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Forensic DNA Technological Advancements as an Emerging Perspective on Medico-Legal Autopsy: A Mini Review

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Abstract

The importance of biological traces and evidences related to a criminal matter has been recognized for a long time. The examination of the expression of genetic polymorphism has been an integral part of the multidisciplinary field of medico-legal autopsy for over a century. Since the initial application of blood group antigens for personalization of a putative perpetrator in a murder case, the discipline of forensic genetics has evolved as a standard of forensic sciences. The real breakthrough, the application of molecular tools and processes for the in-vitro replication of genetic substances, has increasingly allowed the exploitation of advances of molecular genetics for both forensic and criminal investigations. Although there are certainly many more applications and scientific fields in the medico-legal arena, the relatively fast progress of genetics, which has accelerated recently with state-of-art technologies, can provide ever more relevant information in relation to a corpse or the cause and manner that resulted in the corpse for autopsy. This topic concerns the currently accepted forensic DNA technology, and the last section reviews commonly used markers for nuclear and mitochondrial DNA analysis as well as ongoing research. This review also focuses on the increasingly important non-human sources of DNA, and shortly covers the main aspects of animal forensic DNA examination.

Keywords: forensic genetics, genetic identification, DNA typing, non-human DNA, animal forensics

1. Introduction

The application of genetics using molecular tools to characterize, identify or practically individualize the biological evidence after the medico-legal autopsy has been adopted worldwide [1].

Forensic genetics as an applied science provides those techniques to contribute to the proper examination of different collected samples. The range of associated biological evidence can be fairly wide, including samples from the body and on the body, as well as human and non-human biological remains. In spite of the existing sampling protocols or recommendations, in some situations, optimal sample collection may be pointless. In some cases, the swabbing of skin surface or fingernails can be effective [2–4] but also reveal an uninformative or uninterpretable mixture. In other cases, the optimal sampling tries to avoid an excessive number of samples, but the efficiency of seemingly appropriate samples can vary according to subsequent analytical steps taken [5] or if the samples were provided for another type of examination [6, 7]. In essence, optimal solutions may be successfully obtained by using slightly different procedures depending on the source of the biological samples [8].

The methodology, in order to obtain genetic profiles, haplotypes, specific markers or species specific information, covers examination of both the nuclear and mitochondrial genome. The technical and theoretical foundation of forensic DNA analysis includes formal protocols as well as the use of standard, commercially available kits and reagents to obtain consensus examinations [9]. The desire for development of higher throughput of laboratory examination, the automation and standardization of steps of DNA analysis—the sample preparation/DNA extraction [10, 11], quantitation [12, 13], new devices [11, 14, 15] and yearly new multiplexes [9] for PCR amplification, fluorescent dye detection systems—provides for wide range advantages of its application.

However, despite the evolution in sensitivity and resolution of DNA techniques allowed by significant achievements year after year, the reality of cases present great risk and do have limitations. Although extreme applications and challenges such as low copy number [16, 17], degradation of DNA [18, 19] as well as mixtures [20, 21] or the collective consequences of these can present themselves as fairly complex issues, there is movement toward improving their interpretation [22, 23].

2. Brief insight into the past, present and future of forensic DNA technological advancements

Forensic genetic analysis is routinely used to obtain required information from biological samples of an autopsy for identification of persons associated with criminal casework or in instances of mass disaster. Upon the characterization of DNA samples using the most promising methods and techniques to improve their collection and further examinations via a medico-legal autopsy, the following steps of a DNA examination are performed in a molecular genetic laboratory, which is a significantly different environment from that of the autopsy room, for extraction, amplification, analyzing (profiling) and interpretation of samples. The proper process of sampling and sample storage used in an autopsy most certainly apply to the transportation of samples; however, contamination issues are always present, not only during the autopsy procedure, but also during the DNA analysis.

Several changes in methodology have promoted the genetic examination of samples with the desire to generate information from the smallest possible amounts of DNA. Wide-scale DNA extraction methods are commonly utilized in forensic practice, but have often proven to be insufficient in recovering all of the collected DNA. In many cases, however, this fact is irrelevant, due to the low minimal requirement of most common typing systems. Recently developed, commercially available kits and methodologies are optimized for specific types of samples and robotic systems [24, 25]. The adequate PCR-based methodologies [26–28] allowed for successful analysis from types of samples not previously examined, such as old, burnt, degraded bone and tissue samples [18, 29–31], single human hairs [32, 33], fingernail [2, 3, 34, 35], bite marks/saliva [36, 37] or touched surfaces [38, 39] and nonhuman remains [40].

2.1. DNA analysis of human and human-derived biological samples

2.1.1. Extraction of biological remains for DNA typing

As mentioned above, potential sources of biological samples related to autopsy are fairly wide ranging. The collected samples contain several substances in addition to DNA, therefore DNA molecules have to be separated from proteins and other cellular material as well as possible additional environmental contaminants, which can inhibit the subsequent steps of analysis [8].

For this reason, a number of methods have been steadily improved, upgrading the primary application for purification of DNA and avoiding its further degradation [10, 11]. Owing to the great variability of multiple influential factors, there can be no “best preparative answer,” rather a selection of most suitable ones. A suitable extraction method should be consistent, sensitive and preferably quick and easy to use. It must also be able to deliver as-pure-as-possible DNA samples, ready to be used in downstream molecular applications and should pose minimum risk for possible cross-contamination between samples as well as between samples and users.

The solution-based, organic extraction method variants [41, 42], combined with filtration and concentration devices [43–45], have been used in practice for long time [46]. These methods can obtain high-quality DNA/RNA, and are adaptable for smaller or larger pieces of evidence material, but are relatively time consuming and require several—including some hazardous—chemicals and transfers. Higher-throughput automated DNA analysis promoted the benefits of variants of solid-phase DNA extraction methods [47]. The developed techniques include, for example, employing silica columns for the isolation and formation of complexes between nucleic acid and the silica gel matrix. In these the DNA selectively binds under particular pH and salt content conditions based on the principles of hydrogen binding to a hydrophilic matrix, ionic exchange using an anion exchanger or size exclusion and affinity. The retained DNA is finally eluted from other components in the subsequent washing steps [48]. An alternative, widely-used solid-phase DNA extraction method is based on an anion exchange/chelating resin that has a high affinity for polyvalent metal ions [41]. This procedure is less compound and faster and limits the use of multiple transfer tubes which reduces the risk of contamination. It requires only small sample volumes, and while it may be combined with, for

example, proteinase K digestion and incubation, the high-temperature Chelex denatures the double-stranded DNA. Single-stranded DNA is obtained and remains suspended in the supernatant for downstream PCR [49–51]. Another commonly supplied solid-phase extraction is based on the reversible bonding of DNA to magnetic particles coated with a matrix of polymers or silica with terminal functionalized groups. The DNA bonded magnetic pellet is immobilized using an external magnet, and the discarded DNA are eluted after washing steps [52, 53]. Several commercially available extraction kits have been manufactured using the liquid/solid DNA extraction approach, and these have been incorporated into semi- and fully automated equipment [8, 24, 25, 54, 55]. The desired optimal DNA extraction from minute samples integrated with new technical developments and automation, “sample-in-answer” platforms for various types of tissues, are ongoing research topics [11, 14, 15, 56–58].

2.1.1.1. Extraction of compound and/or challenging samples

In point of fact, many—sometimes most—relevant casework samples belong to, so-called, difficult or challenging samples, which are, for example, more or less mixed and/or have a low copy number (LCN) or include low-template DNA (LTDNA). To process these difficult samples, several approaches have been developed, although the basic idea has not changed much. What would be the optimal selective or differential way of extraction in order to provide the greatest amount of the available target DNA with the least of the potential inhibitors and thus avoid downstream profiling from non-relevant allelic contributors [8]? An early technical solution for the separation of male and female content from mixed samples of sexual assault cases is based on the differential analyses of sperm and vaginal epithelial cells [59, 60]. The traditional differential extraction procedure has undergone modification, improvement and automated platforms, despite improvements in the alternative separation developments, and years later, is still in use today [61, 62].

Other potentially non-mixed, but easily containable low template DNA samples such as burned body or tissue which was subjected to a high temperature [46, 63–65], formalin-fixed samples [66–68], a piece of short hair with no root [69], or fingerprints of a touched surface and body parts [38, 39, 70–72] often require special consideration. Depending on the complexity of various influences, and with lack of valid information concerning the initial environmental circumstances and the chemical and/or physical processes involved, the degree of damage of and/or quality of obtained DNA cannot be, at first, accurately predicted. Since most extraction methods vary in relative efficiency and are incapable of offering an all-encompassing solution to the problem, for the purpose of optimization of a given procedure, it would be reasonable to consider the modification, combination or elimination of certain steps from different methodologies, specifically for trace samples [72].

2.1.2. DNA typing of human and human-derived biological samples of a medico-legal autopsy

2.1.2.1. Quantitation, amplification, sequencing, separation and detection

Although the practice of omitting the extraction and quantitation steps may occasionally seem to present a kind of benefit in regards to time and reduction of costs, for example in the case of disaster-victims [73], there may be multiple reasons for quantitation of the target DNA of

extracted samples [13]. When it cannot be excluded that the extracted samples putatively include additional DNA from other species, or the seemingly homogeneous sample is actually a mixed—for example, male and female epithelial cells at touched body surface—sample, determination of the appropriate amount of template DNA is required. In addition, the assumption that only low amounts of DNA obtainable in touched objects cannot always be correct, and in these situations, quantification can ensure the efficiency of downstream PCR [12]. It can help to provide an indication of hidden inhibitors [74] and to avoid off-scale artifacts or over-amplification. However, since the accuracy of various methods and commercially available kits can be slightly different [75–77], in cases of very low template amounts, quantitation can be often appropriated for indication, rather than merely an absolute measurement of the concentration, consequently, a negative quantitation result should not prevent future downstream amplification [12]. In light of standardized sampling tools and protocol developments as well as different alternative amplification methods—mainly in the field of reference samples—the importance of the quantitation step is partially based on ongoing research [78–80].

Very small amounts of nearly all targeted biological samples and DNA have been made detectable thanks to in-vitro replication—the polymerase chain reaction (PCR) [81]. The standardized amplification process, in increasing number of cycles, is an appropriate method to ensure efficient analysis, even of LTDNA obtained from the corpse belonging to persons other than that of the victim. The efficiency of PCR reaction is higher when the copy number of the entire target DNA is higher and/or the length of the amplified fragment is shorter. The specificity of PCR is directly affected by the primers and the primer binding site at target DNA. The number of markers can be amplified simultaneously in the same aliquot with an optimized mix of primer pairs, but the reaction can be inefficient due to the presence of different inhibitor molecules. When amplifying a low level of sample DNA—with higher probability of mixed samples—an unequal, stochastic fluctuation can manifest, which may lead to a preferential, imbalanced presence of the allelic component, and the number of PCR cycles cannot be increased unlimitedly [17, 82–84].

Since the initial application of the PCR method, several modifications and alternative molecules, e.g., novel DNA polymerases [85, 86], oligomeric mobility modifiers [87, 88] and fluorophores for labeling [89, 90], nonamers, aptamers [91, 92], etc., have been developed [82]. PCR can be performed in a solution, using immobilized amplicon at solid phase (SP-PCR) [93, 94] or compartmentalization of template molecules in water droplets in a water-in-oil emulsion (Em-PCR) [92, 95, 96] to obtain the desired sequence or fragment length of sample DNA. The combination of improvements in chemistry and the evolution in analytical—separation, detection—platforms provides a plethora of applications. Merging advantages of fluorescent tags with mobility modifiers gives a unique electrophoretic signature for each amplified ligation product and enables extensive sample multiplexing for separation by capillary array electrophoresis with laser-induced fluorescence detection on an automated genetic analyzer [82, 97, 98]. The uses of fluorophores sensitized by fluorescence resonance energy transfer (FRET) provided detection kinetics of fluorescence accumulation, and combined PCR amplification with real-time detection [76, 77, 99, 100]. Alternative, or engineered, high-processivity DNA polymerases can have increased resistance to inhibitory factors relating to amplification of target DNA directly from samples without prior extraction or quantification [73, 78, 101, 102].

With the incorporation of more fluorophores as fluorescently labeled ddNTPs with primer extension and chain termination [103] on capillary electrophoresis platforms, a complete genome is sequenceable. An alternative solution for sequence determination is based on sequentially added nucleotides to the synthesis reaction, and real-time detection of an optical signal of released pyrophosphate molecules [104]. Recently, successive generations of massively parallel (MPS) or deep-sequencing methods—also referred to as next (NGS) or current generation sequencing—applying different phase PCR, different molecule-labeling, different—optical, proton or electric—signaling and platforms have revolutionized genomic research. State-of-the-art devices provide sequencing of both whole genomes and that of many individuals simultaneously, even from a single molecule of deoxyribonucleic acid [105–107]. As a result of variance in the error rate of existing MPS assays, platforms and computational techniques, forensic validation of the quality of a NGS provided data is recommended [106]. With the application of MPS technology in the forensic genetic field, the limited number of STR and SNP capable of being [108] can be ignored, while increasing the potent application of SNPs in degraded samples, allowing the simultaneous analysis of different marker types and improving the high throughput for mitochondrial DNA testing as well in caseworking and databasing for laboratories [109]. Additionally, the implementation of MPS on the field of molecular autopsy can increase the genomic and etiological background in cases of sudden death, allowing for new therapies and strategies for treatment or prevention [110]. Although new developments have mostly superseded conventional sequencing, the first-generation Sanger sequencing method is still occasionally used in many, not-only-lower throughput laboratories [111].

Over the last decade, microfluidic DNA analysis and devices—also referred as microfluidic biochips; sample-in to results-out, lab-on-a-chip (LOC) technology—present an enticing technology platform for automating laboratory procedures [112]. DNA biochips enable the miniaturization, integration, and automation of tests, and can perform thousands of biological reactions in a few seconds [113–115]. Integrated and mobile rapid-DNA devices, which are designed for several purposes, can produce quality STR-profiles suitable for reference or as database samples [80, 116]. The ever smaller size of state-of-the-art devices may allow for portable DNA analysis, possibly even meeting the needs of decentralized environments [79]. Despite successful typing results from casework samples which indicate that mobile technologies can provide investigative leads, their implementation in casework also brings along possible risks of losing information concerning crime scene sample profiling [117, 118]. Although the constantly increasing demand for high-throughput and parallel analytical devices assists integration of state-of-the-art technology with the forensic DNA process, many opportunities exist for further improvements.

2.1.2.2. Brief history of markers for individualization of human samples

Those genetic markers which have high mutation rates are appropriately polymorphic and useful for forensic examinations. Polymorphic DNA variation can be fundamentally divided into the branches of sequence polymorphic and length polymorphic markers or loci, both for

human identity. The genome-scattered repeated DNA sequences form—sometimes referred as mini- or microsatellites [9, 119]—is typically designated by the length and number of repetitive units. The medium-length repeat (8–100 bp)—sometimes referred to as variable number of tandem repeat (VNTR)—markers such as D1S80, were mostly commonly used in the first part of the 1990s [120, 121]. Despite the relatively high level of polymorphisms of VNTR loci [122], the ability for easier PCR amplification—avoiding the problems of preferential amplification—has made the shorter-length repeat (2–6 bp)—sometimes referred to as microsatellites, short tandem repeat (STR)—markers more popular. The potency of analysis of degraded DNA using STR is greater due to it being less prone to allelic drop-out and more discriminative than the earlier alternatives.

A plethora of STRs are present in the human genome, and can vary not only in the length but also in the intervening sequence [123, 124]. Despite the complex, hypervariable motifs of some tetramer loci—posing a challenge for appropriate genotyping, among the types of STR markers—the tetranucleotide repetition has been developed for common forensic applications [9, 125–127]. Due to the narrow allele size range of STRs, the monoplex form of PCR has been rapidly replaced by the quadruplex form [9, 128–131]. The increasing number of standardized STR loci and fluorophore molecules combined with capillary electrophoretic separation [90, 97, 132, 133] has established the standard sets of STR markers for the forensic community [9]. Based on the national legislation of “core” loci, numerous national DNA databases have come into existence, and a collaboration on an international level between them has evolved. Existence of databases efficiently supports the recent state-of-the-art developments using database-related markers in further applications [9, 79, 80]. In addition to the application of MPS technology, it may also provide further sequence information for a more in-depth evaluation of STR alleles [106, 109, 134].

The early application of sequence polymorph markers in the forensic field is based on reverse dot-blot hybridization and allele-specific oligonucleotide (ASO) probes [135]. However, there are countries where the use of information of the coding DNA sequences for forensic purposes are restricted in some jurisdictions, which has limited the widespread use of sequence polymorphic markers in the forensic field. Additionally, the multi-allelic polymorphic STR markers have a higher number of possible alleles and a higher discrimination power than that of the early developed sequence polymorph systems such as Polymarker or HLA DQ alpha testing kits [136–138]. In spite of the validation and popularity of these types of polymorphic markers, they have already been phased out of forensic practice.

However, alternative sequence variability and biallelic markers—also referred to as single nucleotide substitution or polymorphisms (SNP)—are the most abundant polymorphism at the genome level, and they provide potent applications in forensic identification [139]. The biallelic polymorphisms have a lower mutation rate than the STRs [140, 141], and can be preferable in case of degraded samples. In spite of the lower discrimination power of a single biallelic marker when compared to a single STR locus, the increased number of simultaneously analyzed SNP loci can be effective for various uses in the forensic genetic field, which in light of advances in massive parallel sequencing can be especially progressive [109, 142–144].

2.1.2.3. *Brief history of uniparental lineage markers*

The Y-chromosome and mitochondrial DNA (mtDNA)—also referred as uniparental/lineage markers—are both haploid entities and, as such, are transmitted from generation to generation without recombination. Consequently, the variability of these markers depends on mutation events [145, 146]. The early implementation of these markers into forensic practice is based on several approaches and is becoming ubiquitous in forensic genetics [147, 148]. Although forensic application of these markers is accompanied by both advantages as well as limitations, their usage does, however, overcome two major challenges commonly encountered by the forensic scientist. In particular, for Y-chromosome specific markers, an increase in the otherwise-limited success of PCR obtained from male/female mixtures and, for mtDNA, obtaining genetic information from samples that are degraded, or nuclear DNA (nuDNA) free.

Although, in numerous cases, jurisdictions make use of the benefits that lineage marker analysis can bring, they do however have strong limitations in forensic applications, specifically, conclusions may not be drawn on the individual level as would be otherwise desirable [149–151]. Although haplotype markers usually are not included as standard markers in police databases, similarly to the Internet-based STR information resource [9, 152], haplotype databases have also been designed to store haplotypes from global populations. These provide a basis for frequency estimations and support data quality requirements to facilitate on-going efforts in forensics DNA investigation [153, 154]. In addition to complete autosomal genetic profiling, the genetic information of lineage markers is especially important in both forensic parental or kinship analyses [155], as well as from an evolutionary and genealogy point of view, in the prediction of potential geographic or ancestral origin [156–158].

The Y-chromosome [159], however, is present with only one copy per normal cell, and has higher diversity than mtDNA in addition to an increasing number of Y-STRs [9, 155, 160] completed by rapidly mutating (RM) markers [155, 160–162], Y-SNPs [155, 160, 163–165] and insertion/deletion polymorph (Indel) markers [166, 167]. Although, the analysis of Y-chromosomal markers can provide complementary information in addition to an autosomal genetic profile [168], the most common application of Y-STRs is in cases of sexual assault, when the female component can greatly overshadow the male component, making autosomal STR profiling frequently difficult, unclear or impossible. Examination of Y-chromosome markers is available in a wide range of commercial kits [12, 169, 170] which perform adequately for identifying male lineages.

Mitochondrial DNA, similar to the Y-chromosome, is not a unique identifier, but its examination in criminal cases can be nevertheless reasonable, for example, when the profiling of nuclear DNA markers fails to produce a profile. The mitochondrial genome evolves relatively rapidly, and newly arising—primarily point—mutations tend to become fixed faster and at a higher rate than that for nuDNA. In the mitochondrial genome, the highest level of genetic variation is located in the control region (CR) [171]. The most common type of polymorphs belong to SNP, which can be found not only in the hypervariable regions (HV I–III), but in the entire mtDNA genome as well [172, 173]. Single base mutations may lead to such a condition

in which both the mutated and original forms coexist as admixture, which is referred to as heteroplasmy [174–176]. Similarly, indel variability is also present in the mitochondrial genome [177, 178]. The higher sensitivity resulting from the order of magnitudes copy number of mtDNA, compared to that of nuDNA, makes it possible to obtain reliable haplotypes, when the DNA gathered from samples is highly fragmented and/or damaged. In addition, due to the inheritance of molecules, the genetic information from maternal relatives as references, e.g., for an unknown or missing person, are suitable for making direct comparisons. In the field of forensic genetics, the mtDNA is also a so-called historical marker analyzed by Sanger sequencing technologies [179]. This method combined with capillary array instrumentation or the application of pyrosequencing technology was previously ubiquitous in laboratories [180, 181] and is currently still in use [182–184].

Although the highest-polymorph regions are the traditionally-analyzed hypervariable regions (HV I–III), in some cases, sequencing of the whole mitochondrial genome is capable of solving even those cases, in which the hypervariable haplotype cannot be differentiated between individuals. The increasing demand for mtDNA examination—missing person cases, natural disasters, human rights investigations, etc.—has revealed the limitation of throughput and cost-benefit relations of conventional technology which had previously focused on only part of the entire mitochondrial genome. The implementation of novel technologies such as MPS, provides an automated workflow and could offer its usage for a wide range of samples. Over the last decade, developments related to the sequencing of entire mtDNA genomes [185, 186] have resulted not only in the high throughput of data, improving the recovery of genetic information from forensic specimens, but have also focused on the discrimination potential of mtDNA evidence. Comparison of whole mitochondrial genomes can provide an understanding of mtDNA mutation and heteroplasmy, and completing it with the forensic validation of MPS platforms may lead to the deconvolution of mixtures, as well as providing solutions to other challenging casework problems [106, 187, 188].

2.1.2.4. Markers for investigation of cause or manner of death: molecular autopsy

Even though the active field of medico-legal autopsy can vary among different countries according to traditional and legislatorial backgrounds, the use of molecular analyses is becoming steadily more frequent and its importance is becoming more recognized in postmortem examinations. Several different forensic sciences—including forensic genetics—are inherently involved in this multidisciplinary molecular investigation. Molecular autopsy is still a relatively new concept in pathology and forensic sciences [189]. In contrast to cases of intentional or suspected violent death, in instances when death occurred either suddenly, unexpectedly, or involving infants and the young [3], autopsy sampling focuses on the corpse's own substances to aid in determination of genetic markers and mutations which could be responsible for the cause or manner of death. From this point of view, the use of forensic genetics is not only strictly limited to purposes of identification, but may also be tasked by performing genetic tests for genes associated with disease [190, 191].

Several specific or rare diseases may be linked to a particular pathological condition in cases of both positive and negative autopsy [189, 192]. Novel MPS technology can be highly informative in detection of all variants of genes affecting unexpected death in epilepsy, adverse drug reactions and metabolism [192–194], as well as the cardiovascular system, e.g., cardiomyopathies and cardiac ion channelopathies, which have been associated with sudden cardiac death [110, 195].

Molecular autopsy is an emerging part of the medico-legal autopsy, and with the incorporation of genome-wide data and computational techniques, its benefit to this multidisciplinary field should continue to increase [192].

2.1.2.5. Markers for supplemental information

An earlier phase in forensic examinations focused on polymorph markers for the construction of forensic databases, as they do not vary over time [196]. The numbers of STR marker-based, legislated databases and their cooperation at the transnational level, as well as the volume of associative data-records and their efficiency are considerable economic factors for their consolidation in forensic practice [197, 198]. Massive parallel sequencing (MPS) techniques have recently provided the opportunity to extend the contemporary investigative role of databases by the sequencing of STRs [199]. A plethora of markers have been recently introduced for forensic applications, although the majority of them have yet to be integrated into forensic databases. The level of polymorphisms is based upon new mutations, which, when they arise in a population, are spread via natural selection, migration and genetic drift. Different types of markers exhibit different rates of mutation; for example, indel mutations occur less frequently than single nucleotide substitutions in both nuclear and mitochondrial genomes [119, 134, 140, 141, 145, 159, 177, 178]. A large number of autosomal and sex-chromosomal STRs, SNPs from both nuclear and mitochondrial genomes, indels and ancestry-informative markers (AIMs) and mRNA and phenotypical markers (FDP) have been introduced and applied by the forensic community. Numerous new markers are being implemented in advancing the desired goals of investigative authorities, specifically, how forensic genetics can help to improve the collection of the most relevant information in terms of individualization and identification from the least amount of biological samples. Due to their abundant distribution in both the nuclear and mitochondrial genome, SNPs increasingly can provide the genetic background for the prediction of physical characteristics [200, 201], or geographical origin [202, 203], and can also provide investigative tools to trace unknown individuals [204, 205] even from historical remains [206–208]. Determination of ancestry [209, 210] and externally visible characteristics, such as skin, eye and hair pigmentation [211, 212], morphology [213, 214], or age [215, 216], is an ongoing research field of forensics, requiring both forensic validation of analytical platforms and computation data.

Although there is a distinct probability of encountering the artificially altered appearance of phenotypes, the phrase “DNA witness” has obtained new perspectives with its potential for providing information of higher accuracy than that of traditional eye witnesses. In light of the

above, forensic DNA phenotyping (FDP), although a promising technique, raises multiple, not only scientific, but also ethical and legislative issues [217, 218].

2.2. DNA analysis of nonhuman remains

There are cases when the victims have been involved in a pet or livestock animal attack with fatal—usually unwitnessed—consequences [40, 219, 220]. In other cases, animal hair from a victim's body helps to reveal the perpetrator [221], or so-called “silent witnesses” such as plant remains [222] on corpses, or even evidence of algae from postmortem tissues, aid in determination of the manner of death [223]. Due to the increasingly particular importance of genetic analysis of nonhuman substances in these types of cases, forensic genetics today is progressively incorporating the examination of nonhuman genetic material to an ever greater extent [224]. In essence, the similarities in analog and ortholog variable components of genomes provide forensic investigation of nonhuman biological substances in the same manner as for human forensics, but distinctions existing in different organisms and species, i.e., genomic architectures, reproductive strategies and genetic diversity, are continuously broadening the dependent scientific areas. The benefits stemming from the extension of forensic genetics toward nonhuman relations were clearly recognized decades ago [225], and the incorporated application of animal, plant or microorganisms has been actualized in a large scale of caseworks, from animal attacks [226, 227] to bioterrorism [228], as well as in wildlife crimes [229, 230], identification of food composition [231, 232], *Cannabis sp.* chemotyping [233, 234], and even the estimation of postmortem interval and skin microbiomes [235, 236]. Other various aspects of possible nonhuman biological evidences found on the corpse, e.g., pollens or plant fragments, can reveal potential indirect links to the perpetrator, and also additional, case-related facts, complementing autopsy information with the modality of death or the crime scene setting for purposes of case reconstruction [237, 238]. The human-related techniques of forensic genetics have been adapted to nonhuman analyses, but the genetic markers implemented frequently originate from the field of conservation biology. This could be a reason that markers and data computation are more varied in nonhuman areas than those in human identification, and comparatively, are rarely standardized for all species. In recent years, genome-wide analyses and MPS technology have revolutionized this broad field in any case, so it would not be practical to introduce the enormous variety of nonhuman forensic DNA analysis here, within this section. Due to the common coexistence of humans and domestic animals, dog violence on humans leading to fatal consequences represents a frequent type of case [224, 239–242]. In these cases, DNA can be separated from saliva from within the biting area, from animal hairs and sometimes blood, or bitten material, including the victims' clothing [40, 219]. The saliva of dogs is a suitable source for DNA extraction [243–245], even when the human victim's blood is present [40, 219]. Although DNA extraction from dog hairs has similarly been solved using well-developed methods [246–249], genetic typing of single dog hairs has often failed [247]. As an analysis concept using shortened amplicons [231, 250, 251], the isolated DNA may be

sufficient for successful amplification. Otherwise, when the amplification of nuclear markers is insufficient due to the low quantity and degradation of nuclear DNA, mitochondrial DNA may be more appropriate [252, 253].

STR-based genetic markers in multiplex forms have been continuously developed for canine individualization [254–257]. Due to the fact that dogs have been involved from relatively early on in the forensic genetic field, positive differences have been made for canines compared to other species in forensic applications. The quality requirement for implementation of canine DNA analysis in forensic practice is as ambitious as in human forensics. The STR marker-sets which have been developed have relevant population studies, occasionally extended with genetic variance [258–264], inbreeding [258, 261] and mutation data [265]. Sequence databases of canine mitochondrial DNA have also been developed [266–268].

Accreditation measures for DNA typing in the nonhuman or animal forensic [269] field are established on a different level, and are occasionally of significant importance, as the potential number of species could be incorporated into legal procedures in a myriad of ways. Although, primarily due to the inherent heterogeneity of existing laboratory environments and the expense and complexity of accreditation, equalization of the nonhuman field to human forensic genetics poses a monumental challenge. Nonetheless, the first steps toward this goal must obviously be taken according to the developed recommendations concerning the standardization of DNA typing of animal species and products [270–272].

3. Conclusions

Although considerably younger, the field of forensic genetics is a distinct part of the forensic arena [1] and its presence has become widespread and is now accepted as the primary forensic method for identifying persons of interest [273]. The modern implementation of genetic analysis within the wider-scale field of autopsy has brought with it an increase in the potentiality of exhibits. The accreditation and quality assurance of the field of forensic genetics, in accordance with professional recommendations [274], legal regulations and harmonization [275], quality standardization and systems [276], as well as the establishment of national and international databases and their subsequent cooperation [197], have caused genetics to occasionally play a pioneer role among scientific fields on the forensic palette. Despite the relative youth of forensic genetics, several generations of methods have already been developed and obsoleted in this field. Recently, sequencing techniques have continued to move ahead at a staggering speed, and the massive parallel sequencing strategy, capable of simultaneously generating thousands of reads on state-of-art devices, will revolutionize genetic analysis [277, 278]. Although the admissibility of MPS results in a court of law will most likely continue to be challenged—as are mostly newly introduced methods—foreseeably raising concerns about privacy [278], the complementary application of MPS genome-wide data, with other innovative and advanced analytical systems—e.g., imaging analysis—can extend the professionalism and efficiency of the multidisciplinary medico-legal field to the ultimate benefit of society [278].

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