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Review

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Forensic DNA Analysis

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Introduction

This review focuses on recent developments in forensic DNA typing. It highlights important recent advances and issues in forensic human identification and identifies representative papers. It is not intended to be comprehensive. The review is divided into several important topic areas. These include developments in forensic serology using RNA, proteomic, and Epigenetic markers, and methods for human identification using short tandem repeats, single nucleotide polymorphisms, and insertion deletions. Sequencing methods for autosomal DNA, sex linked DNA, and mitochondrial DNA are included as well as for the human microbiome. New technologies are also featured, such as real time PCR, microfluidics, integrated rapid PCR systems, and massively parallel sequencing. Expert systems have also been developed to assist with the analysis of data from these complex analytical tools.

Forensic serology

A key issue in DNA typing is sample identification based on serological markers. These tests include both chemical and biological methods. Key issues in serological analysis involve human specificity and sensitivity. Given the high sensitivities available for DNA typing with PCR, it becomes important to match this sensitivity in serological testing. Major research efforts are underway to convert older chemical and enzymatic tests into more sensitive and specific nucleic acid and proteomic based analyses.

Chemical and Spectroscopic methods

A chemical test using luminol and hydrogen peroxide for the detection of blood received a boost in sensitivity through the addition of 8M Urea.[1] This resulted in an increase in sensitivity from 1:4,000 dilution to 1:16,000 dilution. A test for semen involving the protein marker semenogelin was evaluated for its use in sexual abuse of animal cases.[2] In particular the test was examined using simulated samples of numerous canine body fluids and was shown to not produce cross-reactivity with human samples. Other tests have been conducted to differentiate human blood from various animal bloods using Raman spectroscopy and various chemometric models.[3-5] Spectroscopic interpretation models and algorithms were developed to discriminate as human or non-human blood based on patterns of bands in the resulting spectra. Another important human body fluid, semen, was investigated with micro-Raman in order to detect prostate-specific antigen.[6] This test allowed for the identification of semen samples using only a few microliters of sample in various matrices, such as shirts, underwear and swabs. Finally, Raman spectroscopy coupled with chemometric modeling was used to create a workflow capable of differentiating between peripheral blood, saliva, semen, sweat, and vaginal fluids in humans.[7] This model achieved 100% accuracy in the differentiation of each body fluid in a rapid and non-destructive manner that allowed for the samples to continue on to traditional forensic DNA analyses.

Body fluid identification via RNA typing

Messenger RNA has been shown to be a promising biomarker for body fluid identification. As a result, many candidate loci and genotyping systems have been developed. Current efforts mainly involve the development and validation of profiling systems for use in actual forensic casework. Human blood-specific mRNA markers were examined for stability in various environmental conditions and contaminants.[8] Improved target mRNA detection in degraded transcripts has been achieved by using next generation sequencing methods to identify primers which were targeted to more stable transcript regions, These primers were found to more consistently and specifically amplify RNA markers of interest.[9] Body fluid detection was also evaluated by examining the potential of circular RNAs in forensic mRNA. Circular RNAs result from the backsplicing of pre-mRNAs. These makers were shown to be provide improved stability over standard mRNA assays and were tested in mixed and degraded samples[10] An assay system based on massively parallel sequencing (MPS) was introduced for improved mRNA marker analysis. Thirty three target RNA loci were developed including 6 for blood, 6 for semen, 6 for saliva, 4 for vaginal secretions, 5 for menstrual blood and 6 for skin.[11] Probabilistic approaches including the evidential value of RNA profiles were suggested for improvement of the interpretation of body fluid specific-mRNA profiles.[12]

There have been efforts to discover mRNA markers for identifying human tissues from uncommon bodily fluids for casework. The mRNA markers of nasal mucosa were identified and added to a previously developed multiplex system for body fluid identification.[13] Trace evidence from skin or sweat-specific mRNAs were evaluated to determine the specific origin of cellular material present in touched contact traces.[14]

Micro RNA (miRNA) markers are short non- coding sequences involved in gene expression. As such they also have great potential for use in body fluid analysis. A variety of assay platforms have been investigated for this application. Massively parallel sequencing (MPS) has been conducted to identify miRNA biomarkers for forensic body fluid identification.[15] The miRNA markers identified by microarray were validated using quantitative PCR along with stability, mixture and blind testing.[16] A qPCR-based quantitative method was employed to analyze miRNA candidates to distinguish menstrual blood and peripheral blood.[17] For reliable semen detection in forensic work, a combination of several semen-specific miRNAs was recommended.[18]

Proteomic body fluid identification

Body fluid specific proteins have the advantage of being much more abundant in cells and being chemically resistant to degradation.[19] Hemoglobin, α-amylase, semenogelins, prostate-specific antigen, acid phosphatase, and uromodulin were all used as confirmation for the presence of blood, saliva, seminal fluid, or urine in a MALDI-TOF mass spectrometric method.[20] A similar study used a Q-TOF mass spectrometer to screen 23 biomarkers that are specific to 1 of 5 body fluids (peripheral blood, vaginal fluid, seminal fluid, urine and saliva) in a multiplex approach.[21] This method was capable of identifying all samples if single source and nearly all samples when two body fluids were mixed together. The mixture of saliva and blood had some matrix effects that prevented the confirmation of saliva. An immunochromatographic technique for identifying and differentiating menstrual blood and peripheral blood from samples of alleged sexual assault has also been produced.[22] The differentiation of peripheral blood and menstrual blood can be used to demonstrate the presence of trauma, rather than a natural bleeding cause. Proteins and peptides from body fluids in finger marks and under finger nails were examined by MALDI-TOF

MS.[23] This study identified vaginal fluids and blood under finger nails and determined that aluminum containing magnetic fingerprint powders did not interfere with MALDI-TOF, but rather aided in the ionization and eliminated the need for a matrix.

Epigenetics

Epigenetics is an emerging area of interest in the forensic field, and DNA methylation profiling in forensic genomics is regarded as one of the most promising methods for providing investigative leads in casework. DNA methylation profiles have been studied to determine a variety of forensic applications, including age prediction and tissue source identification.[24] A recent review on the application of massively parallel sequencing for DNA methylation profiling has summarized potential benefits and limitations of the application of this technology.[25] Over the past 10 years there has been a steady development of new and promising markers for forensic analysis using DNA methylation data.[26]

Epigenetic studies have been performed to identify novel body fluid identification markers and to validate the systems developed for forensic practice. DNA methylation markers for semen, blood, saliva and vaginal epithelia have been evaluated by pyrosequencing.[27,28] Differentiation of menstrual blood from venous blood or vaginal fluid is more challenging than other body fluids. A number of potential markers for differentiating blood, menstrual fluid, and vaginal fluid have been evaluated.[29] A previously developed multiplex SNaPshot system using DNA methylation markers for blood, saliva, semen and vaginal fluid was modified by adopting novel CpG loci specific to menstrual blood.[30] A new real-time PCR-based method was developed for analyzing the quantitatively semen-specific DNA methylation status of the CpG sites in the DACT1 gene.[31] By examining data produced using the Infinitium Methyl chip, a 10-plex methylation sensitive restriction enzyme-PCR (MSRE-PCR) system was developed using a

biofluid-specific marker with the HhaI recognition sequence. The designed assay was combined and coamplified with a set of mini short tandem repeats for simultaneous body fluid identification and STR typing.[32] The Combined Bisulfite Restriction Analysis (COBRA) technique was employed for assessing a brain tissue-specific CpG locus in EML2 gene in aged samples.[33]

It has been demonstrated that certain CpG loci produce DNA methylation levels that correlate with age. Recently, efforts have been made to improve the systems for DNA methylation analysis for age prediction. A real time PCR assay for age prediction has been developed that does not require the bisulfite conversion step in the workflow.[34] Massively parallel sequencing (MPS)-based assay and methylation-sensitive high-resolution melting assays have been created.[35-37] In order to improve the accuracy of age prediction, multiple approaches involving statistical algorithms have been evaluated.[35,36,38,39]

DNA methylation-based age prediction has also been evaluated for body fluids and specific loci were developed for blood. The methylation patterns of age-associated CpG markers in saliva were investigated.[40,41,37] The potential for using existing DNA methylation markers for blood was analyzed in samples from skeletal muscle, cerebrum, bone and buccal swab.[35,42] Inferring chronological age from DNA methylation patterns in teeth samples was also possible.[43] Semen samples from forensic casework have also been tested using a previously published age-predictive method.[44]

The impact of genetic ancestry on chronological age prediction was also evaluated.[45] The age-predictive power of five DNA methylation markers previously developed was evaluated in groups from different biogeographical regions, including a Korean group and a Singapore local population.[46,47] The different dynamics in DNA methylation between the young and older

individuals has been shown to affect the accuracy of the age prediction models.[35,36,40] A number of candidate markers for children and young adults have been investigated.[48] The effect of post-mortem changes on forensic age prediction has been tested by using cadaver samples from autopsies.[49]

DNA extraction and sample recovery

The sample recovery of genomic DNA is highly dependent on the method of collection, and the storage. Recovery is also dependent on the manner that the DNA or cells are released from the substrate. Bruijns, et.al., demonstrated that polyester, foam, and nylon swabs were more efficient for recovery of DNA, when compared to cotton. [50] A rayon swab has also shown much promise in aiding the collection of samples for direct PCR. [51] Kirgiz, et.al. examined double swabbing with cotton and tape-lifting for collection of touch DNA from steering wheels. Tape was shown to be useful in recovering DNA, however it may not fully dissolve in extraction buffers.[52]

Recovering DNA from fingerprints is a critical process in crime scene analysis. Solomon, et.al., suggested a workflow for dealing with archived latent fingerprints to increase recovery of DNA[53]. Another visualization technique was developed using Diamond™ Nucleic Acid Dye that binds to the backbone of the DNA molecule and fluoresces under a 480nm excitation light source.[54] This method has also been used to visualize DNA collected with on swabs which were stored for up to 4 weeks.[55]

Recovery of DNA from clothing is also an important process in forensic DNA analysis. Hess, et.al, compared taping, scraping, and swabbing as collection methods from clothing. The shedding status of the "perpetrator" impacted the results, as does the type of material.[56] Farash,

et.al. developed a method to enhance the recovery of single source DNA from skin and fabrics. The method focused on collecting individual cells or clumps of cells instead of just swabbing an area of a surface. The cells were collected with water soluble tape attached to a tungsten needle.[57]

The effects of solvents and solutions to improve sample recovery and storage have also been investigated. Using a lysis detergent on the swab to collect DNA from a surface and storing the collected samples at -20°C was shown to produce improved recovery of DNA when compared to those stored at room temperature.[58] Feine, et.al, suggested a method for collecting DNA from electrical tape that uses acetone and water to melt the adhesive.[59]

Ng, et.al. tested the effects of storage temperature and time on the recovery of DNA from urine. Extraction at 4° C and -20°C produced about 90% of alleles after 100 days, with the samples at -20°C showing the highest recovery of DNA over the three-month time course.[60] Eychner, et.al., who compared methods of recovering DNA from chewing gum, suggested that either swabbing or processing the gum whole can result in high amounts of recovered DNA. [61]

Differential extraction involves the isolation of male sperm cells from swabs containing mixtures of male and female cells following a sexual assault. A study of the effectiveness of differential extraction techniques demonstrated the importance of documenting overall recovery.[62] Different methods for differentially lysing the cells have also been explored. Martinez, et.al. demonstrated the use of immunomagnetic cell capture step to effectively remove epithelial cells from a swab prior to further isolation of sperm cells by pressure cycling and alkaline lysis.[63] Cell capture methods were also explored to recover sperm cells. Katilius, et.al. developed an affinity-based sperm purification method using magnetic streptavidin-coated beads with bound ligands modified using biotin. The epithelial and sperm cell mixtures were incubated with these beads to

bind the sperm cells, with the epithelial DNA being removed.[64] Microfluidics has also been used for differential extraction. Inci, et.al. developed a chip that uses an oligosaccharide to bind the sperm cells and isolate them from epithelial cells. 70-90% of sperm cells were captured, depending on concentration.[65]

Genotyping methods using short tandem repeats (STRs)

The current standard method in forensic DNA analysis is STR typing, which utilizes the polymerase chain reaction (PCR) to amplify a set of short tandem repeats present in the DNA template and then applies capillary electrophoresis (CE) to separate the DNA amplicons. A variety of commercial kits have been designed with additional loci that aid in sex determination and improve overlap with existing international databases. These include the Promega PowerPlex Fusion 6C System, the QIAGEN Investigator 24plex QS and the Applied Biosystems GlobalFiler PCR amplification kit.[66-68]

One important challenge facing the modern forensic DNA analyst is reducing turnaround time for sample analysis. Current approaches involve speeding up protocols by using techniques such as reducing incubation times for extracting DNA.[69] Other groups suggest direct PCR as a solution as it removes the extraction and quantitation step entirely.[70] Speeding up amplification or separation and detection has also been examined.[71,72]

Increasing the speed of the PCR amplification step can be done in a variety of ways. Some groups modify commercially available kits and their protocols, while others create entirely new multiplexes specially designed for fast amplification.[72,73] Gibson-Daw used a 7-locus multiplex on a high speed thermocycler and a rapid polymerase to achieve a multiplex amplification in 6.5 minutes.[74] Lower total volumes have been shown to help reduce the time of heating and cooling

of the sample, reducing amplification time by 56-73%.[75] DuVall et al. used a 10 loci multiplex containing a subset of the CODIS loci (all smaller than 350 BP) to achieve amplification in 15 minutes.[76] This microfluidic chip could be coupled with conventional or non-conventional extraction and detection methods to achieve users' requirements.

Rapid Y-STR typing for both screening samples and identification purposes was developed.[74,77,78] Screening methods are useful because much of the biological evidence received by labs often contains body fluids from a number of different contributors. Rapidly mutating Y-STR markers (RM Y-STRs) have been selected for inclusion in multiplexes. These RM Y-STRs have higher mutation rates, so they show higher variability between individuals in a population. In some cases, they can even differentiate between members of the same family.[77] Abuidrees et. al. used a pairing of two fast polymerases (Phusion Flash High Fidelity and Platinum Taq) to amplify a previously designed RM Y-STR multiplex in less than 28 minutes.[77,78]

Direct PCR can speed up analysis times by removing the extraction and quantitation steps. In direct PCR, a portion of the sample is introduced directly into the PCR reagent mix and amplified, with no prior sample prep or cleanup steps. Direct PCR has also successfully been employed for a variety of crime scene samples including blood, hair, fingernail scrapings, touch DNA and even DNA recovered from improvised explosive devices.[79-81]

Rapid and direct PCR methods can also be combined. Procedures involving smaller multiplexes have been developed to screen samples in under 20 minutes.[72,82] Methods have been developed for complete kits that require as little as 47 minutes and could be used for identification purposes.[83] Another way to decrease sample processing time is to reduce the time required for separation and detection of amplified fragments. Microfluidic devices have been developed that can reduce separation times from 30-40 minutes to 80 seconds per sample.[72]

An important area of research is the development of fully automated rapid DNA systems that do not require user input to extract, amplify and detect STR data. Two such instruments are the DNAScan/ ANDE (Accelerated Nuclear DNA Equipment) system and the IntergenX Rapid Hit system. The user simply inputs a buccal swab into the instrument, presses start and a profile will be generated in approximately 90 minutes.[84,85] Recent reports have documented separation of the Powerplex 16 STR kit with the ANDE system and the PowerPlex, GlobalFiler Express and AmpFLSTR NGMSElect kits with the Rapid Hit system.[84,85]

Studies have examined the application of these instruments with samples other than buccal swabs. For example, Turingan, et al. modified the ANDE system to work with low levels of DNA input, such as touch samples found at crime scenes. The described modification included changes to the disposable BioChipSet (BCS) cartridge components as well as the addition of an ultrafiltration module to concentrate the DNA after the extraction steps.[86] It has been noted that careful consideration is needed to determine which samples would be appropriate to run on such a system, due to issues with sample sensitivity and the need to recover sample for later, downstream testing.[87]

Mixtures and probabilistic genotyping

A crime scene sample containing DNA from two or more people is termed a mixture. An increasing share of casework samples are mixtures as DNA evidence becomes utilized in a wider array of evidenceencountered.[70] Contributing to this trend are improvements in the detection of low concentration DNA and improvements in statistical treatment of samples that contain degraded DNA or low concentration of DNA.[88-91] Historically, mixed DNA analysis involved binary peak height thresholds to specify the presence or absence of alleles from a person of interest in a mixture.[92] This binary logic system is rapidly being eclipsed by probabilistic

genotyping (PG) methods using statistical statements of likelihood regarding the inclusion or exclusion of persons of interest.

Probabilistic genotyping methods were developed largely to address the problem of allele dropout that occurs with low template DNA samples. The loss of these alleles greatly complicates mixture interpretation, as it is often difficult to determine if an allele is missing or simply masked by an allele from another individual in the mixture. In probabilistic genotyping models, allele dropout is modeled as a continuous function of DNA concentration rather than a binary one. This results in a more realistic analysis. A second factor in the movement toward probabilistic methods is a trend toward increased complexity in DNA mixtures, driven by expanded multiplexes and increases in the sensitivity of DNA detection. Probabilistic genotyping has been defined as 'the use of biological modeling, statistical theory, computer algorithms, and probability distributions to calculate likelihood ratios (LRs) and/or infer genotypes for the DNA typing results of forensic samples.[93] These methods are probabilistic in two ways. Firstly, probabilistic approaches can provide different statistical weightings for each potential genotype. Secondly, because the techniques are computerized, they can consider vastly more possible genotype combinations than would be possible by manual methods. In contrast, certain historical methods of mixture interpretation (for example combined probability of exclusion) generally consider all potential genotype combinations as equally probable. Secondly, the statistical interpretation of the significance of low-level alleles is based on binary peak height thresholds.

Instead of binary thresholds, dynamic modeling is used when performing probabilistic genotyping. Data utilized by these programs include assumed number of contributors to the mixture, possibility of drop-in or drop-out of alleles, degradation of DNA and considerations

regarding the presence or absence of alleles and stutter. Guidelines were proposed for validating the data models.[93] Probabilistic genotyping methods are often divided into two broad classes termed 'semi-continuous', and 'fully continuous' largely based on how alleles and stutter are modeled. Semi-continuous methods do not model electrophoretic peak heights, while fully continuous methods model quantitative information from peak intensities. Within each of these broad classes, numerous variations exist in the approaches taken. Variation in peak heights were modeled using Normal and Gamma distributions.[94,95] Similarly, stutter is modeled in various ways, where some models used constant stutter ratios, and others modeled stutter based on the length of the parent allele, the length of the longest uninterrupted repeat motif stretch or the lengths of multiple motifs.[94,96] Methods also differ with respect to whether and how allele drop-in and allele degradation is modeled; and on whether and how forward and double stutter modeled is modeled.[94,96] Because of the large number of possible DNA profiles in complex mixtures and the computational complexity involved in multidimensional data, probabilistic genotyping methods use techniques to assure that the algorithms operate in realistic regions of the possible parameter space and that nuisance parameters are efficiently eliminated, through maximum likelihood or integration.

Partly because of the choices of model implementation, probabilistic software can experience run-times varying from minutes to days.[97] However, recent comparisons between software show a convergence of results as measured by likelihood ratios produced using the same data.[98,99] Fully continuous models tend to produce higher likelihood ratios than semi-continuous models for true contributors.[99] A partial list of software utilizing semi-continuous models includes LabRetriever, LRMix Studio, LoComatioN.[100] A partial list of software utilizing fully-

continuous models includes DNAMixtures, EuroForMix, GenoProof Mixture, Kongoh, LikeLTD, MaSTR, STRMix, and TrueAllele.[94,96,97,101-103]

Methods for Estimating the number of contributors

CPI-based methods are not directly dependent upon the number of contributors (NOC) to a mixture. However, NOC is a critical parameter value in the analysis of mixtures using probabilistic genotyping (PG) methods. The most widely used method for estimating the NOC to a mixture is the maximum allele count (MAC) method, which consists of counting alleles at loci and taking account of peak heights for possible allele stacking. Computer-aided methods employing various algorithmic approaches are also available as an alternative to the MAC method. Maximum likelihood methods were developed first, including the method by Haned et al., and the commercial software NoCIT.[91,104] More recently, a machine learning method was developed and commercialized as PACE, and a Markov chain Monte Carlo method was developed as part of the latest release of STRMix software [https://strmix.esr.cri.nz/].[105,106] Computer based methods were shown to achieve higher accuracies than the manual MAC method.[104,105]

X, Y chromosome STRs

In situations such as sexual assault or fingernail scrapings, the presence of the female victim's DNA can overwhelm that of the male assailant. In such situations the use of data from the Y chromosome can be very important as there will be minimal interference from the female victim's DNA. Thus, there have been constant efforts to expand and develop Y chromosome STR loci. Recently a research group from China has developed and validated a typing system

involving a multiplex amplification of 37 YSTRs using Capillary Electrophoresis with 6 dye chemistry. The goal was to increase the power of discrimination between male individuals.[107]

Other researchers have focused investigations on applications of YSTR loci to assess the reliability of Y STR markers for applications in anthropological and lineage studies. The commercial YFiler STR kit containing 17 STR loci was compared with a new YFiler plus kit containing 27 loci. The results demonstrated that increasing the number of loci in the system can improve discrimination power and aid in the confirmation of familiar relationships.[108] Other researchers investigated the robustness of such YSTR kits in order to minimize stochastic effects. Custom software was developed to improve determination of analytical thresholds utilized in system validation and application.[109] Other studies have focused on developing Y based miniSTR loci for improving the analysis of degraded and trace evidence, [110] and using massively parallel sequencing for detection and classification of variant Y STR sequences.[111]

There have also been studies involving the development of X chromosome short tandem repeats. These can be particularly useful in paternity testing. The potential for linkage between different X STR loci was examined by testing a set of 15 XSTRs among 158 families.[112] Addition X STR loci have been developed and validated in order to increase the power of discrimination and assist in the analysis of degraded samples.[113] In 2017 two separate papers focused on the discovery of novel XSTR and the study of the genetic linkage as a useful tool for kinship determination.[114,115] A guideline for applications of XSTR in kinship analysis was also published.[116]

Single Nucleotide Polymorphisms

Single nucleotide polymorphims (SNPs) are particularly useful in situations in which the recovered DNA is badly degraded. The application of these types of markers is seeing a resurgence due to the utility of SNPS in ancestry and phenotyping studies. A recent review presented guidelines on how to perform SNP typing using SNaP shot assays. The work included several helpful tips for mixture deconvolution.[117] Real time PCR with high resolution melting (HRM) can be used for SNP typing and was compared to SNaP shot techniques. The results indicated that although real time PCR with HRM is faster than the SNaP shot, HRM is less useful for mixture deconvolution and performs poorly in regions rich in GC basepairs.[118] There has also been much work on the application of SNPs to predict phenotypic traits Methods for the prediction of eye, hair and skin color have been developed and included in a kit developed by Qiagen. The results obtained by the validation study demonstrated the reliability and sensitivity of the kit to levels as low as 63pg of input DNA.[119] A SNP panel consisting of 1024 SNPs was analyzed using next generation sequencing methods and was tested on a variety of samples, including mixtures and degraded samples. The new panel permitted discrimination of minor contributors.[120] There is also a growing interest in the use of microhaplotypes, a set of short amplicons containing a several SNPs within each sequence. For example, Kidd et al, developed a panel of 182 microhaplotype loci capable of generating high discrimination. These loci were surveyed to determine potential applications in ancestry and mixture deconvolution.[121]

Y- SNPs X- SNPs

Application of single nucleotide polymorphism in sex chromosomes have increased since the introduction of improved sequencing and genotyping techniques. The importance of the Y SNPS has been well established as these loci can be used to determine ancestry as well as discriminate

between individuals.[122] Ancestry and lineage markers are particularly impotent in forensic casework when dealing with unknown perpetrators. The Ampliseq Identity Panel was used for haplogroup assignment and paternity testing.[123,124] Y SNP variants have also been determined in specific populations in order to increase database size and power of discrimination. A study of the Y SNPS in the Flemish population identified the variant alleles in 270 male samples.[125] Fifteen new SNPs were developed with the purpose of performing high resolution subtyping of the haplogroup R1b-DF27, which displays high frequencies in Iberian and Iberian-influenced populations [126]

Similarly research studies on X SNPs have been performed. Two different research groups applied the MALDI-TOF MS techniques to their samples in order to analyze a variety of X SNPs in different populations. The common purpose of these two research studies was to expand population data and thus increase the discrimination power of the technique. The results confirm that the analysis of X chromosomal data can be a useful tool in forensic investigation[127]

Insertion/deletions and mtDNA

Genetic loci containing insertion/deletions, INDELs, are commonly used in medical genetics purposes but can also have applications in ancestry and population studies.[128]A different panel of 14 INDELS was developed to resolve 2 person mixtures.[129] In the past the use of INDELS in forensic applications was limited to biallelic assays. However, recently a set of 17 multiallelic INDELS was developed containing loci that include a mononucleotide homopolymer structure. These multiallelic INDELs can improve discrimination.[130] A panel of 13 INDELS on the X chromosome, were analyzed in different populations using capillary electrophoresis[131] Another research group developed a set of INDEL sequences coupled to downstream SNPs known as

DIP –SNPs. These loci produce amplicons with lengths from 80- 300 bp which only amplify if the specific form of the INDEL sequence is present (S or L). A panel of 14 of these loci were tested using 60 unrelated Chinese individuals. The results showed that the technique was very sensitive and useful for the identification of minor contributors in mixtures due to the specificity of the primer binding.[132]

Mitochondrial DNA

Forensic samples such as bone and hair can benefit from the application of mtDNA. There are 100s of copies of mtDNA in each cell, making the procedure far more sensitive than autosomal DNA. mtDNA also can be used for lineage studies as male mtDNA is not transferred during fertilization. However, it is less probative than autosomal STRs and autosomal single nucleotide polymorphisms (SNPs) as there is no sexual recombination. MtDNA is typically analyzed using sequencing methods, however mtDNA SNPs can also be probed using other techniques such as SNapShot. For example, a SNaPshot procedure was developed to genotype a panel of 52 phylogentically informative mtSNPs. The method resulted in an efficient procedure for classifying haplogroups and could prove useful in forensic analysis.[133] An interesting paper by Strobl et al. analyzed mtDNA present in hairs, bones and teeth previously analyzed using by Sanger sequencing with massively parallel sequencing. The results illustrated that full genome profiles can be obtained for samples stored over a period of years.[134]

A similar procedure was used to determine the effectiveness of massively parallel sequencing for mixture analysis using the Precision ID mtDNA Whole Genome Panel, Ion Chef, and Ion PGM/S5 sequencer (Thermo Fisher).[135] Another study focused on the deconvolution of mixtures by focusing on heteroplasmic sites present in the sequence. This phenomenon involves

mutations in the mtDNA, which result in an individual having two different mtDNA sequences at the same locus. In order to demonstrate deconvoltion of mixed sequences, artificial mixture samples were generated and analyzed to detect heteroplasmy. The results showed that mt DNA heteroplasmy with a PHR above 10% could be distinguished from sequencer noise.[136] Another group analyzed very old skeletal samples with the most recent NGS techniques to evaluate the effects of degradation and deamination. In this project they investigated degraded DNA along with DNA that had been repaired using a special mixture of enzymes. The results showed that enzymatic repair might represent an additional tool for this type of forensic analysis.[137] Yao et al. focused their study on older bloodstains resulting from forensic casework. They compared results with Sanger sequencing and the ION torrent personal genome machine and found that phylogenetic analysis improved the concordance between the two techniques.[138]

Ancient DNA, Bones and Teeth

Teeth and bone frome skeletonized human remains are frequently used as sources of DNA to perform genetic analysis for forensic as well as archaeological and museum specimens. DNA extraction from these samples is a critical first step in the genetic analysis of these remains.[139] However, there are different factors that affect the success of the procedure. Human DNA is subject to a wide range of degradation reactions that take place after death. A common process involves the deamination of cytosine residues into uracils, which mainly takes place on DNA fragments containing single-stranded overhanging ends.[140] Additionally, skeletal remains are exposed to microorganisms, hydrolysis and oxidative damage. As a result, ancient DNA fragments are generally extremely short, with fragment sizes ranging from around 50-100 bp. They may also contain inhibitory substances from the soil.[141] Thus, maximizing the recovery of endogenous DNA is an essential step prior to the genetic analysis of remains.[142]

Improving DNA extraction from teeth and bones. Current approaches.

Huynen et al. compared five bone extraction methodologies, including standard silica purification, sodium acetate/silica purification, HCl/silica purification, and 2 methods using phenol.[143] Although all methods resulted in the recovery of DNA and amplification of mtDNA, the standard silica method provided the lowest yield of DNA. In contrast, the greatest yield was achieved using hydrochloric acid. HCl is very effective at dissolving carbonated hydroxyapatite, the mineral phase of the bone. When the bones are formed, cells deposit tiny crystals of hydroxyapatite in a matrix of collagen fibers. The DNA that remains in this structure, has been shown to be a good source DNA for PCR amplification.[144] HCl releases this DNA into the solution, which later binds strongly to silica, providing a good substrate for downstream applications.

Following this line of research, Boessenkool et al. combined washing the bone powder with bleach along with a pre-digestion treatment with EDTA, proteinase K and laurylsarcosyl and then followed the protocol of Gamba et al., including silica columns.[142,145-147] Posterior library preparation and high-throughput sequencing demonstrated that these combined methods provided higher DNA yields and more successful sequencing than if these methods were used alone.

Another method for improving sensitivity involved the use of carrier molecules to increase the yield of nucleic acids during the extraction process. Among the carriers used, Poly-A carrier RNA is present in certain commercially available silica-based DNA extraction kits. The carrier RNA potentially increases the amount of DNA binding to the silica and reduces DNA loss during extraction. Higgins et al. compared four DNA silica-based extracted methodologies with modifications: 1) not combined with carrier RNA; 2) addition of carrier RNA; 3)

demineralization + silica-based extraction, no added carrier RNA; 4) demineralization + silica-based extraction + carrier. The result of this study demonstrated that demineralization plays a key role in recoveries of nuclear DNA, while carrier RNA had no significant effect on results.[139] It appears that demineralization is the crucial step in efficient DNA extraction of bone.

The use of nanoparticles for DNA has found broad application in the extraction of genomic DNA. Lodha et al. used copper nanoparticles for DNA extraction from bones, starting with a demineralization step, and then immobilized the DNA with the nanoparticles, followed by a cleanup step, to finally elute the DNA.[148] This methodology was examined using both blood and bone samples and gave high quality DNA yields, making it suitable for downstream genetic applications. Zapico & Ubelaker applied a silica-based methodology without a demineralization step for DNA extraction from dentin and pulp, and encountered variability on the DNA yields between these two substrates.[149] Despite this fact, this methodology demonstrated successful DNA recovery for subsequent downstream genetic applications.[149-151]

Non-human DNA

The use of non-human DNA has expanded rapidly in the past few years due the fact that microorganisms, plants, and animal traces can help investigators associate the suspect to the crime scene. Moreover, non-human DNA can be an important tool in determining geographical origin of drugs, solving wildlife crimes and detecting animal cruelty. Forensic investigations can also be aided through an association of biological material with a victim or suspect in a crime.

Feces, hair, saliva, or blood from domestic animals has been used in criminal proceedings to link perpetrators to crimes. A single nucleotide polymorphism (SNP) assay was developed to

determine feline-derived biogeographical ancestry to phenotype using SNaPshot technology. 64 feline SNPs were combined into 6 miniplexes containing 39 intergenic SNPs and 26 phenotypic SNPs, as well as a sex marker (ZFXY). The procedure was found to work well for degraded DNA.[152] A non-coding region of mitochondrial control region (mtCR DNA) was used by Arcieri and colleagues to build a database of Canadian feline mitotypes and by Głażewska and Kijewski for use with Polish domestic cats.[153,154] Ottolini and colleagues created a domestic cat mtDNA database for the UK by examining a 402-bp region of the mtCR DNA.[155]

In Argentina, DNA from dog feces, found at a crime scene, was isolated and two hypervariable regions in the mtDNA were used to genotype the dog. This short fragment of the canine mtDNA produced useful evidence to connect the suspect with a victim and a crime scene, supporting the prosecutor´s hypothesis.[156] mtDNA markers were also used to build a rapid 2 step multiplex real-time PCRs with high resolution melt (HRM) to simultaneously identify nine domestic and four wild animals. The assay worked well with low levels of DNA template and could be useful in screening samples containing DNA from unknown animal origin.[157] Another study examined the potential use of mtDNA markers for species identification of trace levels of biological materials when autosomal DNA was too low for detection.[158]

A16 loci short tandem repeat (STR) system recommended by International Society for Animal Genetics (ISAG) was used to successfully genotype 1421 domestic pigeons (*Columba livia domestica*). Due to the difficulty in determining the sex of an adult bird, a sexing marker was added to the panel as an extra quality control. The results demonstrated the applicability of the panel in parentage verification and identity control for the domestic pigeon in both routine laboratory settings as well as casework.[159]

Investigations of threatened animal are important, as there can be a link between animal abuse and human violence. For this reason, DNA techniques have been often used to assist in animal cruelty investigations.

Thirteen STR loci were used to genotype 19 harvested boars (*Sus scrofa*) in order to solve a case in which a trained hunting dog (*Canis lupus familiaris*) was accidentally killed during a wild boar hunt in central Germany. During the surgery, wild boar hairs were found in the dog's abdominal cavity, suggesting that the bullet first hit a wild boar and then the dog. Since it was known who harvested each of the 19 bagged animals, a DNA genotype was used to identify the person responsible for shooting the dog.[160] In another study, newly-designed non-specific and specific mtDNA primers were used to develop and validate a simple and affordable DNA-based method for species authentication in furs. This method had the main purpose of enforcing a regulation within the European Union (EU), that banned the use and trade of dog and cat furs.[161] In Korea, 600 dogs were genotyped using 10 STR markers. This genetic population study assisted in the development of a canine database to help solving crimes such as animal cruelty, dog-attacks, and missing or abandoned dogs.[162]

Plant material can also be used to as evidence to link a suspect to a crime scene. Botanical traces from outdoor environments can be transferred to tools, vehicles or clothing. Consequently, molecular biology techniques for plant genotyping have been developed and successfully applied to forensic cases. A study on the development of a forensic 6 STR kit for two species of Birch, (*Betula pendula and Betula pubescens*) indigenous to and abundant in North West Europe was recently published for forensic application in the analysis of plant residue from these species.[163]

Methods for the detection of pollen have also been developed. Given pollen's ubiquity in the environment. It has great potential to resolve events both spatially and temporally, due to its

long-term durability. However, the taxonomic resolution of pollen is relatively poor. Bell and collaborators assert that the identification of pollen through DNA barcoding has the potential to overcome these limitations. In their paper, they outlined directions for future research to improve the technology and increase its applicability to a broader range of samples and situations.[164] A study evaluating the persistence and stability of shortleaf pine (*Pinus echinata)* pollen on a cotton jacket for a 14-day period was published by Schield and collaborators.[165] They combined the use of a new forensic device for pollen collection, a high-throughput method for DNA extraction, and a newly developed 9 multiplex STR system. This study showed that pollen can be a stable source of forensic DNA evidence and may persist on cotton clothing for at least 14 days of wear.

Endangered Species and wildlife forensics

DNA metabarcoding has been successfully applied to the illegal orchid trade as well as in the identification of endangered species in complex samples.[166-168] DNA mini-barcoding was utilized for the identification of highly processed animal skin and fur, wildlife skin samples, and samples of animal claws.[169-171] The forensic analysis of cytochrome b (cyt b), a mtDNA gene, has become an essential tool for species identification in routine practice, and for this reason, a number of different primers were designed with species identification capabilities.[172-174] Another study combined a morphometric approached with DNA analysis using cyt b and 16S rRNA genes to identify seized samples of tiger claws in India.[175] Illegal trading of ivory is responsible for the decrease in elephant populations. To verify the origin of the ivory and its processed products as well as the identity of the species origin of elephant, 7 mitochondrial SNPs and cyt b genes were used to build a mini-SNaPshot multiplex assay. The method was validated according

to the recommendations of the International Society for Forensic Genetics (ISFG) and showed excellent identification accuracy.[176]

Two animals at risk of extinction in Brazil had their complete mitochondrial genome sequenced. *Sporophila maximiliani*, commonly known as Great-billed Seed-Finch or 'bicudo', is a trafficked bird in Brazil due to the species' beauty and singing, which is appreciated by breeders and collectors.[177] *Myrmecophaga tridactyla*, popularly known as giant anteater from Brazilian savanna, an illegally hunted and traded animal was also sequenced.[178] Another study amplified a short sequence of ~230 base pairs (bp) of mtDNA from 24 canid skins that were illegally imported from Mongolia to Denmark. Additionally, this group developed DNA-based species identification based on genetic identification and morphological traits. They used this data to clarify the relationship between the haplotypes of the investigated samples and published sequences from known wolves from Europe and Mongolia.[179]

A fully-regulated mtDNA database of species currently targeted in the international illegal wildlife trade was proposed by a consortium of researchers from different countries. This project (ForCyt) is funded by the U.S. Agency for International Development (USAID), and has established a protocol to generate and share genomic data. ForCyt will allow confidence in future species identification in forensic laboratories worldwide.[180] Ribosomal and chloroplast DNA markers were selected to develop a tracking tool in the context of illegal logging of *Gonystylus bancanus*, an endangered species used as incense as well as ramin timber.[181]

Parrots are currently involved in illegal traffic for the pet supply, and many are threatened with extinction. For this reason, Jan and Fumagalli developed, characterized and tested 106 polymorphic microsatellite loci (mostly tetranucleotides) for seven endangered parrot species (*Amazona brasiliensis, A. oratrix, A. pretrei, A. rhodocorytha, Anodorhynchus leari, Ara*

rubrogenys and Primolius couloni). The variability displayed by these microsatellite loci demonstrates their potential utility to perform individual genotyping and parentage analyses.[182] Another study assessed 16 microsatellite markers specifically designed for the South African endemic Cape Parrot (*Poicephalus robustus*) to determine if a bird was bred in captivity, so it can be legally traded, or if it was illegally removed from the wild. This approach can be used to aid in the management of the captive population.[183]

Drug sourcing

Determining of the source of an unknown drug sample (forensic geosourcing), such as marijuana and heroin, is vital to informing domestic and foreign policy related to counter narcoterrorism. Furthermore, DNA fingerprint techniques can aid in determining the geographic origin of such plants. Houston and colleagues used chloroplast DNA and mtDNA markers to build up a multi-loci system to predict biogeographical origin and discriminate between individual *Cannabis sativa* plants.[184] A 13 loci STR multiplex method to genotype marijuana (*Cannabis sativa L.*) was developed, optimized, and validated according to relevant ISFG and Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines. The system accurately genotyped 101 *C. sativa* samples from three seizures provided by a United States Customs and Border Protection crime lab and displayed a power of discrimination of 1 in 55 million.[185] A 13-loci STR multiplex system was used to genotype 72 samples of marijuana seized in Brazil. The system permits sample individualization and origin differentiation and can be used as a tool to help trace trade routes.[186] 11 new highly polymorphic simple sequence repeat (SSR) markers were used to differentiate hemp and marijuana. A unique molecular profile for each individual sample was obtained, and a clear differentiation between hemp and marijuana varieties was observed.[187] A Loop-mediated isothermal amplification (LAMP) assay was used to detect

marijuana and hemp by targeting the conserved region of tetrahydrocannabinolic acid (THCA) synthase gene. THCA is the decarboxylated form of the tetrahydrocannabinol (THC), the primary cannabinoid responsible for the psychoactive effects of marijuana.[188] The THCA synthase gene was also combined with the internal transcribed spacer (ITS) region of the 45S rRNA gene to develop a fluorescence in situ hybridization (FISH) assay to identify trace levels of cannabis.[189] Plants such as Morning glory (*Ipomoea purpurea*), Jimson weed (*Datura stramonium*), Hawaiian woodrose (*Merremia tuberosa*), and marijuana, have been found in teas, capsules, and chewable material. A multiplex real-time PCR high resolution melt (HRM) assay was developed to simultaneously identify those four "legal high" plant species. The assay had the advantage of not requiring post-PCR gel processing or follow-up DNA electrophoresis, and it allowed the identification of multiple species in under 2 hours.[190]

There are limited genomic DNA sequences of opium poppy (*Papaver somniferum L.*) available in the public database, however, an in-silico analysis has identified more than 500 microsatellites, including tri-, tetra-, penta- and hexanucleotide tandem repeats that could aid to identified geographic origin of such plants.[191] The principal barrier for heroin origin identification by STR is the limited amount of damaged and degraded opium poppy present in the samples. (*Papaver Somniferum L.*) DNA. A method published by Marciano and colleagues represents the first time that DNA from the opium poppy (*Papaver somniferum L.*) was successfully isolated from heroin samples. This genetic information, was obtained by next generation sequencing (NGS), and may prove useful in sourcing the material.[192]

Massively Parallel Sequencing

One of the most significant advances in DNA technology in the last 20 years has been the introduction of Massively Parallel Sequencing (MPS).[193] Massively parallel sequencing (MPS)

systems enable simultaneous analysis of forensically relevant genetic markers to improve efficiency, capacity, and resolution, and provide a dramatic improvement in the capabilities of forensic DNA laboratories to solve crimes.[194,195] MPS provides the ability to generate results on nearly 10-fold more genetic loci than current technology. In cases where the evidence is limiting, and multiple tests are indicated, the ability to multiplex molecular tests (autosomal STRs and YSTRs) into one test reduces consumption of evidence and total assay time.[196] The ability to perform sample-specific indexing/barcoding permits multiplexing of up to 96 samples per analysis using 1 ng or less of template DNA. The ability to detect sequence variants of STR alleles of the same size (isometric heterozygotes) not detected by CE provides higher discrimination, improved mixture resolution and more accurate results.[196] In addition, enhanced results can be obtained on degraded and inhibited samples as many MPS loci target small amplicons (<200 bp).[197] The compatibility of the STR data with worldwide CODIS DNA databases facilitates the use of already established databases, and the ability to investigate familial relationships and personal identification using X and Y STRs without iterative testing. Recent concordance studies were performed and demonstrate concordance of MPS with capillary electrophoresis STR results.[198,199] Global MPS population studies have provided data on suites of microhaplotype loci that were shown to be highly informative for individual identification, ancestry prediction and for mixture identification and deconvolution.[200-202]

MPS has significantly improved the resolution power of the analysis of mtDNA heteroplasmy.[203-204] This technology permits the development of investigative leads using SNPs for phenotype and ancestry prediction in cases with no observed database hits and epigenetic analysis can provide information on tissue origin, smoker status, age, and even the capability to distinguish monozygotic twins.[205-207] The ability to sequence multiple forensic type samples for

multiple genetic markers from minute amounts of DNA, provides a method for higher genetic resolution and efficiency to solve more cases.

Two recent reviews on massively parallel sequencing in forensic genomics were published in a special issue of the journal Electrophoresis on Novel Applications of Massively Parallel Sequencing (MPS) in forensic analysis.[208] The first contains a description of the first, second, and third generation sequencing techniques along with an overview of the MPS STR and SNP technologies.[195] The second includes reviews and summaries of forensic MPS STR validation and implementation studies, available panels, platforms, bioinformatics tools, population sequencing studies and international projects and standardization group efforts toward standardizing nomenclature.[194] These review articles provide up-to-date information and overviews of the state of MPS STR sequencing and validation in forensic genomics. MPS STR technology has also been applied to paternity testing.[209,210] Silva et al 2018 tested 29 trios (mother-child-father) using an autosomal and Y STR MPS commercial kit resulting in increasing in the paternity index values as compared to capillary electrophoresis length-based approaches. The authors also report allele inconsistencies (mutations) between child and parents may be resolved with MPS by assessing the core repeat and flanking region sequences thus resulting in increased resolution for the trios/families tested.[210]

MPS has also been shown to increase the typing capability on degraded DNA. The authors evaluated the sensitivity of MPS STR sequencing results on serially diluted DNA down to 5 pg as well as degraded DNA. They reported allele call frequencies of greater than 80% using 50 or more pg and when the degradation index was lower than 72.28.[211] These results may assist laboratories in the design of validation studies and in additional performance comparisons of MPS STR sequencing systems. One major advantage of MPS over CE based approaches is the

capability to perform large scale sample multiplexing. Moreno et al demonstrated this using an MPS STR sequencing system from Verogen.[212] The authors conclude that consistent sequencing results were obtained by using up to 40 single source 1 ng samples pooled into a sequencing reaction.

MPS STR population sequence studies provide pivotal data for the basis of statistical calculations of power of discrimination. This is particularly useful for isoalleles, alleles of the same size but different sequence. Borsuk et al 2018, conducted sequencing on a set of an additional 1036 new loci with the Illumina ForenSeq DNA Signature Prep Kit, and reported the detection of additional alleles in the SE33, DXS8377, DXS10148, DYS456, and DYS461 loci.[213] Variation within the autosomal STR marker SE33 was evaluated resulting in the identification of 53 unique alleles by length and 264 by sequence. 100% concordance with CE data was determined, after manual review and confirmation sequencing of three flanking region deletions. The authors reported a number of challenges in interpreting the data, including high sequence noise, allele-size dependent variance in coverage, and heterozygote imbalance.[213]

Phillips et al., studied 944 individuals of the CEPH human genome diversity panel (HGDP-CEPH), from 51 globally distributed populations using 58 forensic STR loci with the ForenSeq™ system.[214] Alignment of the sequence data to a human reference sequence, required reversal and re-alignment of STR allele sequences in 20 of 58 STRs. The authors assessed the frequencies of population-specific sequence variants and singleton observations, in order to provide for laboratory implementation of this MPS STR system.[214] Kim et al, determined genotypes using the MiSeqFGx™ forensic signature kit, comprised of amelogenin, 27 autosomal STRs, 24 Y-STRs, 7 X-STRs, and 94 SNPs for identification, ancestry and phenotyping. (Verogen, San Diego, CA, USA) 209 unrelated Koreas were examined and compared to results

obtained using capillary electrophoresis.[215] 26 novel sequence variations in autosomal STRs were detected increasing the discriminatory power of individual identification using this approach.[202] Zhang et al, focused on constructing a multiplex PCR system with fusion primers for one-directional PCR for MPS of 15 commonly used forensic autosomal STRs and amelogenin.[211] Samples from 554 unrelated Chinese Northern Han individuals were typed. The number of alleles increased in 12 of 15 loci compared to CE based data and more than 2-fold increases were observed for D2S1338, D5S818, D21S11, D13S317, vWA, and D3S1358. Heterozygosity, discrimination and paternity exclusion probability were determined [211]

Emerging forensic applications using MPS hold great promise for increasing the capability to successfully bring resolution to complex mixtures as well as provide intelligence data for cold cases. As the technology is brought to bear on casework, it is important to consider the interface of these forensic MPS applications with legal and ethical issues.[216] This issue is underscored by the extensive capabilities of the procedure for long range familial searching of genealogical databases resolution.[217,218]

The Microbiome as a source of DNA

Forensic applications of microbiome analysis are not new. Important historical areas of interest include the determination of the cause of death, identity of soils, postmortem interval, human identity, life style determination, body fluid identification, and potential use of microbes as biological weapons.[219-222] Metagenomics involves the study of a wide range of genetic material recovered directly from environmental matrices. Forensic applications can include studies of the human, soil, dust, or plant microbiota, which might indicate exposure to new or additional sources of genetic material. Examples might include criminal assaults or other physical contact and transfer between objects containing sources of microbial and genetic material.[223] The

average human has almost equal numbers of non-human (microbial) and human cells.[224] The transfer of this microbial material creates an opportunity to detect and verify a victim's statement concerning the crime.

Presently almost all microbiome projects are studied by the use of the 16S ribosomal RNA (or 16S rRNA) (bacteria), 18S rRNA (eukaryotes) and the internal transcribed spacer region (ITS) by use of amplicon sequencing.[222] More advanced methods are currently being developed to increase the specificity of these studies.[225] One such method involves the use of K-mer matching which allows the analyst to utilize shotgun sequences as the first step in the analysis. K-mer matching permits strain identification as well as providing a knowledge set for samples containing, virus, fungi, protists, and virulence/antibiotic resistance genes.[226,227] There are unsupervised and supervised machine learning algorithms that can be used in classification. In this context supervised learning seem to give excellent results based on some soil studies.[228] The interpretation of bacterial 16S sequence data can be performed using a variety of statistical methods including: Linear Discriminant Analysis (LDA), and Partial Least Squares (PLS).[229,230] Large data sets from the Human Microbiome Project (HMP), American Gut Project (AGP), and the Earth Microbiome Project (earthmicrobiome.org) provide basic reference sources and sequence repositories for most Microbiome data.

The earliest applications of microbiome analysis involved investigations of the microbial causes of death. Studies have shown that microbes have the potential to change toxicology results, alter questions of causes of death, and even place a suspect at the scene of a crime.[222,231,232] Other efforts have been made to develop a "microbial clock" determination of the circumstances of death and to estimate a time of death. This work is based on the assumption that as a body decomposes certain microbes will appear at specific intervals as the body decomposes. [233,234]

More recently certain investigators have begun to examine the presence of microbiome which might point to a cause of death. Lee et al examined the lungs of drowning victims and using 16S ribosomal RNA sequencing to suggest that presence of aquatic microbiota in the closed organs may provide marker for a diagnosis of drowning.[235] Rivera-Pérez et al has examined "accidental pathogens," which are previously nonpathogenic and/or environmental microbes that have inadvertently experienced an evolutionary shift toward pathogenicity [236] The published work of agricultural scientists have increased our knowledge concerning soil microbial communities which may indicate the potential location of a sample.[228,231,237] However, questions still arise involving the confounding effects of season, temperature change, rainfall, and other factors. Recent efforts have begun to address these factors with the aid of machine learning algorithms.[228] Another study compared the success and consistency of procedures for the bacterial characterization of soil samples. Methods examined included ribosomal intergenic spacer analysis (RISA), terminal restriction fragment length polymorphism (TRFLP) of the rpoB gene, and methods using the 16S rRNA gene.[237]

Microbial forensics has been defined as “a scientific discipline dedicated to analyzing evidence for attribution purposes from a bioterrorism act, biocrime, hoax, or inadvertent microorganism/toxin release.”[225] A new domain in microbial forensics involves identity testing using the human microbiome. In 2017, to illustrate the power of the new sequencing tools and new statistical advances, Walker et al sequenced Boston, New York City, and Sacramento, California DNA subway systems using 16s microbial DNA sequences. Data was then parsed using Principal component analysis (PCA) demonstrating that the bacterial signature from each city strongly showed that they were different from each other.[238] Other recent studies have dealt

with characterization of the skin microbiome through the development of a preliminary marker panel. Schmedes et al. demonstrated stable clade-specific markers could be used to classify skin microbiomes from a particular individual with up to 100% accuracy at three body sites."[239] These and other experimental panels show promise for further development of applications of the skin microbiome to touch evidence. Supervised learning algorithms have been used by a number of authors for individualizing touch microbiomes .[240-242] Hair can provide similar results to those obtained from the skin microbiome. A study in 2018 investigated the human hair microbial environment and found that it may be possible to determine the source and geographical origin of hairs collected at a crime scene using the microbiome.[243] A landmark study appeared in 2014 by a number of authors which used a Bayesian method of analysis which significantly matched individuals to their residences.[244] Of interest were the relationships between the microbiota from individuals living in the same household and visitors within the same dwelling. This study suggested that dwellings harbor a distinct microbial "fingerprint" of individuals living within the household that includes a microbial relationship between pets and humans living within the residences. Schmedes et al have introduced clade-specific markers from the skin microbiome using supervised learning which can predict individuals with high accuracy. They introduced hidSkinPlex comprising 286 bacterial (and phage) family-, genus-, species-, and subspecies-level markers. This may present a start to the introduction of this technology in court, based on a specific set of loci and sequences.[240]

Post mortem interval

Forensic scientists are building a "clock" from the bacteria and other microscopic scavengers that make up the postmortem microbiome.[233] Microbes respond to environmental conditions in a predicable manner which can possibly be measured and timed.[231] A publication by Metcalf et al.

demonstrated that soil types where a body is found are not a dominant factor driving community microbial development, and that the decomposition process is sufficiently reproducible to offer new opportunities for forensic investigations[222]. Their results show a prediction estimate for time of death with an error rate of +/- 3 days in a 25-day period. A multidisciplinary team of experts in various fields of microbiology and autopsies have performed a review of the literature in Medline in order to develop an operational procedure for the detection of unexpected infections causing sudden death, identify emergent pathogens, and recognize medical errors. Additionally, they will evaluate the use of the microbiome for the estimation of PMI.[245] Finley et al has analyzed microbial signatures of grave soil during the decomposition of a cadaver. This study used soil microbial communities that were surveyed from 18 human cadavers placed on the surface or buried that decomposed over a range of decomposition time periods (3-303 days). Because this study involved a large time period as well as human specimens, a much better understanding of microbial community structure and its shifts over time was developed.[232] Singh et al. investigated the temporal and spatial impact of human cadaver decomposition on soil bacterial and arthropod community structure. This study added much needed data necessary to develop an understanding of the ecosystem surrounding carrion decomposition islands and thus could be applicable forensic study of PMI.[246]

The identification of body fluids by use of the microbiome has become an interesting application. Several groups have studied the use of the 16S locus to identify body fluids. In a study using standard 16S rRNA gene sequencing, Hanssen et al placed saliva on the skin of various individuals as a test for the potential to differentiate between skin and saliva microbiomes. The study successfully classified samples from saliva vs that of the skin 94% of

the time.[230] An advanced set of statistical and taxonomic tools achieved an optimal overall accuracy close to 98% for specificity of fecal, oral, vaginal, and skin and nasal samples.[229]

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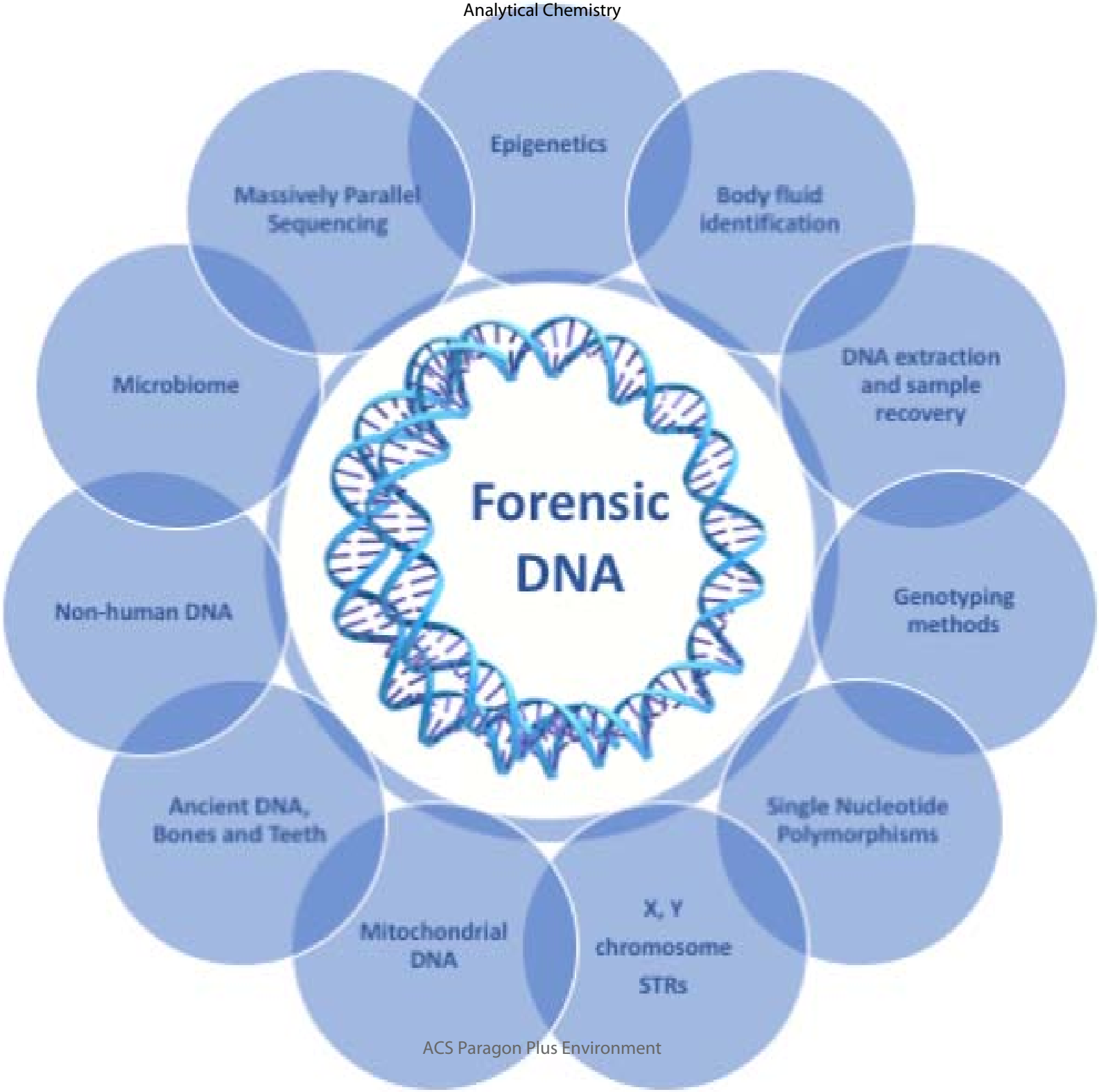
Forensic DNA
Epigenetics
Body fluid identification
DNA extraction and sample recovery
Genotyping methods
Single Nucleotide Polymorphisms
X, Y chromosome STRs
Mitochondrial DNA
Ancient DNA, Bones and Teeth
Non-human DNA
Microbiome
Massively Parallel Sequencing