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Genetic Individualization of Domestic Cats Using Feline STR Loci for Forensic Applications

REFERENCE: Menotti-Raymond M, David VA, Stephens JC, Lyons LA, O'Brien SJ. Genetic individualization of domestic cats using feline STR loci for forensic applications. *J Forensic Sci* 1997;42(6):1039-1051.

ABSTRACT: A group of ten short tandem repeat (STR) loci suitable for PCR typing from DNA of domestic cats is evaluated for genetic individualization using blinded samples of eight putative feline blood specimens. The ten loci were also typed in a 70 member cat pedigree to demonstrate Mendelian inheritance and independent assortment. A "match window" or measurement precision estimate was empirically established by determining the maximum gel migration difference among alleles identical by descent in different individuals of the pedigree. Hardy-Weinberg equilibrium and abundant heterozygosity was observed for each locus in cat population samples from Canada and the USA. The probabilities of two unrelated individuals matching by chance (P_m) at all ten loci was estimated as 1.35×10^{-10} . We present a conservative approach to compute, for forensic consideration, the mathematical likelihood of a chance genotypic match between DNA evidence from a crime scene and the suspect composite STR genotypes for species or populations when genotype frequency information is not available.

KEYWORDS: forensic science, feline short tandem repeat forensic panel, genetic individualization of domestic cats

One third of households in the United States provide homes to about 65 million cats (1). Hairs of domestic animals are sometimes associated with crime scenes and morphological criteria have typically been utilized to identify hairs on a species or breed level (2-4). However, genetic fingerprinting of animal specimens in forensic analysis has not been reported for three reasons: 1) the limitation of DNA quantity that can be isolated from hairs relative to the amount required for minisatellite (VNTR) analysis, 2) the dearth of animal single locus VNTR probes, and 3) the lack of population data bases for animal species required to compute statistical match likelihoods.

The report of STR loci as a source of polymorphism in human DNA (5-7) has revolutionized the forensic community in the past several years (8-10). The short length of STR loci, generally less than 100 basepairs (bp), is well suited for amplification using

polymerase chain reaction (PCR) (6) based technology. The potential of genotyping multiple STR loci in multiplex analysis with a single nanogram of genomic DNA opened the forensic potential for biological material considered inappropriate due either to the age of the sample, quality, or quantity of DNA yield (11-15). The development of STR loci in a range of domestic animals and their incorporation into genetic recombination maps (16-22) has created the potential for forensic identification of domestic animals with STR loci. Species-specific STR markers have recently been employed for genetic individualization in paternity analyses of grizzly bear populations (23) and for paternity exclusion in free-ranging chimpanzees, the Ethiopian wolf, koalas, and cattle (24-27).

In an effort to generate a genetic recombination map of coding (Type I) and highly polymorphic (Type II) loci (28,29) in the domestic cat, we have isolated and characterized 400 dinucleotide repeat $(dC \cdot dA)_n \cdot (dG \cdot dT)_n$ STR loci from genomic DNA of *Felis catus* (30). One hundred and fifty loci have been genotyped in a three-generation, 70-member interspecies backcross pedigree between the domestic cat and the Asian leopard cat (*Prionailurus bengalensis*). The pedigree was generated between separate species to maximize genetic differences that could be followed in gene mapping exercises.

We report here on a PCR-based method of genetic individualization of domestic cats which employs a panel of 10 STR loci. We demonstrate the potential of the felid panel for forensic evaluation through the correct identification of matching blood samples supplied in a blind proficiency test, discuss their application to single hair analyses and present a useful approach toward the development of a "match window" for allelic identity. Finally, we present a conservative method for computing the likelihood of a chance genotypic match between suspect and evidentiary composite STR genotypes in animal species or populations in which a population genetic database is unavailable.

Materials and Methods

Materials

Blood samples: Eight coded blood samples were supplied as dried specimens on Whatmann 3 mm filter paper by Dr. Ron Fourney, Royal Canadian Mounted Police (RCMP), Central Forensic Laboratory, Ottawa, Canada as test samples for the proficiency test.

Population survey: 6-mL blood samples from 19 unrelated domestic cats from Prince Edward Island (PEI), Canada were supplied by Dr. Jane Bondt, DVM.

Interspecies pedigree: A three-generation, 70-member interspecies backcross pedigree between the domestic cat and the Asian

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The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names imply endorsement by the U.S. Government.

Received 1 Oct. 1996; and in revised form 25 Feb. 1997; accepted 28 Feb. 1997.

leopard cat included 14 domestic cats, 4 Asian leopard cats, 10 F_1 interspecies hybrids and 42 backcross progeny (28).

Amplification of STR Loci

Primer pairs were previously designed to amplify dinucleotide repeat STR loci in the domestic cat, generating products of from 100–300 base pairs under a standard set of conditions (30). PCR amplifications of individual STR loci were performed in 10- μ L reaction volumes containing 1X Boehringer Mannheim PCR buffer containing 10 mM Tris-hydrochloric acid (HCl)(pH 8.3), 50 mM potassium chloride (KCl), 1.5 mM magnesium chloride ($MgCl_2$) with additional 0.5 mM $MgCl_2$, (final concentration of $MgCl_2$ = 2.0 mM); 250 μ M each of the four deoxyribonucleoside 5'-triphosphates (dATP, dCTP, dGTP, and dTTP) (Pharmacia), 0.4 Unit *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT), and 4.0 picomoles each of forward and reverse primer (Life Technologies, Gaithersburg, MD and PE Applied Biosystems Inc. (ABI), Foster City, CA). One member of each primer pair was labeled with a fluorescent dye phosphoramidite. Sequences for primer amplification, expected PCR product sizes and molecular characterization of the STR loci are described in Table 1 and (30). PCR amplification was performed in a Perkin Elmer Model 9600 Thermocycler using the following set of conditions: one cycle of 3 min at 93°C, 10 cycles of 94°C for 15 s, 55°C for 15 s, 72°C for 30 s, 20 cycles of 89°C for 15 s, 55°C for 15 s, 72°C for 30 s, and one cycle of 72°C for 10 min. PCR products were diluted 1:10 with sterile deionized water (Quality Biological) in individual tubes in a Perkin-Elmer 9600 tray assembly. Two mL of diluted product was mixed with 4 μ L of a gel loading buffer/standard mixture composed of 6:1:1 ratio of formamide (Sigma), ABI Prism™ Genescan-350 TAMRA internal lane standard, and ABI Genescan loading buffer, respectively. Samples were denatured 3 min at 94°C and placed on ice. Two mL of sample were loaded per lane and electrophoresed in 6% denaturing polyacrylamide gels in an ABI Model 373A Automated DNA Sequencer Apparatus for 3.5 h at 2500 V, 40 mA, 25 W. Allele sizes were estimated with ABI Genescan (Version 1.2.2-1) and Genotyper (Version 1.1) software applications using the Local Southern method (31) to generate a best-fit curve from the size standards electrophoresed in each lane.

Isolation of DNA from Blood Stains

DNA was extracted from the blood stains as described in the RCMP protocol Isolation of DNA from Body Fluid Stains in the Biology Section Methods Guide (32). Half of each blood stain was placed in a 15-mL screw-capped Sarstedt tube (Sarstedt, Inc; Newton, NC) with 2 mL of digestion buffer including 10 mM Tris-hydrochloric acid (HCl)(pH 8.0), 100 mM sodium chloride (NaCl), 10 mM ethylenediaminetetraacetic acid (EDTA), disodium salt, 0.5 mg/mL Proteinase K (Life Technologies), 2% sodium dodecyl sulfate (SDS), and 39 mM dithiothreitol (DTT) and digested for 18 h at 56°C. The digest was extracted two times with equal volumes of phenol, once with phenol/chloroform, once with chloroform and once with *n*-butanol. The aqueous phase was concentrated in a Microcon 30 microconcentrator (Amicon Inc., Beverly, MA) according to the manufacturer's suggestions and the retained volume was desalted by the addition of 500 μ L of sterile distilled water followed by centrifugation at 14,000 g for 7 min. Sterile distilled water was added as needed to bring all final sample volumes to 50 μ L. A reagent blank and digest of a human blood stain were also processed. The quality and quantity of DNA was estimated by electrophoresis of 10 μ L of each product in a 1% TBE agarose (FMC) gel and compared to a known quantity of DNA standard (Lambda DNA, Hind III digest, Life Technologies).

Population Survey

A χ^2 was performed on observed genotype frequencies of PEI cats to detect deviations from expectations consistent with Hardy-Weinberg equilibrium (33). In one case, a rare heterozygote was grouped with a more common genotype (34).

Results

Selection of STR Loci for Forensic Analyses

Ten loci were initially selected as candidates for forensic analyses from 150 feline STR loci which had been typed in the interspecies pedigree and assigned to linkage groups. The ten loci were unlinked, exhibited Mendelian inheritance in a three-generation pedigree, exhibited high heterozygosity, and amplified with as little as one nanogram of DNA generating strong, clean electrophoretogram profiles. Under limiting template conditions, certain STR loci do not amplify both alleles

TABLE 1—Molecular characterization and primer sequences for PCR amplification of feline STR loci.

STR Locus	Dye Label	Repeat Length	Primer Sequences (5' to 3')	PCR prdt Size (bp)	HET
FCA026	6-FAM	(CA) ₂₁	*TGACACGCACCAAAAACAA GGAGCCCTTAGAGTCATGCA	150	0.75
FCA058	TET	(CA) ₂₁	*GTGAAGAAAGCTGGTGTGCA CATCCCTGACTAGCCTGAGC	230	0.62
FCA080	TET	(CA) ₂₂	AAGCTCTGTCATGTTCTGC *TGTAAGGCCCAAAAATCTGC	257	0.68
FCA088	6-FAM	(CA) ₂₂	*TTTTTCTTTTCCCGTAATACACA AGGAAAATGAAGTCAAGAAAATGG	116	0.84
FCA132	6-FAM	(CA) ₂₃	*GATGCCTCATTAGAAAATGGC ATCAAGGCCAACTGTCCG	150	0.82
FCA149	TET	(CA) ₁₇	*GTCTACCATGTGTGGGATG CCTATCAAAGTTCTACCAAAATCA	129	0.80

*The starred member of each primer pair was labeled with one of the fluorescent dye phosphoramidites 6-carboxyfluorescein (6-FAM) or tetrachloro-6-carboxyfluorescein (TET). HET is heterozygosity determined from a population survey of unrelated domestic cats in Canada. Primer sequences and locus characterization for FCA043, FCA090, FCA096, FCA126 were reported in Menotti-Raymond and O'Brien (30). FCA043 was labeled with TET; FCA090, FCA096, and FCA126 were labeled with 6-FAM. The repeat length reported for each STR locus was determined from sequence analysis of recombinant clones selected from an M13 genomic cat DNA library screened with radiolabeled (dC-dA)_n·(dG-dT)_n oligonucleotide probes (30).

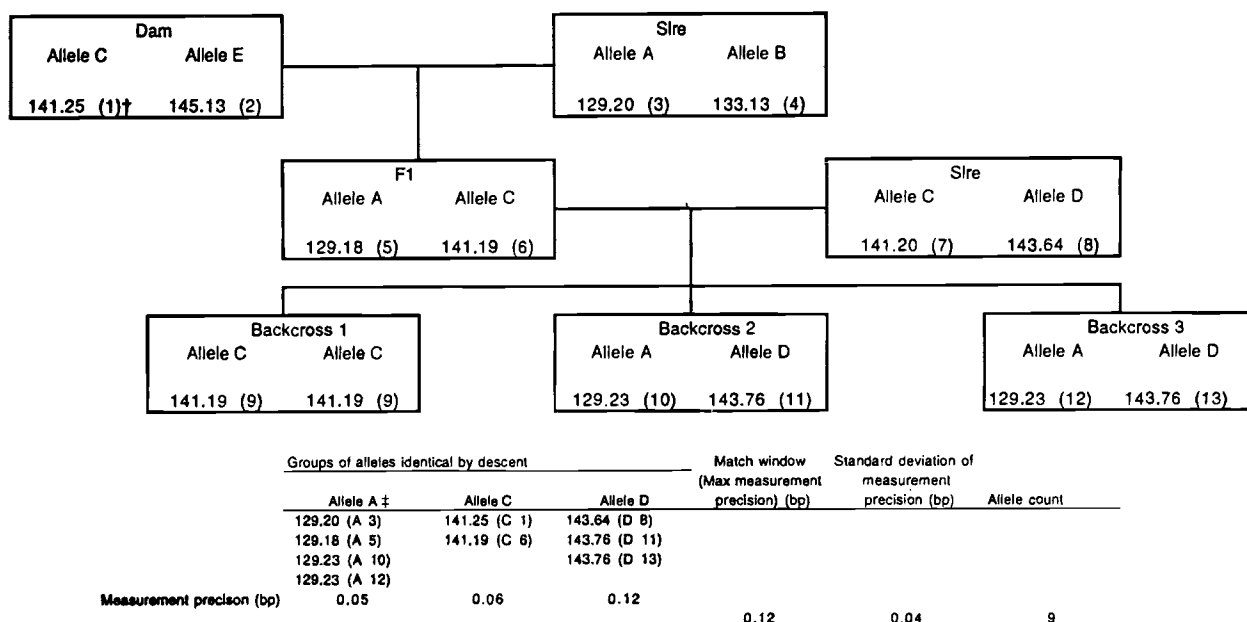


FIG. 1—Example* of how a match window was determined for STR locus FCA 126 by examining size estimates of alleles unambiguously identical by descent.

*One of 16 family groups used to derive match window for STR locus FCA 126 is shown above. The match window for the entire 70 member pedigree, defined as the maximum pairwise identical allele migration difference, was 0.53 bp.

†Each allele from every individual is sequentially numbered for Mendelian reference.

‡Only those cases in which alleles can be tracked unambiguously through the pedigree are shown. Alleles are not shown for homozygous individuals such as Backcross 1, since the allele scored could represent a combination of two different alleles.

of a heterozygous individual (13). With one nanogram of genomic DNA, the ten STR loci consistently generated strong diallelic profiles.

Independence Across Loci

The ten loci had been genotyped in a 70-member feline pedigree. None of the ten loci were observed to be linked within limits of detection (≤ 10 cM) in the 70-member kindred using the M-link option of LINKAGE (35). The possibility of non-independence of the selected test loci, due to linkage disequilibrium or other factors was evaluated in a population survey of 19 unrelated domestic cats from Prince Edward's Island, Canada using Fisher's exact test for independence of all pair wise combinations of alleles among loci. Using the convention described for multiple tests (36), the allelic variation displayed patterns consistent with locus and allele independence for this population.

Establishing a "Match Window" for STR Loci Using a Feline Kindred

In order to establish criteria for declaring a match between STR alleles of similar size (those in the same bin), human forensic laboratories empirically determine a size difference threshold ("match window") which defines acceptable variation in migration between any two measured alleles to conclude that they match (37). The match window provides an empirical determination of precision for each STR locus from a sample of multiple ascertainment of identical alleles. The magnitude of the match window is based on the difference observed in the largest and smallest size estimation for identical alleles which were electrophoresed on the same gel.

We determined the match window for the ten feline STR loci empirically by comparing migration (size) estimates of alleles identical by descent in a 70-member feline pedigree. The difference in size estimation between multiple individuals for each allele was

determined. (All size comparisons were made of multiple cat DNA samples electrophoresed on the same gel). The maximum size difference observed after examination of all allele size classes determined the level of precision or match window for that particular STR locus. In forensic comparisons, alleles which differ in size by a quantity less than or equal to the match window were judged to be a match.

Figure 1 illustrates the determination of match window for STR locus FCA 126 for a three-generation family group in our pedigree. Alleles which are identical by descent are grouped and listed in the table below. Observe that neither of the 141 bp alleles of backcross animal 1 are listed in the table, as the 141.19 bp genotype is a composite estimate of the comigration of two alleles. The difference in size between the largest and smallest size estimation of the 129 bp allele, bands 3, 5, 10, and 12, is 0.05 bp (129.23 bp-129.18 bp). The difference in size between the largest and smallest size estimation of the 141 bp, and 143 bp alleles is 0.06 bp, and 0.12 bp, respectively. If the match window had been determined from this one family group, a level of precision of 0.12 bp would constitute the match window for STR locus FCA 126. The match window computed for each STR locus based upon 16 three-generation kindreds of the feline interspecies backcross pedigree, is presented in Table 2.

Test of the Feline STR Forensic Panel

To evaluate the feline STR forensic panel, we determined composite genotypes of 8 coded blood stains of putative feline origin to assess whether any of the blood samples came from the same cat. One STR locus (FCA 058), initially amplified from 1 and 4 μ L of product isolated from the 8 test samples (5-60 ng DNA), a reagent control, a human control, and the positive cat DNA control (NCI 123), generated amplification products from 1 μ L but not from 4 μ L of the extraction

TABLE 2—Match window determined using maximum difference among alleles identical by descent in a feline kindred.

STR Locus	Number of Allele Size Classes	Number of Alleles Counted	Allele Range (bp)	Average Size of Measurement Precision (bp)	Standard Deviation of Measurement Precision (bp)	Match Window (bp)*
FCA 026	8	87	133.30–157.96	0.14	0.10	0.37
FCA 043	8	65	114.22–130.19	0.17	0.14	0.59
FCA 058	7	66	210.37–230.92	0.13	0.08	0.36
FCA 080	5	35	250.00–259.35	0.15	0.10	0.30
FCA 088A	8	75	112.58–129.02	0.15	0.10	0.42
FCA 090	8	67	93.26–120.64	0.14	0.10	0.46
FCA 096	6	43	184.09–223.23	0.11	0.09	0.25
FCA 126	6	37	129.13–148.25	0.13	0.14	0.53
FCA 132	10	74	136.00–175.79	0.10	0.09	0.27
FCA 149	6	91	120.56–132.27	0.10	0.07	0.29

*Match window is the maximum measurement precision or greatest size difference estimated in all pairwise comparisons.

products, suggesting the presence of PCR inhibitors. A potential source of inhibition of PCR amplification from the blood-extracted samples was residual heme, a powerful inhibitor of PCR (38). All samples were “washed” two times by addition of 2 mL of sterile distilled water followed by concentration in a Centricon 100 concentrator (Amicon Inc., Beverly, MA) using the centrifugation protocol recommended by the manufacturer. The “washed” samples amplified successfully with primer pair FCA 058 using either 1 or 4 μ L of sample (5–60 ng DNA). Bovine serum albumin (Sigma #A-3294) was additionally added to the PCR master mixture (0.16 mg/mL final concentration).

The 9 additional STR loci were then amplified individually from 5–25 ng of each DNA sample. For each STR locus, amplification products amplified for the 8 test samples and controls were electrophoresed in separate lanes on the same polyacrylamide gel. Table 3 is a summary of amplification product sizes observed for the 10 STR loci in the 8 coded samples and positive cat control (NCI 123) (#9). Figure 2 presents an electrophoretogram of amplification products of locus FCA 080. The PCR product sizes obtained are graphically presented in histograms which demonstrate the range and distribution of allele sizes observed for each locus (Fig. 3). Allele sizes for each locus fall into discrete size classes distinct from one another by more than one base pair. Each allele size class was assigned an identifying bin letter. A table beneath each histogram in Fig. 3 identifies the bin designation for each allele size class, the size range for each bin, and standard deviations. Table 3 presents the composite genotypes as allele sizes and assigned bin designation for each sample. The results indicated that samples 2, 5, and 7 had unique feline composite STR genotypes while samples 1 and 4 shared an identical feline composite genotype as did samples 3 and 8. The identical pairs produced a perfect match for all 10 loci including 17 alleles for samples 1 and 4, and 18 alleles for samples 3 and 8.

A forensic comparison of composite STR genotypes for matching sample pairs, 1, 4 and 3, 8, is presented in Table 4. Measurement differences in allele size estimations for the matching sample pairs are listed and compared to the match window computed for each locus. The two pairs are each judged to be a match because the differences between matched allele sizes are in every case less than the match window presented in Table 2.

Sample 6, a Sample with a Nonfeline STR Genotype Profile

For sample 6, no amplification products were observed for STR loci FCA 043, FCA 026, FCA 149, FCA 088A, or FCA 090, while amplification products for STR loci FCA 132, FCA 096, FCA 080, FCA 126, and FCA 058 were not of a size, pattern or amplitude consistent with feline STR amplification products. As an illustration,

sample 6 has a single amplification product for locus FCA 080, identical in size to that obtained for the human sample (Fig. 2). The amplitude of the peak is significantly less than the other seven coded samples and does not demonstrate the characteristic stutter bands of dinucleotide repeat amplification products (39). We observed that all amplification products of sample 6 were in a size range, amplitude and signal pattern similar to those observed for the human control. To test for the possibility of human DNA in sample 6, a human dinucleotide STR locus, d9S171 (40) was amplified in the 8 samples, controls, and DNA of the 2 investigators who came in contact with the samples in the laboratory. The results revealed successful amplification of sample 6 with products in size, range, and amplitude of human DNA controls, but of a genotype distinct from that of either investigator (data not shown). We conclude that sample 6 was non-feline, and likely of human origin.

Estimating the Probability of a Match due to Chance Using an ad hoc Population Database

In association with a forensic case, we have developed two small population STR databases to estimate feline STR allele frequencies. The first included a sample of 19 unrelated domestic cats, collected by a veterinary clinic in Prince Edward Island, Canada; the second was a group of 9 domestic cats collected in the eastern United States. Fifty nanograms of DNA from each cat was amplified for each of the 10 STR loci. The allele distribution for the two populations is presented in Table 5. A χ^2 test performed on observed genotype frequencies in the PEI population did not detect any deviations from expectations consistent with Hardy-Weinberg equilibrium (33).

Under the presumption of locus independence and Hardy-Weinberg (genetic) equilibrium, it is possible to estimate the incidence of a composite STR genotype in the database populations using the “product rule” (41). Generally, the estimated incidence of the specific genotype is a composite product of the frequency for each homozygous and heterozygous locus in the population using the Hardy-Weinberg equilibrium per locus frequency derived from the allele frequency (p and q) of the allele(s) for each locus expressed in the individual. In cases in which an individual was homozygous at a particular locus, the frequency was estimated as $2p$ based on recommendations of the Technical Working Group on DNA Analysis Methods (TWGDAM) (41). Minimum default allelic frequencies were determined for each STR locus as described by Chakraborty (42) which adjust for data base sample size and locus heterozygosity. Using this method to compute the likelihood of a

TABLE 3—Genotypes for STR amplifications of coded bloodstain samples.

STR Locus FCA 026					STR Locus FCA 090				
Sample	Allele 1		Allele 2		Sample	Allele 1		Allele 2	
	bp	Bin	bp	Bin		bp	Bin	bp	Bin
1	145.77	E	152.05	H	1	109.87	D	119.86	H
2	148.00	F	157.87	J	2	93.78	A	112.04	—
3	143.54	D	145.57	E	3	93.55	A	115.75	F
4	145.79	E	152.02	H	4	109.99	D	119.90	H
5	145.57	E	147.79	F	5	110.11	D	120.09	H
7	143.54	D	143.54	D	7	107.92	C	107.92	C
8	143.65	D	145.65	E	8	93.77	A	115.91	F
NCI 123	143.56	D	152.01	H	NCI 123	109.80	D	115.80	F

STR Locus FCA 043					STR Locus FCA 096				
Sample	Allele 1		Allele 2		Sample	Allele 1		Allele 2	
	bp	Bin	bp	Bin		bp	Bin	bp	Bin
1	120.54	C	120.54	C	1	210.89	E	212.86	F
2	126.33	D	126.33	D	2	197.09	C	211.03	E
3	120.59	C	126.32	D	3	210.86	E	210.86	E
4	120.90	C	120.90	C	4	211.00	E	212.95	F
5	126.45	D	126.45	D	5	211.04	E	211.04	E
7	120.54	C	120.54	C	7	211.03	E	219.03	G
8	120.76	C	126.52	D	8	211.03	E	211.03	E
NCI 123	118.89	B	128.37	E	NCI 123	210.92	E	210.92	E

STR Locus FCA 058					STR Locus FCA 090				
Sample	Allele 1		Allele 2		Sample	Allele 1		Allele 2	
	bp	Bin	bp	Bin		bp	Bin	bp	Bin
1	229.03	D	233.00	F	1	141.06	C	143.33	D
2	223.20	B	229.21	D	2	140.83	C	151.85	H
3	229.24	D	229.24	D	3	141.01	C	151.84	H
4	229.12	D	233.16	F	4	141.06	C	143.33	D
5	229.20	D	233.22	F	5	140.83	C	140.83	C
7	221.16	A	229.16	D	7	141.01	C	141.01	C
8	229.05	D	229.05	D	8	141.06	C	152.02	H
NCI 123	221.34	A	223.34	B	NCI 123	138.80	B	141.06	C

STR Locus FCA 080					STR Locus FCA 132				
Sample	Allele 1		Allele 2		Sample	Allele 1		Allele 2	
	bp	Bin	bp	Bin		bp	Bin	bp	Bin
1	249.21	A	259.34	F	1	150.66	E	152.61	F
2	255.17	D	259.19	F	2	135.99	A	135.99	A
3	253.26	C	255.17	D	3	150.87	E	152.82	F
4	249.21	A	259.16	F	4	150.87	E	152.82	F
5	259.00	F	259.00	F	5	137.28	B	137.28	B
7	238.87	*	249.21	A	7	152.76	F	152.76	F
8	253.40	C	255.30	D	8	150.86	E	152.77	F
NCI 123	259.43	F	263.39	H	NCI 123	136.11	A	152.92	F

STR Locus FCA 088A					STR Locus FCA 149				
Sample	Allele 1		Allele 2		Sample	Allele 1		Allele 2	
	bp	Bin	bp	Bin		bp	Bin	bp	Bin
1	116.24	C	116.24	C	1	128.55	C	128.55	C
2	122.09	F	122.09	F	2	128.32	C	128.32	C
3	112.58	A	122.09	F	3	128.33	C	132.11	E
4	116.31	C	116.31	C	4	128.33	C	128.33	C
5	116.31	C	122.09	F	5	122.54	A	130.09	D
7	122.21	F	122.21	F	7	132.13	E	132.13	E
8	112.68	A	122.10	F	8	128.13	C	131.89	E
NCI 123	120.28	E	122.10	F	NCI 123	130.16	D	132.15	E

*Allele not present in PEI or US survey.

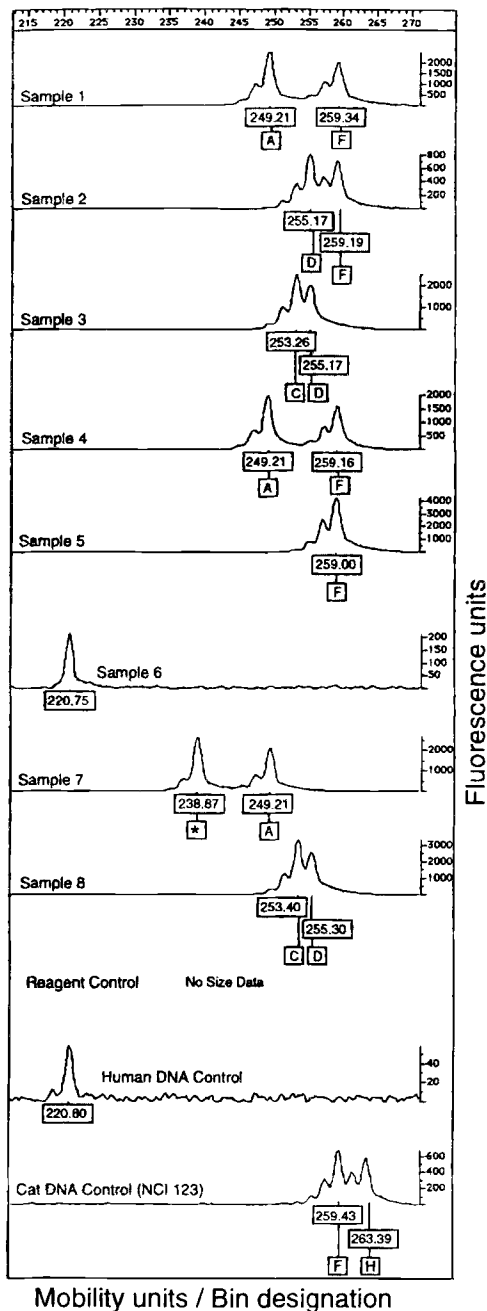


FIG. 2—Electrophoretogram of amplification products of STR locus FCA 080 in proficiency test samples (samples 1–8), reagent control, human control, and positive cat DNA control, NCI 123 (sample 9). No size data is reported for the reagent control as no amplification products were observed. Sizes of amplification products are reported in mobility units sized relative to the migration of internal lane standards on an ABI 373A Sequencer Apparatus measured by Genescan and Genotyper software applications. Boxed values are mobility units which correspond to estimated nucleotide length or basepair sizes. Boxed letters values are assigned bin or allelic classes (see text and Table 3).

chance match of samples 1 and 4 in the proficiency test based upon the PEI frequencies, the estimated frequency of the composite STR genotype for samples 1 and 4 would be 7.95×10^{-13} . For samples 3 and 8 the estimated frequency would be 1.04×10^{-13} . Had all cat samples been drawn from Prince Edward Island, these frequencies would be equivalent to the probability of a chance match of samples given the first cat's genotype and drawing the

second randomly from the cat population. This probability would be higher if sampled cats were close relatives or if a significant substructure occurred between populations (e.g., between PEI and the sample pool of the proficiency test). However, when we compare the Maryland sample with PEI (Table 5), there are only slight differences in allele distribution and frequencies revealing little substructure between these populations.

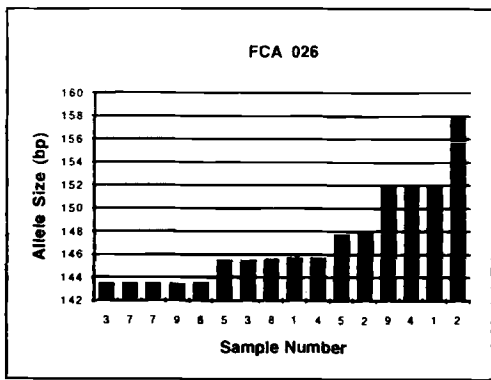
Feasibility of Genetic Individualization from Single Cat Hair Specimens

Hairs are frequently deposited at crime scenes and have historically been a valuable resource of forensic analysis (43). Higuchi et al. (44) first demonstrated that DNA genotyping could be accomplished with single human hair specimens using the polymerase chain reaction while Morin has reported on genetic individualization of single hair specimens of free-ranging chimpanzees using human STR primer pairs (24). Feline STR loci have been successfully utilized in forensic evaluation of multiple puma hairs associated with two puma attacks on joggers in California (Culver, in preparation). As the forensic utility of domestic cat specimens is most likely to be applied to hair specimens, we examined single cat hairs for the feasibility of genetic individualization of domestic cat hair specimens with the feline STR panel. DNA was extracted from several individual hairs, both plucked and shed with a visible root following the protocol of Higuchi et al. (44). We obtained 15–30 ng of high quality DNA per root as compared to 250–500 ng of DNA extracted from a human hair root. The forensic panel of 10 feline STR loci was successfully amplified and genotyped from single hair extraction products (data not shown). Although the quantity of DNA from a cat hair is an order of magnitude less than that obtained from a human hair root, the feline STR loci amplify well with a single nanogram of DNA. We examined six primer pairs of the STR panel for the feasibility of multiplex amplification. Five STR loci, including FCA 043, FCA 090, FCA 132, FCA 080, and FCA 149, generated products with a signal and amplitude identical to that observed in single amplifications from either 1, 5, or 25 nanograms of DNA (NCI 123). No artifactual bands were generated in the multiplex amplification reaction.

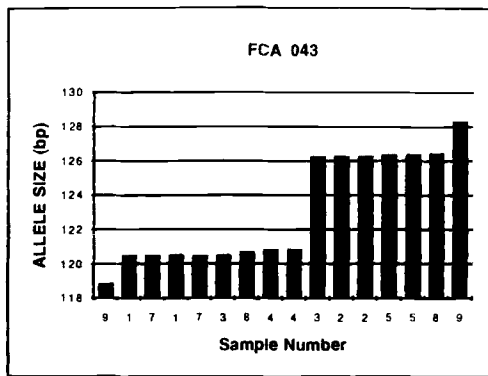
Estimating the Probability of Composite STR Genotypic Matching in the Absence of a Population Database

Primer pairs for amplification of STR loci are published for a wide range of mammalian species (45). Genetic recombination maps of STR loci are published for the cow, pig, mouse, sheep, and rat (16–22) and are in progress in a number of species including cat, dog, horse, and deer. Although the availability of STR primers for individual identification and forensic purposes in novel species can be anticipated, current application requires the construction of a reference population database of allele frequencies. The ideal forensic genotypic database would consist of more than 200 individuals from the population located geographically around the crime scene (46). For animal specimens, this would not always be available and because of expense, even small *ad hoc* databases such as detailed here will not always be feasible.

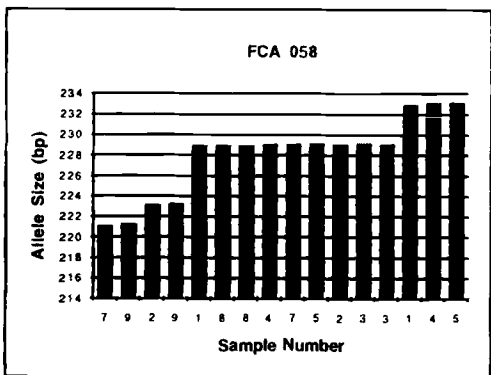
We propose a conservative method for estimating chance STR genotypic matches in the situation where no database exists for the species, but there occurs a putative match at N loci between the "evidence" and the "suspect." The high heterozygosity of STR loci in all species allows us to take a very simple and conservative approach toward estimating the likelihood of genotypic matching



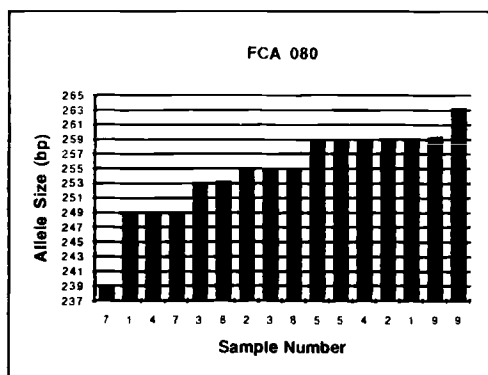
Bin*	Min (bp)	Max (bp)	Bin Range (bp)	Std Dev (bp)
D	143.54	143.65	0.11	0.05
F	145.57	145.79	0.22	0.11
F	147.79	148.00	0.21	0.15
H	152.01	152.05	0.04	0.02
J	157.87	NA†	NA	NA



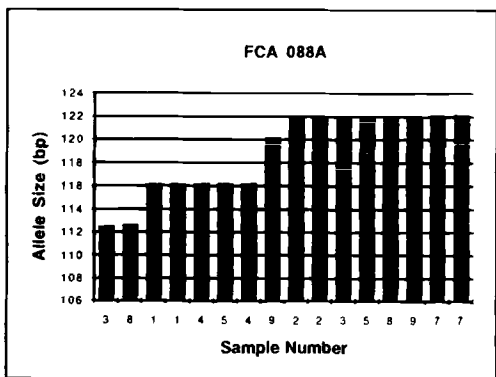
Bin	Min (bp)	Max (bp)	Bin Range (bp)	Std Dev (bp)
B	118.89	NA	NA	NA
C	120.54	120.90	0.36	0.15
D	126.32	126.52	0.2	0.10
E	128.37	NA	NA	NA



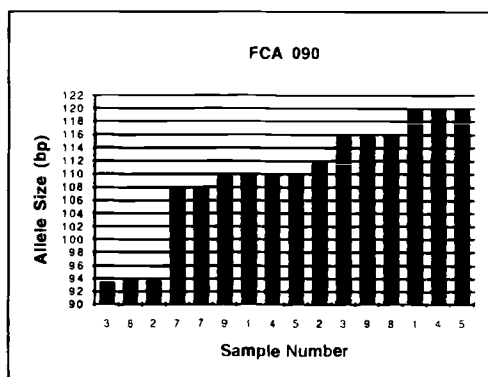
Bin	Min (bp)	Max (bp)	Bin Range (bp)	Std Dev (bp)
A	221.16	221.34	0.18	0.13
B	223.20	223.34	0.14	0.10
D	229.03	229.24	0.21	0.08
F	233.00	233.22	0.22	0.11



Bin	Min (bp)	Max (bp)	Bin Range (bp)	Std Dev (bp)
†	238.87	NA	NA	NA
A	249.21	249.21	0	0
C	253.26	253.40	0.14	0.10
D	255.17	255.30	0.13	0.08
F	259.00	259.43	0.43	0.17
H	263.39	NA	NA	NA



Bin	Min (bp)	Max (bp)	Bin Range (bp)	Std Dev (bp)
A	112.58	112.68	0.10	0.07
C	116.24	116.31	0.07	0.04
E	120.28	NA	NA	NA
F	122.09	122.21	0.12	0.05



Bin	Min (bp)	Max (bp)	Bin Range (bp)	Std Dev (bp)
A	93.55	93.78	0.23	0.13
C	107.92	107.92	0	0
D	109.80	110.11	0.31	0.14
†	112.04	NA	NA	NA
F	115.75	115.91	0.16	0.08
H	119.86	120.09	0.23	0.12

FIG. 3—Histograms and tables of allele size distributions for 10 feline STR loci amplified in proficiency test samples (1–8) and a positive control (sample 9). *: Bin designations are based on PEI and US reference surveys (Table 5) †: Allele not present in PEI or US survey. Min: minimum size observed for alleles in bin; Max: maximum size for alleles in bin; std dev: standard deviation; ‡: not applicable, as only a single allele was observed. For sample 6, product sizes observed were not in a size range, amplitude or profile characteristic of a feline STR amplification product and were suspected to be of non-feline species origin.

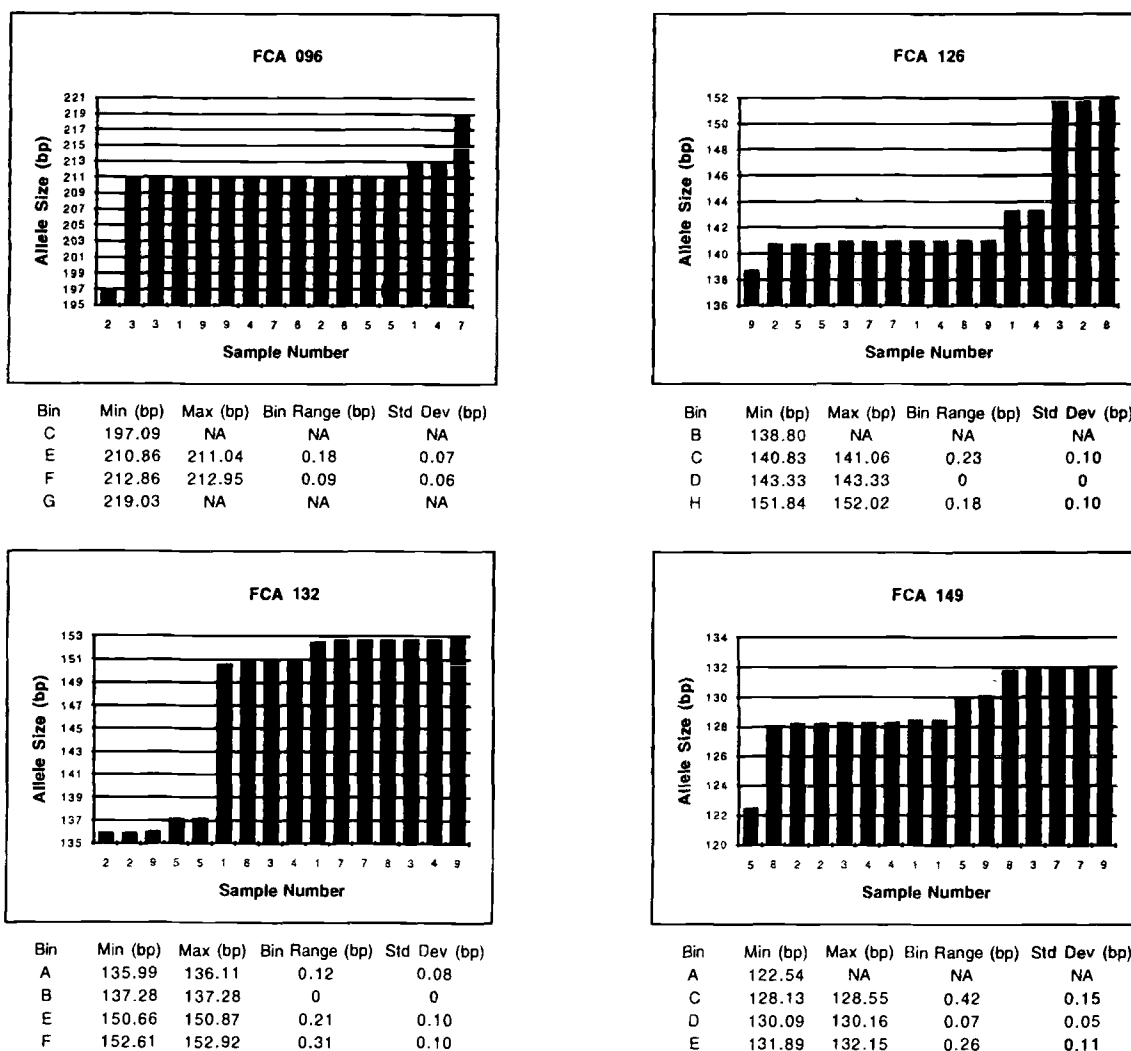


FIG. 3—Continued

by chance. First, we assume that the evidentiary sample is drawn from a population whose gene pool has exactly the gene frequencies found in that sample. That is, all homozygous loci in the evidence are considered to be monomorphic and uninformative in the population and all heterozygous loci are assumed to be polymorphic for two alleles found at an equal frequency of 0.5. We then can compute how often a second sample drawn from this gene pool will have the same genotype as the evidentiary sample. Each heterozygous locus has a 50% chance of exclusion, since a random sample would be homozygous for one of the two alleles 50% of the time. Hence, we can exclude non-matching genotypes with a high probability that increases as a function of the number of loci (Table 6).

Formally, we desire k heterozygous (informative) loci such that 2^{-k} is below a predetermined threshold, for example 0.05, 0.01, or 0.001. In this example, $k = 5, 7,$ and 10 heterozygous loci to achieve probabilities less than 0.05, 0.01, and 0.001, respectively (Table 6). How often these thresholds would occur is a function of the true population heterozygosity of STR loci and the number of loci surveyed. Mathematically, if L loci are sampled and each locus has probability h of being heterozygous, we can appeal to the binomial distribution:

$$\text{Prob} (k \text{ heterozygous loci}) = C(L,k)h^k(1 - h)^{L-k}$$

where $C(L,k)$ is the binomial coefficient. Since this is the probability of exactly k heterozygous loci among L loci typed, we want the upper tail of this distribution to be assured of having enough heterozygous loci to be confident when declaring matching genotypes. In Table 7 we show the probabilities of obtaining 5, 7, or 10 heterozygous loci for population heterozygosities from 0.5 to 0.9 and for sample sizes (L) from 10 to 30 loci. We see that for low population heterozygosities, we need to type 15 or more loci for modest significance ($k = 5$) and 25 or more loci to be reasonably sure of high significance ($k = 10$). On the other hand, if population heterozygosities are higher than 0.7, 20 loci is enough to almost guarantee high significance ($k = 10$). For the 10 loci selected here, PEI and Maryland have composite heterozygosities of 0.85 and 0.87 respectively, which means for 10 loci the two lower levels of significance are readily achieved ($p \geq 0.999$ for threshold of $p \leq 0.05$; $p \geq 0.95$ for threshold of $p \leq 0.01$). A highly significant threshold of $p \leq 0.001$ is not likely to be reached unless one is willing to sample more loci.

Clearly, our method sacrifices a large portion of the potential information in the true (but unknown) population allele frequencies. Yet, there remains sufficient information to achieve robust inclusion/exclusion probabilities. With continued improvement in the methodology, we view $p < 0.001$ as a reasonable target threshold for statistical significance since it comes from such a conserva-

TABLE 4—Comparison of STR genotypes for matching sample pairs 1,4 and 3,8.

STR Locus	Sample	Allele 1 (bp)	Allele 2 (bp)	Sample	Allele 1 (bp)	Allele 2 (bp)	Match Window (bp)
FCA 026	1	145.77	152.05	3	143.54	145.57	0.37
	4	145.79	152.02	8	143.65	145.65	
	Difference	0.02	0.03	Difference	0.11	0.08	
FCA 043	1	120.54	—*	3	120.59	126.32	0.59
	4	120.9	—	8	120.76	126.52	
	Difference	0.36	—	Difference	0.17	0.20	
FCA 058	1	229.03	233.00	3	229.24	—*	0.36
	4	229.12	233.16	8	229.05	—	
	Difference	0.09	0.16	Difference	0.19	—	
FCA 080	1	249.21	259.34	3	253.26	255.17	0.30
	4	249.21	259.16	8	253.40	255.30	
	Difference	0	0.18	Difference	0.14	0.13	
FCA 088A	1	116.24	—*	3	112.58	122.09	0.42
	4	116.31	—	8	112.68	122.10	
	Difference	0.07	—	Difference	0.10	0.01	
FCA 090	1	109.87	119.86	3	93.55	115.75	0.46
	4	109.99	119.90	8	93.77	115.91	
	Difference	0.12	0.04	Difference	0.22	0.16	
FCA 096	1	210.89	212.86	3	210.86	—*	0.25
	4	211.00	212.95	8	211.03	—	
	Difference	0.11	0.09	Difference	0.17	—	
FCA 126	1	141.06	143.33	3	141.01	151.84	0.53
	4	141.06	143.33	8	141.06	152.02	
	Difference	0	0	Difference	0.05	0.18	
FCA 132	1	150.66	152.61	3	150.87	152.82	0.27
	4	150.87	152.82	8	150.86	152.77	
	Difference	0.21	0.21	Difference	0.01	0.05	
FCA 149	1	128.55	—*	3	128.33	132.11	0.29
	4	128.33	—	8	128.13	131.89	
	Difference	0.22	—	Difference	0.20	0.22	

*Not applicable.

tive, sharply-defined database—that of the evidentiary sample itself. Under our procedure, 10 heterozygous loci in the evidentiary sample is enough to achieve this level of statistical significance. Family-law courts in Texas require any method of paternity testing to provide a power of exclusion of $\geq 99\%$ (47). Furthermore, STR studies in many different organisms show heterozygosities well above 0.5, suggesting that sample sizes on the order of 20 loci would ensure unequivocal results for the vast majority of studies. Typing of 20 loci should be readily achievable in future settings, even for relatively small evidentiary samples. While our proposed method might not be an adequate substitute when a well-defined population database exists, it would seem to be a viable alternative for exotic populations or species, or even for familiar but genetically uncharacterized species.

Results of Test

Dr. Ron Fournay, RCMP, who supplied the proficiency test of 8 specimens confirmed that all matching cat samples were correctly identified and that sample 6 was a human blood sample.

Discussion

We have developed and tested a method for genetic individualization of domestic cats based on the generation of composite genotypes from a panel of 10 feline STR loci. The STR panel has the discriminating potential to identify matching feline DNA genotypes from a blind panel of coded blood samples and the sensitivity for genetic individualization from single hair analyses. We have calculated the probability of match (P_m), the probability of two unrelated individuals matching by chance, at all ten loci

as 1.35×10^{-10} using the convention of Jones (48) based on allele frequency estimates of a small sample of cats from Prince Edward Island, Canada. Multiplex amplification of five loci of the STR panel can be accomplished with as little as one nanogram of DNA. The P_m of the five STR multiplex set is 5.24×10^{-6} .

A “match window” was empirically established for each STR locus based on determining the measurement precision among size estimations of allele fragments judged to be identical by descent in a 3-generation 70-member feline pedigree. In a population survey, alleles in the same bin are contributed by individuals who are unrelated, while the match window was calculated from a comparison of alleles judged to be identical from a Mendelian reference. It is interesting to note that the size of the match window for each STR locus was in many cases smaller than the bin size range for that STR locus in the population survey (Table 2 and Table 5). The greater size range of the bin may reflect migrational differences due to sequence variation in the region flanking the STR locus among alleles contributed by unrelated individuals.

STR primer pairs have been observed to amplify DNA of species separated by large evolutionary distances (49). The unanticipated inclusion of a human sample in the proficiency test allowed us to demonstrate that the feline STR panel does not generate amplification products from human DNA which are of a size, amplitude or signature of a feline STR locus. Hence contaminating human DNA would not complicate a feline genotype profile.

Tetranucleotide repeat STR loci are typically utilized in the forensic genotyping of humans due to the minimal generation of artifactual “stutter” bands in PCR amplification (39). “Stutter” bands are generally more pronounced in the amplification of dinucleotide STR loci and can potentially complicate the genotyping

TABLE 5—Allele frequency distributions for 10 feline STR loci in 2 reference surveys.*

STR Locus	Allele Size	Bin	PEI Survey		US Survey		Allele Frequency	
			Allele Count	Range	Allele Count	Range	PEI Survey	US Survey
FCA 26	127	A	1	NA†	0	NA	0.03	0.00
	135	B	1	NA	2	0.41	0.03	0.11
	139	C	1	NA	0	NA	0.03	0.00
	143	D	7	0.41	5	0.22	0.18	0.28
	145	E	6	0.12	2	0.29	0.16	0.11
	147	F	16	0.31	5	0.26	0.42	0.28
	149	G	2	0.25	0	NA	0.05	0.00
	151	H	2	0.05	2	0.27	0.05	0.11
	153	I	2	0.00	1	NA	0.05	0.06
	157	J	0	NA	1	NA	0.00	0.06
FCA 43	118	A	0	NA	2	0.00	0.00	0.11
	119	B	7	0.23	2	0.15	0.18	0.11
	120	C	5	0.19	2	0.10	0.13	0.11
	126	D	20	0.35	9	0.36	0.53	0.50
	128	E	1	NA	3	0.36	0.03	0.17
	130	F	1	NA	0	NA	0.03	0.00
	132	G	4	0.29	0	NA	0.11	0.00
FCA 58	220	A	7	0.34	4	0.17	0.18	0.22
	222	B	7	0.25	5	0.10	0.18	0.28
	224	C	1	NA	0	NA	0.03	0.00
	228	D	21	0.36	6	0.79	0.55	0.33
	230	E	1	NA	2	0.86	0.03	0.11
	232	F	1	NA	1	NA	0.03	0.06
FCA 80	249	A	2	0.05	0	NA	0.05	0.00
	251	B	2	0.18	2	0.01	0.05	0.11
	253	C	0	NA	3	0.77	0.00	0.17
	255	D	3	0.30	2	0.19	0.08	0.11
	257	E	18	0.54	8	0.33	0.47	0.44
	259	F	11	0.32	2	0.26	0.29	0.11
	261	G	2	0.03	0	NA	0.05	0.00
	263	H	0	NA	1	NA	0.00	0.06
	263	I	0	NA	1	NA	0.00	0.06
FCA 88A	111	A	3	0.16	1	NA	0.08	0.06
	113	B	5	0.30	6	0.01	0.13	0.33
	115	C	3	0.14	1	NA	0.08	0.06
	117	D	0	NA	1	NA	0.00	0.06
	119	E	10	0.41	2	0.46	0.26	0.11
	121	F	5	0.47	3	0.53	0.13	0.17
	123	G	2	0.22	0	NA	0.05	0.00
	125	H	7	0.21	3	0.09	0.18	0.17
	127	I	3	0.00	1	NA	0.08	0.06
	93	A	7	0.26	4	0.15	0.18	0.22
	104	B	2	0.18	0	NA	0.05	0.00
FCA 90	107	C	2	0.15	0	NA	0.05	0.00
	109	D	4	0.31	3	0.01	0.11	0.17
	113	E	3	0.25	0	NA	0.08	0.00
	115	F	14	0.31	6	0.19	0.37	0.33
	117	G	1	NA	2	0.00	0.03	0.11
	119	H	3	0.15	3	0.23	0.08	0.17
	121	I	2	0.12	0	NA	0.05	0.00
	183	A	0	NA	2	0.00	0.00	0.11
	185	B	2	0.00	0	NA	0.05	0.00
	197	C	4	0.05	0	NA	0.11	0.00
FCA 96	209	D	2	0.19	0	NA	0.05	0.00
	211	E	28	0.32	11	0.26	0.74	0.61
	213	F	1	NA	1	NA	0.03	0.06
	219	G	1	NA	1	NA	0.03	0.06
	221	H	0	NA	1	NA	0.00	0.06
	223	I	0	NA	2	0.01	0.00	0.11
	137	A	1	NA	0	NA	0.03	0.00
	139	B	5	0.21	4	0.71	0.13	0.22
	141	C	18	0.29	6	0.61	0.47	0.33
FCA 126	143	D	6	0.11	2	0.10	0.16	0.11
	145	E	1	NA	3	0.30	0.03	0.17
	148	F	0	NA	1	NA	0.00	0.06
	150	G	4	0.00	2	0.00	0.11	0.11
	152	H	3	0.23	0	NA	0.08	0.00
	136	A	12	0.26	9	0.22	0.32	0.50
	138	B	3	0.22	2	0.27	0.08	0.11
	140	C	8	0.43	2	0.01	0.21	0.11
FCA 132	143	D	1	NA	2	0.00	0.03	0.11

TABLE 5—Continued

STR Locus	Allele Size	Bin	PEI Survey		US Survey		Allele Frequency	
			Allele Count	Range	Allele Count	Range	PEI Survey	US Survey
FCA 132	149	E	3	0.04	0	NA	0.08	0.00
	151	F	2	0.00	1	NA	0.05	0.06
	153	G	4	0.04	1	NA	0.11	0.06
	155	H	3	0.00	1	NA	0.08	0.06
	157	I	2	H‡	0	NA	0.05	0.00
FCA 149	122	A	6	0.25	0	NA	0.16	0.00
	124	B	5	0.17	5	0.14	0.13	0.28
	128	C	10	0.19	5	0.16	0.26	0.28
	130	D	3	0.00	2	0.19	0.08	0.11
	132	E	10	0.07	6	0.13	0.26	0.33
	134	F	4	0.02	0	NA	0.11	0.00

*A control sample (NCI 123), which was used as the positive cat DNA control in the proficiency test, was included as a reference sample in both population surveys.

†NA—not applicable.

‡H—the 2 alleles come from a single homozygous individual.

TABLE 6—Frequency of composite STR genotypes in a hypothetical population based upon allele frequencies identical to that of a single individual.

Number of Heterozygous Loci in Evidence	Frequency of Composite Genotype = Probability of a Chance Match	Number of Heterozygous Loci in Evidence	Frequency of Composite Genotype = Probability of a Chance Match
1	0.5	11	4.88×10^{-4}
2	0.25	12	2.44×10^{-4}
3	0.125	13	1.22×10^{-5}
4	6.25×10^{-2}	14	6.10×10^{-5}
5	3.13×10^{-2}	15	3.05×10^{-5}
6	1.56×10^{-2}	16	1.53×10^{-5}
7	7.81×10^{-3}	17	7.63×10^{-6}
8	3.91×10^{-3}	18	3.81×10^{-6}
9	1.95×10^{-3}	19	1.91×10^{-6}
10	9.77×10^{-4}	20	9.54×10^{-7}

TABLE 7—Probabilities of obtaining a minimum number of heterozygous loci (k = 5, 7, or 10) for representative population heterozygosities (h, 0.5–0.9) and for a range of loci typed (L, 10–30).

L	Population Heterozygosity (h)								
	0.50	0.55	0.60	0.65	0.70	0.75	0.80	0.85	0.90
<i>k</i> = 5 (p < 0.05)*									
10	0.623	0.738	0.834	0.905	0.953	0.980	0.994	0.999	1.000
15	0.941	0.975	0.991	0.997	0.999	1.000	1.000	1.000	1.000
20	0.994	0.998	1.000	1.000	1.000	1.000	1.000	1.000	1.000
25	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
30	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
<i>k</i> = 7 (p < 0.01)*									
10	0.172	0.266	0.382	0.514	0.650	0.776	0.879	0.950	0.987
15	0.696	0.818	0.905	0.958	0.985	0.996	0.999	1.000	1.000
20	0.942	0.979	0.994	0.998	1.000	1.000	1.000	1.000	1.000
25	0.993	0.998	1.000	1.000	1.000	1.000	1.000	1.000	1.000
30	0.999	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
<i>k</i> = 10 (p < 0.001)*									
10	0.001	0.003	0.006	0.013	0.028	0.056	0.107	0.197	0.349
15	0.151	0.261	0.403	0.564	0.722	0.852	0.939	0.983	0.998
20	0.588	0.751	0.872	0.947	0.983	0.996	0.999	1.000	1.000
25	0.885	0.956	0.987	0.997	1.000	1.000	1.000	1.000	1.000
30	0.979	0.995	0.999	1.000	1.000	1.000	1.000	1.000	1.000

*Based on Table 6.

of mixed samples. The feline typing system was selected from 400 dinucleotide STR markers which are being incorporated into a genetic recombination map of the cat. Utilizing a high resolution genotyping system which quantitates PCR products, we have had no difficulty in discriminating "stutter" bands versus allele bands in genotype measurements for over 25,000 samples. Feline forensic material is likely to consist of discrete, single hair specimens, as opposed to mixed samples and as such the dinucleotide STR markers would be adequate. The utility of the feline forensic typing system will be largely dependent on the quality of hair specimens and the presence of a hair root, as STR loci are amplified from genomic DNA isolated from the hair root and surrounding sheath cells. In a direct application of the feline panel, 19 of 28 cat hairs associated with a crime scene were observed with roots; DNA was extracted and the ten STR loci amplified from one of four single hair extractions (50). Although report has been made of nuclear sequences amplified from hair shaft extracts (51), mitochondrial DNA (mtDNA) sequences are more successfully amplified from hair specimens without roots (44,52). MtDNA polymorphic sites are a proven source of forensic utility in human beings (53-55). The complete sequence of the cat mitochondrial DNA genome, including regions of high sequence polymorphism has recently been reported (56,57). Under circumstances in which only feline hair shafts are available, selected feline mtDNA regions could be amplified and examined on an exclusion basis. STR genotyping of human samples is rapidly becoming an invaluable resource for forensic analyses. Prior to PCR-based methods of genotyping, Southern blot format VNTR typing typically required tens of nanograms of high molecular weight genomic DNA to generate composite genotypes. STR genotyping is now being applied to a range of biological material which would not have been considered appropriate for analysis before due to the quantity or quality of DNA which could be extracted from the sample. The implementation of STR in forensic applications offers an important advance over other methods in sensitive ascertainment and statistical power. The growing availability of species-specific STR loci incorporated in genetic maps of common domestic animals creates enormous forensic potential for the genetic individualization of species which are integral parts of our daily lives.

Acknowledgments

We gratefully acknowledge the assistance of Dr. Ron Fourney, RCMP, Central Forensic Laboratory for supplying the proficiency test samples, and evaluating the results of the test. We acknowledge A. E. Evers for critical evaluation of the manuscript, Dr. Jane Bondt for collection of the PEI domestic cat blood samples, and to Constable Roger Savoie, RCMP who initiated our interest in the forensic application of cat STR loci. We also acknowledge Deborah Lomb for conducting the Fisher's exact test analysis for independence of STR loci, and Dr. Lisa Forman for helpful advice. The primer pair for amplification of a human STR locus was generously donated by Dr. Michael Smith.

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