Forensic DNA Identification of Animal-derived Trace Evidence: Tools for Linking Victims and Suspects

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Aim
To evaluate the population substructure of purebred dogs and cats in order to estimate the true significance of a microsatellite-based DNA match for use as evidence in legal proceedings. The high frequency of animal hair as a forensic evidence submission necessitates the development of mitochondrial analysis tools as well.

Methods
Random samples from a large convenience collection of veterinary diagnostic submissions from the western USA were used, as well as contributed samples of unrelated purebred cats and dogs. Dogs (n = 558) were profiled with 17 microsatellites and the data evaluated for Hardy Weinberg and linkage equilibrium. The mitochondrial control region (D loop) of dogs (n = 348) and cats (n = 167) was sequenced to determine the haplotype distribution.

Results
Domestic dogs in the western United States showed significant population substructure with marked associations within loci but no disequilibrium between loci. A population substructure coefficient \( F_{ST} = 0.11 \) is recommended for calculating genotype frequencies. Mitochondrial haplotypes in cats and dogs show less variation than human haplotypes.

Conclusion
Although population substructure occurs in domestic dogs (and can be inferred in cats), the discriminatory power of microsatellite analysis is dramatic with even partial DNA types, strongly supporting the prosecution of perpetrators in five discussed cases. Mitochondrial analysis, while less powerful, adds a layer of evidence in four discussed cases.

Animal hair is a common finding at crime scenes, however, it is often overlooked as a potential form of evidence. A large percentage of people in western countries own cats and dogs. Approximately 50% of US households own at least one dog or cat. In European countries, from 5-10% of people own at least one dog and 8-16% own at least one cat. Moreover, people are often very close to their pets. Pet hair can be found on clothing, in homes, and in cars, and is easily transferred in daily activities. With the number of household pets in western countries, it is reasonable to predict that an exchange of pet hair between a crime victim and a suspect is likely. Depending on the circumstances, the finding of pet hairs may be probative, ie, animal hair can link a suspect to a crime scene.

Hair evidence has been made more powerful with the advancement of DNA testing; identification of an individual from a single hair is feasible. Human DNA identification using short tandem repeat loci (STRs or microsatellites) is the method of choice due to the high information content of these markers, the standardization of STR loci in the forensic community, and the commercial availability of STR kits. Moreover, canine identification with STR loci has been widely re-
searched and applied in pedigree verification. Since 1996, STR analysis of canine trace evidence has been performed in over 20 criminal investigations and presented in 7 homicide trials. As investigators become aware of this technology, the number of cases in which this evidence plays a role is expected to increase.

Most animal hairs recovered as trace evidence are naturally shed and are in the telogen stage of development. They generally have root ends but no adherent hair bulb material. If STR typing fails, the control region (D loop) of the mitochondrial genome can be typed as a means of exclusion or inclusion. There are hundreds to thousands of mitochondria in the hair-forming cells of the hair root bulb that are deposited in the hair shaft. Thus, the mitochondrial DNA genome is present in higher copy numbers than nuclear DNA and is often less degraded in aged samples. Mitochondrial analysis may be useful in an investigation when STR amplification is unsuccessful.

The first technology used for genetic identification and parentage verification in animals was based on blood group analysis. Immunologic methods for cattle and horses were developed in the 1970s and typing was gradually adopted as a requirement of registration. Thousands of animals were typed annually at relatively low cost. Early DNA-based typing methods, such as restriction fragment length polymorphism (RFLP), could not compete with blood typing on a cost basis. However, the Irish Coursing Club, the first canine breed registry to adopt DNA typing, contracted with a laboratory that performed restriction fragment length polymorphism (RFLP) with Jeffrey’s probes (1). This early adoption of DNA in dogs was due to a lack of sufficient blood group variation for canine typing and the greater discriminatory power afforded by DNA analysis.

In 1990, microsatellites were first described in the human genome as a string of dinucleotide repeats with flanking unique DNA sequences amenable to amplification as a single locus (2). The utility of these markers for gene mapping was readily apparent and they were soon described in many other species. Horses and cattle were the focus of early development; the first report of canine microsatellite loci appeared in 1993 (3). These early investigators were concerned that, in contrast to humans, the inbreeding common in domestic animals would reduce the variation found in microsatellites. However, as more loci were developed, the polymorphic variability and ease of use demonstrated both their utility and their higher discrimination. The animal identification community quickly phased out blood typing technologies in favor of DNA typing for animal parentage testing, just as the human parentage and forensic communities have done.

Parallel to the development of microsatellite markers, the reagents and instruments for their analysis underwent dramatic improvements. The refinement of PCR amplification, such as the development of “hot-start” Taq polymerase to reduce stutter, made PCR multiplexing possible. Multiple dye systems for maximizing the information content per gel lane or capillary, as well as software development for allele scoring and databasing, contributed to the gradual adoption of animal DNA typing on a routine basis.

The presence of tetranucleotide repeats in dogs was first reported in 1996 (4). Taking its cue from the human forensic community, Zoogen Inc., a private service laboratory in Davis, California, began developing canine tetranucleotide repeat loci in 1994. The larger repeats can be scored less ambiguously and have less stutter. By the time the first reports on canine tetranucleotides appeared in the literature, Zoogen Inc. had already reported a single-lane multiplex with 10 high-quality tetranucleotide loci (5). From 1998 to 2001, the American Kennel Club (AKC) conducted a large study (n=9,548 dogs from 107 breeds) to evaluate the STR loci in commercial kits manufactured by Applied Biosystems (6).

Despite their utility in the general scientific community, publications in peer-reviewed literature on the use of canine microsatellites in forensic analysis are limited. The frequency variation of STR alleles has been reported to tentatively identify wolf-dog hybrids, an application of note to law enforcement (7,8). Shutler et al (9) reported the first case involving a human suspect. In 1991, an elderly man and his dog were killed by blunt trauma. The suspect had mixed bloodstains on his clothing, but the amount was insufficient for the restriction fragment length polymorphism variable number tandem repeats (RFLP-VNTR) testing of the time. In 1996, the case was reopened and the stains were tested with both human and canine microsatellites. The human results matched the victim and the canine results matched the victim’s
There are two reports of canine STR typing to identify the canine perpetrator(s) in attacks on people (one fatal) and another on zoo animals (10-12). A report on canine STR reagents, databases, canine population structure, and casework reported that, despite the inbreeding found in US dog populations, the canine STR loci have similar discriminatory power as forensic STR loci for humans (13).

STR loci have also been developed for domestic cats and a validated reagent protocol and database have been reported (14).

Methods

A total of 558 dogs were included in the Zoogen STR study. A total of 395 samples had been submitted for routine diagnostic testing by veterinarians throughout northern California and southern Oregon. The breed, as indicated by the veterinarian, was the only information accompanying the sample. The samples belonged to fifteen breeds (n=17-32 per breed) and mixed breed dogs (n=40). In addition, Dr Candy Gaiser (Zoogen, Inc. Davis, CA, USA) provided extracted DNA samples from unrelated dogs collected throughout the US. These consisted of a panel of 96 purebred dogs, each from a different breed, panels of 19 unrelated Whippets and 19 unrelated Greyhounds, and 29 mixed breed dogs. The samples were amplified with two PCR multiplexes of 10 and 7 loci (Stockmarks® for Dogs Canine I and II [no longer commercially available]), electrophoresed on ABI 377s, and analyzed using GeneScan 3.0 software and Genotyper 2.0 software. Population substructure was analyzed with GDA software (15) by examining the associations within loci (Hardy-Weinberg disequilibria) and between loci (linkage disequilibria). Inbreeding coefficients were estimated for each locus. A population substructure coefficient \( \theta \) was estimated using standard methods (16).

Sequence analysis of 655 basepairs of the canine mitochondrial control region (Genbank Account. CFU96639: 15,431 to 16,085) was performed on 348 dogs from 88 pure breeds (n=303) and mixed breeds (n=45). Sequence analysis of 945-1,105 base pairs (length varies due to number of tandem repeats present) of the feline mitochondrial control region (Genbank Account U20753: 16,287-475) was performed on 167 cats from 14 pure breeds (n=86) and of mixed breed origin (n=81).

Results

The Hardy Weinberg proportion describes the relationship of alleles within loci, ie, between the allele frequencies and genotype frequencies at a single locus. The Zoogen study database involves 17 loci in 17 breeds and mixed-breed dogs. This entails 306 independent tests of the Hardy-Weinberg equilibrium. For statistical tests at the five-percent significance level, approximately 5% (15 tests) would be expected to show disequilibria just by chance alone. Instead, there were 57 tests showing disequilibria; this is far more than one would expect if the loci were in Hardy-Weinberg equilibrium in these populations.

Linkage disequilibrium refers to associations of alleles or genotypes between loci. If there is equilibrium across loci, then the genotypes between loci sort randomly and the genotypic frequencies can be multiplied to get a profile frequency. The number of tests showing linkage disequilibrium at the 5% probability level is 103, which is close to the 122 tests expected by chance, indicating that the study population is in linkage equilibrium.

In order to estimate the significance of a mitochondrial match in dogs and cats, the frequencies of mitochondrial control region haplotypes in a database of dogs (n=348) and cats (n=167) were determined and are shown in Figures 1 and 2.

![Figure 1. Canine mitochondrial haplotypes are defined by patterns of sequence variation in 655 base pairs of the canine control region (D loop) in 348 dogs from 88 pure breeds (n=303) and mixed breeds (n=45). Sixty-three types were seen; types with a population frequency greater than 1% are shown. There were another 46 haplotypes with frequencies less than 1%.](image-url)
Discussion

Canine STRs and Population Substructure

To effectively use canine microsatellites for forensic identification, it is imperative to assess the population substructure in dog populations. Purebred dogs, estimated to comprise approximately half of the American dog population, do not mate randomly and could be expected to exhibit significant substructure. Even mixed breed dogs may show some degree of population substructure from recent purebred ancestry. If population substructure exists, then matching genotypes between two different dogs is not entirely random. In order to estimate the significance of a DNA match, the association of alleles within loci and between loci must be addressed. The Zoogen STR database of 558 dogs has been evaluated for these associations (13).

Overall statistical results are consistent with populations in equilibrium between loci but in disequilibrium within loci. This is consistent with the high levels of inbreeding in the dog populations. It is appropriate, therefore, to multiply the genotype frequencies to compute the overall probability of a DNA test, provided that corrections are made for the allelic disequilibria. From the Zoogen study, the best estimate of θ in dogs is 0.106, which is about ten times the conservative estimate from the human population. Our common approach in casework today is to report likelihood computed with a θ value of 0.11.

Likelihood Ratios and DNA Match Significance

The 1996 US National Research Council report suggests that the weight of a DNA match between an evidence sample and a reference sample be expressed as a likelihood ratio (LR) (17). The likelihood ratio requires the formulation of two contrasting hypotheses for the DNA match. The first hypothesis forms the numerator of the ratio and is usually that the DNA profiles match because they came from the same source. This would generally be the prosecution’s explanation of the match. An example of a second hypothesis (the defense explanation) might be that the DNAs did not come from the same source but matched by random chance. The defense may have other explanations of a DNA match; a likelihood ratio can be devised for any alternative explanation.

If the evidence sample has a limited amount of DNA, the profile obtained may exhibit allelic dropout. Currently, there is no quantitation assay for small amounts of canine DNA. In casework with telogen hairs, amplification is attempted without the opportunity to optimize template input. Research with human forensic kits has shown that allele dropout is common with template input in the range of 100-250 pg (18). The impact of potential allelic dropout in forensic cases is currently handled by appropriate probabil-
ity calculations that, like the $\theta$ value, will increase
the denominator (13).

Many forensic analysts utilize a 4-step interpretation score to state the significance of a
DNA match. Once the two hypotheses of the like-
lihood ratio have been clearly defined, this verbal
score tells the deciders of fact (judge or jury) the
extent to which the evidence supports the hypo-
theses (19). Although there are exceptions, the pros-
ecution’s explanation of the DNA match typically
forms the numerator of the ratio. If so, a positive ra-
tio supports the prosecution’s case. The conven-
tion of expressing the degree of support is as fol-
lows; 1) a LR equal to 1-10 provides limited sup-
port, 2) a LR equal to 11-100 provides moderate
support, 3) a LR equal to 101-1000 provides strong
support, and 4) a LR exceeding 1000 provides very
strong support.

With the advent of STR typing, however,
these verbal scores are inadequate to express the
high discrimination of STR-based DNA identifica-
tion. Moreover, as the lay public is increasingly
exposed to the power of STR typing (often by the
popular media), the modest likelihood ratios asso-
ciated with partial STR types or mitochondrial typ-
ing seem inadequate even though they do, in fact,
support the prosecution’s case.

**Casework with Canine STR DNA Identification**

*Office of the Prosecuting Attorney of
King County Washington, 1998. The State v.,
Leulualahi (97-C-08256-9) and State v. Tuilefano
(97-C-01391-3) for the murder of Jay Johnson and
Raquel Rivera. Gang members looking for drug
money murdered a young couple during a home
invasion. The couple’s dog Chief attacked the
invaders at the door and was shot twice at short
range; he died later in surgery. The suspects were
still wearing blood-spattered clothing when ar-
ested later that day. Canine STR profiles (10 loci)
from bloodstains found on the clothing of the sus-
pects could not be excluded to Chief. The likeli-
hood ratio supporting the prosecution’s hypo-
thesis that the blood spatters were from Chief was
$4.8 \times 10^9$ (Fig. 3A). The use of the canine DNA re-
results was successfully appealed in 2003, based on
the lack of an admissibility hearing and of peer-re-
viewed publications on the Stockmarks canine
STR markers available at the time of the appeal.

Since then, several reports have been published in peer-reviewed journals (6,13).

Twelfth Judicial District State of New
Mexico. State v. Charles Martinez (Cause No.
CR-99-108) and State v. Chris Faviel (Cause No.
CR-98-64), 1998-1999. A woman’s body was
found in the New Mexico desert. The woman had
been harassing a former boyfriend; the police sus-
ppected that the ex-boyfriend and his new lover had
murdered her. A partial canine STR profile (8 loci
with some allele dropout) from a single hair found
on the victim’s sock could not be excluded to the
dog owned by one of the suspects. The likelihood
ratio for the match was $7.6 \times 10^4$ and very strongly
supported the prosecution’s case that the hair
came from the suspect’s dog (Fig. 3B). The lover,
Charles Martinez, was tried and convicted. The
ex-boyfriend, Chris Faviel, pled guilty.

People v. Laykham, Ventura County,
CA, 2002. A small dog’s determined barking
alerted his elderly owner to the presence of an in-
truder. The suspect, Soum Laykham, attempted to
rape and rob the victim. A telephone call caused
the suspect to flee. Dog hairs found on the sus-

\[\text{Figure 3. Likelihood ratios and support interpretation}
\text{scores from 5 criminal cases using canine microsatellite}
\text{identification. The significance of canine STR profile}
\text{matches from bloodstains (A) and hairs (B-E) is shown with}
\text{both likelihood ratios and verbal support scores. Likeli-
hood ratios are calculated from the estimated genotype}
\text{frequencies of the matching samples with the population}
\text{substructure coefficient ($\theta$) and appropriate probability}
\text{modifications for allelic dropout. The five cases are desig-
nated by the name of the primary defendant and are as fol-
lows: A. The State of Washington v. Leulualahi and}
\text{Tuilefano (13). B. State of New Mexico v. Charles Martinez}
\text{and Chris Faviel. C. People v. Laykham, Ventura County. D.}
\text{Commonwealth of Pennsylvania v. Stephen Treiber, and E.}
\text{Regina v. Daniel McGowan, Leeds, United Kingdom (13).}
\text{Documentation for casework is in the author’s possession}
\text{and in the public trial records.}
\]
pect’s clothing and in his closet matched the victim’s dog by both mitochondrial typing and STR typing (9 loci). The likelihood ratio for the STR match was $7.7 \times 10^8$ and very strongly supported the prosecution’s case that the hairs on the suspect’s clothing had come from the victim’s dog (Fig. 3C).

Commonwealth of Pennsylvania v. Stephen Treiber, 2002. The 2-year old daughter of Stephen Treiber died in a house fire in Mill Creek, Pennsylvania. During the investigation of the fire, Treiber showed the police a threatening letter made by gluing words cut from newspapers to a sheet of paper. The trace evidence examiner noticed a dog hair encased in the glue with just the root end protruding. Alleles from a partial STR profile (7 loci with significant allele dropout) from the hair could not be excluded to one of Treiber’s dogs. Ironically, this dog also died in the fire, but its body was not consumed. The likelihood ratio for the match was 575 and strongly supported the prosecution’s case that Treiber had fabricated the letter (Fig. 3D). He was convicted on all counts.

Regina v. Daniel McGowan, Leeds, United Kingdom, 2003. Brian Keating was beaten and abducted from his home while his family was held at gunpoint. He was found bludgeoned to death the next day. Numerous dog hairs were found on his clothing. The West Yorkshire Police believed that he had been transported during the night in a van owned and driven by Daniel McGowan. A STR profile with 16 STR loci and minor allelic dropout matched the dog owned by Daniel McGowan. The likelihood ratio for the match was $5.3 \times 10^{14}$ and very strongly supported the prosecution’s case that McGowan had been involved in the abduction and murder (Fig. 3E). He and three other defendants were convicted.

**Mitochondrial Typing of Dogs and Cats**

If STR typing fails or does not meet the minimum scoring criteria, the control region (D loop) of the mitochondrial genome can be typed as a means of exclusion or inclusion. There are hundreds to thousands of mitochondria in the hair-forming cells of the hair root bulb that are then deposited in the hair shaft. Mitochondrial DNA is present in higher copy numbers than nuclear DNA and is often less degraded in aged samples. Mitochondrial typing, however, has some significant disadvantages as a means of forensic identification. An animal’s mitochondrial type is inherited from its mother, so mitochondrial typing can not distinguish maternal relatives. In addition, the variation seen in mitochondrial typing is scored as a single locus (haplotype). These variations occur entirely by mutation, hence the reduced variation seen in dogs and cats compared to humans reflects the shorter time scales of their origins as species. In comparison to human beings, dogs and cats have far fewer haplotypes and higher frequencies of certain common haplotypes. QuestGen’s canine database of 348 dogs of diverse pure breeds and mixed breeds contained a total of 63 mitochondrial haplotypes. Approximately 50% of the dogs have the four most common types with frequencies ranging from 9-17%. Approximately 25% have more unusual types with frequencies ranging from 2-5%, and approximately 25% are rare with frequencies less than 1% (Fig. 1). These frequencies can be divided into 1 to produce a likelihood ratio. Application of the population substructure coefficient $\theta$ (0.11), calculated from the Zoogen canine STR database, to the likelihood ratios for mitochondrial typing provides a conservative estimate of the significance of a DNA match.

Mitochondrial typing has also been explored in domestic cats. A database of 167 pure and mixed breed cats was sequenced for the control region. Sequencing of the control region in cats is made more challenging by the presence of a long tandem repeat in the middle. This repeat introduces substantial polymorphism, but is difficult to sequence through in forensic samples such as hair. With the tandem repeat included the frequency distribution of feline mitochondrial haplotypes in QuestGen’s feline database is similar to dogs with the highest frequency equal to 12.6%. Without the tandem repeat, 70% of cats fall into the 3 most common types (Fig. 2). Although there are no available estimates of inbreeding in the domestic cat, most are of mixed breed origin and are more likely to mate randomly than dogs. Therefore, the $\theta$ estimated from dogs is probably a conservative adjustment when calculating the likelihood ratio.

In casework involving a single animal, only the finding of a rare mitochondrial haplotype will provide a likelihood ratio verbal score beyond “limited support” or “moderate support” of the prosecution’s case. In most of the cases described
below, prosecutors used the mitochondrial evidence as “another piece of the puzzle” at trial. However, many households own more than one pet and probability calculations from multiple animals may increase the power of mitochondrial identification. In the future, larger databases and research into additional regions of mitochondrial sequence polymorphism will further enhance mitochondrial typing as a powerful tool for animal DNA identification.

**Casework with Mitochondrial Analysis**

State of Illinois v. Cecil Sutherland, 2004. Sutherland was retried for the abduction, rape, and murder of 10-year old Amy Shultz. He had been convicted before, but had appealed based on an incompetent defense. Mitochondrial typing of a human hair found on Amy was matched to Sutherland and excluded the other suspect in the case. Numerous dog hairs found on Amy’s clothing matched Sutherland’s dog Babe. The frequency of Babe’s haplotype in QuestGen’s database is 2.6% and the resulting likelihood ratio is 34.3 (Fig. 4A).

State of Iowa v. Ben O’Donnell, 2003. Tracy Carson disappeared following a birthday party in a local bar. Her body was found 6 months later; she had been strangled, wrapped in a bolt of fabric, partially burned and then buried in a creek bed. Her body was unearthed by spring flooding. Examiners noted animal hairs on the fabric. Ben O’Donnell had “partied” with Tracy the night she disappeared and was the primary suspect. Police conjectured that the fabric wrapping the body had been taken from the home of O’Donnell’s grandmother. Mitochondrial haplotypes of cat hairs found on the fabric (a common type with a frequency of 28.5% and an uncommon type with a frequency of 1.3%) could not be excluded to the three household cats. These frequencies are independent and can be adjusted with $\theta$, multiplied together, and divided into 1 to produce a likelihood ratio of 239 (Fig. 4B). Other evidence was developed when investigators used luminol to find blood traces with matching DNA profiles to Tracy Carson in the truck of O’Donnell’s car. He pleaded guilty to second-degree murder.

State of Florida v. Brent Robert Huck, 2003. April Misty Morse was kidnapped and murdered. Her body, bound with duct tape, was found in a coastal river. Several dog hairs were found on the duct tape. Police suspected an ex-boyfriend, Brent Robert Huck, had murdered her on his boat. The mitochondrial haplotype of the dog hairs matched that of Huck’s dog. The frequency of the type was common at 10.4%; the likelihood ratio is 8.5. Brent Huck was convicted on both counts of kidnapping and murder (Fig. 4C).

State of California v. David Westerfeld, 2002. Seven year-old Danielle Van Dam was abducted from her home and murdered. Her body was found two weeks later. Danielle had been very close to the family’s Weimeraner dog. The police suspected the Van Dam’s neighbor, David Westerfeld, and searched his house and motor home. The mitochondrial type from dog hairs found in the motor home, on a quilt and in the lint-trap of his dryer matched the Van Dam’s dog. The frequency of the type was common at 9%; the likelihood ratio is 9.9 (Fig. 4D). This was the first trial in the US to admit canine mitochondrial DNA analysis as evidence. David Westerfeld was convicted on all counts.

In conclusion, DNA identification of animal-derived samples can provide a powerful link between victim and suspect. Animal hairs are often found at crime scenes and, depending on the crime and the relationship of the participants, may...
be probative. Animal hairs from cats and dogs can be typed by either STR analysis or mitochondrial sequence analysis. Evidence from both has been admitted as evidence in US courthouses and has contributed to crime investigations that led to plea agreements.

Conflict of Interest Statement

Some of the work on canine STRs was performed while the author was an employee of Applied Biosystems or its affiliate Celera Genomics. She is no longer employed there. QuestGen Forensics did the remainder of the work with internal funding. QuestGen Forensics is a service laboratory performing forensic casework using the techniques developed.

References


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