Phylogenetic Assessment of Introns and SINEs Within the Y Chromosome Using the Cat Family Felidae As a Species Tree

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The cat family Felidae was used as a species tree to assess the phylogenetic performance of genes, and their embedded SINE elements, within the nonrecombining region of the Y chromosome (NRY). Genomic segments from single-copy X-Y homologs *SMCY, UBE1Y*, and *ZFY* (3,604 bp) were amplified in 36 species of cat. These genes are located within the X-degenerate region of the NRY and are thought to be molecular "fossils" that ceased conventional recombination with the X chromosome early within the placental mammal evolution. The pattern and tempo of evolution at these three genes is significant in light of the recent, rapid evolution of the family over approximately 12 Myr and provides exceptional support for each of the eight recognized felid lineages, as well as clear diagnostic substitutions identifying nearly all species. Bootstrap support and Bayesian posterior probabilities are uniformly high for defining each of the eight monophyletic lineages. Further, the preferential use of specific target-site motifs facilitating SINE insertion is empirically supported by sequence analyses of SINEs embedded within the three genes. Target-site insertion is thought to explain the contradiction between intron phylogeny and results of the *SMCY* SINE phylogeny that unites distantly related species. Overall, our data suggest X-degenerate genes within the NRY are singularly powerful markers and offer a valuable patrilineal perspective in species evolution.

Introduction

The eutherian Y chromosome is distinctive because the majority of its genes are located within the non-recombining region (NRY) and by definition do not undergo conventional recombination with the X chromosome. Consequently, the NRY was originally presumed to be a "functional wasteland," but subsequent results from human and mouse analyses (Lahn and Page 1997, 1999; Burgoyne 1998; Lau and Zhang 2000; Bachtrog and Charlesworth 2001; Skaletsky et al. 2003) define distinct categories of genes within the Y chromosome. Full-length sequence analysis of the Y chromosome in humans (Skaletsky et al. 2003) currently identifies (1) genes within the pseudoautosomal region that undergo conventional X-Y recombination during meiosis; (2) X-degenerate single-copy genes considered to be molecular "fossils" from when X and Y were autosomal pairs early within mammalian evolution; (3) X-transposed genes that are more recently acquired from the X chromosome; and (4) ampliconic genes that exist in multiple copies on the Y chromosome and are expressed exclusively in the testis and may undergo a unique form of recombination termed Y-Y gene conversion to maintain function.

The mechanisms for maintaining gene function within the X-degenerate regions of the Y chromosome are less clear. Without the benefits of either conventional recombination or Y-Y gene conversion, X-degenerate genes would, thus, be expected to either become specialized for male function (Graves 1995, 1998) or gradually degenerate from the accumulation of deleterious mutations through processes such as Muller's ratchet (Charlesworth and Charlesworth 1997), hitch-hiking with favorable mutations at other Y-chromosome genes (Charlesworth 1996), back-ground selection (Charlesworth 1996), and insertion of retroposable elements (Charlesworth 1991). However, expression studies reveal that these genes are functional, expressed in multiple tissues, and typically perform a "housekeeping" role (Skaletsky et al. 2003; Pecon-Slattery et al. 2000b).

One useful empirical approach to examining these paradoxical theoretical considerations is comparative phylogenetic analyses of sequence data across species within a reference taxonomic group. The cat family Felidae is a useful reference phylogeny for estimating patterns of NRY evolution. Modern felid phylogeny is derived from consistent concordance among trees derived from analyses of multiple independent genetic markers and partitions the 37 species into eight evolutionary lineages (Collier and O'Brien 1985; Modi and O'Brien 1988; Pecon-Slattery et al. 1994; Janczewski et al. 1995; Masuda et al. 1996; Johnson et al. 1996; Johnson and O'Brien 1997; Pecon-Slattery and O'Brien 1998; Johnson et al. 2001; Johnson et al. 2004). Our previous analyses of the single-copy X-Y homologous pair *ZFX* and *ZFY* (zinc finger protein on Y) in Felidae corroborated the utility of the cat family as a reference phylogeny in sex-chromosome evolution. Using the cat species tree as a template for studying patterns of substitution in sex-chromosome genes, we have demonstrated (1) faster overall rates of substitution in the Y chromosome than in the X chromosome (Pecon-Slattery and O'Brien 1998), supporting the hypothesis of male-driven evolution (Miyata et al. 1987); (2) an alternate form of recombination, ectopic gene conversion, that may be a mechanism for maintaining function in X-degenerate genes within the NRY (Pecon-Slattery, Sanner-Wachter, and O'Brien 2000b); and (3) a phylogenetic depiction of the distinct lineages of SINE retroelements on the Y chromosome in felids and additional carnivore taxa (Pecon-Slattery, Murphy, and O'Brien 2000a).

Here, we conduct phylogenetic analysis of intron regions sequenced from the single-copy Y-chromosome genes *ZFY, SMCY* (select mouse cDNA on Y), and *UBE1Y* (ubiquitin activating enzyme E1 on Y) in 36 species of

Felidae. Although each gene has an X-chromosome homolog, there is no longer any form of conventional recombination between them because of the Y-chromosome homolog position within the MSY. However, these genes are thought to be fully functional (Koopman, Ashworth, and Lovell-Badge 1991; Agulnik et al. 1994; Mazeyrat et al. 1998). *ZFY* and *SMCY* are located within the X-degenerate region of the MSY in humans (Skaletsky et al. 2003) and the corresponding region of the NRY in mouse and cat (Murphy et al. 1999). Likewise, *UBE1Y* is in the NRY of cat and mouse but is seemingly lost from the human Y chromosome during primate evolution (Murphy et al. 1999; Mitchell et al. 1998).

In addition, we reexamine the evolution of short-interspersed elements (SINEs) within single-copy Y-chromosome genes in the NRY. SINEs are scattered throughout the genome and are thought to proliferate by the transcription of a master-copy SINE followed by reverse transcription into DNA and reinsertion at a new site within the host genome (Deininger et al. 1992; Brookfield 1994; Smit 1996; Kidwell and Lisch 1998; Shedlock and Okada 2000). Originally SINEs were thought to be perfect markers for phylogenetic analyses because they integrate within the genome in a stochastic fashion and have no known mechanism of excision (Shedlock and Okada 2000). However, this assertion is challenged in theoretical terms by Hillis (1999) and by evidence that nonrandom target sites may be preferentially selected for SINE insertion (Jurka and Klonowski 1996; Tatout, Lavie, and Deragon 1998), as well as by empirical data describing parallel insertion of SINEs (homoplasy) unrelated to phylogeny in rodents (Cantrell et al. 2001) and in our previous research in carnivores (Pecon-Slattery, Sanner-Wachter, and O'Brien. 2000*b*).

Using a phylogenetic approach, we provide useful insights into the power of Y-chromosome markers to resolve patterns of speciation with minimal phylogenetic "noise." We provide sequence data from select portions of three NRY genes and assess phylogenetic inferences based on introns and SINEs inserted within these regions.

Methods
DNA Specimens

DNA was purified from either blood or skin cell fibroblasts from at least one male of 36 species of Felidae representing eight defined evolutionary lineages (see review in Johnson et al. [2001]) (Johnson et al. 2004). Species from the ocelot lineage included ocelot (*Leopardus pardalis*), tigrina (*L. tigrina*), margay (*L. weidii*), pampas cat (*Lynchailurus colocolo*), kodkod (*Oncifelis guigna*), and Geoffroy's cat (*O. geoffroyi*). Representatives from the domestic cat lineage were domestic cat (*Felis catus*), Chinese desert cat (*F. bieti*), jungle cat (*F. chaus*), European wildcat (*F. silvestris*), African wildcat (*F. libyca*), black-footed cat (*F. nigripes*), and sand cat (*F. margarita*). The panthera lineage consisted of leopard (*Panthera pardus*), tiger (*P. tigris*), jaguar (*P. onca*), snow leopard (*P. uncia*), lion (*P. leo*), and clouded leopard (*Neofelis nebulosa*). The puma lineage comprised the puma (*Puma concolor*), cheetah (*Acinonyx jubatus*), and jaguarondi (*Herpailurus*

yagouaroundi). The lynx lineage included bobcat (*Lynx rufus*), Siberian lynx (*L. lynx*), Canadian lynx (*L. canadensis*), and Iberian lynx (*L. pardina*). The Asian leopard cat lineage consisted of the Asian leopard cat (*Prionailurus bengalensis*), flat-headed cat (*Ictailurus planiceps*), fishing cat (*P. viverrina*), and rusty-spotted cat (*P. rubiginosa*). The caracal lineage was represented by caracal (*Caracal caracal*), African golden cat (*Profelis aurata*), and serval (*Leptailurus serval*). Asian golden cat (*Profelis temmincki*) and marbled cat (*Pardofelis marmorata*) represented the bay cat lineage. No males were available for the bay cat (*Pardofelis badia*) of the bay cat lineage and Andean mountain cat (*Oreailurus jacobita*) of the ocelot lineage. The final species included, pallas cat (*Otocolobus manul*), is a monotypic lineage that is sister taxa to domestic cat and Asian leopard cat lineages.

PCR Analyses of Intron Sequences (2,462 bp) Amplified from Three Y-Chromosome Genes

Primers were designed for intronic regions for the single-copy Y-chromosome genes of *ZFY, UBE1Y*, and *SMCY*. For *ZFY*, we used published felid sequences from the terminal zinc-finger intron and added new taxa using the same primers (Pecon-Slattery and O'Brien 1998). For *SMCY*, we developed primers from conserved regions within exon 4 and exon 5 from published *SMCY* sequences for human and mouse. Additional primers internal to these were developed specifically for the cat family (table 1 in Supplemental Material online). Similarly, we designed primers from mouse *Ube1Y* sequence spanning intron 17, exon 18, and intron 18 (Levy et al. 2000) with additional internal primer sets specific to felids. Y-chromosome specificity of all PCR reactions was confirmed by the presence of the expected product in males and its absence in females. Sequences are deposited in GenBank under accession numbers AY219653 to AY219681 (*SMCY*), AY518597 to AY518631 (*UBE1Y*), and AY518632 to AY518667 (*ZFY*).

PCR reactions consisted of 25 μl reactions containing 62.5 ng of genomic DNA, 50mM KCl, 10mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 uM of each primer, and 2.5 units Taq polymerase. Typical cycling conditions used a hot start of 3 min at 95°C, followed by 45 cycles of 15 s at 95°C, 30 s at 55°C, and 45 s at 72°C, ending with a final extension of 72°C for 5 min. To better increase primer binding in some species, the annealing temperature was adjusted between 56°C and 58.6°C. Reactions were run in either an ABI Perkin-Elmer 9700 or in a Biometra T1 thermocycler. Products were visualized on 1% agarose gels run in 1× TAE buffer at 120 volts for 60 min using 5 μl of 5 mg/ml ethidium bromide in the gel and in the buffer. Products were cleaned using Microcon Centricon filters or Microcon PCR filters, and sequenced using ABI BigDye sequencing reagents and ABI automated sequence machine models 377, 3700, and 3730.

Genetic and Phylogenetic Analysis

Sequences amplified from 36 species of cat from *SMCY* (1,395 bp), *UBE1Y* (1,210 bp), and *ZFY* (999 bp)

for a total of 3,604 bp, were compiled using Sequencher version 4.1 (Gene Codes Corporation) and aligned using ClustalX (Thompson et al. 1997). Before phylogenetic analysis, ambiguous sites and all SINEs were removed resulting in gene fragments sizes of 783 bp (*SMCY*), 958 bp (*UBE1Y*), and 721 bp (*ZFY*). The program ModelTest (Posada and Crandall 1998) was used to determine the optimal model of substitution for phylogenetic analyses for the concatenated multiple sequence file (2,462 bp).

Phylogenetic reconstruction of intron sequences in Felidae was performed by PAUP* (Swofford 1998) using three different optimality criteria of minimum-evolution (ME), maximum-likelihood (ML), and maximum-parsimony (MP) methods. The GTR + gamma model was derived by ModelTest for ML and ME methods. The parameters for the ML and ME analyses used (1) rate matrix AG = 1.191600, AC = 3.871200, AT = 0.672200, GC = 2.699700, GT = 2.362700, and CT = 1.0; (2) estimated nucleotide frequencies A = 0.29933, C = 0.16400, G = 0.18990, and T = 0.35280; and (3) gamma = 0.782. Specific search conditions for the ML analysis use starting trees obtained by stepwise addition and branch-swapping using the tree-bisection-reconnection (TBR) algorithm. Specific conditions for the ME heuristic search included starting trees obtained by neighbor-joining, TBR branch-swapping algorithm, and no collapsing of zero-length branches. The heuristic search with MP coded gaps as "missing," with stepwise addition of taxa and the TBR branch-swapping. Support for specific clades was assessed by bootstrap analysis using identical settings established for each method of phylogenetic reconstruction and retention of node bootstrap values greater than 50%. For the ME and MP analyses, 1,000 iterations of bootstrap were performed with heuristic tree searches employing TBR branch-swapping. For ML bootstrapping, 100 iterations were implemented using nearest-neighbor interchange for branch-swapping. A fourth method utilizing a Bayesian approach for computing credibility values for nodes within the tree was implemented using the MrBayes version 2.01 program (Huelsenbeck and Ronquist 2001). Specific parameters included (1) four Markov chains run for 700,000 generations, (2) empirical estimates of stable likelihood values to set the burn-in at 50,000 generations, and (3) trees sampled every 20 generations. Two runs were performed to confirm the stability of posterior probabilities and parameters. Alternative topologies for the Y-chromosome intron phylogeny were tested using the approximate uncertainty test (AU test) and the multiscale bootstrap technique implemented in the computer program CONSEL (Shimodaira 2002).

A separate analysis of SINE sequences derived from *ZFY, UBE1Y*, and *SMCY* utilized the above phylogenetic reconstruction methods and programs. The newly described SINEs were compared with previously published Y-chromosome SINEs within Felidae (Pecon-Slattery, Sanner-Wachter, and O'Brien 2000*b*) (GenBank accession numbers AF221612 to AF221618) and two outgroup SINEs from domestic cat MHC (Yuhki et al. 2003). The alignment of 23 SINE sequences (194 bp) excluded the poly-T/A tail to remove ambiguous sites. The substitution model selected for ML and ME methods was a variant of

HKY with the transition:transversion ratio set to 3.875 and corrected for the proportion of invariant sites (I = 0.4946). Heuristic search and bootstrap analyses used the same conditions listed previously. The parameters used in the Bayesian analyses were the same as above except for burn-in = 60,000 generations. Alternative topologies for the SINE phylogeny were tested for significance by the AU test in CONSEL and the Shimodaira-Hasegawa test (RELL option, 1,000 bootstraps) in PAUP*. Multiple male individuals were sequenced to test for intraspecific variation in SINE inserts located within *SMCY* in domestic cat–lineage species *F. catus* (N = 18), *F. silvestris* (N = 5), *F. chaus* (N = 2), *F. libyca* (N = 2), *F. margarita* (N = 3), and *F. nigripes* (N = 2), as well as for the lynx lineage species *L. rufus* (N = 28), *L. lynx* (N = 6), *L. canadensis* (N = 2), and *L. pardina* (N = 2).

Results
Felid Evolution Defined by Intron Sequence from *ZFY, SMCY*, and *UBE1Y*

Felid phylogeny was derived by sequence analyses (excluding SINEs and ambiguous sites) from three Y-chromosome genes, *ZFY, UBE1Y*, and *SMCY*. The complete alignment is presented in figure 1 of Supplementary Material online and consists of *SMCY* (sites 1 to 1395) *UBE1Y* (sites 1396 to 2605), and *ZFY* (sites 2606 to 3604). *UBE1Y* did not amplify in *Herpailurus yagouarondi* (jaguarondi). Imbedded within each of the three segments are SINE retroposons. Two SINEs are located in *SMCY* at sites 405 to 640 and 1073 to 1373, one is located in *UBE1Y* at position 2323 to 2560, and one is located in *ZFY* at position 2827 to 3071 of the total alignment (figure 1 in Supplementary material online). A 42-bp insert unique to *Lynx canadensis* (canadian lynx) is located within position 750 to 792 of *SMCY*. An additional 80 bp were identified as phylogenetically ambiguous. The SINEs and ambiguous sites were removed from the final data set used for all subsequent phylogenetic analyses, resulting in a data set of 2,462 bp (figure 2 in Supplementary Material online). Of the 2,462 bp sequenced, 376 sites were variable. Of these sites, 235 were singleton substitutions and 141 were designated as parsimony informative.

Despite a low number of variable sites (15%) within Felidae, the combined sequences were highly informative in both species identification and organization into the expected eight monophyletic lineages (fig. 1). Strong support for each lineage is suggested by bootstrap values of 88% or higher and Bayesian probabilities of 1.0. Four lineages (domestic cat, lynx, ocelot, and panthera) all had values of 100% by the four measures of node support used. Additional insights are possible by examination of the numbers of steps diagnostic for each lineage corresponding to the branch length (BrL) versus the portion of those that were homoplasies (convergent, reversal, or parallel substitutions) as derived by the MP method of analysis (fig. 1). The MP tree (based on a consensus of 60 equivalent trees of identical length), has a consistency index (CI) of 0.9081; excluding uninformative sites, the CI = 0.7919. Lineages exhibiting one
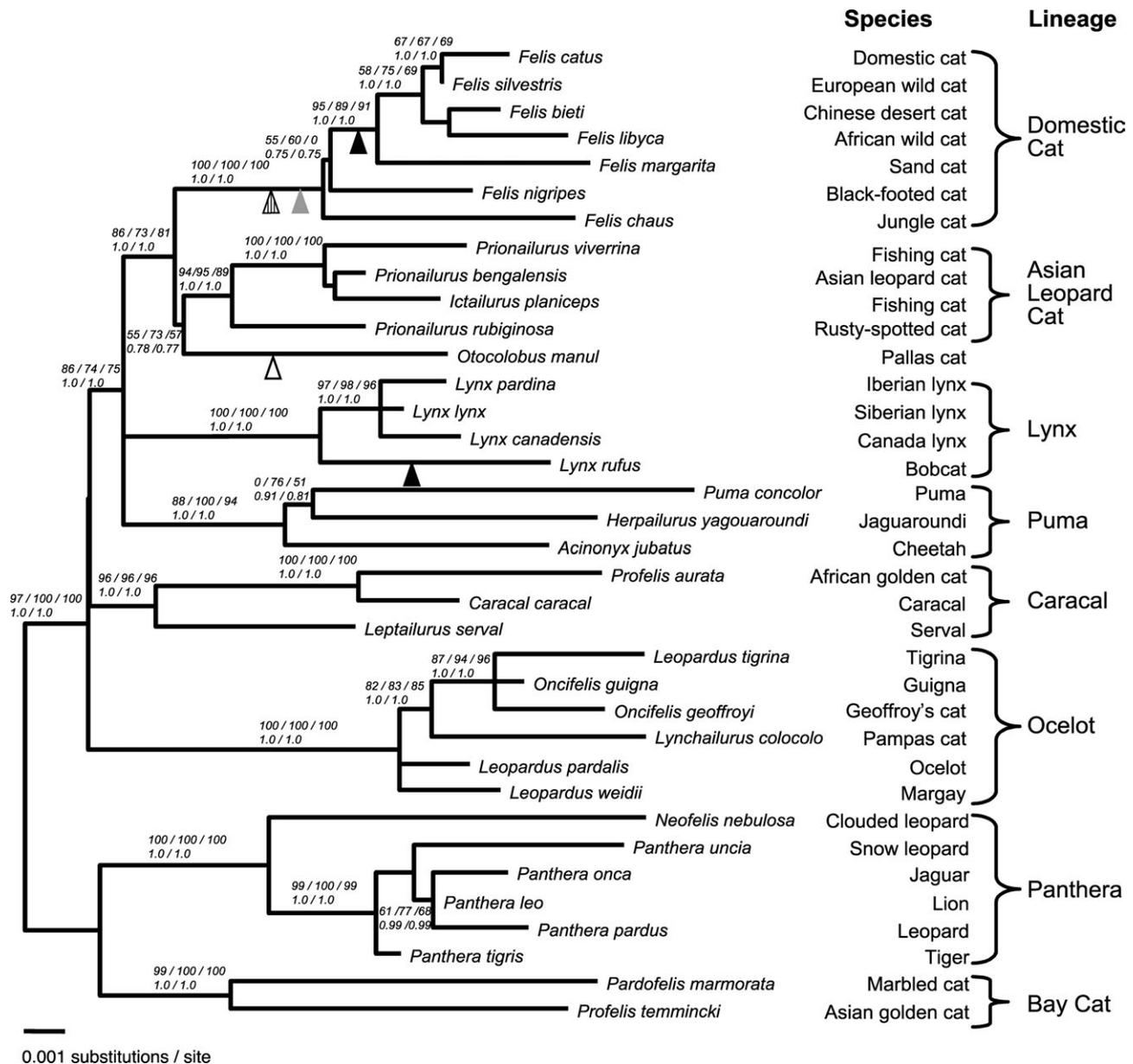
FIG. 1.—Phylogenetic tree of combined data from three Y-chromosome genes *SMCY, UBE1Y,* and *ZFY* (2462 bp) sequenced from 36 species of felids. Shown is the maximum-likelihood tree (–Ln likelihood score = 6594.88761; 13,685 rearrangements tried). Nearly identical topologies to this tree were recovered using minimum evolution (ME) (GTR distances) and maximum parsimony (MP). Phylogenetic analyses using ME resulted in a single tree (tree score = 0.18787; 13,064 rearrangements tried). Phylogenetic analyses using MP criteria recovered 60 equivalent trees (length = 445 steps; 818,973 rearrangements tried). Numbers in italics reflect bootstrap support for adjacent nodes as ME (1,000 iterations)/MP (1,000 iterations)/ML (100 iterations) and are followed by Bayesian posterior probability values (two independent runs). The tree is midpoint rooted. Striped triangle = *ZFY* SN244@2827 insertion point in a common ancestor to species of the domestic cat lineage. Grey triangle = *SMCY* SN231@405 insertion point in a common ancestor of the domestic cat lineage. Black triangle = *SMCY* SN327@1073 insertion point in five closely related species within the domestic cat lineage and in *Lynx rufus* of the lynx Lineage. Open triangle = *UBE1Y* SN237@2323 found only in *Otocolobus manul.*

homoplasy were puma (BrL = 11), bay cat (BrL = 8), Asian leopard cat (BrL = 3), and caracal (BrL = 4). The remaining lineages of ocelot (BrL = 17), lynx (BrL= 12), domestic cat (BrL = 9), and panthera (BrL = 9) had two homoplasies each (data not shown).

The distribution of sites diagnostic for each lineage was examined for each intron separately (figure 2 in Supplementary Material online and table 1). We define sites diagnostic for a given lineage as (1) exclusive to that lineage

and (2) shared by all species within the lineage. Of the total number of sites (N = 68) that met the lineage diagnostic criteria, *SMCY* (N = 20) and *ZFY* (N = 20) contained equivalent numbers of diagnostic sites whereas *UBE1Y* (N = 28) had the greatest number.

Although the relative branching order between the eight lineages is not well resolved, there is strong support (86/73/81, ME/MP/ML, respectively and Bayesian posterior probabilities of 1.0), for a common ancestor uniting the

domestic cat lineage with the Asian leopard cat lineage (fig. 1). Within this association, the pallas cat is consistently placed within the Asian leopard cat lineage, although support for this is not high. Using midpoint rooting, the panthera lineage and bay cat lineage appeared separated from the other felid lineages, with bootstrap support of greater than 97% and Bayesian probabilities of 1.0.

In addition, *SMCY*, *UBE1Y* and *ZFY* introns were informative as species-diagnostic markers within each lineage. With the exception of *Panthera leo* (panthera lineage), *Felis silvestris* (domestic cat lineage), and *Lynx lynx* (lynx lineage), all other species were distinguished by two or more unique autoapomorphic substitutions. Moreover, the three methods of phylogenetic analyses and Bayesian analyses were completely consistent in deriving the same relative branching order between species within each lineage, although several polytomies exist (fig. 1). These polytomies include a trichotomy between *Panthera leo*, *Panthera pardus*, and *Panthera onca* in the panthera lineage; a trichotomy between *Leopardus tigrina*, *Oncifelis geoffroyi*, and *O. guigna* in the ocelot lineage; and a trichotomy within the lynx lineage.

Genetic and Phylogenetic Analyses of
SINE Retroelements

Each of the three gene introns contains SINE retroelements. As described previously (Pecon-Slattery, Murphy, and O'Brien 2000*a*), the SINE in *ZFY* is unique to all species within the domestic cat lineage (*Felis catus*, *F. silvestris*, *F. margarita*, *F. libyca*, *F. bieti*, *F. chaus*, and *F. nigripes*) (figures 1 and 3 in Supplementary Material online). In *UBE1Y*, a single SINE occurs as an autoapomorphic insertion for the pallas cat within intron 18 (figures 1 and 3 in Supplementary Material online) and was described previously (Pecon-Slattery, Murphy, and O'Brien 2000*a*). The fourth intron of *SMCY* contains two SINE insertions that both occur within the domestic cat lineage. SN231@405 (231 bp) is located at position 405 within the alignment (figure 1 in Supplementary Material online) and is shared by all seven domestic cat–lineage species and is newly described in this paper (figure 3 in Supplementary Material online). The second insertion, SN327@1073 (327 bp) occurs at position 1073 of the alignment and is in inverse orientation relative to SN231@405 and is shared by five species within the domestic cat lineage (*F. silvestris*, *F. catus*, *F. libyca*, *F. bieti*, and *F. margarita*) and the unrelated bobcat (*Lynx rufus*), which is a member of the lynx lineage (fig. 1). This result confirms our earlier findings (Pecon-Slattery, Murphy, and O'Brien 2000*a*) but differs by expanding the number of species in the domestic cat lineage that share the SN327@1073 insertion (figure 3 in Supplementary Material online).

The presence of SN327@1073 within two separate felid lineages may represent an ancestral polymorphism that is not fixed within and between species. We tested this hypothesis by sequencing *SMCY* in multiple male individuals (see *Methods*) of six species of the domestic cat lineage and all four lynx-lineage species. The results confirmed the fixation of SN327@1073 among all individuals examined

Table 1
The Distribution of Diagnostic Sites Located in the *SMCY*, *UBE1Y*, and *ZFY* Alignment That Defines Each of the Eight Monophyletic Lineages

Lineage	SMCY (783 bp)	UBE1Y (958 bp)	ZFY (721 bp)	Total sites (2,462 bp)
Domestic cat	N = 0	N = 6 839 909 1216–1217(I) 1317 1381 1444	N = 2 2141 2423	N = 8
Asian leopard cat	N = 2 97 730	N = 0	N = 1 2416	N = 3
Lynx	N = 1 528	N = 5 885 1102 1281 1336 1709	N = 5 1975–1980(D) 2058 2059 2099 2120	N = 11
Ocelot	N = 4 15 95 295 658	N = 5 897 913 1113 1283 1728	N = 6 1791 1792 1811 1915 2218 2358	N = 15
Panthera	N = 5 50 123 131 252 305	N = 3 883 1076 1371	N = 2 2128 2241	N = 10
Bay cat	N = 3 125 305 771	N = 4 858–875(D) 1223 1432 1464	N = 1 2179	N = 8
Puma	N = 5 67 252 407 671 732	N = 4 1012 1278 1294 1582	N = 1 2065	N = 10
Caracal	N = 0	N = 1 1303	N = 2 1836 2053	N = 3
TOTAL	20	28	20	68

Note.—I = insertion, D = deletion, N = number of sites (see figure 2 in Supplementary Material online).

from the five species within the domestic cat lineage and in bobcat and its absence in the remaining species.

We performed phylogenetic analyses to determine the evolutionary relationships of the Y-chromosome gene SINE retroelements and to ascertain whether the bobcat SN327@1073 shared a common origin with SN327@1073 in the domestic cat lineage. The results define three monophyletic lineages for SINE sequences (fig. 2) that are clustered by a common insertion within the genome. The first lineage is composed of *ZFY* SN244@2827 inserts
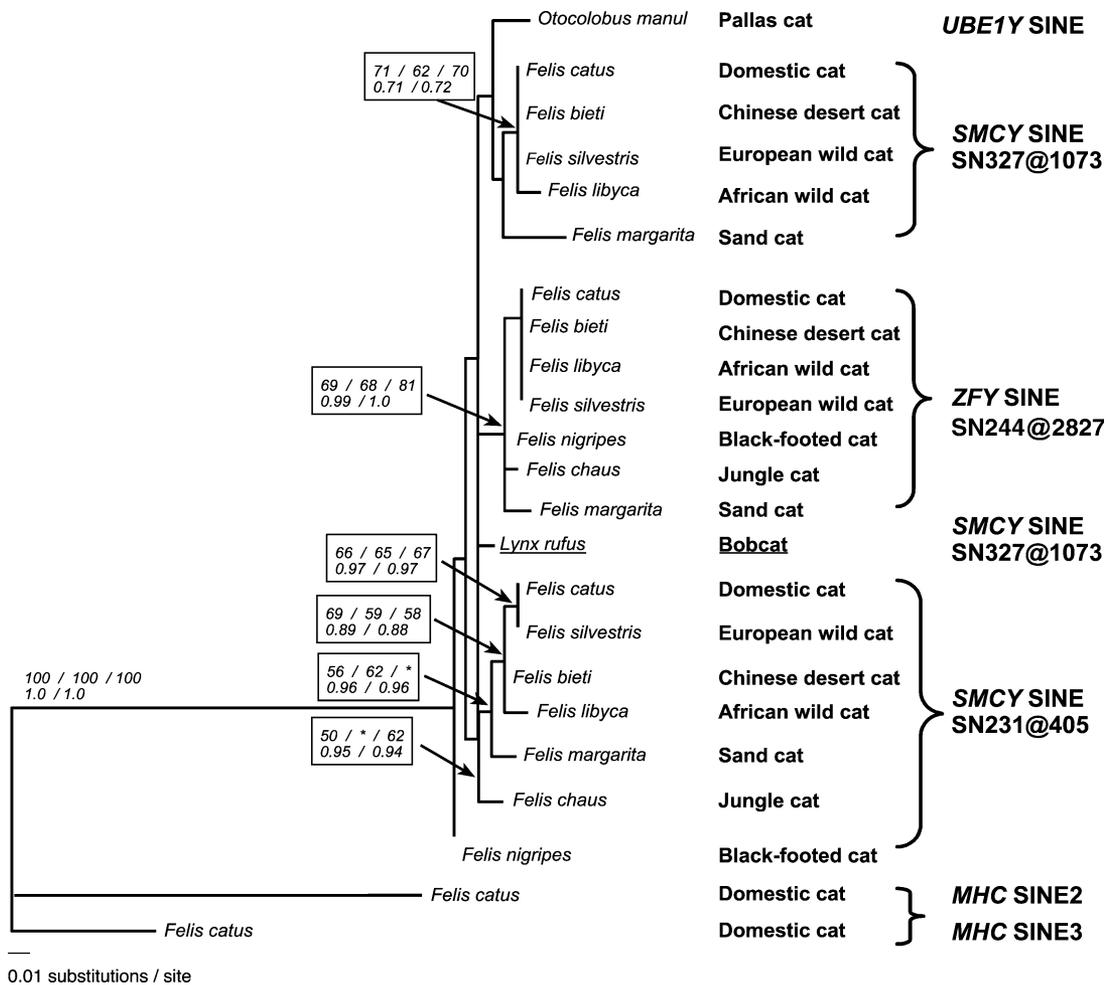
Fig. 2.—Phylogenetic tree of SINE elements isolated from three Y-chromosome introns in Felidae. Alignment and phylogenetic analysis excluded the poly-A/T tails. Trees are rooted using two SINEs isolated from the domestic cat MHC (Yuhki et al. 2003) as outgroups. Shown is the maximum-likelihood (ML) tree (–Ln likelihood = 649.62883; 2,742 rearrangements). Nearly identical topologies were recovered using minimum evolution (ME) (HKY85 + I model) and maximum parsimony (MP). The ME heuristic search constrained branch lengths to be nonnegative and resulting in three identical trees (score = 0.65535; 8,626 rearrangements). The MP analyses resulted in 19 equivalent trees (length = 83 steps; 54,292 rearrangements tried). Numbers in italics reflect bootstrap proportion support for adjacent nodes arranged as ME (1,000 iterations)/MP (1,000 iterations)/ML (100 iterations) and followed by Bayesian posterior probability values (two replicate runs). An asterisk denotes bootstrap proportions less than 50%.

shared by all domestic cat–lineage species, the second includes *SMCY* SN231@405 within the domestic cat lineage, and the third is formed by SN327@1073 from *SMCY* within domestic cat lineage.

In addition, SINE sequences from bobcat *L. rufus* *SMCY* SN327@1073 and pallas cat *UBE1Y* are phylogenetically unaligned within the tree. The lack of affiliation for the SINE in pallas cat *UBE1Y* is not unexpected, given that this was a unique event not observed in any other cat species. However, the ambiguous phylogenetic status of bobcat *SMCY* SN327@1073 within the phylogeny suggests that it is not closely aligned with the domestic cat–lineage *SMCY* SN327@1073, even though they share the same insertion site. We considered alternative hypotheses for the origin of the bobcat *SMCY* SN327@1073 by creating three constraint trees that test the following associations: (1) bobcat *SMCY* SN327@1073 + ZFY SN244@2827; (2) bobcat *SMCY* SN327@1073 + domestic cat–lineage *SMCY* SN231@405; and (3) bobcat *SMCY*

SN327@1073 + domestic cat–lineage *SMCY* SN327@1073. Our results detected no significant differences (AU test $P = 0.179$; Shimodaira-Hasagawa tests $P < 0.394$) among the alternative trees, suggesting that the bobcat *SMCY* SN327@1073 sequence cannot be conclusively assigned to a particular SINE sublineage.

To better examine the possiblity that the bobcat SINE is merely an outcome of ancestral lineage sorting, we tested an alternative Y-chromosome intron tree that unites the lynx lineage and domestic cat lineage as sister taxa against the derived phylogeny (fig. 1). The resultant AU test ($P = 0.163$) could not distinguish a significant difference between the two trees. However, as discussed previously, few substitutions within the Y-chromosome introns support and define internal nodes among lineages. In particular, the presumed sister taxa relationship of Asian leopard cat lineage with domestic cat lineage is supported by two sites (727 and 1849), whereas there are no sites that support the alternative topology uniting lynx lineage and

Table 2
Microsatelite Allele Structure and Frequency Within Poly-A/T Tail of SN327@1073 Among Five Species of Domestic Cat Lineage and in Bobcat *L. rufus*

Species	Lineage	N	Allele Structure	Allele Frequency
Felis catus	Domestic cat	17	$TTTAA_5\ TTTTA_4$	0.82
			$TTTAA_4\ TTTTA_5$	0.18
Felis silvestris	Domestic cat	5	$TTTAA_5\ TTTTA_4$	0.20
			$TTTAA_6\ TTTTA_3$	0.40
			$TTTAA_7\ TTTTA_5$	0.20
			$TTTAA_7\ TTTTA_4$	0.20
Felis margarita	Domestic cat	3	$TTTTA_{14}$	1.0
Felis libyca	Domestic cat	2	$TTTAA_7\ TTTTA_4$	1.0
Felis bieti	Domestic cat	1	$TTTAA_6\ TTTTA_1$	1.0
Lynx rufus	Lynx	28	No repeat	—

domestic cat lineage (figure 2 in Supplementary Material online). Thus, the inability of the AU test to determine differences between the two topologies may reflect an overall paucity of informative sites defining higher-order relationships between lineages in Felidae.

Lastly, in the five domestic cat lineage–species, the poly-A/T tail of *SMCY* SN327@1073 has been expanded into a pentamer microsatellite locus. Two of the species examined, *F. silvestris* and *F. catus*, were polymorphic for this microsatellite locus (table 2). In contrast, the bobcat *SMCY* SN327@1073 poly-T tail consisted of a 38-bp monomer repeat interrupted at six sites by transition substitutions and exhibited no resemblance to the pattern within the five domestic cat–lineage species (figure 1 in Supplementary Material online). In addition, no intraspecific variation within the bobcat *SMCY* SN327@1073 poly-T tail was detected (N = 28) compared with the polymorphism observed with *F. silvestris* and *F. catus*.

Discussion

Sequences from *SMCY, UBE1Y*, and *ZFY* genes located in the NRY are highly informative markers in defining patterns of speciation. The location of *SMCY* and *ZFY* within the X-degenerate regions of the human Y chromosome (*UBE1Y* is absent in humans) may also be true for felids because of observed high homology in gene order and content between human and cat (Murphy et al. 1999). Our findings suggest Y-chromosome intron nucleotide substitutions are highly informative in both species identification and organization within the eight recognized monophyletic felid lineages. In addition, the pattern of Y-chromosome SINE diversification within the well-characterized species phylogeny of Felidae expands and revises our previous findings on the pattern of insertion and sequence diversification of these retroelements.

Phylogenetic Assessment of Y-Chromosome
Intron Sequences

By using the established Felidae phylogeny as a reference phylogeny, phylogenetic analyses suggest evolution within X-degenerate genes in the NRY is tightly linked with speciation. Because of its autosomal location

in marsupials (Sinclair et al. 1988) *ZFY* may have become Y linked at a later time within mammalian evolution than either *UBE1Y* or *SMCY*, as these genes are both conserved across marsupial and placental Y chromosomes (Toder Wakefield, and Graves 2000). In any event, all three NRY genes are thought to have been evolving independently from their X-chromosome homolog after cessation of conventional recombination before the placental mammal radiation approximately 105 MYA (Springer et al. 2003). Consequently, these genes have likely been transmitted paternally on the same DNA molecule since the divergence of placental mammals.

The pattern and tempo of NRY evolution within Felidae, represented by X-degenerate genes, is significant in light of the recent, rapid evolution of the cat family approximately 12 MYA. The most notable feature of the derived NRY phylogeny is the exceptional support of the each of the eight recognized lineages and clear diagnostic substitutions identifying nearly all species. Bootstrap proportions supporting the monophyletic nodes of each lineage are uniformly high. With the exception of the puma and Asian leopard cat lineages, support for each lineage are greater than 96% for ME/MP/ML methods. Further, Bayesian posterior probabilities of 1.0 were obtained in support of each lineage. These eight distinct monophyletic felid lineages are concordant with previously established phylogenetic results derived from multiple mitochondrial and nuclear markers (Collier and O'Brien 1985; Modi and O'Brien 1988; Pecon-Slattery et al. 1994; Janczewski et al. 1995; Masuda et al. 1996; Johnson et al. 1996; Johnson and O'Brien 1997; Johnson et al. 2001). Moreover, a concurrent study (Johnson et al. 2004), based on over 24 kb of genomic sequence from genes located across cat chromosomes, fully supports the previously established results and those of the three Y-chromosome regions presented here.

The exact concordance between the Y-chromosome phylogeny with the expected felid lineage phylogeny is even more notable because of very low incidence of homoplasy (convergent, parallel, or reversal substitutions). For example, the MP consistency index averaged across all sites within the tree was CI = 0.9081 and suggests the overwhelming majority of substitutions to be phylogenetically informative. A comparison of the numbers of lineage-specific, diagnostic sites versus numbers of homoplasies in the MP analysis indicated that the preponderance of substitutions were shared derived sites unique to each lineage (table 1). Thus, of the 141 phylogenetically informative sites, 61 were unambiguous markers of lineage classification within Felidae. The distribution of lineage-specific substitutions was fairly consistent among the three genes (table 1) with values of 2.5% (*SMCY*), 2.9% (*UBE1Y*), and 2.7% (*ZFY*).

The three NRY introns do not completely resolve the relative branching order among the eight felid lineages. Internodes uniting lineages within the phylogeny are short (fig. 1). However, the Y-chromosome intron phylogeny supports the monophyly of the domestic cat lineage + Asian leopard cat lineage and the separation of the panthera and bay cat lineages from other felids (node support >73% and >97%, respectively). The incomplete

resolution of the relative associations between lineages is consistent with a recent, rapid evolutionary burst, and the NRY introns sampled here may be inadequate for recovering deeper patterns of divergence within Felidae.

However, X-degenerate Y-chromosome introns are powerful diagnostic markers in species identification and are moderately successful in recovering the relative branching order of species within each lineage. With the exception of *Felis silvestris* (domestic cat lineage) and *Panthera leo* (Panthera lineage) (fig. 1), all species were defined by multiple, unique, autoapomorphic substitutions with very few homoplasies. Intralineage species associations were less robust, as indicated by collapsed nodes. Trichotomies occur within the panthera lineage (*Panthera leo*, *P. onca*, and *P. pardus*), the ocelot lineage (*Leopardus tigrina*, *Oncifelis guigna*, and *O. geoffroyi*), and the lynx lineage (*Lynx pardina*, *L. lynx*, and *L. canadensis*). Similarly, the relative branching order was indeterminate between *Felis nigripes* and *F. chaus* (domestic cat lineage) and between *Leopardus pardalis* and *L weidii* (ocelot lineage). Likewise, placement of the pallas cat basal within the Asian leopard cat lineage is marginally supported by bootstrap and Bayesian analyses and does not convincingly alter its status as phylogenetically unaligned.

Phylogenetic Insights Based on SINE Insertions Within *SMCY*, *ZFY*, and *UBE1Y*

Inserted within each of the three gene introns are SINE retroposons. The relatively high frequency of SINE insertion is reminiscent of findings within humans that reveal nearly 60% of the X-degenerate region is composed of Alu repeats (Skaletsky et al. 2003). Unlike Alu repeats, but consistent with SINEs from other carnivore taxa (Pecon-Slattery, Murphy, and O'Brien 2000a; Vassetsky and Kramerov 2002), the structure of cat SINEs consists of an internal promoter for *pol III*, a taxon-specific region followed by a dinucleotide repeat of variable length, and a poly-A/T–rich tail. Likely derived from tRNA molecules (Okada 1991; Oshima et al. 1993; Oshima and Okada 1994), the felid SINEs most resemble tRNA-*lys* or tRNA-*arg* (Pecon-Slattery et al. 2000a).

Two of these SINEs, located in Pallas cat *UBE1Y* and in *ZFY* in seven species of the domestic cat lineage were characterized previously (Pecon-Slattery, Murphy, and O'Brien 2000a), and our conclusions remain unchanged by the present analyses that incorporated additional felid taxa. Therefore, the SINE phylogeny (fig. 2) supports the uniqueness of the *UBE1Y* SN237@2323 in Pallas cat and corroborates the conclusion that the *ZFY* SN244@2827 inserted into a common ancestor of the extant species within the domestic cat lineage.

However, our complete sequence of the fourth intron in *SMCY* uncovered results that enable our revision and expansion of our previous study. In *SMCY*, two SINEs had inserted within the fourth intron during felid evolution. The first *SMCY* SINE, located at position 405 (SN231@405) is newly described in this study. Similar to the SINE within *ZFY, SMCY* SN231@405 likely inserted into a common ancestor of all seven species of the domestic cat lineage and serves as a precise phyloge-

netic marker of that lineage. In contrast, the second *SMCY* SINE (SN327@1073) inserted in inverse orientation at position 1073 within the alignment (figure 1 in Supplementary Material online). This SINE, initially described in Pecon-Slattery, Murphy, and O'Brien (2000a), is common to five species known to be closely related within the domestic cat lineage (*F. catus, F. bieti, F. libyca, F. silvestris,* and *F. margarita*). However, *SMCY* SN327@1073 is also found in the exact site within 28 males sampled from the bobcat *Lynx rufus*, but it was not found in any other related lynx species or in any other felid species. These new data alter our previous view that *SMCY* SN327@1073 was found in only two species, *L. rufus* and *F. silvestris* (Pecon-Slattery, Murphy, and O'Brien 2000a). Thus, our initial conclusion that the shared SINE between these two unrelated species represent a parallel insertion, or homoplasy, is strengthened by the additional data presented here.

Phylogenetic analyses of felid SINE sequences from Y-linked genes indicates that the insertion of *SMCY* SN327@1073 into *Lynx rufus SMCY* is a homoplasy. The phylogenetic tree defines three monophyletic lineages (fig. 2). Each lineage is composed of SINEs from the same gene and clustered in accordance with species relationships defined by genomic flanking regions. However, bobcat *SMCY* SN327@1073 is phylogenetically unaligned within the tree. Thus, it is not clear whether the bobcat *SMCY* SINE was derived from a different master copy, or represents a more ancestral version of *SMCY* SN327@1073.

At the very least, the differences between bobcat and domestic cat lineage *SMCY* SN327@1073, combined with information from the genomic flanks, can be used to reject alternative hypotheses of incomplete lineage sorting of an ancestral SINE insertion (Miyamoto 1999; Takahashi et al. 2001) or a consequence of ancestral or ongoing episodes of interspecies hybridization. Incomplete lineage sorting of *SMCY* SN327@1073 would be possible if the domestic cat and lynx lineages were closely related sister taxa. However, in the Y-chromosome intron phylogeny, the closest relatives to domestic cat lineage are pallas cat and the species within the monophyletic Asian leopard cat lineage but not the lynx lineage. It should be noted that topology tests (AU test $P = 0.163$) failed to find significant differences between the Y-chromosome intron tree (fig. 1) and an alternative-user tree that united domestic cat lineage with lynx lineage. However, all other measures of phylogenetic support (bootstrap and Bayesian posterior probability values) of the three Y-linked genes (fig. 1 and table 1) presented here combined with the results of a concurrent study of over 24 kb of genomic and mitochondrial sequence (Johnson et al. 2004) identify the sister taxa of the domestic cat lineage is the Asian leopard cat lineage.

In addition, ancestral or ongoing hybridization between species is not supported by phylogenetic analyses of both genomic and SINE sequences. The consistent placement of bobcat within the lynx lineage by both mitochondrial (maternal lineage) and Y-chromosome (paternal lineage) sequences does not support hybridization. No intraspecific variation in the presence or absence of *SMCY* SN327@1073 is detected, as might be expected

if episodes of hybridization were taking place. Last, the structure of the T-rich tail of *SMCY* SN327@1073 differs between bobcat and the five domestic cat lineage species. The bobcat sequence consists of a mononucleotide poly-T track interrupted stochastically by single adenosine nucleotides. In contrast, the poly-A/T tail of *SMCY* SN327@1073 has been modified postintegration into a complex microsatellite repeat that is polymorphic in two of the five domestic cat species. The difference may reflect the relative time since insertion as research with Alu elements suggest those with long uninterrupted stretches of monomer repeats are likely more recent (Batzer et al. 1990; Ellgren 1993; Arcot et al. 1995). Thus, the bobcat *SMCY* SN327@1073 may be a relatively recent, autoapomorphic insertion that occurred after the divergence of bobcat within the lynx lineage.

Previous research suggests that SINEs are highly informative and excellent cladistic markers of evolution because once inserted, no mechanism is known for subsequent excision (Miyamoto 1999). Although rare, a finding similar to the shared SINE homoplasy between bobcat and domestic cat SN327@1073 was observed in *Peremyscus* evolution (Cantrell et al. 2001). These instances of parallel insertion of SINEs within unrelated species may be possible because of target-site selection within the genome. Evidence suggests that nicks occur in DNA associated with a suite of small hexamer motifs of A/T tracks (Jurka 1997; Tatout, Lavie, and Deragon 1998; Stenger et al. 2001; Jurka and Klonowski 1996) for subsequent SINE integration. Likewise, the flanking regions of *SMCY* SN231@405 and SN327@1073 are composed of similar candidate motifs for target sites (see figure 1 in Supplementary Material online). The most-parsimonious interpretation of the felid *SMCY* data is that the target site may influence the patterns of SINE proliferation and integration. Thus, the optimal phylogenetic usage of SINE insertion patterns should include sequence data from the adjacent genomic flanks and the SINEs themselves.

In conclusion, sequence analysis of X-degenerate introns in the family Felidae reference phylogeny illustrates the utility of the NRY as a patrilineal marker of speciation. The putative ancient association linking X-degenerate single-copy genes of *ZFY, SMCY,* and *UBE1Y,* transmitted to descendent males in an analogous manner to mitochondrial DNA in females, is corroborated by the precise phylogenetic results observed within Felidae. Retroelements such as SINEs, which may be preferentially embedded within the X-degenerate regions of the Y chromosome, are shown to be valuable phylogenetic markers both in pattern of insertion and in sequence. However, SINEs may preferentially employ target-site insertion, resulting in SINE insertion at identical sites unrelated to a shared ancestry. Thus, it remains useful to rely upon multiple markers for phylogenetic reconstruction.

Acknowledgments

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