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Development of Y Chromosome Intraspecific Polymorphic Markers in the Felidae

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Abstract

Y chromosome haplotyping based on microsatellites and single nucleotide polymorphisms (SNPs) has proved to be a powerful tool for population genetic studies of humans. However, the promise of the approach is hampered in the majority of nonhuman mammals by the lack of Y-specific polymorphic markers. We were able to identify new male-specific polymorphisms in the domestic cat *Felis catus* and 6 additional Felidae species with a combination of molecular genetic and cytogenetic approaches including 1) identifying domestic cat male-specific microsatellites from markers generated from a male cat microsatellite-enriched genomic library, a flow-sorted Y cosmid library, or a Y-specific cat bacteria artificial chromosome (BAC) clone, (2) constructing microsatellite-enriched libraries from flow-sorted Y chromosomes isolated directly from focal wildcat species, and (3) screening Y chromosome conserved anchored tagged sequences primers in Felidae species. Forty-one male-specific microsatellites were identified, but only 6 were single-copy loci, consistent with the repetitive nature of the Y chromosome. Nucleotide diversity (π) of Y-linked intron sequences (2.1 kbp) was in the range of 0 (tiger) to 9.95×10^{-4} (marbled cat), and the number of SNPs ranged from none in the tiger to 7 in the Asian leopard cat. The Y haplotyping system described here, consisting of 4 introns (*SMCY3*, *SMCY7*, *UTY11*, and *DBY7*) and 1 polymorphic microsatellite (SMCY-STR), represents the first available markers for tracking intraspecific male lineage polymorphisms in Felidae species and promises to provide significant insights to evolutionary and population genetic studies of the species.

The mammalian Y chromosome consists of a small pseudoautosomal region (PAR) and a large (95% in human) nonrecombining or male-specific region. The analysis of male-specific Y chromosome (MSY) markers along with mitochondrial and autosomal markers provides a means to estimate sex-specific population genetic parameters and to explore questions such as the role of different sexes in breeding and dispersal (Seielstad et al. 1998; Oota et al. 2001; Petit et al. 2002). The abundance of mitochondrial DNA polymorphism has made it one of the most popular markers used in population genetic studies (Avise 2000). By contrast, until recently the use of Y chromosome markers has been restricted almost exclusively to humans.

Although early attempts at describing Y chromosome polymorphism in human populations appeared to indicate a paucity of variation (Dorit et al. 1995), a variety of Y-specific polymorphisms (e.g., short interspersed nuclear element (SINE)/long interspersed nuclear element (LINE) insertions, single nucleotide polymorphisms [SNPs], microsatellites, and minisatellites; Kayser et al. 2004) have been identified and used successfully to estimate male-mediated gene flow, to disentangle the effects of selection and historic demography, and to document sex differences in admixture events (reviewed in Petit et al. 2002; Jobling and Tyler-Smith 2003). In particular, studies have demonstrated the power of the combined haplotyping of slowly (e.g., SNPs) and more rapidly (e.g., microsatellites) evolving polymorphic markers on the Y chromosome to resolve recent population ancestry in humans (de Knijff 2000; Hill et al. 2000).

Surveys of Y chromosome polymorphisms in natural populations of nonhuman mammals have been hampered by the lack of available Y-specific markers for 2 main reasons. First, there is the common assumption that the Y chromosome has a low genetic variability resulting from the effect of selective sweeps and/or a reduced effective population size (Dorit et al. 1995; Hellborg and Ellegren 2004). As in mitochondrial DNA, the nonrecombining Y chromosome has an effective population size one-quarter that of autosomal DNA and therefore is predicted to be sensitive to genetic drift (Petit et al. 2002). Second, there are technical difficulties in finding Y-specific genetic markers due to the high occurrence of gene conversion, degeneration, and repetitive sequences on this chromosome (Rozen et al. 2003; Skaletsky et al. 2003). Nevertheless, efforts are currently underway to complete the chimpanzee (Hughes et al. 2005) and mouse Y chromosome sequences, and Y-specific variation has been reported in several species, including the house mice *Mus musculus* (Boissinot and Boursot 1997), field voles *Microtus agrestis* (Jaarola et al. 1997), macaques monkeys *Macaca* spp. (Tosi et al. 2003), horses *Equus* spp. (Wallner et al. 2004), bovids *Bos* spp. (Hanotte et al. 2000), Scandinavian wolves *Canis lupus* (Sundqvist et al. 2001; Vila et al. 2003), common shrews *Sorex araneus* (Lugon-Moulin and Hausser 2002), greater white-toothed shrew *Crocidura russula* (Handley, Berset-Brandli, and Perrin 2006), North American deer *Odocoileus* spp. (Cathey et al. 1998), and hamadryas baboons *Papio hamadryas* (Handley, Hammond, et al. 2006). A limitation of these studies was that most of the Y markers were used to address species-level introgression and interspecies hybridization issues but were not polymorphic enough to assess variation among populations within a single species.

In the domestic cat *Felis catus*, 8 coding loci and 2 microsatellites have been mapped on the Y chromosome (Murphy et al. 1999; Menotti-Raymond, David, Agarwala, et al. 2003; Menotti-Raymond, David, Chen, et al. 2003). In addition, sequences from *SMCY*, *SRY*, *UBE1Y*, *ZFY* genes and SINEs within the Y chromosome have been used to elucidate the Felidae phylogeny and have provided a valuable patrilineal perspective in species evolution in the cat family (Pecon-Slattery and O'Brien 1998; Pecon-Slattery, Murphy, and O'Brien 2000; Pecon-Slattery, Sanner-Wachter, and O'Brien 2000, Pecon-Slattery et al. 2004). However, a survey of 3.5 kbp of Y-linked gene introns in the Eurasian lynx *Lynx lynx* did not reveal any polymorphism (Hellborg and Ellegren 2004), and no other study is currently available that has described Y chromosome polymorphism in any of the other 37 felid species.

With recent advances in cytogenetic technologies and comparative genomics, several techniques have been developed that facilitate the discovery of Y-specific polymorphism. These strategies include database mining and cross-amplification of Y chromosome sequences previously known in a related species or designed from conserved regions across species (Hellborg and Ellegren 2003; Erler et al. 2004), isolation of SNPs and/or microsatellites from partial genomic libraries constructed from Y chromosomes isolated directly from the focal species using flow sorting (Bergstrom et al. 1998) or microdissection (Shibata et al. 1999), and subtractive hybridization to enrich Y chromosome fragments (Bergstrom et al. 1997; Wallner et al. 2004).

In this study, we used 5 methods (Figure 1, Methods A to E) to identify Y chromosome intraspecific polymorphic markers in 6 Felidae species. We screened microsatellites isolated from a male domestic cat partial genomic library (Method A), a cosmid library constructed from domestic cat flow-sorted Y chromosomes (Method B), and a BAC clone that was mapped to the domestic cat Y chromosome (Method C) for markers that are male specific and examined cross-species applicability. We also constructed microsatellite-enriched libraries from flow-sorted Y chromosomes of the Asian leopard cat *Prionailurus bengalensis* and tiger *Panthera tigris*, respectively, and attempted to directly isolate species-specific Y microsatellites (Method D). Finally, we applied Y chromosome conserved anchored tagged sequences (YCATS) primers or exonic primers flanking different intronic regions of single-copy Y-linked genes (Lyons et al. 1997; Hellborg and Ellegren 2003) to 6 wildcat species that represented 3 of the 8 evolutionary divergent lineages in modern felids (tiger, leopard *Panthera pardus*, Asian leopard cat, fishing cat *Prionailurus viverrinus*, Asian golden cat *Pardofelis temmincki*, and marbled cat *Pardofelis marmorata*; see Johnson et al. 2006) and compared their specificity and polymorphism in different species (Method E). The final Y haplotyping system, consisting of 4 intronic DNA fragments (*SMCY3*, *SMCY7*, *UTY11*, and *DBY7*) and 1 microsatellite (SMCY-STR), represents the first available markers for detecting the male lineage polymorphism at the intraspecific level in Felidae and will provide significant insights to evolutionary and population genetic studies of the species.

Materials and Methods

Screening of Y Microsatellite Markers from the Domestic Cat *F. catus* (Method A, B, C)

Three hundred and eighty dinucleotide (CA) repeat microsatellites were isolated from genomic DNA of a male domestic cat as previously described (Sarno et al. 2000) from construction of a microsatellite-enriched library using capture hybridization techniques (Figure 1, Method A). Most of the microsatellites were isolated to be integrated onto the genetic linkage maps of the domestic cat (Menotti-Raymond et al. 1999, Menotti-Raymond, David, Agarwala, et al. 2003; Menotti-Raymond, David, Chen, et al. 2003; Pontius JU, Mullikin JC, Smith D, Lindblad-Toh K, Gnerre S, Clamp M, Chang J, Stephens R, Neelam B, Volfovsky N, Schäffer AA, Agarwala R, Narfström K, Murphy WJ, Giger U, Roca AL, Antunes A, Menotti-Raymond M, Yuhki N, Pecon-Slattery J, Johnson WE, Bourque G, Tesler G, and O'Brien SJ in preparation). Y chromosome specificity of candidate markers was tested by comparing the polymerase chain reaction (PCR) amplification patterns between males and females using a high-throughput fluorogenic 5′ nuclease assay as described previously (Green 1997; Jouquand et al. 2000). Each marker

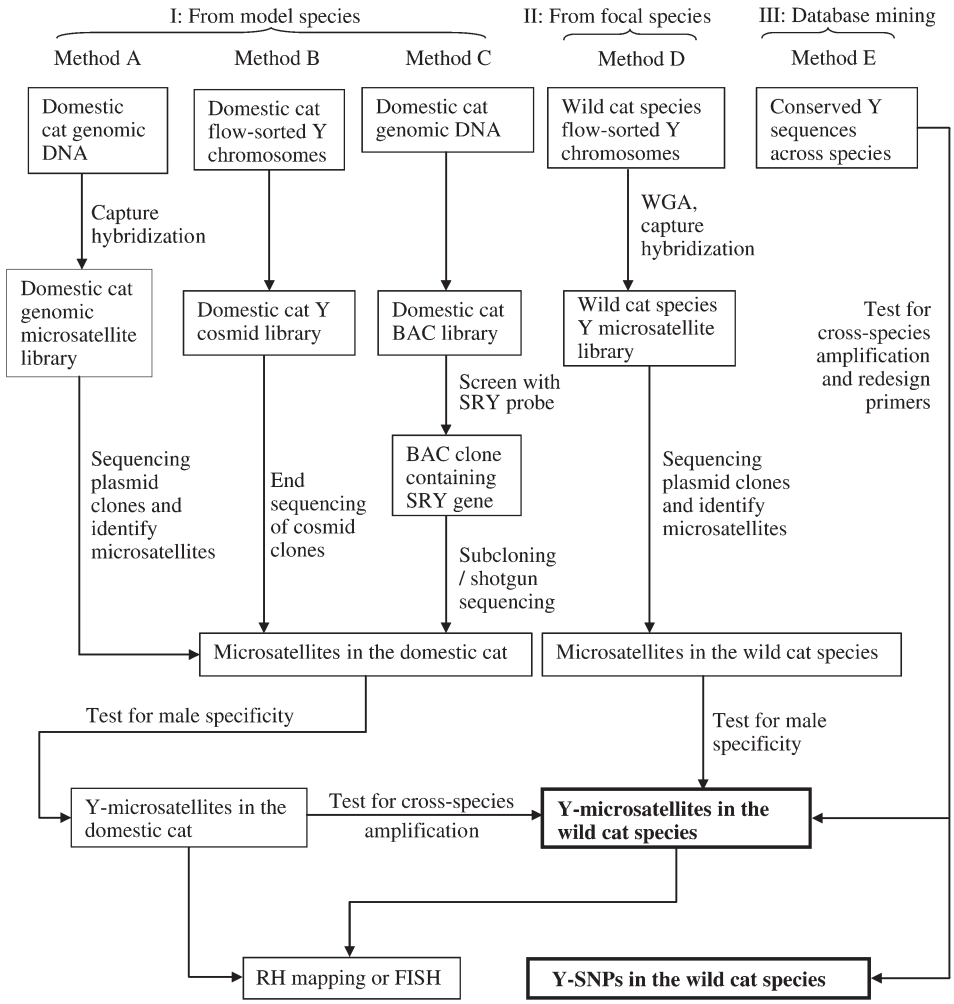

Figure 1. Strategies used in this study for developing Y-specific polymorphism in the Felidae. I: Screening of Y microsatellite markers from the domestic cat *Felis catus*. II: Screening of microsatellites from species-specific flow-sorted Y chromosomes. III: Screening of SNPs and microsatellites from single-copy Y-linked gene introns (YCATS; Hellborg and Ellegren 2004).

was tested in 2 male and female domestic cats and 1 male and female tigers. PCR amplifications were performed as described by Van Etten et al. (1999), and fluorescence was detected on an ABI PRISM™ 7700 Sequence Detection System (Applied Biosystems, Foster City, CA).

Additional Y microsatellite candidates were identified from partial sequencing of a flow-sorted domestic cat Y cosmid library (Figure 1, Method B). The library was constructed as previously described (Longmire et al. 1993) and contained 3648 cosmid clones, representing a 4.3-fold sequence coverage of the cat Y chromosome. Colonies were picked after an overnight incubation at 37 °C in 2× YT broth with 50 μg/ml ampicillin. Cosmid DNA was extracted using Qiagen REAL Prep 96-well Kit (Qiagen, Valencia, CA). Each colony was sequenced with both T3 and T7 primers using BigDye terminator kit (Applied Biosystems) and run on an ABI 3730 sequencing apparatus. Microsatellite-containing sequences were identified with Repeat Masker at http://www.repeatmasker.org, and primers were designed from clones containing di-, tri- and tetranucleotide repeat microsatellite sequences using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

To examine the relative abundance of tri- and tetranucleotide microsatellites on the cat Y chromosome, the domestic cat Y cosmid library was screened for repeat-containing clones with 6 ^{32}P-labeled oligonucleotide probes ($[GATA]_{10}$, $[GAAA]_{10}$, $[GGAA]_{10}$, $[GTT]_{10}$, $[AGA]_{10}$, and $[ACT]_{10}$). The library was arrayed and plated onto 10 nitrocellulose filters and screened with techniques described previously (Ostrander et al. 1992). Hybridization temperatures with ExpressHyb solution (Clontech, Mountain View, CA) were adjusted according to the GC content in different oligonucleotide probes (55 °C for $[GATA]_{10}$ and $[GAAA]_{10}$, 52 °C for $[GTT]_{10}$, $[AGA]_{10}$, and $[ACT]_{10}$, and 65 °C for $[GGAA]_{10}$).

A domestic cat BAC library (Beck et al. 2001) was screened using a probe derived from a Y-linked *SRY* gene (Figure 1, Method C). BAC clone 278g21 was identified, and

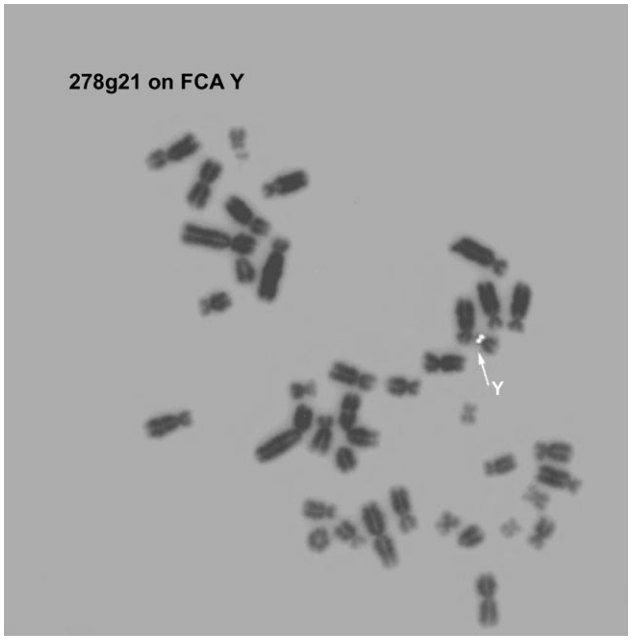

Figure 2. FISH of the BAC clone 278g21 DNA on the metaphase chromosomes of the domestic cat *Felis catus*. The result shows that 278g21 was localized on the short arm of the *F. catus* Y chromosome, and no obvious contamination from other chromosomes.

isolated DNA was mapped by fluorescent in situ hybridization (FISH) to the short arm of the Y chromosome (Figure 2). The BAC clone was subsequently sequenced with a shotgun library approach as described by Yuhki et al. (2003). Microsatellite markers were identified from the sequences obtained and tested.

Screening of Microsatellites from Species-Specific Flow-Sorted Y Chromosomes (Method D)

Metaphase chromosomes from fibroblast cell cultures of the tiger (*P. tigris*), leopard (*P. pardus*), and Asian leopard cat (*P. bengalensis*) were prepared after standard cytogenetic procedures and chromosome isolation techniques for flow sorting (Yang et al. 1995). Approximately 200 000 Y chromosomes (Figure 1, Method D) from each species (20–30 ng, 300 chromosomes/μl of water) were sorted using a dual-laser cell sorter (FACS DiVa; Becton-Dickinson, Palo Alto, CA). To verify the chromosome purity of the flow-sorted materials, aliquots of 2 μl were amplified and labeled by degenerate oligonucleotide primed PCR (Yang et al. 1995). FISH was performed on the metaphase chromosomes of each species after standard methods (Stanyon et al. 1999).

Whole genome amplification (WGA) reactions were performed with the GenomiPhi DNA amplification system (Amersham Biosciences, Little Chalfont, UK) using 10–15 ng of the flow-sorted DNA. Microsatellite-enriched partial genomic libraries were produced with 15 μg WGA, Y chromosome DNA according to a modified protocol for nonradioactive microsatellite detection based on capture hybridization (Refseth et al. 1997) as previously described (Sarno et al. 2000). Plasmid DNA was purified and sequenced using both forward and reverse primers, and primers designed from clones containing di- and tetranucleotide repeat microsatellite sequences as described above.

YCATS and Microsatellite Markers from Single-Copy Y-Linked Gene Introns (Method E)

We tested the specificity and polymorphism of 8 YCATS (Figure 1, Method E), reported previously from the Eurasian lynx *L. lynx* (Hellborg and Ellegren 2003, *DBY4*, *DBY7*, *DBY8*, *DBY12*, *SMCY3*, *SMCY7*, *SMCY17*, and *UTY11*) in 6 Felidae species (tiger *P. tigris*, leopard *P. pardus*, Asian leopard cat *P. bengalensis*, fishing cat *P. viverrinus*, Asian golden cat *P. temmincki*, and marbled cat *P. marmorata*). Four markers, *DBY7*, *SMCY3*, *SMCY7*, and *UTY11*, which could be amplified across all the target species, were selected for large-scale examination. One CA repeat microsatellite locus was detected from the seventh intron of the *SMCY* gene, and SMCY7-STR primers were designed for specifically amplifying the locus about 230–290 bp in size (Table 1). A total of 83 Asian leopard cats, 24 fishing cats, 29 Asian golden cats, 8 marbled cats, 55 tigers, and 75 leopards were screened for intraspecies polymorphism of the YCATS and SMCY7-STR.

Screening of Y Chromosome SNP and Microsatellite Polymorphism in the Felidae

All male-specific microsatellite candidates (Table 1) were PCR amplified in a 10-μl system that contained 20 ng DNA, 2.0 mM $MgCl_2$, 0.4 units AmpliTaq Gold DNA polymerase (Applied Biosystems), 1× PCR buffer II containing 10 mM Tris–HCl (pH 8.3) and 50 mM KCl, 250 μM of each of the 4 dNTPs, 4 pmole reverse primer, 0.26 pmole M13-tailed forward prime, and 4 pmole fluorescently labeled M13 primer (5′-CACGACGTTGTAAAACGAC-3′; see Boutin-Ganache et al. 2001). The reaction was performed in a Perkin Elmer 9700 thermal cycler and analyzed as previously described (Ishida et al. 2006).

Y chromosome intron nuclear DNA sequences were amplified following a modified protocol from Hellborg and Ellegren (2003). For *DBY7* and *SMCY7*, PCR was carried out in a 15-μl system that contained 40 ng DNA, 2.0 mM $MgCl_2$, 0.75 units AmpliTaq Gold DNA polymerase (Applied Biosystems), 1× PCR buffer II containing 10 mM Tris–HCl (pH 8.3) and 50 mM KCl, 1.0 mM dNTPs, and 3 μM forward and reversed primers. For *SMCY3* and *UTY11*, the conditions were the same except for 2.5 mM $MgCl_2$. The amplification protocol was denaturation 10 min at 95 °C, a touchdown procedure of 95 °C for 30 s, 60–50 °C for 1 min, 72 °C for 1 h 30 min, with 2 cycles at each annealing temperature, then 35 amplification cycles of 95 °C for 30 s, 50 °C for 1 min, and 72 °C for 1 h 30 min, followed by an extension of 10 min at 72 °C. Sequences were inspected using SEQUENCHER 4.6 (Gene Codes, Ann Arbor, MI) and aligned using ClustalX 1.83. Measures of

Table 1. Forty-one male-specific microsatellites and 4 Y chromosome intron DNA markers identified in the Felidae

				Cross-species utility[b]		
Locus ID	Type	Primer sequences	Method[a]	FCA	PBE	PTI
19.008-2	(CA)n	F:TGTGCACATGGCAACACCTGC R:TGGCAGCCTTGAAGAGTTG	A	Multicopy	Multicopy	Multicopy
16.08-2	(CA)n	F:CCAATATAGTTGAAGCTGAAGTGC R:GCCTGCTGCCTCAGTAAGTA	A	Multicopy	Did not amplify	Duplication
17.094	(CA)n	F:GCCCACAATACACACCAGC R:GTACCAGGACTTTCCACGGA	A	Multicopy	Did not amplify	Multicopy
31.019	(CA)n	F:ACTTGCATCCTGGGACTGAC R:GTGGACTGGGAGCTGTGG	A	Multicopy	No data	Did not amplify
18.20	(CA)n	F:CACCTGTCCCACACCAGTC R:CGCGATGTTTATGTGGACAC	A	Multicopy	No data	Did not amplify
29.017	(CA)n	F:TCGAGCAGTGTCAAGTGGTC R:TCTCACACCGCTAGCATGTC	A	Multicopy	No data	Amplified in both sex
8H7_T7-4	(ATT)19	F:TGGTACTTACCTGTTGTCTATGTGG R:CCCTGACCTCCTCACTTCAG	B	Multicopy	Duplication	Did not amplify
17G9_T3-4	$(TTG)_9$	F:CGGGGAATTCTTCGTTTCAT R:TCCAAAACACACCTCCTGAA	B	Multicopy	Did not amplify	Multicopy
16G4_T3-4	$(TAA)_{22}$	F:GGTATTCTCTGTCCTGTCCTGTTT R:GGTGTATGATCATCGGCACA	B	Multicopy	Multicopy	Did not amplify
19C8_T7-4	$(CT)_{40}(ACAT)_{15}$	F:CTCTCTCTCTGCCCCTACCC R:TGGGTACCCTGTGGAGTAGC	B	Multicopy	Multicopy	Multicopy
19F8_T3-4	$(ATT)_{19}$	F:GGCCAATGGGTTCAAGTAAA R:CCAGTCAGAAATACTTTCAAATAAGG	B	Multicopy	Did not amplify	Did not amplify
1E6_T7-4	$(AAAT)_{15}$	F:TGGAGCCTGTTTTGGATTCT R:GGTCAAAGGGGGTTCTTCTC	B	Multicopy	Did not amplify	Multicopy
1H9_T7-4	$(ATT)_{19}$	F:TCCTTGTTTGGTACTTACCTGTTG R:CCAAATAGACAAGCCCCTGA	B	Multicopy	Did not amplify	Did not amplify
20C1_T3-4	$(TAT)_{20}$	F:CAGTATACAGGGGCCAAAGG R:CCAGTCAGAAATACTTACAACTAAGC	B	Multicopy	Did not amplify	Did not amplify
37F10_T3-4	$(AAAT)_{19}$	F:CCCGTTCAGGATCTGTCTCT R:GGTCAAAGGGGGTTCTTCTC	B	Multicopy	Did not amplify	Did not amplify
32G1_T3-4	$(ATT)_{14}$	F:AGGATCCCAATCCGTGAGAT R:TGATGATACTTTTTAAGCCAGTCA	B	Multicopy	Did not amplify	Not amplified
33B10_T3-4	$(TAT)_{20}$	F:TGAAACATAGCAAACACCCCTTA R:CAGGAAATTCATTTGTTTGCAT	B	Multicopy	No data	No data
33B12_T3-4	$(ACAA)_9$	F:CACATAGGAGAGACCCACAGC R:TGCTGCAGCTAAGACTTCCA	B	Duplication	Duplication	Did not amplify
4F4_T3-4	$(TCC)_9$	F:GCACCCGAACATTCACTCTT R:GTTGAGGAGGACGAGAGCAG	B	Multicopy	No data	No data
6C3_T3-4	$(ACA)_{12}$	F:GAAAACAAAACAACGACAACG R:TGCTCCGCCTATTACCACTT	B	Multicopy	Did not amplify	Did not amplify
6H7_T3-4	$(TTG)_9$	F:TGCTGTCCTGCTTCTAGTTCC R:GAAAACCCTTCAGGATGCAA	B	Triplication	Did not amplify	Did not amplify
7A12_T7-4	$(AAAC)_{10}$	F:TCCCGTTCAGGATCTGTCTC R:GGTCAAAGGGGGTTCTTCTC	B	Multicopy	Multicopy	Multicopy
8B11_T7-4	$(TAAA)_{13}$	F:GAGTGTCGCGTCCACATTTA R:TTCAACAGATAAAAATTCTGTTTCTCA	B	Multicopy	No data	No data
9F10_T7-4	$(ACA)_{11}$	F:TCTTTTTACTGACTTTACAAAATGACG R:GGGATCCCAGAAGGAAAAGA	B	Multicopy	Single-copy monomorphic	Single-copy monomorphic
2A11_T7	$(GT)_{16}$	F:GGGAGCTGAGATGGGTTACA R:GTTCCAGGCCCACAATACAC	B	Multicopy	Multicopy	Multicopy
2E6_T3-4	$(AC)_{18}$	F:ATGTCAGGGCTGTGCTATCC R:GAAGCCTGCTTTGGATTCTG	B	Multicopy	Multicopy	Multicopy
2F1_T3-4	$(GA)_8$	F:GGTGTGACTTAGGGGTCTGC R:TCAGAGCCTGTAGCCTGCTT	B	Single-copy monomorphic	Single-copy monomorphic	Single-copy monomorphic
17F12_T3-4	$(AG)_{18}$	F:ATGCTGGGTTATGCTTCCTG R:AGGGGAAAATTCTGGAGCAG	B	Multicopy	Multicopy	Multicopy
4F6_T7-4	$(AC)_{30}$	F:TCCCTCCCCTCTCACTCTCT R:TGACACAGGCTGTTTTCTTCC	B	Multicopy	Did not amplify	Did not amplify
4G10_T7-4	$(AC)_{27}$	F:GGAGGGGCAGAGAGACAGA R:GCTCAGGGGTGTAAGCATCT	B	Multicopy	Multicopy	Did not amplify

Table 1. Continued

Locus ID	Type	Primer sequences	Method[a]	Cross-species utility[b] FCA	PBE	PTI
278g21-1	$(GA)_{40}$	F:CAGTGATGAAGGTGAGAGCAG R:GCTTGATCCGAGCTTTATGC	C	Multicopy	No data	No data
278g21-2	(GAAA)n	F:CAGATATTCGCCCAACTTGA R:TTCATTTTTGTCTGCCAACTG	C	Duplication	Did not amplify	Did not amplify
278g21-3	$(GA)_{18}$	F:TAGAATGGGTCCCACATTGA R:GACGTCAGTCCGACACAACT	C	Triplication	Did not amplify	Did not amplify
278g21-4	$(CAAA)_{11}$	F:ATAGATTGCACAGGGCACAG R:CTGCATGTGACGAAGGAATC	C	Triplication	Single-copy 3 alleles	Single-copy monomorphic
278g21-5	$(AC)_{16}$	F:CATTTCCCAAACCTTGGAGT R:TGCAGACAAAAGGGAGACAG	C	Multicopy	Did not amplify	Quadruplication
278g21-6	$(GT)_{17}$	F:ATTGCTAGGGTGAAGGGAAA R:TCCCATTTTTAAGTATGCAGTTG	C	Multicopy	Did not amplify	Not amplified
278g21-7	$(CGTG)_6(TG)_8$	F:AGAGGCTCCCGCATAACA R:CTATAAAGGCAGGGGCTTCA	C	Single-copy monomorphic	Did not amplify	Quadruplication
278g21-8	$(TCCAC)_8$	F:AGAGCAGGAGGGAGAAAACA R:GCCCTGCATTTAACCCTTTA	C	Triplication	Did not amplify	Did not amplify
278g21-9	$(GA)_{13}$	F:CTGGGTGTTATGGAACAACG R:TTCTACATGAAGGCCAGACG	C	Multicopy	Did not amplify	Did not amplify
278g21-10	$(CAT)_{13}$	F:ACTCCTTGGAAATGCGAGTT R:TACTGTTGGCGGAATGAGAC	C	Single-copy monomorphic	Did not amplify	Did not amplify
SMCY7-STR	(CA)n	F:TCTCACGGTTGGTGAGTTTG R:GTCTGTCCAACCACTGTTGC	E	Single-copy	Single-copy 10 alleles	Single-copy 2 alleles
SMCY3	Y intron	F:ATTTACCCTTATGAAATRTTT R:TCAAATGGGTGWGTGTYACAT	E	Single-copy	Single-copy	Single-copy
SMCY7	Y intron	F:TGGAGGTGCCCRAARTGTA R:AACTCTGCAAASTRTACTCCT	E	Single-copy	Single-copy	Single-copy
DBY7	Y intron	F:GGTCCAGGAGARGCTTTGAA R:CAGCCAATTCTCTTGTTGGG	E	Single-copy	Single-copy	Single-copy
UTY11	Y intron	F:CATCAATTTTGTAYMAATCCAAAA R:TGGTAGAGAAAAGTCCAAGA	E	Single-copy	Single-copy	Single-copy

FCA, *Felis catus* domestic cat; PBE, *Prionailurus bengalensis* Asian leopard cat; PTI, *Panthera tigris* tiger.

[a] Methods correspond to Figure 1. A: Y-loci identified from domestic cat microsatellite library from male cat genomic DNA. B: Y-loci identified from domestic cat Y chromosome cosmid library. C: Y-loci identified from domestic cat BAC Clone on Y chromosome. E: Y chromosome introns from conserved anchored tagged sequences across mammal species (Hellborg and Ellegren 2003).

[b] Single-copy microsatellite loci identified in the species were underlined.

genetic diversity parameters were estimated in ARLEQUIN 3.01 (Excoffier et al. 2005).

Results

Novel Y Microsatellites Identified from the Domestic Cat and Other Felidae Species

The fluorogenic 5′ nuclease assay for rapid detection of positive PCR amplification was shown to be sensitive, accurate, and highly reproducible (Figure 3). Of the 380 dinucleotide (CA) repeat microsatellite markers randomly isolated from male domestic cat genomic DNA and tested in the domestic cat and tiger of both sexes (Method A), 10 failed to amplify in both species and 284 (76%) could be amplified in the tiger. Seven loci (7.29, 19.008-2, 16.08-2, 17.094, 31.019, 18.20, and 29.017) were confirmed to be male specific in the domestic cat. Because loci 7.29 and 19.008-2 were found to contain the same flanking sequences, the total number of domestic cat male-specific microsatellite markers decreased to 6 (Tables 1 and 2), accounting for about 1.6% of the genomic microsatellite loci screened. Only 3 of the 6 markers could be amplified and were male specific in tigers.

Because subcloning and shotgun sequencing of multiple cosmid clones to obtain microsatellite markers as described in White et al. (1999) is time consuming and labor intensive, we used a direct sequencing strategy to rapidly acquire a large amount of sequences from a domestic cat flow-sorted Y chromosome cosmid library (Method B). A total of 1.8 Mb sequences (3073 segments) were obtained by sequencing with T3 and/or T7 primers from the cosmid vector into the end portions of the inserts. We screened these sequences for microsatellites that had a repeat unit size of $\geq$2 and a repeat count of $\geq$7. The 464 identified microsatellites were clustered into groups by sequence similarity (threshold 90%), as the library contained 4.3-fold of the cat Y chromosome sequences and it was likely that the same segment was sequenced more than once, or existed with multiple copies. For each group, a random sequence was selected and 34 primers

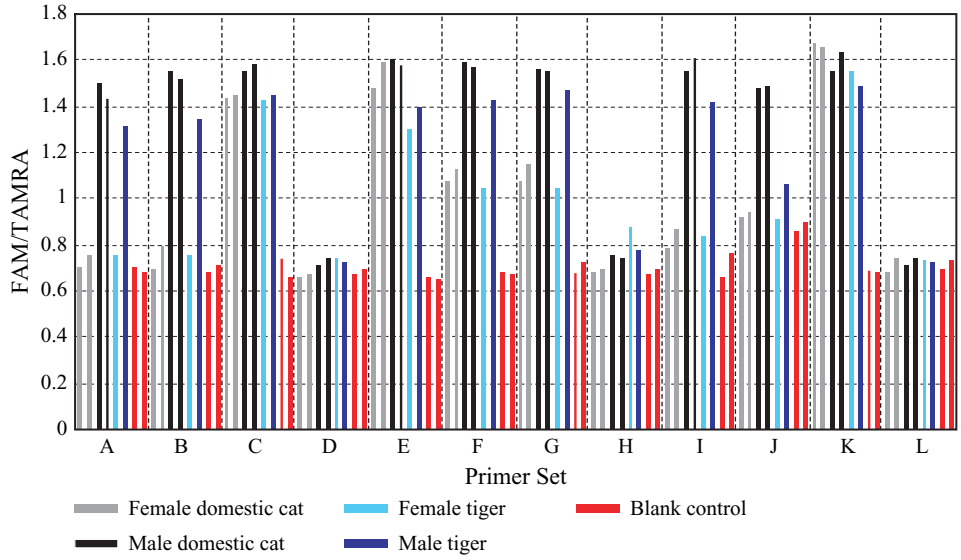

Figure 3. A panel of 12 markers tested with fluorogenic 5′ nuclease assay for rapid screening of male-specific microsatellites isolated from a male domestic cat microsatellite-enriched library. Values on the *x* axis indicate the well number in a 96-well polypropylene PCR plate and *y* axis the ratio of the reporter dye (FAM) to the quenching dye (TAMRA) for each PCR product. This panel includes 12 loci, with 1 locus every 8 wells, following the order of 2 female domestic cats (gray), 2 male domestic cats (black), 1 female tiger (light blue), 1 male tiger (dark blue), and 2 PCR negative controls (red). Loci tested in this panel are A-7.29, B-7.29 (duplicate), C-17.037, D-17.050, E-16.15, F-17.094, G-17.094 (duplicate), H-19.019, I-19.008-2, J-31.019, K-31.017, and L-17.050-2. Loci D, H, and L did not amplify, C, E, and K were X-linked or autosomal, A, B, F, G, and I were potential Y-linked microsatellite candidates in both the domestic cat and tiger, and J was Y-linked in the domestic cat only and did not amplify in the tiger.

were designed. Two failed in amplification, 8 were not sex specific, and 24 generated positive male-specific PCR products, including 6 di-, 12 tri-, 5 tetranucleotide repeat, and 1 complex microsatellite markers (Table 1).

Hybridization screening of the Y cosmid library with 6 tri- or tetranucleotide repeat oligonucleotides identified different numbers of positive clones (Table 3), indicating a wide range of microsatellite frequency on the cat Y chromosome. We identified 369 cosmids containing $(GATA)_{10}$ repeats, based on which we estimated that there are approximately 68 GATA microsatellite loci present on the domestic cat Y chromosome. This frequency is lower than that found on the human Y chromosome, whose euchromatic region only contains almost 100 GATA microsatellites (Table 3). GAAA and AGA repeats have similar abundance as GATA loci, whereas the GTT repeat microsatellites seem to be much more frequent on the cat Y chromosome than on human.

The domestic cat BAC clone 278g21 contains the *SRY* (sex-determining region Y) gene, which is telomeric in human near the PAR and has been located to the short arm of Y chromosome in cat. Shotgun sequencing of the BAC clone (Method C) resulted in 39 730 bp of sequences, which included 18 microsatellite loci with repeat units of 2–6

Table 2. Summary of Y microsatellites obtained using different screening methods

Method	Source	Number loci or sequences tested	Repeat type	Number of male-specific loci	No. of multicopy loci	No. of single-copy loci
A	FCA (male) genomic microsatellites	380	Dinucleotide (CA)	6	6	0
B	FCA Y cosmid library	1.8 Mb, 34 loci	Di-, tri-, tetranucleotide	24	22	2
C	FCA Y BAC clone (*SRY*)	40 kbp, 18 loci	Di-, tetranucleotide	10	7	3
D	PTI/PBE flow-sorted Y chromosomes	14 loci PTI, 29 loci PBE	Tetranucleotide (GATA)	0	0	0
E	Y single-copy gene intron sequences	2.2 kbp, 1 locus	Dinucleotide (CA)	1	0	1
Total				41	35	6

FCA, *Felis catus* domestic cat; PBE, *Prionailurus bengalensis* Asian leopard cat; PTI, *Panthera tigris* tiger.

Table 3. Estimated abundance of tri- and tetranucleotide microsatellites on the cat Y chromosome based on hybridization screening of the domestic cat Y cosmid library

Marker	Hybridization temperature in 3× standard saline citrate (SSC) buffer (°C)	Number of positive clones	Estimated abundance on cat Y chromosome[a]	Abundance on human Y chromosome euchromatin[b]
$(GATA)_{10}$	55	369	68	95
$(GAAA)_{10}$	55	288	53	139
$(GGAA)_{10}$	65	208	38	56
$(AGA)_{10}$	52	357	66	21
$(GTT)_{10}$	52	>900	>166	18
$(ACT)_{10}$	52	0	Extremely low	3

[a] Estimated microsatellite abundance = number of positive clones × 85%/4.6, assuming each positive clone contains one microsatellite locus. The formula reflects that the cosmid library contains 4.6-fold sequence coverage of the cat Y chromosome and 85% Y chromosome purity.

[b] Human Y chromosome (~60 Mb) = 24 Mb euchromatin + 30 Mb heterochromatin + 3 Mb pseudoautosome. Microsatellite abundance listed here is only based on the 24-Mb published euchromatic sequences.

nucleotides. Ten primers were designed and confirmed to be male specific (Table 1 and 2).

Our approach of combining flow sorting of the whole Y chromosomes, WGA, and subsequent construction of microsatellite-enriched libraries from non–domestic cat felid species (Method D) represents the first attempt of using such techniques in directly isolating Y microsatellites from a target species without prior genome sequencing or genetic mapping information. We sorted 108 000 Y chromosomes from the leopard *P. pardus*, 279 000 from the tiger *P. tigris*, and 201 000 from the Asian leopard cat *P. bengalensis*. We used the *P. bengalensis* and *P. tigris* flow-sorted Y chromosomes as templates for WGA, producing microgram quantities of Y chromosome DNA. The flow-sorted materials were mapped by FISH to the euchromatic region on the Y chromosome in both species (Figure 4), confirming their chromosomal purity. Two plasmid libraries enriched with tetranucleotide (GATA) repeat microsatellites were constructed, respectively, with the WGA Y chromosomes from the 2 species. After sequencing 384 colonies in each library, 14 microsatellites were identified in *P. tigris* and 29 in *P. bengalensis*. However, when tested in both male and female individuals, none of the markers was male specific, indicating that the GATA repeat microsatellites were perhaps derived from the PAR or single-copy X–Y homologues within the nonrecombining regions on the Y chromosome.

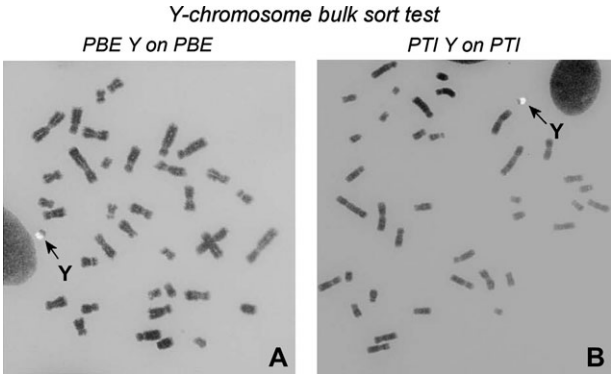

Figure 4. FISH of the flow-sorted Y chromosomes on the metaphase chromosomes of the Asian leopard cat (PBE) and tiger (PTI). Results show that the flow-sorted materials originated from the Y chromosome in both species with no obvious contamination from other chromosomes.

Amplification Patterns of Y Microsatellites in the Felidae

Forty-one Y microsatellite candidates identified from various sources as described above (summarize in Table 2) were tested for polymorphism and cross-species utility in 10 males and 2 females in 3 cat species (Table 1): the domestic cat (FCA), Asian leopard cat (PBE), and tiger (PTI). In the domestic cat, all microsatellite markers consistently generated male-specific amplification and were negative for females. With the exception of 4 loci (2F1_T3-4, 278g21-7, 278g21-10, and SMCY7-STR), the other 37 or 90% of the candidate markers were repetitive in nature, with 2 (duplication), 3 (triplication), 4 (quadruplication), or more than 4 (multiple-copy) alleles present in a single male individual (Figure 5). Most of the observable multiple alleles were one repeat unit apart, probably because single-step mutations are most common for microsatellite markers. The single-copy microsatellite loci 2F1_T3-4, 278g21-7, and 278g21-10 were monomorphic across the 10 domestic cats tested. Among the 10 microsatellite loci isolated from a BAC clone (insert size ≥150 kbp) containing the *SRY* gene, there were 2 single-copy, 1 duplicated, 3 triplicated, and 4 (40%) multiple-copy loci in the domestic cat. The number of copies of repetitive elements in the region near a single-copy gene such as *SRY* is apparently much less than that of random sequences from the Y chromosome cosmid library, from which 88% (21 of 24) of the microsatellite loci identified have more than 4 copies.

Of the 14 male-specific microsatellite loci identified in the Asian leopard cat (Table 1), 4 (2F1_T3-4, 9F10_T7-4, 278g21-4, and SMCY7-STR) were single-copy loci. Two markers (278g21-4 and SMCY7-STR) were polymorphic with 3 and 9 alleles, respectively. Seventeen Y-specific microsatellite loci including 4 single-copy ones (2F1_T3-4, 9F10_T7-4, 278g21-4, and SMCY7-STR) were found for the

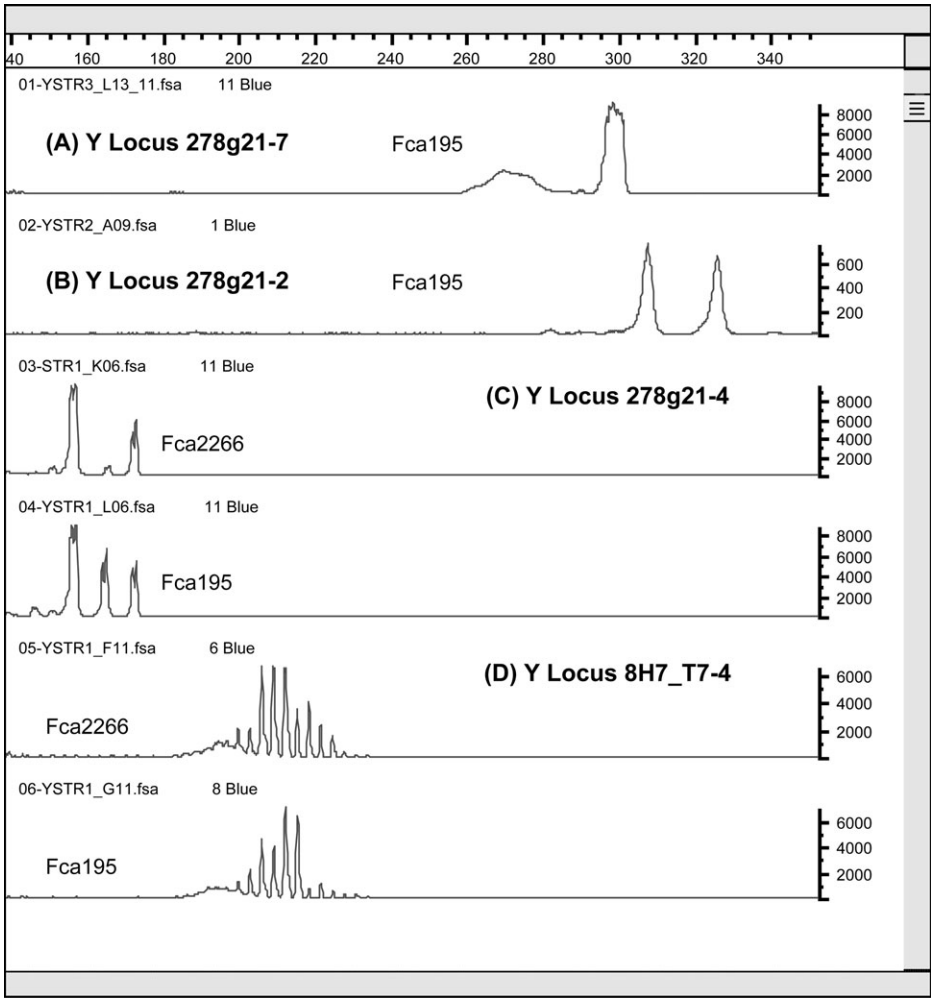

Figure 5. Electropherograms of male domestic cat DNAs amplified at 4 Y microsatellite loci. None of the loci amplified in female cat DNA samples, and data were not shown. (**A**) Locus 278g21-7, a single-copy Y microsatellite. (**B**) Locus 278g21-2, a Y microsatellite locus likely derived from segmental duplication along the Y chromosome in the domestic cat. (**C**) Triplication at locus 278g21-4. (**D**) A multiple-copy Y microsatellite locus 8H7-T7_4, note that most observable multiple alleles should be a single repeat unit apart because single-step mutations are most common for microsatellite markers.

tiger, and SMCY7-STR was polymorphic. Two single-copy microsatellite loci, 2F1_T3-4 and SMCY7-STR, can be amplified across 3 species, but the first is monomorphic in all species examined.

The microsatellite locus SMCY7-STR is located within the seventh intron of a single-copy gene *SMCY* on the Y chromosome and is highly conserved across species with diverged evolutionary histories. To further examine the utility and variability of this marker in Felidae, 6 species were examined. Polymorphism was observed in 4 out of the 6 species (Figure 6), ranging from 9 alleles in the Asian leopard cat, 3 in the fishing cat, and 2 in the tiger and marbled cat. Overall, the difference in allele size range among species corresponded roughly to the depth of their divergence and each species has unique alleles. For example, alleles found in the fishing cat (269, 271, and 273 bp) fell within the range of the Asian leopard cat (263–285 bp), a closely related species that shared a common ancestor about 2.55 million years ago (Johnson et al. 2006).

Intraspecific Polymorphism of Single-Copy Y Nuclear DNA Markers (YCATS) in the Felidae

The relative positions of the Y chromosome genes from which we derived intron markers were previously located on a domestic cat RH map (Murphy et al. 1999). Of the 8 YCATS markers, *DBY4*, *DBY12*, and *SMCY17* did not amplify or were not male specific in at least one species. *DBY8* was invariable in the pilot screening of 10 males across 4 species (Asian leopard cats, tiger, fishing cat, and Asian golden cat) and was excluded from subsequent large-scale examination. The final set of 4 markers, *DBY7*, *SMCY3*, *SMCY7*, and *UTY11*, were screened for intraspecific polymorphism in a panel of individuals in 6 cat species (Table 4). Among the 5

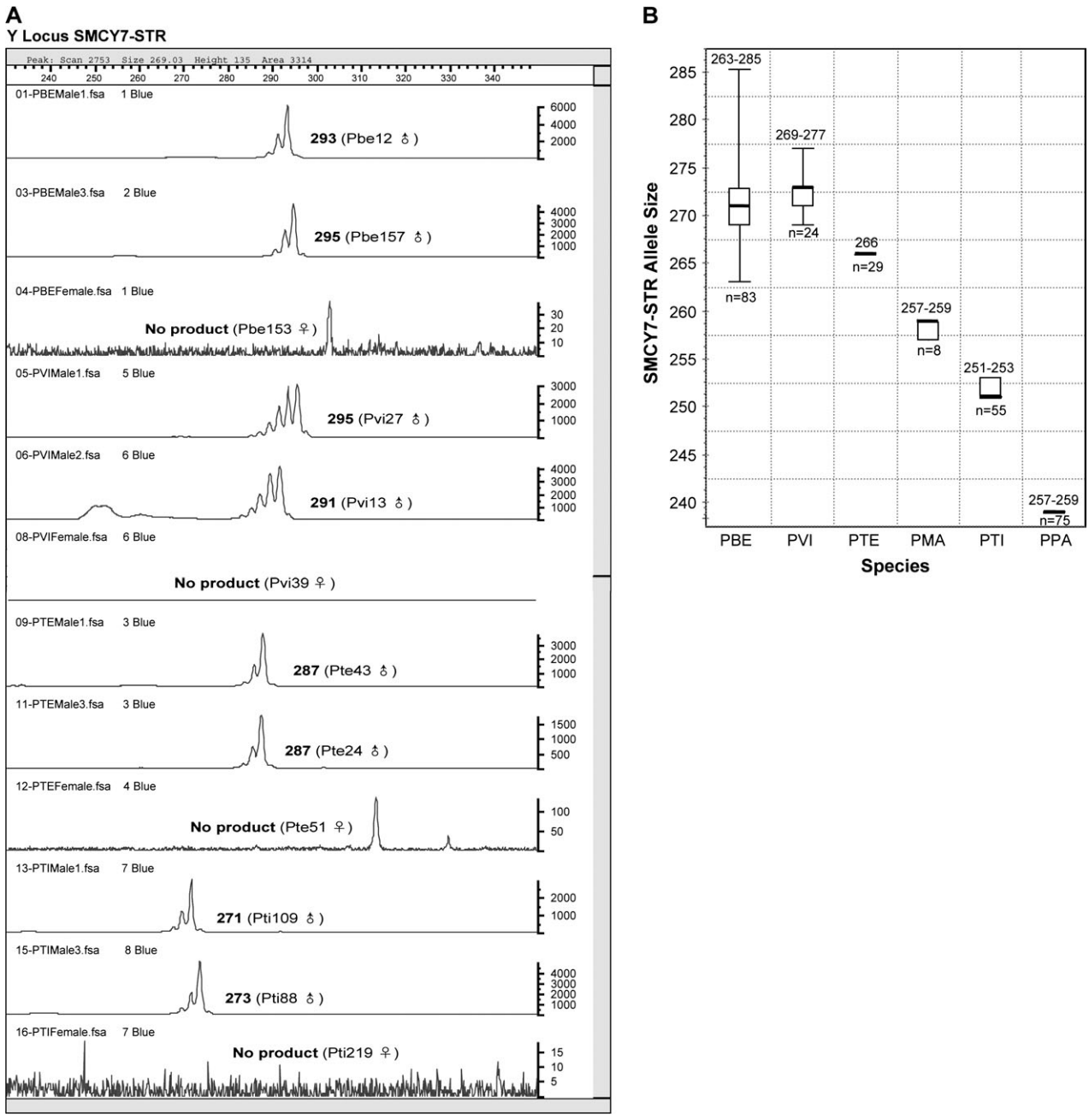

Figure 6. Amplification of a single-copy Y microsatellite locus SMCY7-STR in Felidae species. (**A**) Representative electropherograms of microsatellite locus with 2 males and 1 female for 4 species that are polymorphic for the locus. (**B**) Allele size range distribution of *SMCY7-STR* in 6 Felidae species, numbers above the bar are allele size range for each species and numbers below the sample sizes. PBE, *Prionailurus bengalensis* Asian leopard cat; PVI, *Prionailurus viverrinus* fishing cat; PTE, Asian golden cat *Pardofelis temmincki*; PMA, marbled cat *Pardofelis marmorata*; PTI, tiger *Panthera tigris*; PPA, leopard *Panthera pardus*.

species where SNPs were found (83 Asian leopard cats, 24 fishing cats, 29 Asian golden cats, 8 marbled cats, and 75 leopards), nucleotide diversity (π) ranged from 0.89×10^{-4} in the Asian golden cat to 9.95×10^{-4} in the marbled cat. The highest number of haplotypes and SNPs were found in the Asian leopard cat, and the highest mean number of pairwise differences and nucleotide diversity were found in the marbled cat. Seven segregation sites, including 5 transitions and 2 transversions, were discovered in 2.1 kbp of combined Y chromosome sequence in the leopard cat, or, about 1 SNP every 300 bp. In contrast, no variation was observed among 55 unrelated male tigers in the same sequence.

Table 4. Characterization of intraspecific polymorphism of Y nuclear DNA intron markers and SNPs found in 6 Felidae species

	Size (bp)	PBE	PVI	PTE	PMA	PTI	PPA
Sample size		83	24	29	8	55	75
Number of variable sites (SNPs)							
Smcy3	830	2 C/T, G/A	0	1 A/G	1 A/G	0	2 A/G, C/T
Smcy7	550	1 C/T	2 A/C, A/G	0 —	— —	0	1 G/A
Dby7	280	3 T/C, G/T, C/T	1 T/C	0 —	1 G/A	0	0 —
Uty11	510	1 T/A	1 T/G	0 —	2 C/T, G/C	0	0 —
Total	2170	7	4	1	4	0	3
Number of haplotypes		6	2	2	2	1	3
Nucleotide diversity π ($\times 10^{-4}$)		6.81 ± 4.62	6.39 ± 4.54	0.89 ± 1.29	9.95 ± 6.96	0	3.80 ± 3.05
Mean number pairwise differences		1.47 ± 0.90	1.38 ± 0.88	0.19 ± 0.25	2.14 ± 1.31	0	0.82 ± 0.59

PBE, Asian leopard cat *Prionailurus bengalensis*; PVI, fishing cat *Prionailurus viverrinus*; PTE, Asian golden cat *Pardofelis temminckei*; PMA, marbled cat *Pardofelis marmorata*; PTI, tiger *Panthera tigris*; PPA, leopard *Panthera pardus*.

Discussion

Isolation of Y-Specific Microsatellites

With a combination of multiple molecular genetic and cytogenetic approaches, we were able to develop 41 new male-specific microsatellite markers including 6 single-copy ones and 4 biallelic YCATS markers in the domestic cat *F. catus* and its related species (Tables 1 and 2). The Y haplotyping system, consisting of 4 intronic DNA markers (*SMCY3*, *SMCY7*, *UTY11*, and *DBY7*) and 1 microsatellite (SMCY-STR), describes intraspecific male lineage polymorphism in Felidae species and promises to provide significant insights to evolutionary history of the species.

Our results suggest that targeting regions near the Y-linked single-copy genes increases the chance of locating single-copy microsatellites. Microsatellite markers identified from one BAC clone containing *SRY* gene are significantly lower in copy number than the randomly isolated ones from Y cosmid or genomic microsatellite library. SMCY7-STR locus within intron 7 of *SMCY* gene can be amplified across evolutionary diverged cat species and displays both species-distinct allele size range and intraspecific polymorphism.

Because genomic libraries (BAC, PAC, fosmid, or cosmid) are publicly available for many species, low–copy number and Y-specific regions from a target or related species can be identified with the YCATS (Hellborg and Ellegren 2003) and male-specific markers can be subsequently isolated by subcloning and shotgun sequencing or by constructing microsatellite-enriched libraries. Two recent studies have isolated Y-linked microsatellites in the horse (*Equus* spp.) and the greater white-toothed shrew (*C. russula*) with such a combined strategy (Wallner et al. 2004; Handley and Perrin 2006), although the success rate was not high (5 markers in the horse and 1 in the shrew, respectively).

There are at least 2 possible reasons why we did not find male-specific (GATA)n tetranucleotide microsatellite from flow-sorted Y microsatellite-enriched libraries in the tiger and Asian leopard cat. First, the flow-sorted materials may have contained a small portion of contaminating autosomes, which could have been enriched during the WGA and capture hybridization process. However, this seems unlikely because the purity of Y chromosome origin was verified by FISH and positive PCR amplifications of previously mapped Y chromosome gene sequence tagged sites (*SRY* and *SMCY*, data not shown). Alternatively, the density of (GATA)n tetranucleotide repeat loci may have been low originally, and therefore the chance of enriching this type of microsatellites from the nonrecombining Y would be small. This second rationale was supported when an exhaustive search of (GATA)n microsatellites (repeat number $n \geq 9$) from the 24 Mb of published human euchromatic and pseudoautosomal Y chromosome sequences resulted in only 95 GATA repeat microsatellites, compared with 615 (CA)n dinucleotide repeat loci. Y chromosome content may vary greatly among species, and from a sampling of 1.8 Mb domestic cat Y cosmid sequences, no GATA repeat microsatellites were found, suggesting its low density in cat Y genome. From our hybridization screening of the Y cosmid library, there are an estimated 68 domestic cat Y chromosome GATA microsatellites, which is lower than that of the human Y chromosome (Table 3). It is therefore reasonable to estimate that in order to obtain male-specific loci with this method, more plasmid clones from a flow-sorted Y tetranucleotide repeat microsatellite library need to be sequenced or libraries enriched with a potentially more frequently distributed microsatellite should be considered.

Multiple-Copy Microsatellite Loci on the Y Chromosome

Six of the 41 male-specific microsatellites were single-copy loci in one or more species of the Felidae. This modest success rate is not unexpected because one of the most prominent features of the Y chromosome is its repetitive nature, which makes the discovery of useful Y-specific markers from a nonmodel species particularly difficult. The multicopy feature has been hypothesized to be a mechanism for maintaining Y chromosome genes in a nonrecombining environment and may arise by a number of mechanisms, including sexual antagonism, genomic conflict, and hemizygous exposure (Vallender and Lahn 2004; Murphy et al. 2006). About 30% of the human MSY euchromatin contains sequence pairs with greater than 99.9% identity (Skaletsky

et al. 2003). Because few genes occur on the Y chromosome, less constraint exists to maintain a particular sequence context, and thus insertion polymorphisms of large sections within the chromosome are more likely. Multiple-copy Y chromosome microsatellites commonly occur on these palindromes. When a section is duplicated, they may acquire a mutation at the microsatellite locus over time, resulting in a new allele (Butler et al. 2005). Primers designed from the relatively conserved microsatellite flanking region would amplify both copies and yield 2 alleles (or more if further duplications occurred) from the same male individual. The value of microsatellite loci of detectable multiple copies remains to be explored in population genetic studies.

Y Chromosome SNPs in Felidae Species

Biallelic markers such as SNPs and insertion/deletions (indels) represent another important class of Y chromosome polymorphism. Nucleotide diversity on the human Y chromosome sequences is significantly lower than that of the rest of the genome (for summary, see Miller et al. 2005), and similarly reduced genetic variability occurs in other mammalian species (Hellborg and Ellegren 2004) and *Drosophila* (Bachtrog and Charlesworth 2000), even after taking into account differences in effective population size and sex-specific mutation rates. The number of SNPs found from 4 Y intron nuclear DNA markers spanning 2.1 kbp of sequences ranged from 0 to 7 in the 6 Felidae species examined in this study. Five Asian cat species in the study, or the Asian leopard cat ($\pi = 6.81 \times 10^{-4}$), fishing cat ($\pi = 6.39 \times 10^{-4}$), Asian golden cat ($\pi = 0.89 \times 10^{-4}$), marbled cat ($\pi = 9.95 \times 10^{-4}$), and leopard ($\pi = 3.80 \times 10^{-4}$), have higher levels of nucleotide diversity than the 2 previously reported carnivore species (Hellborg and Ellegren 2004), lynx (*L. lynx*, $\pi = 0$) and wolf (*C. lupus*, $\pi = 0.4 \times 10^{-4}$). Four of them also have nucleotide diversity higher than that in human ($\pi = 1.5 \times 10^{-4}$). The overall high genetic variability of the Y-linked SNPs in the Asian cat species may reflect relatively large historic effective population sizes, deep divergence, and high levels of subdivision within the species.

By contrast, the tiger is invariable across the Y chromosome region examined, consistent with the observed low mitochondrial DNA variability which corresponds to a relatively recent common ancestor for all modern tigers (72 000–108 000 years ago; Luo et al. 2004). The lower SNP frequency found on the tiger Y chromosome may also reflect a recent genetic homogenization in the species relative to the Asian leopard cat. A combined study with other independently evolved genetic markers such as mitochondrial and autosomal DNA will further reveal the historic phylogeography and demographic processes, shaping patterns and rates of genetic differentiation between or within species. Ideally, these initial data from cat species will lead to further development of Y chromosome markers across a wide range of taxa and will stimulate efforts to better understand the comparative evolution of Y chromosome genomes, including more emphasis on whole genome sequencing of male-linked markers.

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