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Abstract: Short tandem repeat polymorphismus (STRP), or microsatellites, are widespread among vertebrate genomes and are useful in gene mapping and population studies due to their high level of length polymorphism. The authors describe the isolation, characterization, and PCR amplification of 10 microsatellite loci from the domestic cat, Felis catus. The flanking primer sequences were conserved among other Felidae species, and amplification products demonstrated abundant polymorphism in puma, lion, cheetah, and domestic cat. The cheetah sample exhibited the lowest level of polymorphism for these loci among felid species.

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Evolutionary Conservation of Ten Microsatellite Loci in Four Species of Felidae

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Short tandem repeat polymorphisms (STRP), or microsatellites, are widespread among vertebrate genomes and are useful in gene mapping and population studies due to a high level of length polymorphism. We describe here the isolation, characterization, and PCR amplification of 10 microsatellite loci from the domestic cat, *Felis catus*. The flanking primer sequences were conserved among other Felidae species, and amplification products demonstrated abundant polymorphism in puma, lion, cheetah, and domestic cat. The cheetah sample exhibited the lowest level of polymorphism for these loci among felid species.

Microsatellite loci are short repetitive elements that exhibit a tandem repeat of a 1–6 base pair (bp) motif. Variations in the number of repeat units result in loci of high polymorphic information content. Since the heterozygosity of these loci was first described in humans (Weber and May 1989), they have been found to be abundant, randomly distributed, and highly polymorphic in all eukaryotic organisms examined to date. Their short length, generally less than 100 bp, leads to facilitated genotyping by PCR (polymerase chain reaction) technology. These loci have rapidly become the polymorphic marker of choice in genetic recombination maps and in the fine-scale mapping desirable in positional cloning. Genetic recombination maps are published or in progress in human, mouse, cow, pig, sheep, cat, dog, and numerous other animal and plant species (Bishop et al. 1994; Dietrich et al. 1992; Gyapay et al. 1994).

Recently, the highly polymorphic nature of microsatellites and the need for only nanogram quantities of even degraded DNA for genotype analysis have led to their successful application to the examination of dynamics of population biology on a molecular genetic level (Bruford and Wayne 1993). Microsatellite loci have been used in recent examination of the social structure of pilot whales (Amos et al. 1993), genetic diversity in the bottlenecked wombat and endangered Ethiopian wolf (Gottelli et al. 1994; Taylor et al. 1994) and in assessing paternity in chimpanzees and ant colonies (Evans, in press; Morin et al. 1993).

We report on the isolation and characterization of 10 highly polymorphic $(dC{\cdot}dA)_n{\cdot}(dG{\cdot}dT)_n$ dinucleotide repeat loci in the domestic cat genome. We demonstrate that the 10 cat microsatellite primer pairs amplify products of predicted size in lion, cheetah, puma, Asian leopard cat, and Geoffrey's cat, suggesting their evolutionary conservation across all Felidae. Individual loci exhibit ample heterozygosity even in the genetically impoverished cheetah to serve as useful molecular genetic markers.

Materials and Methods

Genomic DNA

We extracted genomic DNA from leukocytes or tissue specimens (Sambrook et al. 1989) from the following species: domestic cat—10 unrelated individuals were used; cheetah—five unrelated captive individuals of the southern subspecies *Acinonyx jubatus jubatus*, collected from Kruger Park, Transvaal, or Namibia and five free-ranging individuals of the east African subspecies *A. j. raineyi*, collected in Tanzania and Kenya: puma—10 unrelated individuals from throughout the geographical range of the species from North America to South America: lion—two lions from each of four populations, (1) Serengeti National Park. Tanzania, (2) Ngorongoro Crater, Tanzania, (3) Kruger Park, South Africa, and (4) Namibia.

Construction of a genomic library, screening with radiolabeled oligonucleotide and wash conditions were as in Dietrich et al. (1992). We monitored counts of radiolabeled hybridization filters of the library screen after each wash. When the average number of counts per lift approximated 1,000–2,000 cpm on a handheld monitor, we blotted filters and exposed them to X-OMAT AR film overnight. Following a secondary screen, we prepared single-stranded DNA from recombinants using a Qiagen M13 mini kit and sequenced them using a Prism Ready Reaction Dye Primer Cycle Sequencing Kit (Applied Biosystems) and an Applied Biosystems 373A DNA Sequencer. Primer pairs were designed in unique sequence flanking the microsatellite using a sequence analysis program (Primer; vers. 0.5; Lincoln, Daly and Lander, Whitehead Institute for Biomedical Research, Cambridge, MA). All primer pairs were designed for uniform amplification conditions and a Tm of 60°. Amplification products for individual loci

Table 1. Characterization of 10 microsatellite loci in four species of Felidae

Locus	No. of repeats	PCR product size (bp)	Heterozygosity/(no. of alleles in parentheses)			
			Domestic cat	Cheetah	Puma	Lion
Fca 8	$(CA)_{24}$	144	0.89 (7)	0.84 (8)	0.49 (4)	0.73 (4)
Fca 23	$(CA)_{17}$	148	0.75 (7)	0.43 (4)	0.50 (4)	0.51 (3)
Fca 35	$(CA)_{18}$	148	0.60 (4)	0.60 (3)	0.50 (4)	0.79 (5)
Fca 43	$(CA)_{17}$	130	0.70 (7)	0.00 (1)	0.85 (8)	0.40 (2)
Fca 45	$(CA)_{15}$	143	0.86 (8)	0.43 (3)	0.10 (2)	0.23 (2)
Fca 77	$(CA)_{20}$	150	0.63 (5)	0.00 (1)	0.62 (3)	0.76 (4)
Fca 78	$(CA)_{19}$	199	0.76 (6)	0.27 (2)	0.71 (6)	0.85 (6)
Fca 90	$(CA)_{17}$	113	0.85 (8)	0.00 (1)	0.83 (7)	0.77 (5)
Fca 96	$(CA)_{17}$	213	0.85 (6)	0.74 (7)	0.82 (7)	0.74 (5)
Fca 126	$(CA)_{24}$	143	0.77 (5)	0.64 (4)	0.66 (4)	0.85 (7)
Average			0.77	0.39	0.61	0.66

Primer Pairs: (5′ to 3′)

Fca 8: ACTGTAAATTTCTGAGCTGGCC
TGACAGACTGTTCTGGGTATGG

Fca 23: CAGTTCCTTTTTCTCAAGATTGC
GCAACTCTTAATCAAGATTCCATT

Fca 35: CTTGCCTCTGAAAAATGTAAAATG
AAACGTAGGTGGGGTTAGTGG

Fca 43: GAGCCACCCTAGCACATATACC
AGACGGGATTGCATGAAAAG

Fca 45: TGAAGAAAAGAATCAGGCTGTG
GTATGAGCATCTCTGTGTTCGTG

Fca 77: GGCACCTATAACTACCAGTGTGA
ATCTCTGGGGAAATAAATTTTGG

Fca 78: TGAACTGAAGTCAGATGCTTAACC
CGGAATCAGCTATTTTTACGG

Fca 90: ATCAAAAGTCTTGAAGAGCATGG
TGTTAGCTCATGTTCATGTGTCC

Fca 96: CACGCCAAACTCTATGCTGA
CAATGTGCCGTCCAAGAAC

Fca 126: GCCCCTGATACCCTGAATG
CTATCCTTGCTGGCTGAAGG

were initially examined in 4% agarose gels to ensure product fidelity.

Genotyping of Unrelated Individuals

One primer of each pair was 5′ end-labeled in a 15-μl reaction that included 75 μCi of [γ-^{32}P]ATP at 6,000 Ci/mmol (New England Nuclear), 10× reaction buffer (Sambrook et al. 1989) and primer concentration of 1.5 μM by T4 polynucleotide kinase for 30 min at 37°C. We found that due to instability of the isotope to freeze-thaw, it was best to incorporate isotope into a PCR product on the day of isotope delivery. Radiolabeled PCR products could be visualized following electrophoresis in polyacrylamide gels up to 2–3 weeks later. Amplification of microsatellite loci proceeded in a 10-μl reaction, including the 10× buffer and dNTP concentration recommended by Perkin Elmer Cetus, 1.2 units of *Taq* DNA polymerase (Boehringer Mannheim), 0.1 Unit of Perfect Match (Stratagene), and 50 ng of DNA with primer concentrations of 200 nM for each unlabeled primer and 1.3 nm of end-labeled primer. Reactions were ampli... MJR Programmable Thermal ... (MJ Research) as follows: initia... ation at 94°C for 3 min, followed ... cles of 94°C for 1 min, 55°C for 2... 72°C for 3 min, with a final cycle ... for 10 min. We denatured product... ing an equal volume of formamid... buffer and electrophoresed them ... TBE, 6% denaturing polyacrylami... (National Diagnostics) in 0.6× T... ning buffer for 2–3 h. Gels were tra... to Whatmann 3MM blotting pap... ered with plastic wrap, and expo... OMAT RP film for 6–24 h.

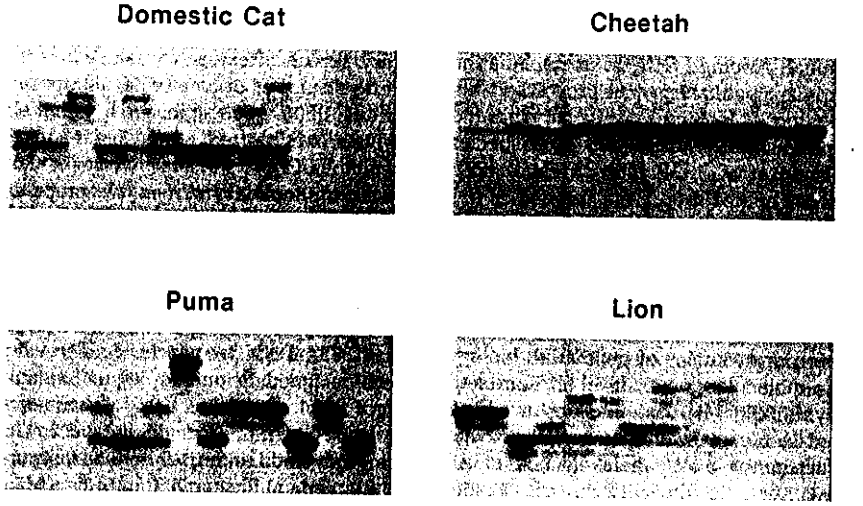

Figure 1. Autoradiographs of PCR-amplified microsatellite locus *Fca 77* in four species of Felidae. Radioend-labeled PCR amplification products of 10 unrelated individuals in four species of Felidae were electrophoresed in a 6% polyacrylamide denaturing gel. Stutter bands two bases shorter than each allele are visible.

Results and Discussion

Ten microsatellite clones were ... from a domestic cat M13 library, ... primers were designed based on t... ing sequence. The 10 primer pa... used to amplify microsatellites fro... samples of six species of Felidae: do... cat, *Felis catus*; puma, *Puma concol... Panthera leo*; cheetah, *Acinonyx ...* Asian leopard cat, *Prionailurus be... sis*; and Geoffrey's cat, *Oncifelis geo...* PCR products of similar size (da... shown) were amplified for each lo... the six species and exhibited a ra... polymorphism (Table 1, Figure 1). ... species the products of amplificat... hibited the classic "stutter" bands ... are a characteristic artifact of PCR ... fication of microsatellite loci (Figur... These data would suggest the evolu... ary conservation for these loci acro... Felidae, as the Geoffrey's cat and the ... represent the oldest and most recen... eages, respectively, of the Felidae ... tion spanning approximately 13 mi... years (O'Brien 1986).

There is a broad range of heterozyg... observed both between species ... among loci within a single species. ... age heterozygosities are highest i... large outbred species, lion and puma ... hibiting 86% and 79%, respectively, ... heterozygosity observed in the cat ... lowed by the cheetah, exhibiting 51... the heterozygosity observed in the ... This profile of genetic diversity in the ... felid species parallels that which has b... observed for nuclear coding loci and m... satellite loci in these species. Electrop... retic analyses of isozyme and soluble p... teins have shown abundant genetic va... tion in large outbred populations of ... mestic cat, lion, and puma, with estimat... average heterozygosities of 7.0%, 3.7... and 1.8%–6.7%, respectively (Newman ...

1985; O'Brien 1980; Roelke et al. 1993). In contrast, the cheetah exhibits a near monomorphic profile for conventional coding loci (O'Brien et al. 1983, 1987; Wildt and O'Brien 1990), the result of a demographic contraction or bottleneck (Menotti-Raymond and O'Brien 1993; O'Brien et al. 1987). Genetic diversity determined for minisatellite DNA detected with a multilocus feline-specific probe (Gilbert et al. 1991) is approximately equivalent in the three large outbred populations of domestic cat, lion, and puma with average heterozygosities of 46%, 48.1%, and 52.9%, respectively (Gilbert et al. 1991; Roelke et al. 1993). Slightly reduced levels relative to the other felids were observed in the cheetah (41.5%) (Menotti-Raymond and O'Brien 1993), consistent with observed levels of heterozygosity for microsatellite loci. This profile of genetic diversity in the cheetah is consistent with an ancient bottleneck, estimated at approximately 10,000 years ago (Menotti-Raymond and O'Brien 1993). Reconstitution of diversity has generated a profile of near monomorphism for coding loci (allozymes, MHC Class 1 genes) which evolve at a relatively slow rate, moderate in diversity for the more rapidly evolving mitochondrial DNA, and moderate to high levels of heterozygosity for the most rapidly evolving minisatellite and microsatellite loci.

There is a wide range in heterozygosity levels observed for the 10 microsatellite loci in the cheetah—from 0 to 0.842. Assuming that all loci were reduced to monomorphism at the time of the bottleneck, this suggests a large range in the rate of mutation for individual microsatellite loci. Little is known about the mutation rate of microsatellites. Weber and Wong (1993) recently reported an average rate of 1.2×10^{-3} per locus per gamete/generation in humans after genotyping 28 short tandem repeat polymorphisms in 20,000 parent-offspring allele transfers. Twelve $(GATA)_n$ tetranucleotide STRPs, one trinucleotide STRP, and 15 dinucleotide STRP loci were included in the study. The mutation rate for tetranucleotide STRPs was nearly four times higher than the average rate for dinucleotide STRPs, and a broad range in mutation rate was observed for individual STRPs—from 0 to 8×10^{-3}. Dallas (1992) also reports a range of mutation rates for microsatellite loci from 10^{-2} to 10^{-4} for three loci scored in recombinant inbred mouse strains.

Microsatellite loci offer potential as a molecular marker in subspecies identification. Five of the cheetahs examined were of the East African subspecies (*A. j. raineyi*), and five were of the South African subspecies (*A. j. jubatus*). Fifteen of the 34 alleles (44%) scored in the two subspecies were unique to one subspecies or the other. Under the assumption that all loci were reduced to monomorphism following the most recent population bottleneck 10,000 years ago and that microsatellite diversity accumulates at a uniform rate, this would suggest a period of approximately 4,400 years of separation to generate the observed diversity between the two subspecies.

These loci also offer promise in assessing genetic diversity and paternity. A small sample of Asiatic lions, a population that experienced a severe population bottleneck less than 100 years ago, exhibited a depressed average heterozygosity of 0.15 for the 10 loci. Figure 2 demonstrates classic Mendelian inheritance of one locus examined in a three-generation pedigree using interspecific backcrosses of the domestic cat and Asian leopard cat currently being used in construction of a genetic linkage map of the domestic cat. Paternity is clearly demonstrable.

The amplification of microsatellites across a broad species range has previously been demonstrated by Moore et al. (1991), who observed successful amplification of 27 of 48 ovine primer pairs in bovine DNA, 42% of which exhibited polymorphism, and by Bowcock et al. (1994), who amplified human primer pairs in chimpanzee, gorilla, and organ utans but found that allele frequencies could not be used to generate genetic distances. The success of amplification of microsatellites across species boundaries depends on the conservation of primer sequences. Caution needs to be taken in interpreting results in that null alleles (alleles for which there is no discernible product due to the lack of conservation of primer sequence) could result in lack of detection of heterozygous individuals, skewing the data toward a higher frequency of homozygous individuals. To test for the presence of null alleles, it is necessary to examine if the number of homozygotes is significantly greater than that expected under Hardy-Weinberg equilibrium.

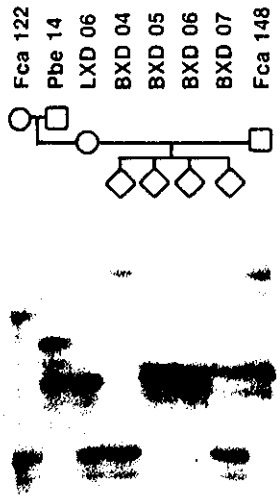

Figure 2. Autoradiograph of PCR-amplified microsatellite alleles for locus *Fca 23* in a three-generation interspecific backcross pedigree of domestic cat × Asian leopard cat. This cross is being constructed as an aid for constructing a gene map of the domestic cat, including both coding loci (Type I) and highly polymorphic microsatellite loci (Type II) (Lyons et al. 1994). Radioend-labeled PCR amplification products were electrophoresed in a 6% polyacrylamide denaturing gel. Segregation of alleles clearly shows Mendelian inheritance. Fca = domestic cat; Pbe = Asian leopard cat; LXD = F_1 generation (leopard cat × domestic); BXD = backcross individuals.

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