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Aim To conduct developmental validation studies on a polymerase chain reaction (PCR) based short tandem repeat (STR) multiplex typing system, developed for the purpose of genetic individualization and parentage testing in domestic cat samples.

Methods To evaluate reproducibility of the typing system, the multiplex was amplified using DNA extracted from hair, blood, and buccal samples obtained from the same individual (n = 13). Additional studies were performed to evaluate the system's species' specificity, using 26 North American mammalian species and two prokaryotes *Sacchromyces* and *Escherichia coli*, sensitivity, and ability to identify DNA mixtures. Patterns of Mendelian inheritance and mutation rates for the 11 loci were directly examined in a large multi-generation domestic cat pedigree (n = 263).

Results Our studies confirm that the multiplex system was species-specific for feline DNA and amplified robustly with as little as 125 picograms of genomic template DNA, demonstrating good product balance. The multiplex generated all components of a two DNA mixture when the minor component was one tenth of the major component at a threshold of 50 relative fluorescence units. The multiplex was reproducible in multiple tissue types of the same individual. Mutation rates for the 11 STR were within the range of sex averaged rates observed for Combined DNA Index System (CODIS) loci.

Conclusion The cat STR multiplex typing system is a robust and reliable tool for the use of forensic DNA analysis of domestic cat samples.

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In the field of forensic DNA analysis, new methods and technology have revolutionized the analysis and detection of genetic variation for human identification (1-3). These advances have been extended to the analysis of DNA extracted from non-human specimens such as plants, bacteria, viruses, and domesticated animals (4). Genetic individualization of animal specimens has increasingly been included as key evidence in criminal investigations (5-11). It has been reported that pet hairs are invariably transferred to the clothing of those visiting the home of a pet owner (12). With approximately 73 million cats residing in one third of households in the United States (13), it is not surprising that cat hairs are often part of the physical evidence associated with crime scenes.

Recently, a polymerase chain reaction (PCR) based short tandem repeat (STR) multiplex typing system has been developed for the use in genetic individualization and parentage testing of domestic cat specimens (14). The system simultaneously amplifies 11 polymorphic tetranucleotide STR loci and one gender identifying sequence tagged site on the Y chromosome Sex-Determinig Region Y gene (SRY gene). The STR markers have been mapped in radiation hybrid and/or genetic linkage maps of the domestic cat (15,16) and were selected for forensic analysis as they are unlinked, amplify robustly, exhibit Mendelian inheritance, and exhibit a high degree of heterozygosity in cat breeds (14).

Following the standard 8.1.2.2 of the quality assurance standards for DNA analysis recommended by the DNA Advisory Board (DAB) (17) and recommendations made for animal DNA forensic and identity testing (18), developmental validation studies were conducted on the cat STR multiplex typing system. These validation studies are required prior to the application of a new genetic forensic typing system to analysis of evidentiary samples, to ensure the accuracy, precision, and reproducibility of the system.

Material and methods

Samples

Blood, buccal, and hair samples were obtained from each of 13 mixed breed domestic cats acquired from Liberty Laboratories, (Waverly, NY, USA) and maintained as members of the cat colony of the Laboratory of Genomic Diversity at the National Institutes of Health Animal Care Facility, Frederick, MD, USA. The mixed breed domestic cat samples included individuals; Fca 926, Fca 915, Fca 2976, Fca 4405, Fca 4084, Fca 4048, Fca 4415, Fca 2996, Fca 4081, Fca 2450, Fca 2454, Fca 2455, and Fca 2457. DNA samples Fca 215 and Fca 123 were supplied from the National Cancer Institute-Frederick DNA stocks, extracted from tissue samples and maintained as stocks for use as standard reference samples. FCA 123 is a mixed breed domestic; Fca 215 is DNA extracted from a tissue sample of a Persian cat supplied by Dr Stephen J. DiBartola, Ohio State University; Columbus, OH, USA.

For the estimation of the mutation rate of the STR loci, DNA samples were obtained from a large multi-generation domestic cat pedigree (n=256) supplied by the Nestlé Purina PetCare Company (19). For the study of species specificity of the multiplex, ocelot and puma DNA samples were provided by Dr Warren Johnson, National Cancer Institute, Frederick, MD, USA. Dr William Murphy, Texas A &M University; College Station, TX, USA, kindly provided the remainder of the carnivore samples. *Saccharomyces cervisae* genomic DNA was obtained from Invitrogen (Carlsbad, CA, USA) and *E coli* strain B DNA was obtained from Sigma (St. Louis, MO, USA).

To confirm the species' identity of each sample, the 16S mitochondrial region was amplified with universal PCR primers and sequenced as previously described (20). The sequences obtained were compared to sequences

available on the NCBI database of amplified and sequenced mitochondrial DNA from the 16S region (20).

DNA extraction and quantitation

DNA from blood and buccal samples were extracted using Qiagen QiAmp® DNA Blood Midi and Mini Extraction Kits (Qiagen, Hilden, Germany) following the suggested protocols of the manufacturer. DNA was quantified using a Hoefer DyNA Quant 200 Flurometer (Amersham BioSciences, Piscataway, NJ, USA).

DNA was extracted from hair samples essentially as described in the Department of Defense DNA Registry protocol Organic Extraction of DNA from hair roots (21). In a 2 mL microcentrifuge tube, a single hair with root was washed twice with 1 mL of 5% Terg-azyme (Alconox, White Plains, NY, USA) for 10 minutes, then with 1 mL of absolute ethanol, and finally with 1 mL of deionized water. Hair samples were washed by gentle agitation several times and solutions were removed between each wash. Then, 2 mL of lysis buffer containing; 10mM Tris-HCL (pH8.0), 100 mM NaCl, 50 mM EDTA (pH 8.0), 0.5% SDS, 40 mM dithiothreitol (DTT), and 0.5 mg/mL Proteinase K (Invitrogen), was added to hair samples and digested for 2-24 hours at 56°C. The digest was extracted once with 200 µL phenol/chloroform/isoamyl alcohol (25:24:1) (Invitrogen) and once with 200 µL *n*-butanol. The aqueous phase was washed four times with TE buffer (pH 8.0) and concentrated in a Microcon YM-50 Centrifugal Filter Device (Amicon Inc., Beverly, MA, USA).

PCR amplification was conducted as reported for the cat multiplex (14).

Desalting of PCR products

To remove degradation products of the fluorescent dyes which can interfere with genotyping, samples were centrifuged through a 96 well Multiscreen HV Plate (Millipore, Billerica, MA, USA) containing G-50 Sephadex (Amersham BioSciences). The 96 well Multiscreen HV plates were prepared by loading G-50 Sephadex into each well, followed by the addition of 300 µL molecular grade water (Quality Biological, Gaithersburg, MD, USA) to allow the resin to swell. Plates were then incubated at room temperature for 3 hours and used immediately or stored in the refrigerator at 4°C overnight.

To remove the water, the Multiscreen HV plates were placed on top of a standard 96-well microplate and centrifuged at 2000 rpm for 2 minutes. A clean standard 96-well microplate was replaced and the PCR product samples were then carefully added to the center of the Multiscreen HV plate wells and centrifuged at 2000 rpm for 2 minutes. The elute was retained.

Allele detection and data analysis

After filtration, 3 µL of a 1:15 dilution of each amplified product were combined with 0.22 µL GS500 LIZ (Applied Biosystems) and 8.78 µL Hi-Di formamide (Applied Biosystems). The samples were electrophoresed on an ABI 3100 Genetic Analyzer using the following parameters; Dye Set: G5, Default Run: Module GeneScan36_POP4DefaultModule, which performs an electrokinetic injection for 10 seconds at 3000 V, allele separation at 15 000 V for 30 minutes, Run temperature: 60°C using the 3100 POP 4 sieving polymer (Applied Biosystems), 1X Genetic Analyzer Buffer with EDTA and a 36 cm array. Data was collected using the ABI 3100 Data Collection Software, version 1.0.1 and analyzed using the GeneScan, version 3.7 Analysis Software and GenoTyper Version 2.5 Software. Allele calls were binned using the Allelogram software (*http://groups.yahoo.com/group/allelogram/*). Pedigree genotypes were analyzed for any Mendelian inconsistencies using PedCheck analysis software developed by O'Connell and Weeks (22).

Results

Species specificity studies

Due to the potential difficulty in ascertaining the species identity of hair samples, as well as the possibility of trace contamination of samples with DNA from other species and microorganisms, we first determined the species specificity of the multiplex. The eleven STR loci were examined in a range of North American mammalian species and two prokaryotes. The primers were amplified under the amplification conditions routinely used in the cat multiplex in genomic DNA of dog, deer, rabbit, guinea pig, hamster, mouse, horse, cow, pig, ferret, mink, sheep, goat, brown bear, fox, badger, wolf, human, beaver, otter, raccoon, possum, skunk, mole, coyote, chipmunk, and prokaryotic species, *Sacchromyces* and *Escherichia* coli (web table 1).

The multiplex displayed a high degree of specificity for DNA in the felid family with products observed in domestic cat (11 loci), puma (7 loci), and ocelot (8 loci) (Figure 1). Additionally, amplification products were observed for two loci (FCA723, FCA733), in the brown bear, another member of the Carnivore order (Figure 1). Although these products were not sequenced for verification, they demonstrated products in the expected size range, and the characteristic "stutter band" amplification artifacts of an STR product (23). No amplification products were observed, under the standard amplification conditions of the multiplex, in any other mammalian species or in *E. coli* or *S. cervesiae*.

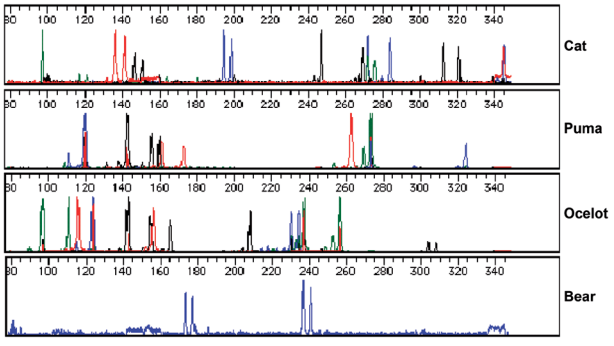

Figure 1. Amplification products observed in domestic cat, puma, ocelot, and bear DNA samples using the domestic cat short tandem repeats (STR) multiplex genotyping system.

Sensitivity studies

Under conditions of limiting DNA template, stochastic effects are often observed, in which a single allele of a heterozygous individual is amplified (24). It is, therefore, critical in a forensic typing system to determine the "limits of the system," the DNA concentration at which stochastic effects may be observed. Two male cat samples Fca 215 and Fca 926 were serially diluted from 10 ng to 8 fg and amplified with the STR multiplex. For all loci, STR product amplitude decreased with decreasing template concentration (Figure 2; web table 2).

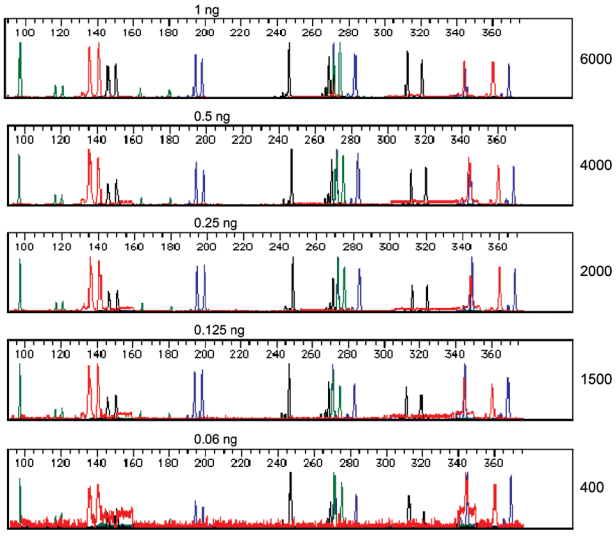

Figure 2. Amplification products from template DNA concentrations of 0.06, 0.125, 0.25, 0.5, and 1.0 ng. The y-axis scale varies in most of the panels and is indicated in relative fluorescence units (RFU) on the right of the figure.

With a minimum threshold of 50 relative fluorescent units (RFU), a quantity of 0.125 ng was required in the two sample DNAs to detect both alleles of heterozygous loci in the 11 STR loci. The gender identifying SRY locus, which has consistently been observed as the most "robust" locus in the multiplex, was detected at 0.06 ng in the Fca 926 sample and 0.03 ng in the Fca

215 sample. "Allele dropout," or the stochastic amplification of one allele of a heterozygote, was observed at DNA concentrations below 0.06 ng in both samples for all 11 STR loci.

Mixture studies

Human DNA evidentiary samples often originate from more than one individual and the sensitivity of the Combined DNA Index System (CODIS) loci to detect all components of mixtures has been carefully investigated (25-29). Although domestic cat specimens are more likely to be discrete samples (hairs), and hence less likely to generate a mixed DNA sample, the grooming practices of cats open the possibility of mixed DNA specimens from domestic cats. The ability and limitations of the multiplex system to identify mixtures was evaluated. Mixtures of two purified DNA samples in defined ratios were amplified and analyzed following the multiplex protocol. Two sets of DNA mixtures were examined both containing ratios at 1:1, 1:3, 1:5, 1:10, 1:20, 1:30, 1:40, 1:50, 3:1, 5:1, 10:1, 20:1, 30:1, 40:1, and 50:1 for the major and minor components (27). The total amount of template DNA was maintained at 2 ng. Set one contained a male cat DNA (Fca 215): female cat DNA (Fca 123) mixture; set two mixture contained domestic cat DNA (Fca 215): human DNA.

Table 1 demonstrates the detection threshold of the minor component as a fraction of the major component for the two cat DNA mixture. Web table 3 presents product profiles for the 2 samples in the two reciprocal mixtures. With the minimal threshold of 50 RFUs, amplification products were detectable for five of the STR (FCA723, FCA733, FCA740, F124, FCA441) if the minor component was present at 10% of the major component. For four STR loci (FCA742, FCA736, F53, FCA731), the minor component was detectable if it was 20% of the major component; while locus FCA749 products were not detected unless the minor component was 50% of the major component. Products of the SRY locus could be detected when the minor component was 2% of the major component in both mixture sets.

In the Fca 215:Human mixture, amplification products were observed only for the domestic cat sample (web table 3). With the minimal threshold of 50 RFUs, amplification products were detectable for five of the STR (FCA723, FCA733, F124, F85, and FCA731) if the domestic cat DNA component was present at 2% of the mixture. In another five of the STR (FCA742, FCA740, FCA736, F124, and FCA441) the domestic cat DNA component was detected if it was 5% of the mixture, while locus FCA749 products were not detected unless the domestic cat DNA component was 10% of the mixture.

Table 1. Minimum detection thresholds (% DNA in mixture) observed for minor component in a two DNA mixture for 12 loci in the domestic cat multiplex

Locus	Threshold FCA 215: FCA 123 (%)*
FCA723	10
FCA733	10
FCA742	20
FCA749	50
FCA740	10
FCA736	20
F124	10
F53	20
F85	20
FCA441	10
FCA731	20
SRY	2

*Fca 215 is the minor component mixed with Fca 123 at ratios from 1:1-1:50 (100%-2% detection threshold). The total amount of template DNA was maintained at 2 ng with a minimal threshold of 50 relative fluorescence units (RFU) required for detection.

Somatic studies

DNA extracted from blood, buccal, and single hair isolates from 13 different domestic cats were examined to confirm that the multiplex amplification profiles generated from DNA of different tissue types of the same individual would be consistent. PCR amplification profiles generated from DNA extracted from blood and buccal samples were identical for the 11 STR loci and the SRY gene for all 13 domestic cat samples (web table 4). Amplification products were observed for only 7 of the 12 loci (FCA733, FCA742, FCA740, F53, F85, FCA731, and

Table 2. Summary of mutation rates and null alleles observed for the 12-plex loci based on meiotic events examined in a multi-generation domestic cat pedigree

Locus	No. of observed mutations	Proposed mutation source*	Mutation rate (sex averaged)	Null alleles observed
FCA723	0		<0.002	No
FCA733	1	Dam-1 rp	0.002	No
FCA742	2	Sire +1 rp /Sire or Dam -1 rp	0.004	No
FCA749	2	Dam +1 rp/Dam +2 rp or Sire -1 rp	0.004	No
FCA740	0		<0.002	No
FCA736	2	Sire -2 rp or Null/Sire +2 rp or Dam -2 rp	0.004	Yes
F124	3	Dam +2 rp/Dam -2 rp/Sire +2 rp or Dam -2 rp	0.006	No
F53	2	Sire +1 rp/Dam -1 rp	0.004	Yes
F85	1	Dam -1 rp	0.002	Yes
FCA441	2	Sire +1 rp/Dam +1 rp	0.004	No
FCA731	0		<0.002	No
SRY	0		<0.002	No

*rp – repeat units

SRY) in DNA isolated from hair isolates (web table 4). Locus FCA733 amplified in DNA extracted from two individuals (Fca 4415 and Fca 2996); the remaining 6 loci, amplified in DNA isolated from a single individual (Fca 2996) (web table 4).

Reproducibility studies

The ability of the multiplex system to be able to reproduce consistent results in repeated trials was examined. DNA samples from a single individual (Fca 215) in a range of concentrations (0.125, 0.25, 0.5, 1.0, and 2.0 ng) were amplified 19 times. All 95 reactions exhibited the identical genotype (web table 5).

Mendelian inheritance and mutation rate studies

The Technical Working Group on DNA Analysis Methods (TWGDAM) committee advises that DNA loci used in forensic testing should be validated by family studies to demonstrate that they exhibit Mendelian inheritance and a low frequency of mutation (28). To verify Mendelian inheritance and directly measure the mutation rate of the individual STR loci, multiplex profiles were generated and examined in a large multi-generation domestic cat pedigree (n = 256 specimens, 483 meiotic events).

Genotypes for all 12 loci demonstrated patterns consistent with Mendelian modes of inheritance. Mutations were observed in seven loci (FCA733, FCA742, FCA749, F124, F53, F85, and FCA441). Null alleles were observed in three loci (FCA736, F53, and F85). Observed mutation rates (sex averaged) and null alleles are reported in Table 2.

Discussion

The developmental validation studies conducted on the domestic cat STR multiplex system demonstrated that the assay is robust, reproducible, and reliable for forensic identification. The multiplex is highly specific for the felid family (domestic cat, puma, ocelot) and a related taxa (brown bear) in the Carnivore order. Amplification products were expected in other members of the Felidae, as STR primers designed in the domestic cat have previously been observed to amplify the homologous site in a range of felid species with high fidelity (29-32). The generation of STR products in brown bear with primers designed in the domestic cat was not unexpected. The brown bear, as the cat, are both members of the order Canivora (33). The practice of using heterologous primers to amplify homologous loci in related taxa is widely observed (31).

Reliable profiles were observed in DNA concentrations as low as 0.125 ng and optimally between 1-2 ng of genomic DNA. These results are similar to those observed with the CODIS loci, which require a minimal amount of 0.2 ng and optimal amount of 1-2.5 ng of sample DNA (34,35). In regard to mixture studies, the domestic

cat STR multiplex system also demonstrated similar results to those observed with the CODIS loci. Both systems can detect a DNA mixture within a 1 ng sample whose minor component is one tenth of the major component (27,35).

Similar to somatic studies conducted on the CODIS loci (36), the domestic cat multiplex loci exhibited no differences in amplification profiles from DNA of different tissue types of the same individual. However, in single hair isolates only 1 of the 13 cat samples produced 7 of the 12 STR loci. The poor amplification observed in hair samples is most likely due to limited amount of DNA template extracted from single hair isolates. The amount of DNA which can be extracted from fresh hair isolates of a domestic cat has been estimated as 10-30 ng using spectrophotometric and gel based assays (6,37).

All the DNA from each single hair extraction was used in a multiplex amplification. The failure of the majority of the loci to amplify is not understood but is under examination. We are presently examining different methods of DNA extraction for single hair isolates. Pfeiffer et al (38) report on a Ca^{2+} improved DNA-extraction method with which they have observed increased success with extraction and amplification of DNA from dog hairs. They report a 0.35 ng average DNA content observed for 10 dog hairs. Additionally, we have developed quantitative PCR based assay which targets the highly repetitive nuclear short interspersed nuclear elements (SINE) elements in the domestic cat to estimate genomic DNA yields from single hair isolates (39).

PCR failure of DNA extracted from hair isolates could result from the presence of PCR inhibitors present in hair pigments (37). We have previously observed significantly different results in amplification success between DNA extracted from hair isolates of different cats (37). The 12 cats that failed repeatedly are mixed breed in nature, unrelated, and do not share any obvious hair color (ie, black hair) or even common hair phenotype. Amplification success in Fca 2996 may reflect a factor associated with the individual's hair root size, color, or personal grooming habits.

As reported in Table 2, results show that the mutation rates for the 11 STR are within the range of sex averaged rates observed for CODIS loci (40,41). The overall ability for detection of mutation rate, given the maximal number of informative meioses is 0.3%. In our study, mutation rates were observed more frequently in females than males. This is contrary to what has been observed in human studies, where STR male mutational events are observed more frequently in males than females (40,41). The cause of this difference is not understood and should be examined. Also, further investigation of loci FCA736, F53, and F85 is required due to the observation of null alleles, which complicate match probability calculations.

In conclusion, we have demonstrated that the cat multiplex is highly specific for felid DNA, reproducible in multiple tissue types of the same individual, and demonstrates a robustness comparable to the CODIS loci with respect mutation rate and the ability to detect mixtures. Additionally, the multiplex is very robust, generating a complete profile with 0.125 ng of genomic DNA. The multiplex system was designed to be easily adaptable to forensic laboratories currently performing human DNA typing.

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