2	Kruger Park, South Africa
Sand cat[a]	*Felis margarita*	Fma 8	Woodland Park Zoo, Seattle, WA
Black-footed cat[a]	*Felis nigripes*	Fni 14	San Diego Zoo, San Diego, CA
Chinese desert cat	*Felis bieti*	Fbi 1	Pan Wen-shi, China
European wild cat[a]	*Felis silvestries*	Fsi 1	Blijdorp Zoo, Rotterdam, The Netherlands
	Panthera group		
Lion	*Panthera leo*	Ple 23	Wildlife Safari, Winston, OR
Leopard[a]	*Panthera pardus*	Ppa 80	San Diego Zoo, San Diego, CA
Tiger[a]	*Panthera tigris*	Pti 77	Knoxville Zoo, Knoxville, TN
Snow leopard[a]	*Panthera uncia*	Pun 19	Cheyenne Mountain Zoo, Colorado Springs, CO
Jaguar	*Panthera onca*	Pon 21	Canas, Las Pumas, Costa Rica
Clouded leopard	*Neofelis neofelis*	Nne 27	Cleveland Metroparks Zoological Park, Cleveland, OH
	Puma group		
Puma	*Puma concolor*	Pco 65	Everglades National Park, FL
Cheetah[a]	*Acinonyx jubatus*	Aju 72	Wildlife Safari, Winston, OR
Jaguarundi	*Herpailurus yagouaroundi*	Hya 17	Buenes Aires Zoological Park, Buenos Aires, Argentina
	Lynx genus		
Bobcat[a]	*Lynx rufus*	Lru 18	United States Fish and Wildlife Service Panther Refuge, Naples, FL
Canadian lynx	*Lynx canadensis*	Lca 3	Catoctin Mountain Zoo, Thurmont, MD
Siberian lynx	*Lynx lynx*	Lly 6	Carnivore Evolutionary Research Institute (CERI), Pittsboro, NC
	Asian leopard cat group		
Asian leopard cat	*Prionailurus bengalensis*	Pbe 32	Tallinn Zoological Park, Estonia
Flat-headed cat	*Ictailurus planiceps*	Ipl 4	Lincoln Park Zoo, Chicago, IL
Fishing cat	*Prionailurus viverrinus*	Pvi 2	Blijdorp Zoo, Rotterdam, The Netherlands
	Caracal group		
Caracal	*Caracal caracal*	Cca 21	Central Florida Zoo, Lake Monroe, FL
African golden cat	*Profelis aurata*	Pau 1	Blijdorp Zoo, Rotterdam, The Netherlands
	Bay cat group		
Asian golden cat[a]	*Profelis temmincki*	Pte 10	Melaka Zoo, Melaka, Malaysia
	Unaligned species		
Serval	*Leptailurus serval*	Lse 2	CERI, Pittsboro, NC
Rusty-spotted cat	*Prionailurus rubiginosa*	Pru 2	Cincinnati Zoo, Cincinnati, OH
Pallas cat	*Otocolobus manul*	Oma 3	Baltimore Zoo, Baltimore, MD
Marbled cat	*Pardofelis marmorata*	Pma 2	Lincoln Park Zoo, Chicago, IL

[a] Species in which two male individuals were sequenced. Preliminary results of a 300-bp fragment exhibit no intraspecific polymorphism (data not shown).

conserved zinc finger exon (Figure 1). Sequences were amplified in 100-μl reactions containing 50–100 ng/μl total genomic DNA, 50 mm KCl, 10 mm Tris, pH 8.3, 1.5 mm $MgCl_2$, 0.01% gelatin, 0.01% NP-40, 0.01% Tween 20, 0.2 μM of each primer, 0.2 mm dNTP, and 2.5 units Taq polymerase. Thermocycling conditions for the first round consisted of a hot start of 10 min (95°), followed by 35 cycles of 1 min at 95°, 1.5 min at 48°, and 2 min 72°, ending with a final extension of 72° for 5 min. The second round consisted of 5 μl volume of the first-round product amplified with primers ZF2F 15 bp upstream and ZF2R 106 bp into the adjacent zinc finger exon (Figure 1). PCR conditions were identical to the first round, but the annealing temperature was changed to 52°. Resultant PCR products were visualized on a 1% agarose gel.

As the nested PCR reaction amplified both Zfy and Zfx introns simultaneously, PCR products for the three felid species were cloned using the protocol of the TA cloning kit (Invitrogen, San Diego, CA). Positive clones were randomly selected, cultured overnight in LB broth, and DNA was prepared. The insert was cleaved from vector by an *Eco*RI digest and visualized on a 1% gel. Variants were screened by the dye terminator kit (Applied Biosystems, Inc., Foster City, CA) using primers ZF2F and ZF2R.

Internal primers were designed specific to felid Zfy and Zfx introns (Figure 1). Specificity of Zfy primers was confirmed by amplification of a single product in males and none in females of the three species.

PCR amplification of felid species: DNA from male individuals representing each of 34 felid species (Table 1) were used for PCR amplification. In a subsample of 12 species, two individuals were sequenced to assess intraspecific levels of variation of Zfy and Zfx in felids. Heminested PCR was used with different primer pair combinations for Zfy and Zfx (Figure 1) but was used with the same thermocycling conditions described above. For Zfy, first-round PCR used primers ZF2F and ZfY1R, which is located ~708 bp inside the intron. The remaining intron segment was amplified by ZFY1F at ~625 bp into the intron and by ZF2R. Additional internal primers ZFY2F and ZFY2R (reverse complement of ZFY2F) were situated at ~325 bp and used in conjunction with ZfY1R and ZF2F, respectively. Heminested PCR amplification of Zfx used primers of ZF2F and ZF2R with the first-round conditions listed above. The second round consisted of ZF2F and ZFX1R (434 bp inside the intron). The remaining half of the intron was amplified with ZFX1F and ZF2R. Verification of overlapping regions used the Dye terminator Prism sequencing kit (Applied Biosystems) and internal primer ZFX2F, which is located ~150 bp inside the intron. Sequences were analyzed using an automated sequencer (model 373; Applied Biosystems) in both forward and reverse directions.

Sequence analyses: Sequences were aligned using the algorithm of Needleman and Wunsch (1970) with the GCG computer package (version 8.0) and verified visually. Computation of nucleotide frequencies, transition:tranversion ratio, and numbers of variable sites among sequences was performed by MEGA (version 1.01; Kumar *et al.* 1993). Mean transition: transversion ratio was computed by averaging across all pairwise values. Genetic distance estimates among all pairs of sequences were computed using the Tajima-Nei model of substitution (Tajima and Nei 1984).

Phylogenetic analysis of the aligned sequences used three major algorithms: minimum evolution estimated by neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML). Although each method used different optimality criteria, the concordance among the resultant topologies was interpreted as evidence of the true phylogeny. Minimum evolution using NJ analysis used the values from the Tajima-Nei distance estimates computed by PAUP*(with permission from David Swofford). Maximum parsimony analysis was performed with PAUP* using search conditions of simple addition of sequences, general heuristic search, and branch swapping using the tree-bisection-reconnection algorithm. Maximum likelihood analysis derived an optimal tree using the DNAML subroutine of PHYLIP (version 3.5; Felsenstein 1993). Bootstrap resampling analyses, consisting of 100 iterations, were used in conjunction with MP and NJ analyses to test the reliability of the data to derive the same tree. Bootstrap proportions >70% were considered strong supports for the adjacent node (Hillis and Bull 1993).

Estimation of male:female mutation rate ratio α_m: Using Tajima-Nei distance matrices, a Y/X ratio was calculated for each Zfy matrix element with the corresponding element from Zfx. Mean Y/X and the 95% C.I. were computed from all possible pairwise estimates ($N = 561$). The male:female mutation rate ratio, α_m, was estimated by substitution into the equation $Y/X = 3\,\alpha_m/(\alpha_m + 2)$ (Miyata *et al.* 1987).

TABLE 2

Sequence variation of final intron of Zfy and Zfx

	Alignment length (bp)	Variable sites	Nucleotide frequency (percent)	Transition: transversion
Zfy	783	167	A 32.5 T 36.2 C 13.6 G 17.7	1.8
Zfx	851	106	A 29.8 T 31.3 C 16.7 G 22.2	1.6

RESULTS

Amplification of the complete final intron within homologous genes Zfy and Zfx in 34 species of cat used sex chromosome–specific PCR primers. For each species, comparison of the genetic variation within the intron from Zfy with that for Zfx revealed minor differences in base composition, sequence length, and average transition:transversion bias (Table 2). Intron sequence length varied among species with values ranging from 752 to 758 bp and 841 to 847 bp for Zfy and Zfx, respectively. Both introns exhibited a frequency bias against G and C nucleotides. Furthermore, all species within the genus Felis (domestic cat lineage) shared a SINE insert of ~270 bp long (not shown) in the Zfy intron. Each intron exhibited low numbers of variable sites among the 34 felid species, but the alignment for Zfx contained considerably less diversity than that for Zfy. (Alignments for both introns are available at the web site http://rex.nci.nih.gov/RESEARCH/basic/lgd/front_page.htm)

Using Ple23 (lion), direct sequence comparison between Zfy with Zfx from the same individual yielded an overall sequence homology of ~69% that was not uniformly distributed. Conserved regions were located

on either end of the introns consisting of the first 190 bp and the last 107 bp. These regions had higher homology between *Y* and *X* introns (85% for both) relative to the third intervening region with a sequence homology of 58%. Under the criteria that shared substitutions within Zfy and Zfx in a given species would indicate gene conversion, no sites were identified in the 5′ and 3′ conserved regions.

Estimation of *Y*/*X* mutation ratio: Matrices composed of Tajima-Nei genetic distance values among all pairs of taxa were estimated separately for Zfy and Zfx (Table 3). The ratio of each Zfy matrix element with the corresponding element from Zfx was averaged across all pairwise comparisons ($N = 561$). In all species except Pallas cat ($Y/X = 0.99$), mean pairwise genetic distance estimates were correspondingly greater for Zfy than for Zfx (data not shown). Subsequently, the estimates of $Y/X = 2.06$ (95% C.I. = 1.96–2.16) and $\alpha_m = 4.38$ (95% C.I. = 3.76–5.14) were obtained.

Phylogenetic analysis of Zfy and Zfx: The resultant trees generated by MP analysis of Zfy sequences exhibited all the expected species groups: Panthera genus, domestic cat lineage, ocelot lineage, puma group, lynx group, Asian leopard cat group, and caracal group (Figure 2). With the exception of the puma group, each node of the predicted cluster was strongly supported by high bootstrap proportions. The internal branching order among these clusters was marked by short limb lengths and little resolution. In contrast, the Zfx tree was less resolved (Figure 3). One group (lynx) of the predicted seven clusters was not supported by MP and NJ, and of the remaining six clades, three had strong bootstrap support. In addition, the placement of the rusty-spotted cat within the Panthera group observed in NJ and MP trees was inconsistent with other results (Johnson and O'Brien 1997; Johnson *et al.* 1996).

Despite low numbers of variable sites across 34 felid species for Zfy and Zfx introns (Table 2), each change was highly informative. Consistency indices were high with 0.813 (Zfy) and 0.874 (Zfx; Figures 2 and 3). In the Zfy analysis, MP identified 16 trees of equivalent length (310 steps) and topology (structure uniting the species) that differed only in relative branch length assignments. In the Zfx analysis, MP retained 60 trees of equivalent length (167 steps). A consensus of the trees revealed disagreement at 4 of 19 internal nodes within the topology (indicated by asterisks in Figure 3).

Multiple MP trees of equivalent length suggest a possible sampling effect between numbers of taxa (34) and low numbers of polymorphic sites (167 and 106; Zfy and Zfx, respectively). To ascertain the influence of these two factors on Zfy phylogeny, a jackknife analysis of taxa consisting of three data subsets composed of 23, 19, and 11 randomly selected taxa used in five replications each was performed (Table 4). The results confirm that this effect exhibits a general reduction in tree numbers and an increase in consistency index with decreased taxa.

Within each evolutionary lineage, individual species were characterized by relatively long branch lengths. In general, MP analysis indicated that >50% of the substitutions were species unique or autoapomorphic. For example, using the Zfy sequences for species comprising domestic cat, Panthera group, and ocelot lineages, 24 of 41, 13 out of 21, and 10 of 19 variable sites were autoapomorphic, respectively. Comparable Zfx autoapomorphies were 20 of 25, 5 of 14, and 12 of 20 for these three groups, respectively.

Concordant topologies (not shown) were obtained with NJ and ML analyses for both Zfy and Zfx sequences both apart and in a combined analysis (Table 5). Bootstrap support for all of the major groups (including the puma group) increased in the combined analysis. In the MP analysis with combined data, a consensus of 1998 trees of equivalent length (511 steps; consistency index = 0.810) recapitulated all the major felid groups but had minor differences in within-group associations. As with either intron, the combined analysis exhibited high consistency among all felid species.

DISCUSSION

Substitution differences within the final introns of Zfy and Zfx across 34 felid species offer new insights on sex chromosome evolution. Results of phylogenetic methods reveal considerable precision and accuracy to the pattern of nucleotide changes within these introns. Furthermore, evolutionary differences between Zfy and Zfx introns in Felidae support both the hypothesis of male-driven evolution (Haldane 1947) and, to a lesser extent, the predicted gradual loss of function for genes located outside the pseudoautosomal region of the *Y* chromosome (Charlesworth 1978, 1991; Graves 1995; Rice 1996).

In Felidae, *Y* chromosome evolution appears to be less conserved than that of the *X* chromosome. Zfy and Zfx intron sequences yield a substitution rate ratio between chromosomes as $Y/X = 2.06$ (95% C.I. = 1.96–2.16) and provide a robust estimate of the male:female mutation rate ratio per generation ($\alpha_m = 4.38$ with the 95% C.I. = 3.76–5.14). Considered together with previous research based on coding and noncoding homologous regions between sex chromosomes (Chang *et al.* 1993; Shimmin *et al.* 1994; Chang and Li 1995; Huang *et al.* 1997), these results clearly support greater mutation rates for the *Y* chromosome relative to the *X* chromosome.

Relatively low values of α_m from intron sequences in felids, rodents ($\alpha_m = 2$; Chang *et al.* 1994), and primates ($\alpha_m = 5.06$; Huang *et al.* 1997) are in accordance with weak, male-driven evolution. Relative differences between the three estimates of α_m imply a positive association with reproductive longevity of rodents, carnivores,

TABLE 3

Tajima-Nei distances (× 100) among all pairs of field taxa using the final intron of Zfy (below diagonal) and Zfx (above diagonal)

Species	Code	LPA	LTI	LWI	LCO	OGE	FBI	FCA	FCH	FLI	FMA	FNI	FSI	PLE	PPA	PTI	PUN	PON	NNE	PCO	AJU	HYA	LRU	LCA	LLY	PBE	IPL	PVI	CCA	PAU	PTE	LSE	PMA	PRU	OMA
Ocelot	LPA		1.08	0.84	1.20	0.84	1.44	1.44	1.32	2.19	2.42	1.44	1.32	1.68	1.93	1.56	1.56	1.44	1.81	1.32	1.20	1.34	1.20	1.20	0.96	1.08	0.96	1.69	1.56	1.56	1.08	1.56	1.33	1.68	2.30
Tigrina	LTI	1.48		0.96	1.32	0.72	1.56	1.56	1.56	2.31	2.54	1.56	1.44	1.80	1.80	1.68	1.68	1.56	1.93	1.44	1.56	1.59	1.32	1.32	1.08	1.20	1.08	1.80	1.68	1.68	1.20	1.68	1.44	1.80	2.41
Margay	LWI	0.67	1.34		1.08	0.72	1.32	1.32	1.32	2.06	2.17	1.32	1.20	1.56	1.81	1.44	1.44	1.32	1.69	0.96	1.32	1.10	0.84	1.08	0.84	0.96	0.84	1.56	1.44	1.44	0.96	1.44	1.20	1.56	2.17
Pampas cat	LCO	0.80	1.21	0.67		1.08	1.69	1.69	1.69	2.43	2.66	1.69	1.56	1.93	2.17	1.81	1.81	1.68	2.05	1.56	1.68	1.71	1.44	1.44	1.20	1.32	1.20	1.93	1.81	1.81	1.32	1.81	1.57	1.93	2.54
Geoffroy's cat	OGE	1.48	1.07	1.34	1.21		1.32	1.32	1.32	2.06	2.29	1.08	1.20	1.32	1.81	1.44	1.44	1.32	1.68	1.20	1.32	1.34	1.08	1.08	0.84	0.96	0.84	1.56	1.44	1.44	0.96	1.44	1.20	1.56	2.05
Chinese desert cat	FBI	2.71	3.41	2.58	2.71	3.41		0.48	0.48	1.20	1.32	0.48	0.36	1.69	1.93	1.56	1.56	1.44	1.81	1.32	1.44	1.34	0.96	0.96	0.72	1.08	0.96	1.69	1.56	1.56	1.32	1.08	1.57	1.69	2.30
Domestic cat	FCA	2.71	3.41	2.58	2.71	3.41	0.67		0.48	0.96	1.44	0.48	0.36	1.69	1.93	1.56	1.56	1.44	1.81	1.32	1.44	1.47	0.96	0.96	0.72	1.08	0.96	1.69	1.56	1.56	1.32	1.32	1.57	1.69	2.30
Jungle cat	FCH	4.31	5.03	4.16	4.31	5.03	2.32	2.46		1.20	1.44	0.48	0.36	1.44	1.93	1.56	1.56	1.20	1.81	1.32	1.44	1.47	0.96	0.96	0.72	1.08	0.96	1.69	1.32	1.32	1.32	1.08	1.57	1.69	2.30
African wild cat	FLI	4.27	4.99	4.13	4.13	4.99	1.75	2.16	3.87		2.18	1.21	1.08	2.19	2.43	2.06	2.06	1.94	2.31	2.06	1.94	2.22	1.69	1.69	1.45	1.81	1.69	2.44	2.31	2.31	2.06	2.06	2.19	2.19	3.05
Sand cat	FMA	3.41	3.84	3.13	3.41	4.12	1.21	1.48	3.17	2.02		1.44	1.32	2.66	2.91	2.54	2.54	2.42	2.79	2.29	2.42	2.46	1.93	1.92	1.68	2.05	1.93	2.66	2.54	2.54	2.17	2.17	2.18	2.66	3.28
Black-footed cat	FNI	2.46	3.16	2.32	2.46	3.16	0.81	0.67	2.35	2.32	1.63		0.36	1.44	1.93	1.56	1.56	1.44	1.81	1.32	1.44	1.47	0.96	0.96	0.72	1.08	0.96	1.69	1.56	1.56	1.32	1.32	1.57	1.69	2.30
European wild cat	FSI	2.57	3.27	2.43	2.57	3.27	0.80	0.67	2.61	2.17	1.48	0.95		1.56	1.81	1.44	1.44	1.32	1.68	1.20	1.32	1.34	0.84	0.84	0.60	0.96	0.84	1.56	1.44	1.44	1.20	1.20	1.45	1.56	2.17
Lion	PLE	2.16	2.86	2.03	2.17	2.85	2.17	2.03	3.75	3.72	3.01	2.05	2.17		0.96	0.60	0.60	0.24	0.84	1.56	1.44	1.71	1.44	1.44	1.20	1.56	1.44	2.18	1.56	1.56	1.56	1.56	1.81	0.72	2.54
Leopard	PPA	2.72	3.42	2.58	2.73	3.42	2.73	2.59	4.33	4.30	3.58	2.61	2.73	0.53		0.60	0.60	0.72	1.08	1.81	1.68	1.96	1.69	1.68	1.44	1.80	1.68	2.42	2.05	1.93	1.81	2.05	2.06	0.72	2.79
Tiger	PTI	2.45	3.15	2.31	2.31	3.00	2.46	2.32	4.05	4.02	3.44	2.34	2.46	0.27	0.81		0.24	0.36	0.72	1.44	1.32	1.59	1.32	1.32	1.08	1.44	1.32	2.05	1.68	1.68	1.44	1.69	1.69	0.36	2.42
Snow leopard	PUN	3.14	3.85	3.00	2.86	3.84	3.15	3.01	4.77	4.73	4.01	3.04	3.15	0.94	1.48	1.08		0.36	0.72	1.44	1.32	1.59	1.32	1.32	1.08	1.44	1.32	2.05	1.68	1.68	1.44	1.69	1.69	0.12	2.42
Jaguar	PON	2.44	3.13	2.30	2.44	3.13	2.45	2.31	4.03	4.00	3.29	2.33	2.44	0.27	0.80	0.54	1.21		0.60	1.32	1.20	1.47	1.20	1.20	0.96	1.32	1.20	1.93	1.32	1.32	1.32	1.32	1.57	0.48	2.30
Clouded leopard	NNE	2.72	3.42	2.58	2.72	3.41	2.45	2.31	4.47	3.72	3.01	2.61	2.31	0.80	1.34	1.08	1.76	1.07		1.69	1.56	1.59	1.56	1.56	1.32	1.68	1.56	2.30	1.93	1.93	1.68	1.93	1.94	0.84	2.79
Puma	PCO	3.28	3.99	3.28	3.28	3.99	3.00	3.14	4.46	4.57	3.85	3.03	3.29	2.73	3.30	3.02	3.73	3.01	3.30		1.08	0.85	0.84	1.08	0.84	1.20	1.08	1.81	1.44	1.32	1.20	1.44	1.45	1.57	2.42
Cheetah	AJU	2.57	2.99	2.44	2.44	3.13	2.17	2.17	3.60	3.71	3.00	2.05	2.59	2.31	2.87	2.60	3.30	2.59	2.87	2.59		1.10	1.20	1.20	0.96	1.32	1.20	1.57	1.56	1.56	1.20	1.56	1.57	1.44	2.54
Jaguarundi	HYA	3.69	4.25	3.55	3.69	4.39	3.69	3.84	5.32	5.29	4.41	3.58	3.27	3.13	3.70	3.56	3.84	3.41	3.69	3.56	3.27		0.97	0.97	0.97	1.34	1.22	1.96	1.59	1.59	1.34	1.59	1.60	1.72	2.59
Bobcat	LRU	3.46	4.04	3.32	3.47	4.04	2.90	2.90	4.52	3.91	3.47	2.64	3.19	2.91	3.34	3.20	3.91	3.18	3.48	3.77	2.76	4.03		0.48	0.24	0.60	0.48	1.20	1.32	1.32	1.08	1.32	1.33	1.44	1.56
Canada lynx	LCA	2.61	3.32	2.33	2.62	3.32	2.06	2.06	3.50	3.62	2.90	1.79	2.34	2.06	2.63	2.35	3.06	2.34	2.63	2.91	1.92	3.46	1.37		0.24	0.60	0.48	1.20	1.32	1.32	1.08	1.32	1.32	1.44	1.81
Siberian lynx	LLY	2.74	3.32	2.47	2.75	3.32	2.47	2.47	4.08	4.04	3.32	2.21	2.47	2.20	2.62	2.49	3.19	2.47	2.76	3.33	2.48	3.60	0.54	0.82		0.36	0.24	0.96	1.08	1.08	0.84	1.08	1.08	1.20	1.56
Asian leopard cat	PBE	1.88	2.57	1.75	1.88	2.57	1.61	1.75	3.02	3.14	2.44	1.49	1.62	1.34	1.89	1.62	2.31	1.61	1.89	2.44	2.03	2.85	2.61	1.78	2.05		0.36	1.08	1.44	1.44	0.96	1.44	1.20	1.56	1.68
Flat-headed cat	IPL	2.30	3.00	2.16	2.30	3.00	1.75	1.89	3.17	3.29	2.58	1.77	2.03	1.76	2.32	2.04	2.74	2.03	2.32	2.59	2.03	3.28	2.77	1.92	2.34	0.40		0.96	1.32	1.32	0.84	1.32	1.08	1.44	1.56
Fishing cat	PVI	2.98	3.69	2.85	2.99	3.68	2.30	2.44	3.58	3.29	3.13	2.32	2.71	2.44	3.01	2.73	3.43	2.72	3.00	3.13	2.44	3.96	3.04	2.47	3.03	1.07	1.07		2.05	2.05	1.20	2.05	1.69	1.93	2.30
Caracal	CCA	3.28	3.83	3.14	3.28	3.83	3.28	3.42	4.76	4.85	3.99	3.17	3.28	2.59	3.15	2.88	3.58	2.86	2.87	3.86	3.43	3.84	4.05	3.20	3.33	2.44	2.73	3.56		0.72	1.44	0.96	1.69	1.81	2.67
African golden cat	PAU	4.83	5.56	4.70	4.84	5.40	3.83	4.26	5.62	5.71	4.83	4.16	4.55	4.13	4.71	4.44	5.15	4.41	4.42	4.99	4.40	5.41	5.06	4.19	4.77	3.98	3.84	4.24	2.31		1.44	0.96	1.69	1.81	2.66
Asian golden cat	PTE	2.30	2.85	2.16	2.31	2.99	2.31	2.45	3.89	3.86	3.01	2.19	2.31	0.94	1.48	1.21	1.90	1.21	1.48	2.87	2.45	3.13	3.05	2.20	2.34	1.48	1.90	2.58	2.73	4.28		1.44	0.96	1.56	2.17
Serval	LSE	2.58	3.28	2.44	2.58	2.58	2.30	2.44	3.74	3.85	3.14	2.32	2.59	2.04	2.60	2.18	3.02	2.31	2.59	2.86	2.31	3.56	2.91	2.06	2.48	1.76	1.76	2.44	2.59	3.70	2.18		1.69	1.81	2.67
Marbled cat	PMA	2.58	3.27	2.44	2.58	3.27	2.59	2.73	4.18	3.99	3.43	2.47	2.59	1.21	1.76	1.49	2.17	1.48	1.75	3.15	2.73	3.56	3.18	2.34	2.47	1.75	2.17	2.86	2.59	4.12	1.07	2.45		1.81	2.43
Rusty-spotted cat	PRU	1.89	2.57	1.75	1.89	2.57	1.34	1.48	2.89	3.00	2.17	1.49	1.62	1.35	1.90	1.63	2.31	1.62	1.90	2.17	1.75	2.85	2.48	1.37	1.92	0.80	0.94	1.61	2.45	3.70	1.48	1.48	1.76		2.55
Pallas cat	OMA	2.31	3.00	2.17	2.31	3.00	1.90	2.04	3.47	3.44	2.73	1.64	2.04	1.76	2.32	2.05	2.74	2.04	2.32	2.74	2.18	3.14	2.48	1.37	2.06	1.21	1.49	2.03	2.88	4.00	1.90	2.04	2.18	0.94	

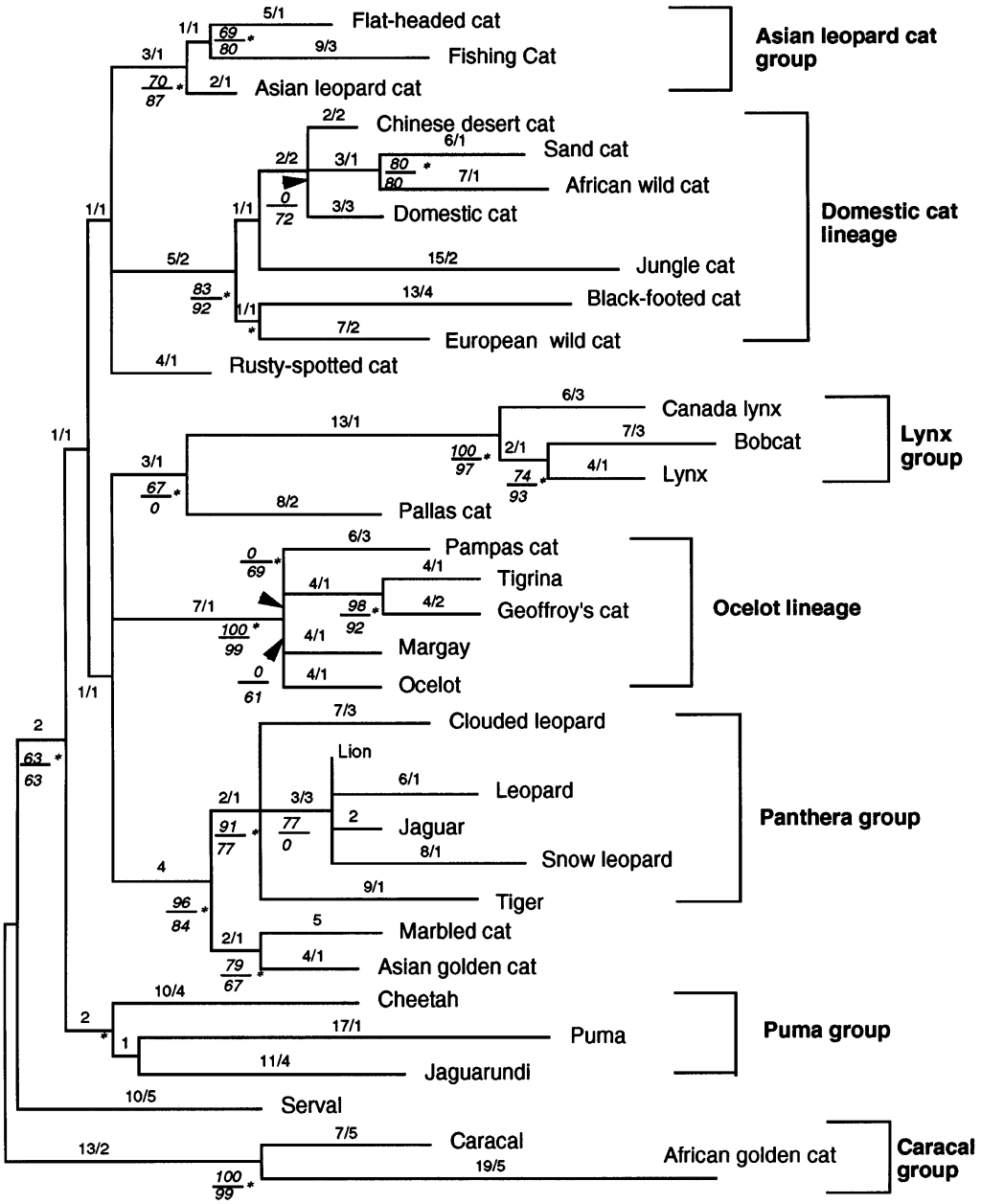

Figure 2.—Phylogenetic reconstruction of the final intron of Zfy in 34 species of Felidae using MP. Shown is the consensus tree of 16 trees of equivalent length and topology generated by 50% majority rule. The numbers on limbs are the number of steps/number of homoplasies. Values in italics are MP (above line) and NJ (below line) bootstrap proportions in support of adjacent nodes >50%. Asterisks denote significant nodes ($P < 0.05$) derived in ML analysis. The arrow indicates the position of an additional node present in the NJ analysis only. Trees are rooted by midpoint rooting.

and primates. This association remains speculative, however, because the number of germ cell divisions per generation are not clearly defined in any of these orders (Chang *et al.* 1994). Furthermore, these values contradict both an estimate inferred from the *X*:autosome ratio of 0.6 ($\alpha_m = \infty$; Miyata *et al.* 1987; Wolfe and Sharp 1993) and the results of a recent analysis that compared synonymous changes in *Y*- and *X*-linked genes with autosomal loci in rodents and found no evidence of enhanced mutation in *Y*-linked genes (McVean and Hurst 1997).

The presence of a SINE insert in Zfy in seven *Felis* species of the domestic cat lineage (J. Pecon Slattery and S. J. O'Brien, unpublished data) represents an evolutionary phenomenon postulated for genes located outside the pseudoautosomal region. Both theoretical arguments (Charlesworth 1991) and empirical data with Drosophila (Steinemann and Steinemann 1992) indicate that the *Y* chromosome, along with other regions within the genome with restricted recombination, is expected to accumulate retroposons. Although not located within an exon, the felid Zfy retroposon pro-

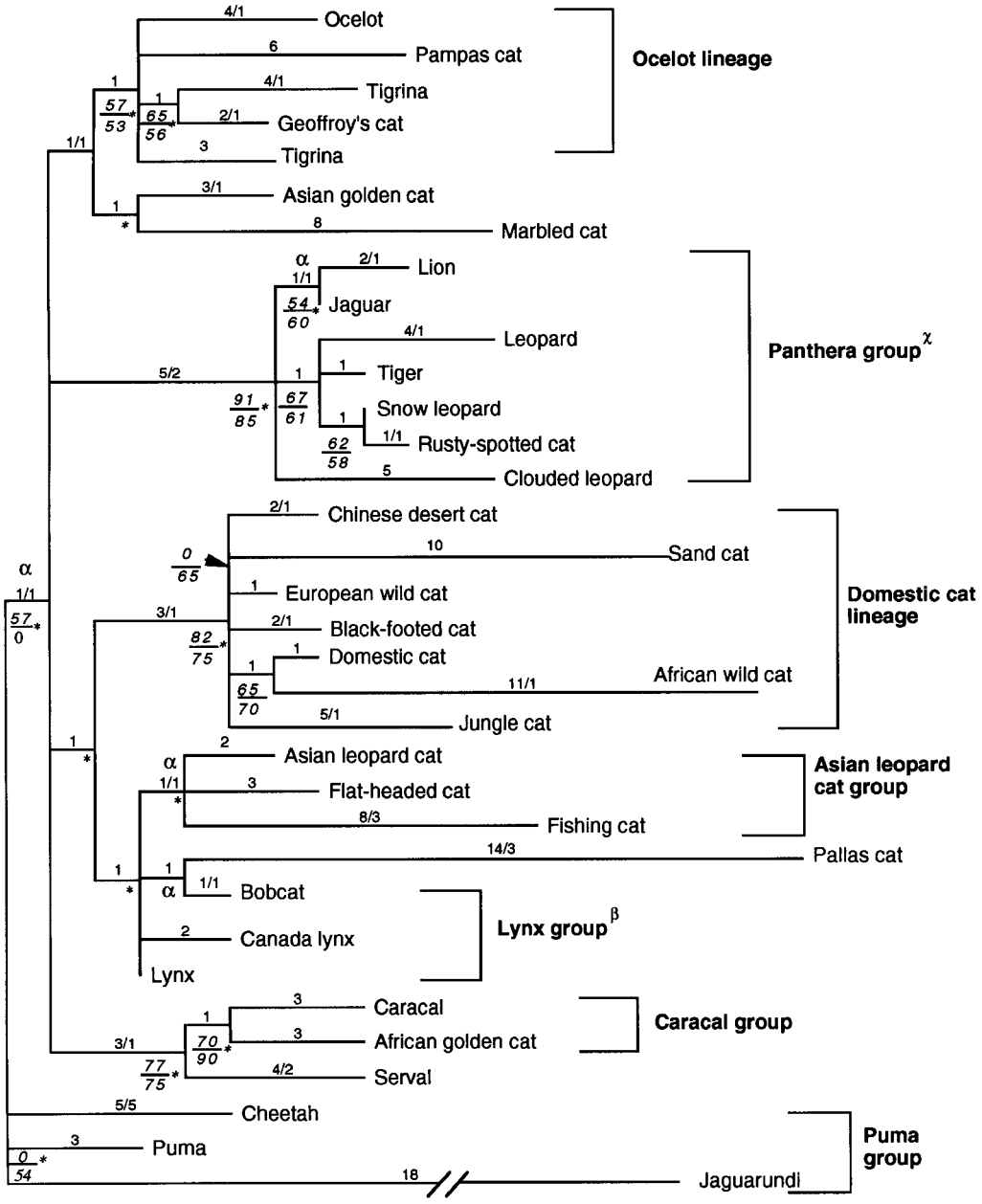

Figure 3.—Phylogenetic reconstruction of the final intron of Zfx in 34 species of Felidae using MP. Shown is the consensus of 60 trees of equivalent length generated by 50% majority rule. An α indicates a node of uncertainty among the 60 trees; β indicates monophyletic lynx group with ML analysis. A χ indicates that the rusty-spotted cat was placed outside of the Panthera group with ML analysis. The numbers on limbs are the number of steps/number of homoplasies. Values in italics are MP (above line) and NJ (below line) bootstrap proportions in support of adjacent nodes >50%. Asterisks denote significant nodes ($P < 0.05$) derived in ML analysis. Trees were constructed during analysis using midpoint rooting.

vides further support for these elements as a mechanism for the degeneration of genes located on the *Y* chromosome. Because it is shared among all seven species within the domestic cat lineage, the insertion mostly likely occurred ~6 mya (Johnson and O'Brien 1997) with the divergence of a common ancestor.

Calibrated by the fossil record, genetic distance estimates between pairs of species from each evolutionary group yielded markedly low rates of substitution for Zfy and Zfx introns. Approximate divergence times indicate that the more ancestral felid clades are the puma group (8.5 mya), domestic cat lineage (6 mya), lynx group (6.7 mya), and Panthera group (6 mya), followed by the divergence of the ocelot lineage (5 mya), caracal group (4.85 mya), and Asian leopard cat group (3.95 mya; Johnson and O'Brien 1997). Substitution rates of 0.11 ± 0.04%/site/millions of years (MY) for Zfy and 0.069 ± 0.03%/site/MY for Zfx are derived by averaging all pairs of Tajima-Nei genetic distances within each defined felid cluster, dividing by 2 and the esti-

TABLE 4

Jackknife analysis of taxa with Zfy intron sequences

No. of taxa	Replicate	No. of trees	Tree length	Consistency index
23	1	16	350	0.923
	2	2	336	0.914
	3	58	366	0.891
	4	2	374	0.888
	5	19	385	0.909
19	1	12	330	0.900
	2	1	323	0.920
	3	1	341	0.930
	4	1	328	0.948
	5	36	295	0.950
11	1	83	271	0.937
	2	3	284	0.951
	3	11	276	0.924
	4	1	268	0.955
	5	5	263	0.966

mated time (in millions of years) that members within each clade last shared a common ancestor listed above. These values are less than those computed for allozymes (1.9%/site/MY) and two-dimensional protein electrophoresis (1.26%/site/MY; Pecon Slattery *et al.* 1994). Additionally, Zfy and Zfx estimates are less than those based on noncoding 12S (0.42%/site/MY) and 16S (0.70%/site/MY) and coding ND-5 (2.43%/site/MY) mitochondrial genes (Lopez *et al.* 1997) in Felidae. The Zfy estimate, however, is comparable to the estimate for primates of 0.135%/site/MY (Dorit *et al.* 1995).

Despite such slow rates of substitution, both introns exhibit high phylogenetic signals maintained by low numbers of polymorphic sites across the 34 felid species. Reconstruction of predicted relationships of the well-characterized Felidae indicate that substitutions within Zfy were highly accurate in recapitulating evolutionary history. In contrast, Zfx had insufficient genetic diversity to completely resolve the felid phylogeny and most likely erred in the placement of the rusty-spotted cat within the Panthera. As defined by Zfy and, to a lesser extent, by Zfx, the 34 species of felid diverge into expected seven major evolutionary groups and three unaligned species (Table 1). The fourth unaligned species, serval, was clearly placed as an early divergence within the caracal group. Strong concordance between Zfy phylogeny with that derived from a combined analysis of mitochondrial genes (Johnson and O'Brien 1997), as well as mitochondrial RFLP data (Johnson *et al.* 1996), indicate Zfy as a promising patrilinear counterpart to mitochondrial DNA in evolutionary analysis.

For both Zfy and Zfx introns, character-based analysis reveal the precision with which each site change reflects evolutionary history. High consistency indices for Zfy (0.813) and Zfx (0.874) indicate low levels of homoplasy (convergent, parallel, or reversals in character changes required for building the phylogenetic tree). Increased consistency indices generated by the taxon jackknife analyses further demonstrate the lack of "noise" within the intron pattern of substitution. Even though most site changes are informative in defining each of the expected clades, the relative branching order among the groups is not clear. Such a pattern implies that the eight present-day felid lineages evolved in a rapid burst,

TABLE 5

Summary of phylogenetic support* for each predicted felid evolutionary association listed in Table 1

	ZFY			ZFX			Combined		
Felid group	NJ	MP	ML	NJ	MP	ML	NJ	MP	ML
Ocelot lineage									
Lpa, Lti, Lwi, Oge, Lco	99	100	$P < 0.01$	53	57	$P < 0.01$	100	100	$P < 0.01$
Domestic cat lineage									
Fca, Fma, Fch, Fli, Fbi, Fni, Fsi	92	83	$P < 0.01$	75	82	$P < 0.01$	99	100	$P < 0.05$
Panthera group									
Ple, Pti, Pon, Pun, Ppa, Nne	84	91	$P < 0.01$	91[a]	85[a]	$P < 0.01$[a]	98	96	$P < 0.05$
Puma group									
Aju, Pco, Hya	NBS	NBS	$P < 0.01$	NBS	57	$P < 0.01$	71	68	$P < 0.01$
Lynx genus									
Lly, Lru, Lca	97	100	$P < 0.01$	NS	NS	NS	99	100	$P < 0.01$
Asian leopard cat group									
Pbe, Ipl, Pvi	87	70	$P < 0.01$	NBS	NBS	NS	89	78	$P < 0.01$
Caracal group									
Cca, Pau	99	100	$P < 0.01$	76	70	$P < 0.01$	100	95	$P < 0.01$
w/Lse	63	63	$P < 0.01$	76	77	$P < 0.01$	93	95	$P < 0.01$

* The phylogenetic methods listed are minimum evolution estimated by NJ, MP, and ML. NS, not supported by topology; NBS, no bootstrap support (node present in topology but collapsed in bootstrap analysis).

[a] Zfx analysis placement of rusty-spotted cat within Panthera genus is inconsistent with other methods.

an interpretation that is consistent with previous genetic analysis.

Within each evolutionary lineage, each species is characterized by multiple unique changes (*i.e.*, long branch lengths) and low levels of homoplasy. However, short internal branches uniting within-group species indicate a paucity of shared derived (synapomorphic) changes. In comparison with mitochondrial data (Janczewski *et al.* 1995; Masuda *et al.* 1996; Johnson and O'Brien, 1997), Zfy and Zfx introns are unusually deficient in synapomorphic changes that are useful for determining intralineage species associations. Such discrepencies may be caused by chance or may indicate these introns reflect the outcome of possible selective sweeps within sex chromosomes during speciation in Felidae. However, further investigation of coding and noncoding regions of sex chromosome genes are warranted to distinguish among alternative evolutionary scenarios.

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