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The present state of forensic identification of animals - a review*

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The use of modern molecular techniques in human forensic genetics can identify individual humans using their DNA profile, yielding estimates of age and external body features, including eye color, hair and skin color, facial shape, and biogeographical origin. Such molecular techniques have been successfully introduced into forensic nonhuman DNA investigations almost as rapidly as into human forensics. This review describes the research methods currently used in the forensic diagnostics of domestic and wild animals and also discusses potential future applications and challenges specific to crime investigation requirements.

KEYWORDS: forensic genetics / animal DNA / evidence

The use of DNA analysis in human identification was a breakthrough in forensic biology, particularly from the time DNA fingerprinting was accepted as legally admissible evidence in civil and criminal proceedings [Jeffreys *et al.* 1985ab]. The use of multiplex PCR of short tandem repeats (STRs) is presently the gold standard for DNA typing in human identification [McCord *et al.* 2019]. The internationally accepted core STR loci included in commercially available kits guarantee comparable results, and DNA profiles obtained by accredited laboratories are registered in national

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DNA databases and exchanged internationally. Additionally, the strength of DNA evidence that is admissible in court has been determined using statistical models.

The usefulness of nonhuman DNA as evidence has also been appreciated [Jeffreys and Morton 1987, Halverson and Basten 2005]. As DNA technology has progressed, species-specific DNA markers have been developed for forensic purposes, encompassing both companion animals such as cats and dogs and a variety of domestic and wild animal species [Menotti-Raymond *et al.* 2005, van de Goor *et al.* 2011a, 2011b, 2009, Ogden *et al.* 2012]. Since then, STRs have been widely used for individual identification parentage testing, breed assessment, phylogenetic studies, food component studies, and forensic applications. Nonhuman biological traces can prove much more diverse in practice than those of humans, and the evidence collected at a single crime scene may include traces of every animal owned by the victim or by the perpetrator and potentially present at the crime scene. This requires an individual approach to the evidence. As in forensic human DNA analysis, nonhuman DNA evidence requires standardized procedures with clear guidelines for evidence collection and preservation, documentation, analysis, and interpretation, and ultimately requires clear conclusions understandable to the court. Animal STR loci need to meet the same high requirements as the human STR loci. Similarly to human forensic genetics, animal forensic markers should consist of unlinked and highly variable microsatellites that can be easily co-amplified. A representative allelic database is also needed to statistically support the DNA match. The majority of STR markers recommended by the International Society for Animal Genetics (ISAG) and the Food and Agriculture Organization (FAO) are dinucleotide or trinucleotide repeats, but their suitablity for forensic purposes has been challenged due to their increased stutter formation [Budowle *et al.* 2005, Linacre *et al.* 2011]. The importance of animal DNA analysis in legal proceedings has led to some debate among forensic researchers. The 2008 ISAG Conference in Amsterdam hosted a special section on animal forensics, in which animal forensic genetics was defined as "the application of relevant genetic techniques and theory to legal matters, for enforcement issues, concerning animal biological material" [ISAG Conference 2008]. Recommendations were presented for animal DNA forensic and identity testing, consistent with the guidelines published by Budowle *et al.* [2005]. Forensic and nonforensic animal DNA tests were defined, interlaboratory collaboration was described, and lab accreditation and certification were outlined. The importance of database availability was also emphasized.

Animals, like humans, may be victims (e.g., of abuse, theft, poaching, illegal trade), witnesses (to certain categories of crime), or perpetrators (e.g., in attacks, property damage, or road collisions) in crimes. Biological material of animal origin may be significant evidence in the investigative and judicial proceedings that connect a human victim with a perpetrator and makes identification of the crime scene possible.

Identification of domestic animals

The first ground-breaking case involving analysis of animal DNA was the homicide of thirty-two-year-old Shirley Duguay on Prince Edward Island, Canada, in 1994 [Menotti-Raymond *et al*. 1997]. White cat hairs were collected from a man's jacket found near the victim's car. There were also stains of the victim's blood on the jacket, but genetic identification of the jacket's owner proved impossible. It was established during the investigation that the parents of Douglas Beamish, the victim's husband, were the owners of a white cat called Snowball. DNA profiles consisting of ten dinucleotide STR loci were amplified from the animal evidence and matched the reference sample from Snowball. Population genetic databases were generated from cats in Prince Edward Island and the Eastern United States to estimate the strength of the feline DNA evidence, which was accepted by the court and presented to the jury in combination with additional human DNA evidence. In 1996, the jury convicted the defendant of second-degree murder [Menotti-Raymond *et al.* 1997].

The feline STR genotyping was developed further. Butler *et al.* [2002] proposed the multiplex PCR approach, which they called "MeowPlex", and which involved eleven cat autosomal tetra repeats. A gender identification marker was also included in the multiplex through the addition of primers specific to the *SRY* gene, which is Y-linked. The PCR products from this 12-plex multiplex amplification fall in the 100–400bp size range and use three dye colors. A forensic genotyping panel of eleven tetranucleotide STR loci from the domestic cat was then characterized and evaluated for genetic individualization of cat tissues [Menotti-Raymond *et al.* 2005]. All loci selected for the forensic panel exhibited Mendelian inheritance in a multigenerational pedigree, were unlinked, and were highly heterozygous in cat breeds.

Another interesting case involved a cat named Tinker, whose genetic evidence helped to solve the brutal murder of David Guy in Hampshire the United Kingdom in 2012 [Cardinali *et al.* 2023]. A dismembered male body wrapped in a shower curtain was found on Southsea Beach in Portsmouth. Eight cat hairs were found on the curtain. A friend of the victim, David Hilder, was suspected of committing the crime. Hilder was also the owner of a cat called Tinker. The mtDNA of the cat hairs from the curtain was analyzed and compared with the American feline reference database containing 493 records, but no similar result was found. The only compatible results were obtained for Tinker's hairs. The court required that the statistical validity of the DNA evidence should be determined by reference to the British cat population. Dr. Jon Wetton of the University of Leicester created an mtDNA database for 152 cats from Great Britain. The three records from the database consisted of the same mtDNA profile as the evidence hairs. That haplotype frequency was sufficient to strongly support the evidence. This was not the only evidence against David Hilder (the victim's blood was found at his home), but the feline DNA evidence strengthened the indictment, and David Hilder was sentenced to life in prison.

Schury *et al.* [2014] proposed fourteen feline hypervariable STR markers, predominantly tetranucleotide repeats, to solve the problem of increased stutter peak

ratio during dinucleotide STR amplification. These authors created an allelic ladder and an allelic database that adhered to the recommendations of the ISFG.

Analysis of DNA from canine companion animals has also played an important role in identifying perpetrators. Shutler *et al.* [1991] reported the first case involving a human suspect: In 1991, an elderly man from Vernon, B.C. and his dog were killed by blunt trauma. The case was reopened in 1996, and the mixed human and dog bloodstains were revealed on the blue jean pants and tested with both human and canine microsatellites. The two human results matched the suspect and the victim, and the canine DNA profile matched the canine victim that with other DNA typing evidence contributed significantly to solving the case.

The murder of Jay Johnson and Raquel Rivera in Washington State was another case involving the use of canine DNA in the late 1990s. The young couple were killed by a gang in their home [Halverson and Basten 2005]. When the gang members entered the house to look for money, they were attacked at the door by the couple's dog, Chief. The dog was shot twice at short range by the intruders and died later in surgery. When the suspects were arrested later the same day, they were still wearing blood-spattered clothing. Canine STR profiles based on ten loci were genotyped from the bloodstains found on the suspects' clothing. The likelihood ratio gave extremely strong support to the prosecution's hypothesis that the blood stains were from Chief. Unfortunately, the use of canine DNA results was successfully appealed in 2003due to a lack of peer-reviewed publications on canine STR markers at the time. This outcome shows how important it is for the justice system to have aresearch confirmation of the methods used in the expert's opinion.

In 2002, the body of seven-year-old Danielle van Dam was found abandoned in a desert area near San Diego, California. A neighbor of the family, David Alan Westerfield, was suspected of raping and killing the child. He possessed a motorhome which he used to travel around the area, and in which dog hair was found on a duvet. While the suspect did not own a dog, Danielle did. The mtDNA of the dog hair from the duvet was found to match the girl's dog, with the mtDNA haplotype judged to be characteristic of 9% of the world's dog population. The key evidence against Westerfield included bloodstains on his jacket and on the motorhome floor, Danielle's fingerprints on the motorhome, hairs consistent with Danielle's on the sheets of the perpetrator's bed, and acrylic fibers found on the child's body consistent with fibers from the perpetrator's home. Alongside these, the canine DNA evidence strengthened the case against David Alan Westerfield, who was convicted and sentenced to death.

The presence of tetranucleotide repeats in dogs was reported for the first time by Francisco *et al.* [1996]. A canine multiplex PCR consisting of nine tetranucleotide repeat STR loci was developed in 2009 for individual identification and parentage testing of dogs [van Asch *et al.* 2009, Dayton *et al*. 2009]. Subsequently, the canine genotype STR panel kit initially recommended by ISAG for the identification and parentage verification of domestic dogs was extended to 18 STR and the Amel gene. At present, the core panel with 21 STR markers is used with three additional markers. ISAG published a 21-locus list of

putative dog STRs (ISAG Canine Marker Panels: http://www.isag.us/Docs/consignment forms/2005ISAGPanelDOG.pdf) The use of the extended panel often does not allow for the amplification of all markers in a single PCR reaction.

A new canine STR test was developed due to the increased stuttering observed in dinucleotide repeats. The *International Society for Forensic Genetics* (*ISFG*) also recommended using tetra repeats for forensic identification. Fifteen unlinked tetranucleotide repeat markers were selected from a pool of 3113 candidate markers and assembled with a sex-linked marker into a multiplex, entitled DogFiler [Wictum *et al.* 2013]. Full validation was performed in accordance with the recommendations of SWGDAM (the Scientific Working Group for DNA Analysis Methods). DogFiler meets the same standards as commercially available kits used for human identification. To analyze the degraded DNA, three mini multiplexes called Mini-DogFiler were also developed by moving the primers closer to the amplicons, shortening the length of allele to under 205 bases [Kun *et al.* 2013].

When developing and implementing recommendations for animal genetic identification, including dogs, differences in marker characteristics between humans and animals must be considered.Particular attention should be paid to the stability and mutation rate of the selected STR markers, which differ depending on the size of the nucleotide repeat motives. It has been reported that human dinucleotide microsatellites have higher mutation rates (1.52×10^{-3}) than tetranucleotides (0.93×10^{-3}), making the other preferred for this analysis [Zhivotovsky *et al.* 2000, Mariat *et al.* 1996]. In dogs the situation is surprisingly reversed, and tetranucleotides show higher mutation rates. These high rates and the presence of complex structures make their resolution difficult, and their use in dogs is thus controversial. They nonetheless remain the markers of choice; however following the trend in human genetics, the stability of tetranucleotide microsatellites should be evaluated prior to their inclusion in a canine panel test, to avoid the risk of errors in paternity assignment and identification [Parra *et al.* 2010].

Each pedigreed population is genetically distinct and can be differentiated from the mixed-breed dog population. The use of the canine allele frequency data conveys the significance of identity testing not only for forensic casework and parentage testing, but also for breed assignments. Kanthaswamy *et al*. [2009] developed a multiplex consisting of eighteen short tandem repeats (STRs) and a sex-linked zinc finger locus for gender determination, to generate the population genetic data necessary to assess the weight of DNA profile evidence. The zinc-finger region (Zfx and Zfy) present on both X and Y chromosomes in carnivores prevented false female results in the analyzed samples. Allele frequencies were estimated for 236 pedigreed and 431 mixed breed dogs from the USA. Breed assignment probabilities were based on gene class, and the random match probability was estimated for breeds with more than 30 individuals, which included American Pit Bulls, Beagles, Dachshunds, German Shepherds, Golden Retrievers, Labrador Retrievers, Miniature Poodles, Standard Poodles, Rottweilers, Shih Tzus, Toy Poodles, Yorkshire Terriers, and mixed breed dogs. Different breeds yield variations in profile probability estimates because of varying allele frequencies. The average random match probability was 1 in 2×10^{33} using the regional database and 1 in 4×10^{39} using the breed dataset.

Another multiplex for 13 hypervariable microsatellite loci was developed by the Canine DNA Profiling group (CaDNAP) of the ISFG using a database of 1,184 dogs from various breeds in Germany, Austria, and Switzerland [Berger *et al*. 2014, 2018, 2019]. The STR marker panel developed by Berger *et al.* [2014] effectively differentiated the 23 most common dog breeds in these countries [Berger et al. 2018]. However, its effectiveness has not been evaluated in the same breeds outside these regions or in less common breeds [Berger et al. 2018].

To determine the evidence value of genetic profiles, allele frequency reference databases have been created for the dog [Kanthaswamy *et al.* 2009, Wictum *et al.* 2013], the cat [Menotti-Raymond *et al.* 2012], the pig [Lin *et al.* 2014], cattle [van de Goor *et al.* 2011a], and the horse [van de Goor *et al.* 2011b]. Commercial DNA typing kits exist for the dog [Wictum *et al.* 2013], the cat [Menotti-Raymond *et al.* 2005], cattle (Thermo Scientific Bovine Genotypes Panel 3.1), and the horse (Thermo Scientific Equine Genotypes Panel 1.1). Unfortunately, there are still many species for which only a small number of STR loci have been described.

Phenotype profiling with the use of DNA markers

DNA phenotyping in a forensic context is a meaningful enhancement to standard human DNA profiling, where STRs are mainly used to identify individuals [Kayser 2015]. In cases where DNA profiles do not match a suspect's profile or a criminal DNA database record, forensic DNA phenotyping (FDP) aims to predict the externally visible characteristics of a person by analyzing appropriate DNA markers. This can provide new investigative leads regardless of the availability of other information, such as eyewitness testimony [Kayser 2015]. DNA-based appearance prediction within forensics began in the early 2000s [Grimes *et al.* 2001] and molecular tests for human iris, hair, and skin pigmentation have been successfully introduced into routine casework investigations [Chaitanya *et al.* 2018, Walsh and Kayser 2016, Kayser 2015, Walsh *et al*. 2014]. Some efforts are currently being made to implement the external visible characteristics estimation ("EVCs") in forensic animal genetics, especially in the dog.

The use of canine DNA phenotyping to predict the external appearance of dogs based on their DNA is a new field of study in animal forensic research. Earlier reports of associations between genotype and phenotype considered the coat color of dogs. [Schmutz *et al.* 2002,2003, Kerns *et al.* 2004]. While additional genetic markers and predictable traits are still sought, the development of molecular genetic tools has begun to support the forensic field with workable assays. In 2021, Berger *et al.* proposed a panel of 21 genetic markers known for their high predictive value of the following six traits: coat color, coat pattern, coat structure, body size, ear shape, and tail length. These markers consist of fifteen SNPs and six INDELS, with three of them belonging to SINE insertions. Berger *et al.* chose six external traits, including the most obvious characteristics of dogs that can easily be recognized and described even by untrained

eyewitnesses: coat color, coat pattern, coat structure, body size, ear shape, and tail length. There were fifteen markers chosen for the coat color and coat pattern, three for the coat structure, and one each for tail length, ear shape, and body size. Single-locus amplification and conventional Sanger sequencing were used to genotype the markers. The practical forensic applicability of the panel was evaluated in a blind test that involved samples from nine dogs. The results demonstrated that prediction of particular trait categories varied considerably. The fifteen markers selected for coat color and coat structure proved to be adequate and showed high predictive accuracy for most of the tested dogs. Body size was tested with the use of a single marker, and the predictions proved correct for seven out of ten dogs, according to a simple classification of "rather small" versus "rather large". The lowest explanatory power showed markers selected to distinguish between drop and non-drop ears. The results of the blind test reflected those of the marker test with a very high accuracy for coat color, coat pattern, and coat structure. The discrepancies observed in the remaining traits may be due to the small number of genetic markers used for these traits, particularly in the case of ear shape, body size, and tail length [Berger *et al.* 2021]. The external visible characteristics that are easy to describe by an eyewitness like coat color, coat pattern, coat structure, body size, ear shape, and tail length have strong support to become the international standard in canine phenotypic testing successfully.

In 2023, the CaDNAP group extended the marker panel for dog phenotyping. They developed and evaluated a massively parallel sequencing (MPS) based molecular genetic assay called the LASSIE MPS Panel, which predicts both external visible features and skeletal traits, covering coat color, coat pattern, coat structure, tail morphology, skull shape, ear shape, eye color, and body size. The panel is based on 44 genetic markers gathered in a single molecular genetic assay [Heinrich *et al.* 2023]. Six traits were selected based their being recognizable by an untrained person. The following traits were considered: coat color (twelve markers, for the phenotypes: black, red, yellow, cream, white, brown, undiluted pigmentation, diluted pigmentation, fawn, black wildtype, albino), coat pattern (six markers, for the phenotypes: no grizzle, grizzle, no eumelanistic mask, eumelanistic mask, no harlequin, harlequin, no merle, merle, no tan points, tan points, solid colored, minimal white spotting, white spotting), coat structure (seven markers, for the phenotypes: short, long, smooth, furnishings, wired curly, and coat present), tail morphology (three markers, for the phenotypes: bobtail, standard, straight, curly), ear shape (three markers, for the phenotypes: prick, drop), skull shape (three markers, for the phenotypes: non-brachycephalic, brachycephalic), eye color (one marker, for the phenotypes: non-blue, blue), and body size (nine markers, for the phenotypes: standard size, short-legged, medium to large, small). Very strong predictive power was observed for coat color, coat pattern, coat structure, skull shape, and strong-to-moderate predictive power was noted for body size, tail morphology, and ear shape.

DNA markers in wildlife crime investigation

The scope and range of wildlife crime are wide, with the term encompassing a variety of criminal activities, such as poaching and illegal hunting of mammals, birds, and reptiles and the use of animal derivatives in traditional medicines. Forensic genetics is widely used to protect endangered wildlife species and to prevent illegal hunting of mammals and illegal trade in animals and animal-derived products [Johnson *et al.* 2014, Mozer and Prost 2023]. International trade in endangered species is monitored and regulated through the recommendations of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). These recommendations are enforced at the national level by legislation.

The analysis of mtDNA is widely established as a standard for forensic taxonomic identification of animals, supported by the high number of mtDNA copies per cell and the absence of recombination. CYTB, COI, 16S rRNA, and 12S rRNA are the most commonly used markers for species identification [Linacre *et al.* 2011]. In 2003, Hebert et al. proposed a 648 bp COI fragment for DNA barcoding, which has since been widely accepted as a reliable method. mtDNA is the only genetic material which traditional medical practices can use for forensic identification of threatened wildlife species. The analysis of mtDNA not only yields information on the species but can also identify illegal trade routes and determine the geographical origin of samples [Pun *et al.* 2009, *Hsieh et al*. 2001, Johnson *et al.* 2014, Tobe *et al.* 2008,2009]. Ivory, rhino horn, and pangolin scales are examples of material evidence whose investigation requires mtDNA analysis. Ewart *et al.* [2018] presented an internationally standardized identification test based on a 230 bp cytochrome-b region *(CYTB)* that allowed correct identification of all five rhinoceros species. Ewart *et al.* [2021] also used the cytochrome-b region to determine the species of pangolin material (of the family Manidae), the most trafficked mammal in the world.

Non-human species identification is only possible if the unknown sample corresponds to a previously well characterized species whose DNA sequence has been submitted to the comparison database.

The DNA Commission of the ISFG published its recommendations in 2014, though there is no standardized locus in mtDNA for species testing, and hence there is a divergence between *CYTB*, *COI*, and other loci such as *ND5* [Parson *et al.* 2014]. To differentiate between closely related species, SNP testing is performed in the known polymorphic mtDNA regions which provide the greatest possible amount of genetic information [Hebert *et al.* 2003, Angleby and Savolainen 2005, Dawnay *et al.* 2007, Kitpipit *et al.* 2012].

SNPs are widely used in forensic investigations of wildlife crimes, focusing on identity testing, ancestry, lineage, and phenotype [Budowle and van Daal 2008, Jordan and Mills 2021]. A commercially available SNP kit (SNaPshot) identifies endangered and trafficked species to support criminal prosecutions, including the identification of tiger and elephant species, as well as differentiating wolves from dogs. [Kitpipit *et al.* 2012, 2017, Fondevila *et al.* 2017, Jiang *et al.* 2020].

Conclusion

Although similar techniques have been developed in human and animal forensic genetics, there are some significant differences between these applications. Human forensic investigations employ tetranucleotide repeats, while investigations on domestic animals predominantly make use of dinucleotide repeats [Budowle *et al.* 2005]. The way in which representative allele frequency databases of sufficient size are built should also be considered. Information on the size and genetic structure of the population concerned is also necessary to appropriately estimate the degree of genetic relatedness between any two individuals [Holsinger and Weir 2009]. For very small populations, inbreeding may be one reason for high relatedness levels [Johnson *et al.* 2014]. Similar to forensic human DNA analysis, presenting animal DNA evidence in court requires standardized procedures for evidence collection, preservation, documentation, analysis, interpretation, and clear conclusions understandable to the court. The lack of uniform recommendations from international societies remains an essential hindrance.

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