Prognoses on DNA-based identification success rates of altered human remains using capillary electrophoresis and Next Generation Sequencing technologies

Inaugural dissertation

to

be awarded the degree of Dr. sc. med.
presented at
the Faculty of Medicine
of the University of Basel

by

Alina Angelika Senst

from Osnabrück, Germany

Basel, 2022

Approved by the Faculty of Medicine

On application of

Prof. Dr. med. Dipl. Phys. Eva Scheurer

Prof. Dr. rer. nat. Sven Cichon

Prof. Dr. sc. hum. Katja Anslinger

Dr. rer. medic. Iris Schulz

Basel, …….

……...

Dean

Prof. Dr. Primo Schär

Acknowledgements

To begin with, I want to express my sincere gratitude to my primary advisor Prof. Dr. Eva Scheurer for her supervision and giving me the opportunity to conduct my PhD at the Institute of Forensic Medicine Basel. I am very thankful for her supporting feedback and guidance throughout this thesis.

My dearest gratitude goes to Dr. Iris Schulz for her exceptional support, coordination, motivation and advice during the recent years. I consider myself extremely fortunate to have her as my supervisor. I am thankful for her dedication in research and all of our inspiring discussions that not only pushed forward my research but also my personal development. Thank you very much for always having an open door and the encouraging words.

I also owe gratitude to Prof. Dr. Sven Cichon for his support and input throughout this thesis. I appreciated each of our meetings and I am very thankful for the exchange and valuable feedback.

My sincere gratitude goes to Dr. Katja Anslinger for evaluating this thesis and her valuable feedback on my publications. I am very grateful for her support and effort.

Additionally, a big thank you goes to the grandiose forensic genetics team for the great times, the constant preparedness to celebrate milestones as well as the assistance and support in the laboratory. Further, I want to thank Janine Schulte and Dr. Melanie Bauer for the support and proofreading of this thesis.

I would also like to thank all collaborating partners for the great cooperation and their valuable contribution to this multicenter study: Dr. Matthias Drum and Christian Cossu (Institute of Forensic Medicine St. Gallen), Dr. Martin Zieger (Institute of Forensic Medicine Bern) and Dr. Amke Caliebe (Institute of Medical Informatics and Statistics Kiel).

Further, I am sincerely grateful for the support of Dr. Kathrin Gerlach and the whole forensic medicine team during autopsies. Many thanks to Katja Heyde for proofreading sections of this thesis and particularly Thomas Rost deserves special thanks for his continuous assistance and support in sample collection.

Last but not least, I want to thank my family and friends for their patience, kindness and always lending an ear in challenging times. Especially my parents and most importantly my mother Elisabeth for her encouraging words and daily morning sun greetings on cloudy days.

"Our genetic differences are at the heart of one of the most fascinating paradoxes of the human condition: that we are all different, yet we are all the same"

Mary-Claire King, geneticist, 1993

Table of contents

Summary

The DNA-based identification success of altered human remains relies on the condition of the collected tissue sample and the associated DNA quantity and quality. Due to tissue-specific differences in post-mortem DNA stability, sampling of the best-suited biological material is essential for successful and rapid identification. However, a large variety and partly contradicting recommendations on optimal material have been published so far. The observed insecurity in sampling strategies revealed the need for a broad and systematic approach in predicting short tandem repeat (STR) genotyping success rates in a wide range of tissue types. Therefore, the overarching aim of this thesis was to improve the DNA-based identification success of altered corpses by presenting novel recommendations and guidance for optimal tissue sampling according to the condition of the body.

First, the current situation of identification processes in forensics casework was assessed by a retrospective study on the identification success of 402 altered human corpses over seven years (*project I*). The evaluation of medical as well as genetic reports revealed an increase in the examination of highly and profoundly decomposed corpses and challenges in molecular analyses of degraded and inhibited samples from altered remains. By comparing the number of successive and parallel PCR amplifications, the most unpredictable typing success and highest number of additional analyses were observed in muscle and bone samples. A comparison with previously published studies highlighted the challenges and insecurity in tissue sampling and the need for standardized guidelines.

Furthermore, during *project II*, the reliability of novel DNA sequencing methods was assessed by validating the MiSeq FGx system for Next Generation Sequencing (NGS) of casework samples and optimizing the sequencing workflow for samples of altered remains. The extensive evaluation of sensitivity, concordance to currently used methods and reproducibility, among others, displayed the technology as robust and implementable in forensic routine casework. Additionally, the applicability of phenotype and biogeographic ancestry prediction was demonstrated in challenging samples of altered corpses. However, as the optimization results revealed, an additional PCR purification step, an increased pooling volume and a reduction of adapter volumes for DNA input concentrations ≥ 31.2 pg is recommended for sequencing highly degraded and inhibited samples.

Finally, based on the outcomes of *projects I* and *II*, the multicentre study concludes with the presentation of novel recommendations on alteration-specific optimal tissue types for first-attempt identification of altered human remains in *project III*. By providing an easy and rapid scoring system, a precise assessment of the corpse alteration progress is enabled. Furthermore, the systematic approach included the comparison of DNA quantity, integrity and resulting STR profile completeness in an exceptional high number of 1698 DNA extracts from 949 samples of 19 different tissue types. Thereby, standard capillary electrophoresis as well as forthcoming NGS methods were used and the impact of DNA extraction methods was assessed. The final and first-time prognoses on genotyping success of a wide range of tissues separated for two DNA extraction methods (purifying and non-purifying) and two sets of STR loci (22 loci and 16 loci of the extended European Standard Set) provide guidance that improves the first-attempt DNA-based identification success of altered corpses.

Zusammenfassung

Der DNA-basierte Identifizierungserfolg zustandsveränderter Leichen ist abhängig vom Zustand der entnommenen Gewebeprobe und der damit verbundenen DNA-Menge und Qualität. Aufgrund gewebespezifischer Unterschiede in Bezug auf die postmortale DNA-Stabilität besteht jedoch eine Uneinigkeit, welches Material für eine effiziente molekulargenetische Identifizierung herangezogen werden soll. Die hohe Anzahl bisheriger Studien mit teilweise widersprüchlichen Empfehlungen und eine damit verbundene Unsicherheit bei der Probenentnahme verdeutlicht die Notwendigkeit eines systematischen Ansatzes zur Prognose des Genotypisierungserfolges von short tandem repeats (STRs) in einer Vielzahl von Gewebetypen. Entsprechend war das Ziel dieser Arbeit, Richtlinien zur effektivsten Entnahme von Gewebeproben in Abhängigkeit vom Leichenzustand zu etablieren.

Zunächst wurde dazu die aktuelle Situation der Identifizierungsprozesse in der Forensik im Rahmen einer retrospektiven Studie zum Identifizierungserfolg von 402 zustandsveränderten Leichen über einen Zeitraum von sieben Jahren erfasst (*Projekt I*). Die Auswertung medizinischer und genetischer Gutachten zeigte eine Zunahme an Untersuchungen von stark fäulnisveränderten Leichen und deutliche Unsicherheiten bei der molekulargenetischen Analyse von degradierten und inhibierten Proben. Die Auswertung paralleler sowie wiederholter PCR Amplifikationen wies besonders bei Muskel- sowie Knochenproben eine hohe Anzahl zusätzlicher Analysen auf. Zusammen mit einem Vergleich bereits publizierter Studien wurde die Unsicherheiten bei der Probenentnahme bestätigt.

Darüber hinaus wurde im Rahmen von *Projekt II* die Anwendbarkeit und Zuverlässigkeit moderner DNA Sequenziermethoden durch die Validierung des MiSeq FGx Systems für das Next Generation Sequencing (NGS) von anspruchsvollen Fallproben untersucht. Die unter anderem erfolgte Bewertung der Sensitivität, Konkordanz mit derzeitigen Methoden und Reproduzierbarkeit zeigte, dass die Technologie für forensische Fallarbeit geeignet ist. Zusätzlich erfolgte für Proben von zustandsveränderten Leichen eine Optimierung des Sequenzierablaufes sowie eine Prognose des Phänotypes und der Herkunft. Entsprechend der Optimierungsergebnisse ist bei der Sequenzierung von Proben zustandsveränderter Leichen ein zusätzlicher PCR Aufreinigungsschritt, eine Erhöhung des Poolingvolumens und eine Reduktion der Adaptervolumina bei DNA Konzentrationen ≥ 31,2 pg empfohlen.

Basierend auf den Ergebnissen von *Projekt I* und *II* konkludiert die multizentrische Studie mit der Präsentation von Empfehlungen optimaler Gewebetypen in *Projekt III*. Neben der Bereitstellung eines Schemas zur Beurteilung des Leichenzustandes erfolgte ein systematischer Analyseansatz, bei dem der DNA Gehalt, die DNA Integrität und die resultierende STR Profilvollständigkeit in einer unvergleichlichen Stichprobengrösse von 1698 DNA Extrakten aus 949 Proben von 19 verschiedenen Gewebetypen verglichen wurde. Dazu wurde sowohl die derzeitige Standardmethode der Kapillarelektrophorese als auch moderne NGS Technologien verwendet. Die erstmalig vorgestellten Prognosen zum Genotypisierungserfolg einer Vielzahl an Gewebeproben, getrennt für zwei DNA Extraktionsmethoden (aufgereinigt und nicht-aufgereinigt) sowie zwei Sets von STR Loci (22 Loci und 16 Loci aus dem extended European Standard Set), bieten Richtlinien, die zu einer Verbesserung der DNA-basierten Identifizierung von zustandsveränderten Leichen beitragen.

Publications

A Senst, A Caliebe, M Drum, C Cossu, M Zieger, E Scheurer, I Schulz. Novel recommendations on the successful identification of altered human remains using standard and forthcoming technologies: results of a systematic approach. Submitted to *Forensic Sci Int Genet.* on the 29th of April 2022. Manuscript number: FSIGEN-D-22-00099

A Senst, A Caliebe, E Scheurer, I Schulz. Validation and beyond: Next generation sequencing of forensic casework samples including challenging tissue samples from altered human corpses using the MiSeq FGx system. *J Forensic Sci.* 67:35318655, 2022; DOI: https://doi.org/10.1111/1556-4029.15028

A Senst, E Scheurer, K Gerlach, I Schulz. Which tissue to take? A retrospective study of the identification success of altered human remains. *J Forensic Leg Med*. 84:102271, 2021; DOI: https://doi.org/10.1016/j.jflm.2021.10

S Egger, **A Senst**, I Schulz. Die Rolle des Speichels in der forensischen Genetik. In: Speichel: Ein Nachschlagewerk für Zahnärzte, Hausärzte, Kinderärzte, Hals-Nasen-Ohren Ärzte, Dentalhygienikerinnen, Zahnmedizinische Prophylaxe- und Fachhelferinnen, Logopäden sowie Studierende der Medizin und Zahnmedizin. A Filippi, T Waltimo. Quintessence Publishing. 2020

A Senst, J Dressler, J Edelmann, M Kohl. Entwicklung eines qPCR-Assays zum Nachweis der Sekretart: Identifikation von Sperma, Vaginalsekret und Speichel über zellspezifische Methylierungsmuster. *Rechtsmedizin*. 29, 94–100, 2019; DOI: https://doi.org/10.1007/s00194-018-0294-y

Presentations and Posters

2022

A Senst, E Scheurer, I Schulz. Validierung und Optimierung von Next Generation Sequencing Methoden für forensische Fallproben mit Fokus auf Proben von zustandsveränderten Leichen. 42th Spurenworkshop. Online. 18th February 2022. Presentation

A Senst, E Scheurer, I Schulz. ePCR1 Puffer. Optimierte Pufferlösung für Inhibitor-belastete Proben zur Identifizierung menschlicher Überreste. 42th Spurenworkshop. Online. 17th February 2022. Presentation

2021

A Senst, E Scheurer, I Schulz. Optimizing Massive Parallel Sequencing methods for challenging samples from decomposed human remains. 7th Department of Biomedical Engineering (DBE) Research Day. Basel, Switzerland. 7th September 2021. Poster

- Awarded 1st place in poster presentation

A Senst, E Scheurer, I Schulz. Validation of the MiSeq FGx System - Optimizing Massive Parallel Sequencing Methods to improve the results of challenging samples from decomposed human remains. Sommertagung Schweizerische Gesellschaft für Rechtsmedizin (SGRM). Arlesheim, Switzerland. 3rd – 4th September 2021. Presentation

2020

A Senst, E Scheurer, I Schulz. Which tissue to take? - A retrospective study of altered human remains. 6th Department of Biomedical Engineering (DBE) Research Day. Basel, Switzerland. 8th September 2020. Poster

2019

A Senst, I Schulz. Post mortem DNA analysis of human remains with different degrees of decomposition – Part 1/4: A retrospective study of unknown human remains at the Institute of Legal Medicine Basel (2014-2019). 28th Congress of the International Society for Forensic Genetics (ISFG). Prague, Czech Republic. 9th – 13th September 2019. Poster

A Senst, I Schulz, I Blank, S Kron, A Lange, R Janscak. Contamination on the outside of allelic ladder containers. 28th Congress of the International Society for Forensic Genetics (ISFG). Prag, Czech Republic. 9th – 13th September 2019. Poster

A Senst, E Scheurer, K Gerlach, I Schulz. Post mortem DNA analysis of human remains with different degrees of decomposition. Department of Biomedical Engineering (DBE) Summer School. 16th – 21st June 2019. Poster

Definitions

DNA degradation	Process of DNA double helix denaturation leading to single and double strand breaks resulting in fragmentation
DNA genotyping	Process of asserting a DNA sequence at specific positions within the genome
DNA phenotyping	Process of predicting a persons' physical appearance, including color of hair, eye and skin
Decomposition	Process of human tissue breakdown into simple organic and inorganic matter after death
ESS	European Standard Set including seven short tandem repeat loci: D3S1358, TH01, D21S11, D18S51, vWA, D8S1179 and FGA
eESS	Extended European Standard Set including 16 short tandem repeat loci: FGA, TH01, vWA, D1S1656, D2S441, D2S1338, D3S1358, D8S1179, D10S1248, D12S391, D16S539, D18S51, D19S433, D21S11, D22S1045 and SE33
Locus	Position of a gene or sequence of DNA on a chromosome
Read	DNA sequence corresponding to a target DNA fragment resulting after DNA sequencing
STR	Short tandem repeat. Microsatellite with DNA motifs repeated at variable numbers. The number of repeats differs among individuals (with exception of monozygotic twins). Sizes range between 100 and 400 bp
SNP	Single nucleotide polymorphism. Substitution of a single nucleotide at a specific position in the genome
VNTR	Variable tandem repeat. Minisatellite differing in number of repeated DNA motifs. Sizes range between 400 and 1000 bp

Abbreviations

ATP	Adenosine tri-phosphate
bp	Base pair
CE	Capillary electrophoresis
CODIS	Combined DNA Index System
ddNTPs	Chain-terminating dideoxynucleotides
DNA	Deoxyribonucleic acid
EDNAIS	Erkennungsdienstliches DNA-Informationssytem
ENFSI	European Network of Forensic Science Institutes
ESS	European Standard Set
eESS	Extended European Standard Set
ID	Identification
ISFG	International Society for Forensic Genetics
NGS	Next Generation Sequencing
PCR	Polymerase chain reaction
PMI	Post-mortem interval
RFLP	Restriction fragment length polymorphism
STR	Short tandem repeat
SBS	Sequencing by synthesis
VNTR	Variable tandem repeat

1. Background

1.1 Identification of human remains in forensic casework

Ascertaining the identity of deceased is one of the most important tasks in forensic casework and of crucial importance for both legal and social matters. Beside answering questions concerning the circumstances, causes and manner of death, it is the examiners responsibility to determine the identity of the corpse based on reliable evidence [1-3]. The process of identification (ID) usually consists of two steps: 1) developing a hypothesis about the unknown corpse as well as gathering information and 2) comparing post-mortem data with presumed ante-mortem data [2, 4]. For the second step, various methods are available depending on the condition of the corpse, the institutional instrumentation and equipment, accessibility of ante-mortem data and the examiners experiences. A common method is the comparison of computed tomography scans to determine the dental status, distinct osseous structures or implants. Additionally, visual ID by potential relatives, comparison of fingerprints or morphological features, such as tattoos, can be performed [2]. However, when none of these methods is applicable or appropriate ante-mortem data is missing, molecular genetic analysis is the most reliable and probably the last method for ascertaining identity [5, 6]. Yet, despite advances in molecular genetic analyses, it is still a challenge to generate sufficient DNA profiles of corpses with advanced signs of decomposition or heat-related alterations [7, 8].

1.2 Post-mortem alterations of the human body

Forensic examiners are frequently confronted with severe alterations and modifications of the human body, which can impede a DNA-based ID [9, 10]. Such altered human remains can be divided in decomposed corpses, corpses found in water and burnt corpses. Since not all deceased are found immediately after death, the body decay can significantly progress with increasing post-mortem interval (PMI), leading to severe degradation at the time of discovery. The rate of decomposition can be highly influenced by environmental factors, particularly water. Corpses decomposed in water can be found after drowning accidents in rivers or seas or after death in a filled bathtub, among others. With about 140.000 reported cases of death by drowning per year all over the world, drowning is a major cause of unnatural death world-wide, indicating the relevance for forensic casework [11]. Furthermore, several crimes or accidents including fires, plane crashes or traffic accidents can cause lethal heat-induced thermal injuries that can affect the examination and further molecular analyses [12, 13].
As the corpses' condition is a decisive factor in DNA stability, the alteration and modification processes leading to DNA genotyping impairments will be summarized in the following sections. For each of the three types of altered remains, the post-mortem characteristics necessary to predict DNA genotyping success will be briefly presented.

1.2.1 Decomposed human corpses

The decomposition of the human body is a dynamic and continuous process, referring to the breakdown of tissues by disaggregating complex macromolecules into their simple organic structures [14-16]. After death, the progress of decomposition is determined by the processes of autolysis (self-digestion of cells), putrefaction (destruction of organic matter) and diagenesis (breakdown of bones) [14, 17, 18].
Although all human bodies undergo essentially the same stages of decomposition, the chronological timing of the processes is highly variable and influenced by intrinsic factors such as body mass (e.g. weight and height), ante- or post-mortem injuries or diseases as well as extrinsic environmental factors such as temperature, humidity, pH value and insect or microbial activity [14, 16, 19]. Of these factors, the ambient temperature has been described as the most decisive influence in accelerating or decelerating the rate of decomposition by affecting all biological activities and biochemical reactions [15 19, 20]. Since the human body contains a significant amount of water, which desiccate upon death, humidity also has a major effect on the rate of decay. Therefore, arid conditions can lead to an increased desiccation and a delay in decomposition [15]. Additionally, low humidity combined with dry heat promotes the dehydration and development of tissue mummification. Mummification usually affects the skin, which turns it into a leathery or parchment-like layer that adheres to the bones [14, 21-23].

Cell death and autolysis

Within minutes after death, human decomposition is initiated by autolysis, a complex series of biochemical and pathological processes, resulting in distinct alterations of the cell's structure and composition [14, 16, 24]. Generally, this self-digestion of cells is executed by endogenous enzymes [25]. Cessation of blood perfusion leads to cellular oxygen and nutritional deprivation, followed by a decrease in adenosine tri-phosphate (ATP) production and a decline in pH value [16, 21, 25]. Due to the shift from oxidative phosphorylation to anaerobic reactions, acidic by-products accumulate and poison the cell. The death of the cell initiates the destruction of lysosomes and consequent the release of high concentrations of cellular enzymes such as lipases, proteases and DNases, which degrade proteins, lipids, carbohydrates and nucleic acids [15]. Hence, the chromatin is irreversibly damaged and the cell membrane degrades, leading to leakage of hydrolytic enzymes into intercellular spaces, which causes cells to detach from each other [21]. As autolysis progresses, external characteristics of post-mortem changes, such as sloughing of skin and hair, loosening of nails, organ liquefaction and fluid accumulation in the skin (post-mortem bullae) can be observed [22].

Concomitant with autolysis is the appearance of three independent post-mortem changes known as rigor mortis, algor mortis, and livor mortis [22].

Rigor mortis describes the stiffening of muscles caused by the breakdown of ATP and the built-up of lactic acid. According to Nysten's rule [26], stiffness is first noticeable in the jaw after approximately two to three hours, reaches a peak after approximately twelve hours and begins to dissipate over the following twelve hours. However, this process can be affected by several factors such as ante-mortem physical activity, body weight as well as ambient temperature [22, 27, 28].

Algor mortis refers to the cooling of the body and begins immediately after death. A decrease in body temperature is caused by the loss of heat producing metabolic processes and reaches ambient temperature after approximately 18 to 20 hours. Yet, factors such as body mass and temperature can accelerate or decelerate this process [27].

Livor mortis describes the physical process of blood settling to the lowest portion of the body caused by cessation of blood circulation and gravity. Even though the process begins immediately after death, resulting discoloration of the skin is first visible after approximately 15 minutes and is fully developed after approximately three to four hours. However, the extent and progress is subject to several variations, such as the body position [12, 27, 29].

Putrefaction

The process of putrefaction is initiated by the rupture of the cell membrane during autolysis, which releases nutrient-rich fluids that serve as an energy source for microorganisms [15, 16, 21, 30]. In contrast to autolysis, which occurs independently from bacterial activity, the process of putrefaction describes the destruction of soft tissues by bacteria, fungi and protozoa [14, 17]. The activity of those microorganisms results in degradation of carbohydrates, proteins and lipids into gases, liquids and their simple organic structures [14, 21]. The catalysis by endogenous bacteria produces sulfur-containing amino acids, resulting in hydrogen sulfide. Further combination with hemoglobin, the oxygen-transport protein found in erythrocytes, results in sulfhemoglobin, which is responsible for the greenish discoloration of the skin [16]. Since putrefactive bacteria predominantly emerge from the gastrointestinal tract into ambient tissues, the first visible discolorations are generally on the right lower abdomen and continue to spread throughout the trunk (Fig.1B)[15-17]. Depending on intrinsic and environmental conditions, those putrefactive signs usually start to appear after twelve to 18 hours in the summer and after 24 to 48 hours in the winter[16]. Bacteria then further invade tissues via the vascular and lymphatic systems and from there continue to spread throughout the whole body [16, 17]. Hemolysis and the spread of bacteria cause "marbling" of the skin, whereas the venous network becomes visible [12]. Due to the increase in microbial decomposition and drying of the tissue, the skin undergoes a color change from pale to green, brown and black (Fig.1A-E)[15]. Putrefaction then progresses with distention of tissues due to the production of gases such as hydrogen, carbon dioxide and methane by bacterial fermentation. Fluid-filled blisters form on the skin and particularly the enclosure of gases in the bowels can lead to an extensive bloating of the abdomen [14, 21]. Internal pressure then results in purging of putrefactive fluids from body openings (Fig.1C).

Subsequently, active decay begins when the combined feeding of maggots and bacterial putrefaction lead to a breakdown of the skin barrier and partial skeletonization (Fig.1D+E). By the end of putrefaction, most of the soft tissue is removed by both aerobic and anaerobic bacteria as well as insect activity, which is dominated by the feeding of Diptera larvae [14, 27].

Diagenesis

Once the soft tissue is completely decomposed and the skeleton is exposed to environmental conditions, diagenesis begins [14, 15] (Fig.1F). Diagenesis is a complex process including loss of organic (collagen) and inorganic substances (hydroxyapatite, calcium and magnesium) [14]. The rate of bone diagenesis depends on environmental conditions such as temperature, humidity or pH value, and access by microbes or scavengers [14, 18]. Since bone preservation is highly dependent on these factors, the time course of dissolution is highly variable and can lead to fossilization under appropriate conditions [22].

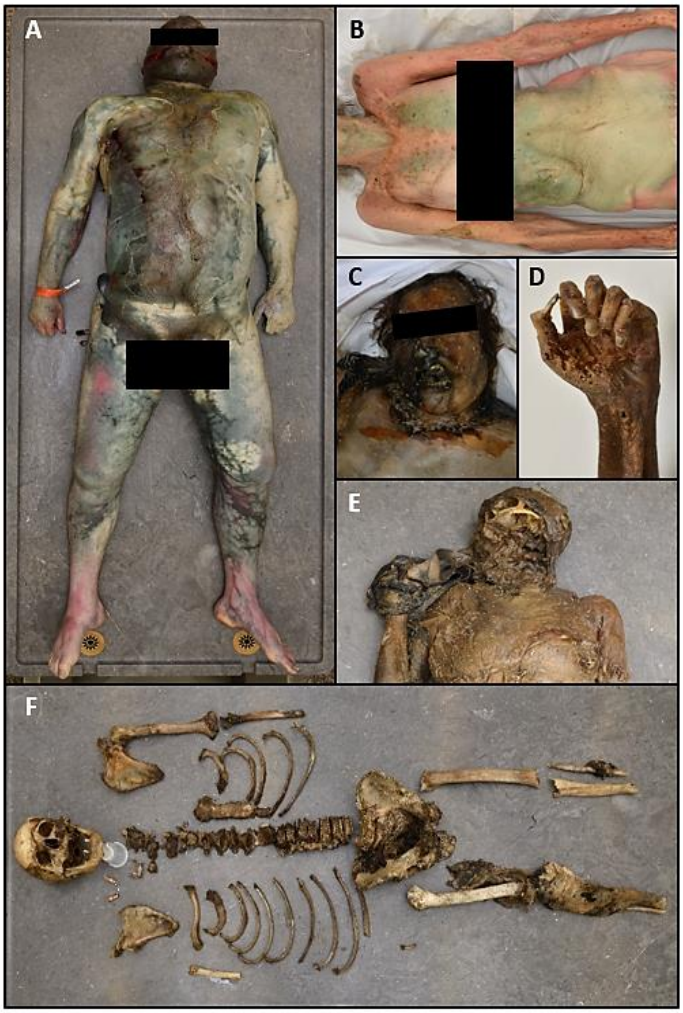

Fig. 1: Decomposed human corpses showing different extents of putrefaction and diagenesis. Green and brown discoloration of the skin (A), greenish discoloration of the abdomen (B), purging of body fluids (C), breakdown of skin barrier and partial skeletonization (D+E), skeletonization (F). Photos: Thomas Rost, Institute of Forensic Medicine Basel.

1.2.2 Human corpses found in water

The decomposition process of human bodies is a dynamic system that interacts with the ambient environment. Water in particular can lead to severe physical and chemical alterations by influencing the osmotic environment of the cells. Comparable to decomposed corpses on land, distinct physical characteristics, including discoloration of the skin, distention of tissues and bloating can be observed (Fig.2A+B). However, post-mortem decay may be accelerated or retarded in human bodies that are fully or partially submerged in water [21]. Compared to a terrestrial position, the aquatic environment is more homogenous, resulting in a more consistent and sequential decomposition progress [31]. Yet, the extent is strongly influenced by factors such as water temperature, stream current, aquatic life, pH value and the presence of salt or fresh water [21, 32, 33]. Generally, the rate of decomposition is decelerated and twice as slow compared to terrestrial environments. This is mainly due to cooler water temperatures and reduced insect activity [23, 34]. In particular, bodies in seawater decompose considerably slower because bacterial activity is inhibited due to high salt concentrations [23]. Compared to the decomposition on land, distinct characteristics such as colonization with algae and the appearance of a so-called "washerwoman's skin" are visible (Fig.2C+D). This phenomenon describes the wrinkled appearance of the skin primarily on hands and feet. Skin wrinkling is usually observed in the early stages of decomposition and results in skin becoming soggier and eventually slipping off, which can be observed as "degloving" [35, 36]. Additionally, the absorption of freshwater into the circulation leads to an abrupt increase in blood volume and consequent rupture of tissue [36]. In rare cases, the decomposition in water can lead to saponi-fication, the formation of a soap-like substance from body fat, also known as grave wax. This decreases pH value and inhibits bacterial growth, facilitating tissue preservation [14, 36, 37].

Fig. 2: Corpses found in water. Bodies displaying green and brown discoloration of the skin and bloating (A+B), washerwoman's skin on hands (C+D). Photos: Thomas Rost, Institute of Forensic Medicine Basel.

1.2.3 Burnt human corpses

In forensic casework, examining and ascertaining the identity of burnt human bodies is crucial since severe impact of heat leads to significant physical and chemical alterations [38]. Modification initially begin with the alteration of the external skin (Fig.3A-E). The extent ranges from superficial burns with damage and blistering of the epidermal and dermal layer, to the damage of the subcutaneous adipose tissue, and leads to black discoloration and destruction of tissues including muscle and bone [2, 39]. A typical post-mortem characteristic is the flexure of limbs to the so-called "pugilistic posture", derived from contractions of the muscles (Fig.3B+E) [39]. Under sustained heat exposure, the muscles contract and enter, with continuing influence of heat, a state of heat stiffening or heat rigor. Since the stiffening begins on superficial muscles, it takes longer for the heat to reach deeper muscles [40]. Subsequently, prolonged exposure leads to shrinkage of the muscles, destruction of internal organs and a retraction of muscles from bones, resulting in the disassociation of the hands and feet. After extensive burn destructions, which include heat-induced bursting of the cranium and complete disassociation of the extremities, the internal bone structure degrades. Finally, remaining bones become discolored, brittle and highly fragmented [39].

Fig. 3: Burnt corpses. Visible alterations and discolorations of the skin (A-E) and typical "pugilistic posture" (B+E). Photos: Thomas Rost, Institute of Forensic Medicine Basel.

1.3 DNA-based identification: Markers, procedures and technologies

Following the examination of characteristic post-mortem alterations of decomposed or burnt corpses or bodies found in water, ascertaining the deceased identity is a common forensic task. In human identification, the development of methods based on the investigation of genetic material has revolutionized forensic sciences and enabled the start of molecular genetic identity testing. Today's DNA profiling is based on Alec Jeffrey's fundamental work on determining individual "DNA fingerprints" by investigating DNA regions with minisatellite markers, also called variable number of tandem repeats (VNTRs) in 1985[41]. As the number of those repeated nucleotide sequence motifs differ among persons, with the exception of monozygotic twins, individual DNA profiles can be generated. Therefore, by introducing a technique called restriction fragment length polymorphism (RFLP), which comprises the digestion of DNA into fragments by restriction of enzymes, to analyze VNTRs, a powerful tool for human identity and crime investigations as well as paternity and genetic genealogy was established [42, 43].
Since the discovery of VNTRs, DNA genotyping has become widespread in the forensic field and evolved rapidly to today's use of short tandem repeat (STR) markers. With the revolutionizing development of the polymerase chain reaction (PCR) method by Kary Mullis in 1985, another milestone was reached in the field of molecular genetics. By providing the ability to replicate one or more regions of the DNA over and over, millions of copies of the same sequences can be yielded within hours [43]. Due to its sensitivity and specificity, the PCR method quickly became an important tool for analyzing genetic markers in forensic samples. At the end of the 1990´s, analyses of STRs became increasingly frequent in forensic casework [43]. Since RFLP typing of VNTRs is time as well labor-intensive and requires large amounts of intact DNA (10-25 ng), the method was not suitable for forensic samples exhibiting degraded or low copy numbered DNA. Therefore, RFLP analyses were replaced by PCR-based assays and typing of STRs [43, 44]. Due to growing demands and complexities of forensic casework, the methods in the field of forensic genetics are becoming progressively sensitive, discriminating, faster and less costly [42, 43]. The technical inventions are constantly improving and expanding the area of applications by enabling complex analyses such as DNA phenotyping and ancestry prediction using novel Next Generation Sequencing (NGS) technologies [45].
In the following sections, an overview of forensic DNA genotyping is provided by briefly summarizing the markers and processes of genetic analyses required for human ID purposes. Thereby, not only the current standard methods of capillary electrophoresis (CE) based genotyping but also the advantages of emerging NGS technologies are introduced.

1.3.1 Short tandem repeats (STRs) and single nucleotide polymorphism (SNPs)

Short tandem repeats are microsatellites in which certain sequence motifs are repeated at variable numbers (Fig.4A). Since the number of repeats differs among individuals, investigation of STRs enables human identity testing [46]. Compared to VNTRs with total sizes of 400 to 1000 bp and sequence motifs comprising 8 to 100 bp repeat units, STRs provide a reduced total size of 100 to 400 bp and shorter repeat sequence motifs with 2 to 7 bp [43, 47, 48]. More than 30.000 separate STRs exit in the human genome showing high polymorphism, however, only 20 are commonly used in forensic casework [49]. Due to their small size, STRs can be reliably amplified by PCR-based assays, which enhance DNA genotyping of degraded samples [48]. Nowadays, multiple STR loci can be combined in one multiplex assay, resulting in high discrimination and, with the exception of monozygotic twins, individual DNA profiles. For ensuring effective comparability and reproducibility across a wide number of jurisdictions, the Federal Bureau of Investigation chose a common set of 13 core STR loci for inclusion within the Combined DNA Index System (CODIS) in 1997 [43]. Around two years later, the European Network of Forensic Science Institutes (ENFSI) decided on a European Standard Set (ESS) including seven loci [50]. However, due to following success and growing numbers of DNA databases, it was revealed that seven ESS loci were no longer sufficient due

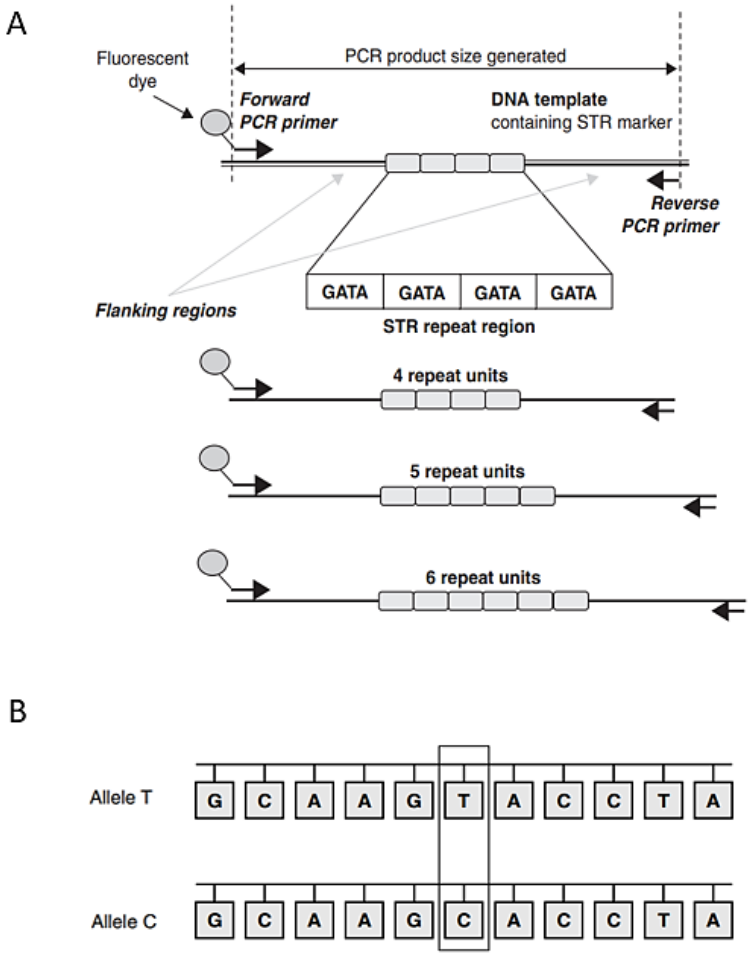

Fig. 4: Forensic DNA markers:
Short tandem repeats (STRs) (A). During polymerase chain reaction (PCR) primers anneal to DNA sequences in flanking regions that enclose the STR region. The size of STR alleles typically differs by the number of repeat units.
Single nucleotide polymorphism (SNP) (B). Majority of SNPs are biallelic with two different alleles (here thymine (T) and cytosine (C)). Source: Modified and adapted from Butler[43] and Goodwin et al.[49].

to the chance of adventitious matches. Therefore, the ESS was extended to 16 loci (eESS): FGA, TH01, VWA, D1S1656, D2S441, D2S1338, D3S1358, D8S1179, D10S1248, D12S391, D16S539, D18S51, D19S433, D21S11, D22S1045 and SE33 [44, 49, 51].

Single nucleotide polymorphisms (SNPs) are single-based sequence variations at specific loci in the DNA sequence (Fig.4B) [43, 52]. Compared to STRs, SNPs exhibit sequence and not length variations and occur at a rate of one in every 1000 bp [52]. Due to their low mutation rate and abundant occurrence in the genome, SNPs can be used as markers for human identity testing. However, since a biallelic SNP provides less discrimination power compared to multiallelic STR markers, it is necessary to analyze a larger number for an individualizing profile. For this reason, SNP markers are not commonly investigated in forensics casework and are primarily subject of research studies. Thereby, the markers used for forensic applications are categorized into four general classes: 1) identity informative SNPs (iiSNPs) for human ID, 2) lineage informative SNPs (liSNPs) for kinship analyses, 3) phenotype informative SNPs (piSNPs) and 4) ancestry informative SNPs (aiSNPs) [46, 47, 53]. For the analyses of SNPs, numerous technologies, including TaqMan assays (based on binding of oligonucleotides complementary to the wild type or variant allele), SNaPshot assays (based on single base extensions) and sequencing methods such as pyrosequencing or NGS are available [51, 52].

1.3.2 Standard procedure of STR genotyping

Forensic DNA typing usually consists of a process starting with sample collection and preparation, followed by molecular genetic analyses involving DNA extraction, quantification and amplification. Subsequent PCR product separation as well as detection leads to the generation and interpretation of DNA profiles (Fig.5) [43].

Prior to STR genotyping, biological material has to be sampled from the corpse for further analyses. In forensic casework, post-mortem tissues such as muscle samples, blood or buccal swabs are routinely collected during medico-legal autopsy's or identification processes [29, 43]. However, due to the lack of standardized guidelines, the choice of the sampling material depends on the institute's routine process and personal experience of the examiner. Further molecular analyses are performed according to the country legislation and usually comprise two separate DNA extractions from the collected tissue sample. For isolating the DNA from the cells, a large variety of methods is available with varying procedures. Thereby, the choice of the optimal method depends on the sample material, resulting DNA yield or whether the sample has to be purified or not [43, 54]. For ensuring the optimal amount of template DNA for the PCR reaction (0.5 to 2.5 ng), the DNA yield can be quantified by using, among others, fluorescence based real-time PCR (RT-PCR) [43, 49]. Yet, a quantification of the DNA yield is usually not performed in case of identity testing of corpses but in the examination of trace samples. Subsequent DNA amplification is performed using commercial kits including at least the 16 STR loci required and recommended by the ENFSI.

For the analyses of PCR products, current capillary electrophoresis (CE) based genotyping has been described as the gold standard for years [55-57]. The technology is based on the separation of fragments by size and fluorescence and therefore allows the discrimination of STR alleles. Due to high voltages, the negatively charged and fluorescently labeled DNA fragments are forced to migrate through capillaries from a negative electrode, known as cathode, to the positive electrode, known as anode. Near the end of the capillary, a laser illuminates the fluorescence dye of the DNA fragments so that a camera can detect and quantify the fluorescent emission signal. The resulting data can be converted by software and represented in the form of peaks on an electropherogram. Since two independent extractions are required in Switzerland, both resulting DNA profiles will be examined for concordance to validate the STR genotype. Subsequently, the profile can be compared with profiles of possible relatives or samples collected from personal ante-mortem items such as tooth- or hairbrushes. In case of missing reference material, a database search can be performed [58]. In Switzerland, the generated DNA profiles will be submitted to the Erkennungsdienstliches DNA-Informationssystem (EDNAIS), which enables the assessment of potential matches with stored DNA profiles. According to the DNA Profilgesetz of the Bundesversammlung der Schweizerischen Eidgenossenschaft, DNA profiles of non-identified bodies will be deleted after successful ID [59].

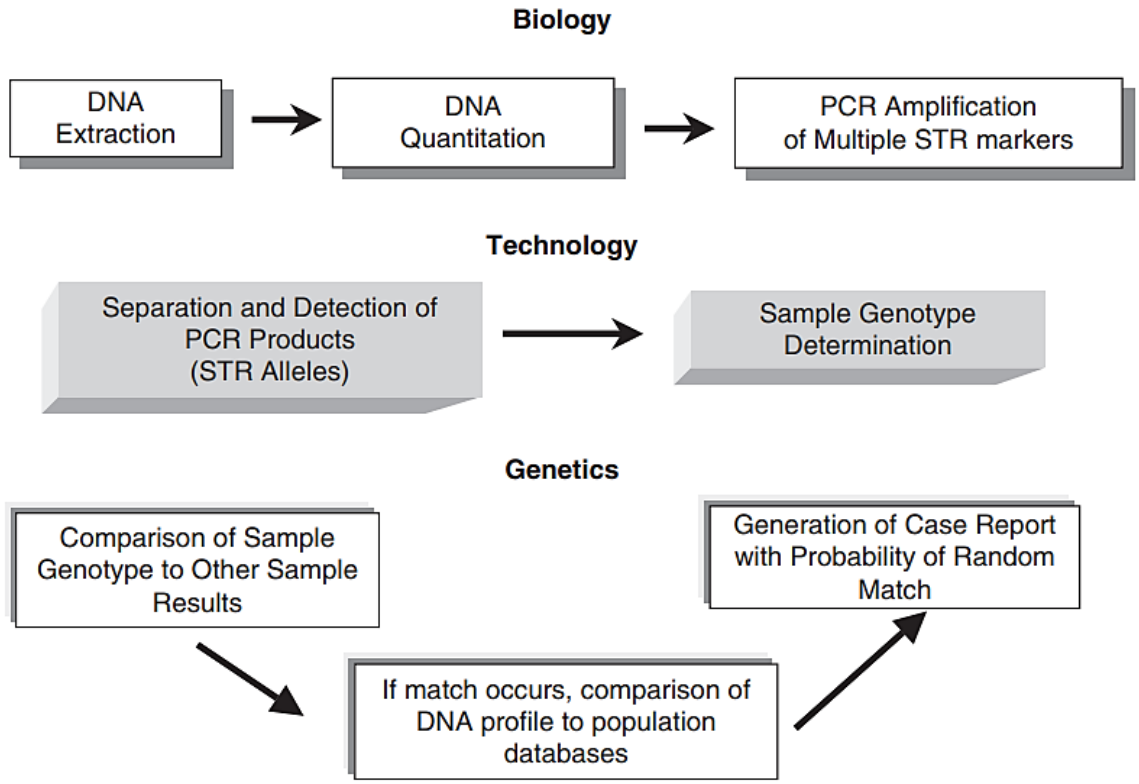

Fig. 5: Standard procedure of STR genotyping. Overview of biological, technological and genetic steps involved in generating a DNA profile. Source: Butler [43].

1.3.3 Novel technologies: Next Generation Sequencing

Molecular genetic analyses have been rapidly advanced in recent years. Particularly in the field of DNA sequencing, constantly new instruments and enhanced methods being introduced due to the significant progress in technology. In forensics, the development of DNA sequencing technologies, starting with Sanger sequencing, marked an essential milestone and evolved to today's application of massive parallel sequencing methods [43, 60].

The start of the so-called first sequencing generation was initiated by the development of the Sanger sequencing technology by Frederik Sanger in 1977 that dominated the market for a long time. The method is based on the incorporation of chain-terminating dideoxynucleotides (ddNTPs) by DNA polymerases, leading to millions of copies of the target DNA sequence with variable length [61-63]. As an alternative sequencing technology, pyrosequencing was presented in 1996. This "sequencing by synthesis" (SBS) method relies on the detection of luminescence emerging from the release of pyrophosphate after nucleotide incorporation [60, 63, 64]. By releasing the first commercial high-throughput platform based on pyrosequencing in 2005, the so-called second sequencing generation, also known as Next Generation Sequencing (NGS), was initiated. NGS enabled the sequencing of an entire human genome within a single day, which, in contrast, took Sanger sequencing a decade [65]. Due to ongoing develop-ments and major improvements in instrumentation, several new platforms with differing sequencing chemistry and technologies was released [63, 66].

As the first fully validated sequencing device specifically designed for forensic genetic applications, the MiSeq FGx system (Verogen) enables massive parallel and high-resolution sequencing of up to 231 STR and SNP loci in a single multiplex reaction [53, 67]. The method is also based on SBS and enables the discrimination of loci based on their DNA sequences [68]. Prior to sequencing on so-called flow cells, the samples have to be prepared into a sample library (Fig.6). As the first step, the extracted DNA undergoes PCR-based target amplification, resulting in fragments of the regions of interest (Fig.6A). Depending on jurisdiction, DNA primer mix A (DPMA, including STRs and iiSNPs of non-coding regions) or DNA primer mix B (DPMB, additionally including aiSNPs and piSNPs located in coding regions) can be used. Within a second PCR, sequencing adapters (i5 and i7) are linked to the amplicons for target enrichment (Fig.6B). The adapters are complementary to the adapters located on the flow cell, allowing them to attach during sequencing reactions [69]. Additionally, each adapter incorporates index sequences, which lead to unique labels after combination and enable pooling of multiple samples. Subsequently, the resulting libraries will be purified, normalized, pooled and denatured (processes not shown). During the following bridge amplification, the library fragments hybridize to the complementary adapters on the flow cell, bend over and hybridize to the adjacent adapters (Fig.6C+D). Subsequent repetition of PCR-based extension, synthesis of new strands and dissociation generates clusters containing thousands of copies of the same DNA fragment (Fig.6E-G). During the sequencing process, each cluster is sequenced as fluorescently labeled nucleotides are incorporated, emitting a series of fluorescent signals that can be detected by a camera (Fig.6H+I). Since each nucleotide emits light at different wavelength, the direct nucleotide sequence can be determined from each read [53, 69-71].

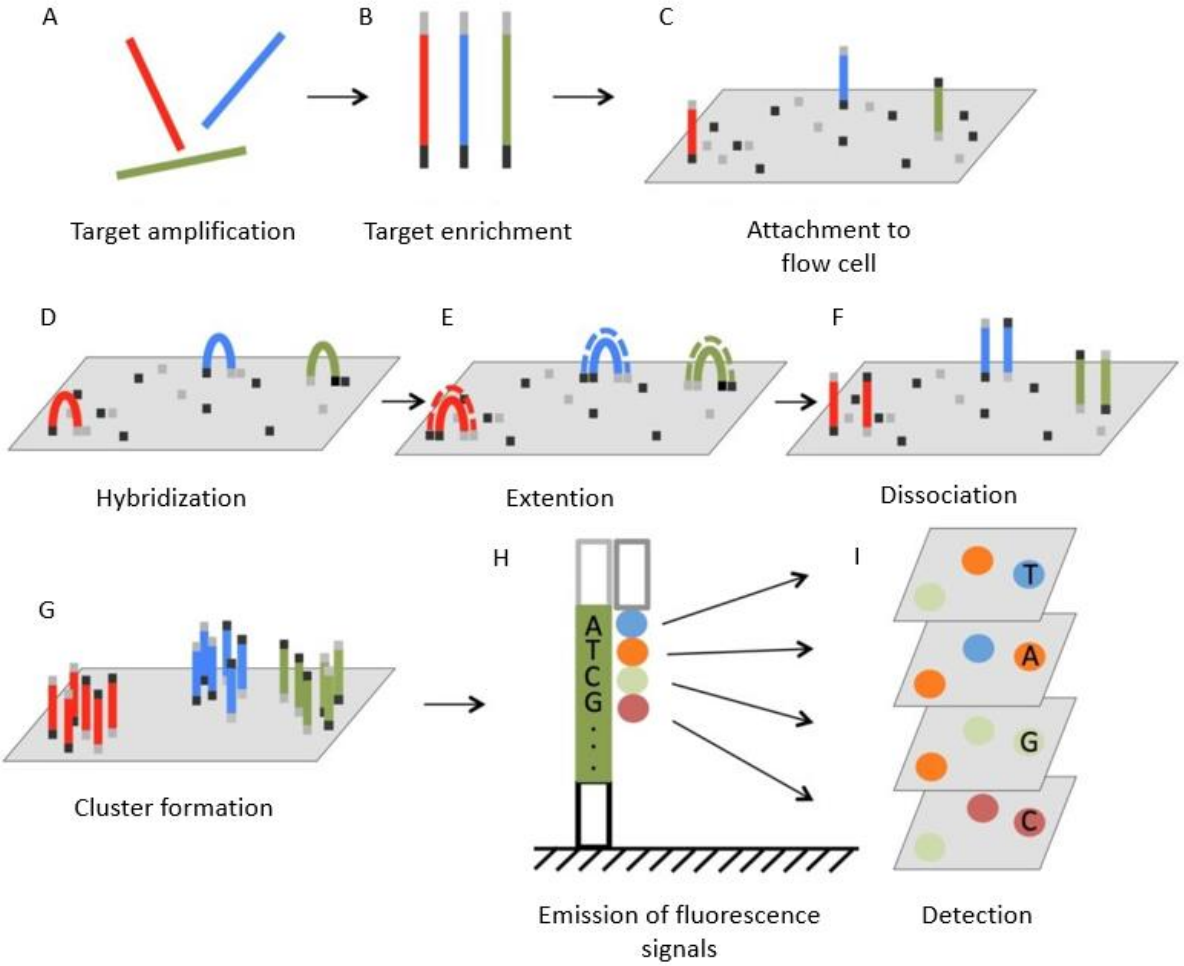

Fig. 6: Overview of NGS library preparation and DNA sequencing using the MiSeq FGx system. Targets of interest are amplified during PCR cycles (A), target enrichment with adapters annealing to the DNA fragments (B), attachment of DNA fragments to a flow cell (C), bridge amplification including hybridization, extension and dissociation that lead to the formation of clusters (D-G), sequencing process including the emission of fluorescence signals after nucleotide incooperation and detection of signals by a camera (H+I). Source: Modified and adapted from Lu et al. [71].

1.4 Challenges in forensic genotyping of altered remains

Although forensic STR genotyping is a widely used and robust method in human ID, post-mortem modifications and alterations of the human body can have negative implications for molecular investigations [9]. In analyzing challenging samples from altered remains, several biological or technological related impairments and limitations have to be considered. Decomposition processes comprising autolysis and putrefaction or influences of heat or water lead to increasing DNA damage, which affects genotyping processes [48]. Therefore, challenges including the analyses of degraded and inhibited samples, tissue-specific differences as well as the impact of DNA extraction and analytical technologies are presented in the following sections.

1.4.1 DNA degradation and inhibition

In vivo, DNA is a macromolecule with a relatively stable structure and tightly bond water molecules per nucleotide residue. However, in post-mortem samples, the stability decreases and results in DNA degradation, the parallel process of DNA denaturation resulting in i.e. single strand and DNA double helix breakdown [43, 48, 72]. Following cell death, the DNA is target for several physical and chemical reactions leading to DNA damage. Loss of enzyme regulation and lactic acidosis enhance the activities of the hydrolyzing enzymes endonucleases and exonucleases (Fig.7). The former are the first agents to initiate fragmentation after death by cleaving phosphodiester bonds within a polynucleotide chain, whereas exonucleases detach nucleotides either from the 5' or 3' end [25, 73]. Furthermore, augmented microbial growth leads to accretive secretion of digestive enzymes and accumulation of metabolism products, which increase the rate of DNA strand breakage [74]. After enzymatic reactions, much slower DNA degradation processes, including hydrolytic and oxidative reactions occur (Fig.7). During hydrolysis, the glycosidic base-sugar bond is dissolved by water, resulting in base loss and single strand breaks. Progressive hydrolysis and single strand breaks within close distance can lead to DNA double strand breaks [25, 75]. Oxidative damage by free radicals and hydrogen peroxide generated through ionizing radiation or microorganisms also causes DNA degradation. The damage mostly includes modification of sugar residues or removal of bases, impairing further PCR reactions [25, 76, 77]. The extent of enzymatic, hydrolytic and oxidative reactions causing degradation relies on microbial activity and environmental conditions such as temperature, pH value and humidity [25]. Especially, the influence of temperature is associated with thermal DNA degradation found in burnt human remains [78, 79].

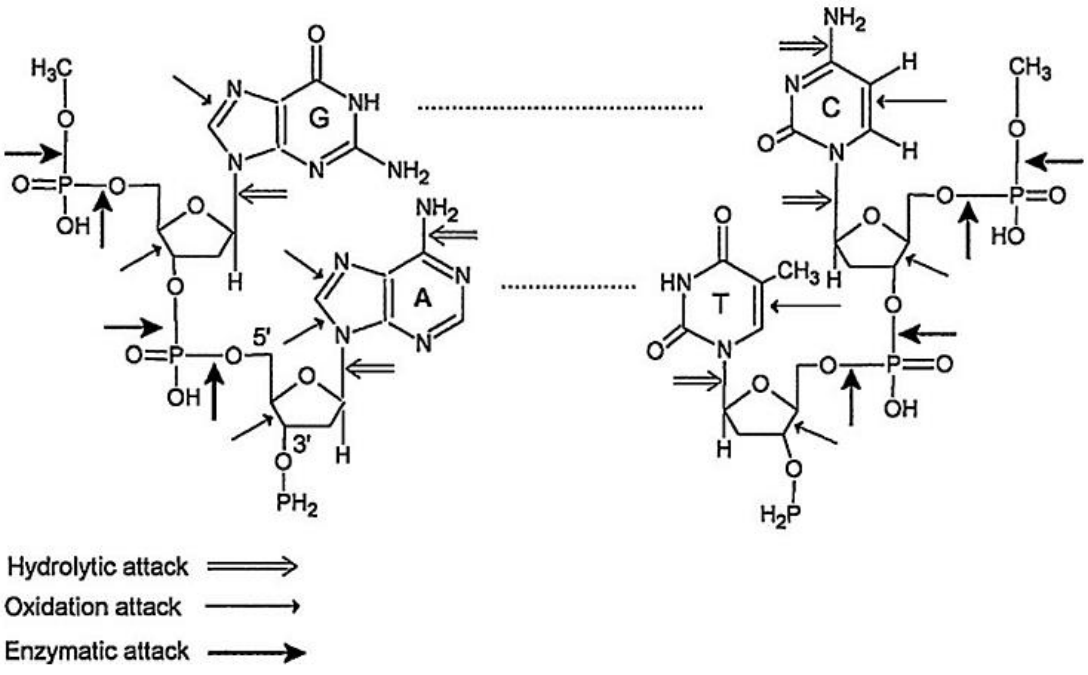

Fig. 7: DNA degradation. Hydrolytic, oxidation and enzymatic attacks leading to DNA damage. Source: Alaeddini et al. [25].

However, regardless of the cause, resulting DNA degradation impairs further PCR-based genotyping. In electropherograms, degradation can be identified by the presence of a distinct downward slope of peak heights, since here larger loci have a lower probability of being amplified and drop out more frequently (Fig.8). This so-called "ski-slope effect" is displayed by smaller loci on the left side of the electropherogram producing significantly higher peaks compared to the larger loci on the right side [8, 51, 80].

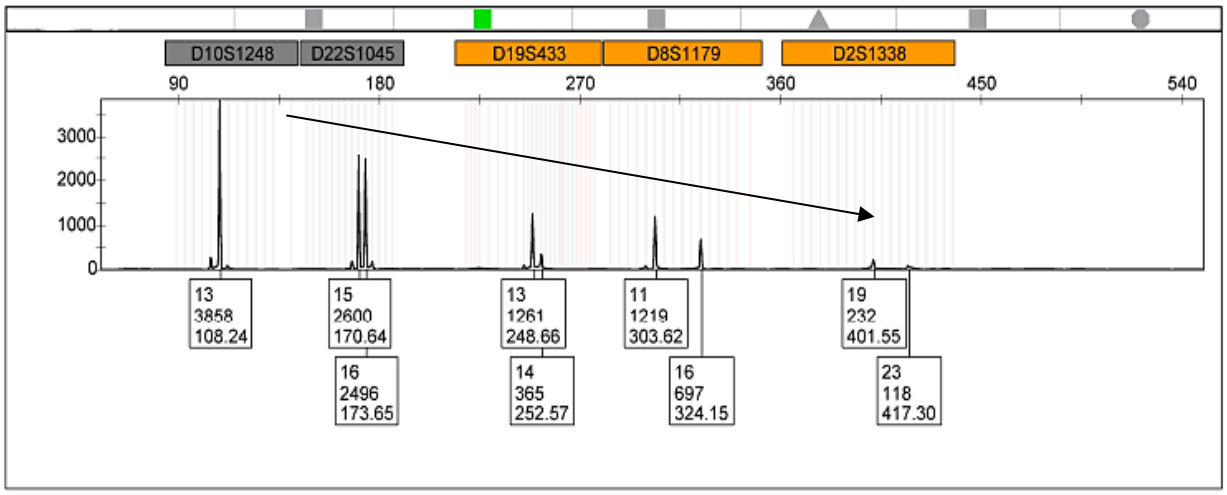

Fig. 8: Electropherogram with distinct "ski-slope effect" identifiable by a downward slope in peak heights (arrow). The shown part of a DNA profile was generated at the Institute of Forensic Medicine Basel by analyzing DNA extracted from a liver sample of a decomposed corpse.

Besides DNA degradation, the presence of inhibitory agents can interfere with PCR amplification and decrease genotyping success [81, 82]. In forensic casework, common co-extracted inhibitors causing PCR failure are humic acid, hematin, indigo dye, calcium ions or collagen. Particularly, humic acids in soil, microbial DNA and calcium ions in bones represent a challenge in STR genotyping of altered remains [83, 84]. During PCR reactions, those substances can interact with the DNA template, nucleotides or amplification primers or bind to the polymerase and block enzyme activity. Furthermore, non-purified inhibitors can impair cell lysis during DNA extraction or degrade nucleic acids. Depending on the extent and severity of inhibitory activities, further STR analysis result in partial profiles with loss of alleles from larger loci or missing profiles [84].

1.4.2 Tissue-specific differences

Although DNA can be extracted from any nucleated cell, DNA stability and preservation depends on the resistance of the tissue against decomposition or influences of heat [85, 86]. Since autolysis and putrefaction are ongoing processes, the extent and state of alteration differs throughout the body and with time [21, 85]. Furthermore, intrinsic and extrinsic influences, including injuries or environmental conditions, can accelerate or decelerate the process, resulting in highly variable degrees of degradation among tissue types [86]. Due to ongoing improvement in reagent sensitivity and robustness as well as enhancement of technologies, analyses of DNA stability in post-mortem materials display increasing relevance in forensic genetics. Since Bär et al.[73] described tissue-dependent degradation rates in 1988, an increasing number of studies compared biological material and presented constantly new tissue types with assumingly high post-mortem DNA stability. In the following, tissue-specific differences and challenges in sampling or molecular analyses, which lead to common recommendations for identifying altered remains are presented.

Organs, muscles and blood

DNA stability has been shown to remain stable in post-mortem organ tissues for a certain time, however, the extent of DNA degradation can be highly variable. Cells with high energy demands and intracellular enzyme activity are primarily and to a greater degree affected by autolysis processes. Subsequent to decomposition of the intestine and stomach, bacteria spread through the vascular system and affect tissues including the heart and blood [21]. However, due to great preservation, Shintani-Ishida et. al.[87] and Watherston et. al.[88] state that the DNA stability in blood is still sufficient for DNA profiling after progressive decomposition. Yet, sampling can be crucial in deceased since the blood ceases to circulate and coagulates [74, 88]. The lung, kidneys and liver are rich in lysosomes facilitating self-destruction of the cell and are therefore highly susceptible to DNA degradation [21, 89]. Though, based on their study results, Schwark et al.[10] and Courts et al.[9] described the kidney as suitable and preferred tissue for genetic analyses of altered remains. In brain tissue, nucleosomal DNA remains attached to the core histones for at least 30 hours after death. This preservation of chromatin structure is beneficial for DNA stability since, under optimal conditions such as cold temperatures, nuclease enzymes may become inactive before they are able to fully cleave the DNA sequence[84, 90]. Hence, van den Berge et al.[91] recommend brain tissue, along with heart tissue, as sampling material from decomposed corpses. Comparably great DNA stability has been described in muscle tissue for up to three weeks after death [73]. Therefore, according to Hansen et al. [92], muscle tissue is a suitable source for post-mortem DNA analyses.

The Achilles tendon, aorta, bladder and intervertebral disc

The Achilles tendon is described as a highly stable tissue and less affected by degradation processes in altered remains. As shown by Roeper et al. [85], samples from the Achilles tendon demonstrated twice as high DNA yields compared to kidney and muscle samples. This was explained by post-mortem changes, which may even facilitate DNA isolation and therefore increase DNA quantities. Comparably, Sato et al.[93] described the aorta as a robust tissue with great resistance to decomposition. According to Helm et al.[94], swabbing of the aortic wall yields highest profile completeness compared to the urinary bladder, brain, liver and muscles. Furthermore, Brito et al. [95, 96] state that DNA preservation is shown to remain stable in the bladder. The internal location of the urinary bladder mucosa reduces the possibility of DNA contamination and degradation. Therefore, the use of bladder swabs is described as a great source with high DNA stability in decomposed as well as burnt corpses [95-98]. As shown by Becker et al. [99], the integrity of the DNA is also provided in post-mortem intervertebral discs. Since the cells are embedded in a matrix comprised of collagen, elastin and other non-collagenous components, the physical barrier might reduce the tissues' susceptibility to DNA degradation processes.

Bones and teeth

Compared to soft tissues, DNA stability is usually considered higher in hard tissues, including bones and teeth [5, 100]. The exterior surface of teeth can reduce the amount of microbial activity and the self-contained structure protects the DNA [101]. In bones, the cells are embedded within a dense bio-mineral matrix, resulting in greater protection of alteration processes. Additionally, comparably lower water content and less extents of digestive enzymes enhance preservation of DNA [48]. Therefore, most studies priory bones as a source for DNA-based ID [5, 97, 100-104]. Usually, DNA quantity and quality is higher in dense bones than in cancellous bones [100]. Consequently, the *pars petrosa* is frequently described as one of the densest bones in the human body, indicating high DNA preservation and a respectively preferred sample material [100, 101, 103, 105-107]. According to the Interpol disaster victim identification guide [102] and the DNA commission of the International Society for Forensic Genetics (ISFG) [97], long, compact bones, healthy teeth and/or other available bones should be the first choice from decomposed corpses. However, osseous preparation and DNA extraction are more labor-intensive as well as time- and cost-consuming compared to soft tissues. This can be a great challenge in cases where time is short and the rapid ID of the corpse is essential [5, 89].

Nails

As further hard tissues, finger- and toenails are recommended biological materials because the DNA not only adheres to the underside of the nail and the nail bed, it is also preserved within the keratin structure [88]. Therefore, the protected location is beneficial for DNA preservation,

leading to great stability even with long post-mortem intervals. Generally, sufficient DNA quantity and quality are described for up to six month after death. As stated by Allouche et al. [6] and Piccini et al.[86], nails are easily to collect and therefore represent an optimal sampling material for human ID processes. However, nails can be negatively impacted by extraneous DNA contaminants, impeding further interpretation. This contamination was observed by Schlenker et al.[108] to be lower in toenails since feet are usually covered by socks or shoes. According to Uerlings et al.[89], finger- and toenails were proven the most suitable tissues for DNA-based ID when comparing nails and five soft tissues (brain, aorta, liver, kidney and muscle).

1.4.3 Insecurities in the choice of the optimal sampling material

Since DNA quantity and quality differ among tissue types, a major challenge in forensic genotyping of altered remains is the choice of the optimal sampling material [85]. The sampling of less ideal materials with high degrees of degradation or PCR inhibitors might lead to PCR failure resulting in time- and cost-intensive repetitions or parallel amplifications. In routine casework, the sample collection usually depends on institutional preferences, laboratory-specific equipment and the examiners own experiences. As described in chapter 1.1.1, several studies analyzed tissue-specific genotyping success rates that might be used as a reference. However, an enormous variety and partly even contradicting recommendations on the presumably most suitable tissues are given. According to the respective study, either hard or soft tissues are preferred. Furthermore, most studies used small sample sizes and highly laboratory-specific methods, including DNA extraction methods, which impede comparability and reproducibility. Additionally, the outcomes are frequently based on comparison of only two to five tissue types and the corpse's condition is usually not scored and sufficiently described. The resulting complexity and diversity of recommendations highlights the uncertainty in selecting the best-suited and most appropriate tissue types, leading to significantly diverging sampling strategies among forensic institutes and potentially more repetitions of DNA amplifications.

1.4.4 Influence of DNA extraction methods

Besides tissue related limitations and challenges, molecular genetic methods and technologies also have an impact on the genotyping success of altered remains. Especially DNA extraction platforms are of great importance as they affect each downstream process of STR profiling. In today's forensic casework, several extraction techniques are used, each based on different methods and non-purifying or purifying systems [43]. Therefore, the choice of the optimal method depends on the type of tissue, the institutional settings and the context of the case. Particularly, when extracting samples of altered remains, it is important to avoid further degradation of the DNA template and to remove PCR inhibitors. Since proteins and other cellular materials can interfere with DNA analyses, adequate separation and purification of DNA molecules is

required [43, 89]. Due to potential soil-derived inhibitors such as humic acid, purification is particularly important in DNA extraction of bone samples. Furthermore, complete demineralization is essential for eliminating inhibiting calcium phosphates [109, 110]. Therefore, the Maxwell® RSC instrument (Promega) is a commonly used instrument for nucleic acid isolation and purification. Subsequently to sample lysis, nucleic acids bind to paramagnetic particles for washing away redundant cellular components [111]. The automated purification platform provides sufficient purification of multiple sample types, however, the procedure is time-consuming and cost-intensive. Commercially available alternatives are non-purifying DNA extraction kits such as the SwabSolution™ Kit (Promega) [112]. Advantages are lower costs, shorter extraction duration and lower contamination risk due to fewer handling steps. Therefore, if the sample and case are feasible, this method is a time-saving and cost-effective alternative [89]. However, DNA is not purified and potential PCR inhibitors remain in the sample, which might decrease genotyping success rates.

1.4.5 Limits of CE-based genotyping and advantages of NGS technologies

Although capillary electrophoresis is described as the gold standard and most widely used technology for STR genotyping, the method still displays limitations and challenges. Since the method relies on differentiation of STR loci by amplicon length and requires labelling with different fluorescent dyes, sequence variations within the amplicon cannot be detected, and the number of multiplexed loci is restricted to a maximum capacity of 25 to 30 [113-116]. In particular, the required amplicon sizes of 80 to 500 bp are a major limitation for degraded DNA from altered human remains. Resulting low peak heights or complete dropouts of larger loci lead to reduced discrimination power of DNA profiles and impairments in identity testing [113, 115].
For overcoming the limitations of CE-based genotyping, major developments in NGS methods offer new approaches by expanding the spectrum of forensic DNA analyses. Compared to CE, the detection of sequencing variants and the ability to multiplex large numbers of autosomal and gonosomal STR as well as SNP loci enhance the discrimination power. By enabling the opportunity to sequence aiSNPs and piSNPs, the deceased biogeographic ancestry and phenotype can be predicted [117, 118]. This additional prognosis can provide valuable information for identifying highly altered corpses and can assist investigations within given legal frameworks [116, 117]. Furthermore, a major advantage for analyzing degraded samples is the reduction in STR amplicon size, as the amplicons will not require a separation by length [119-121]. However, there are still limitations with NGS in terms of costs, the labor-intensive workflow, the need for internal validations, and trained staff. Additionally, the potential formation of adapter dimers and restrictions of DNA input volumes to 5 µl is a drawback, especially for low concentrated samples [122, 123].

2. Motivation, study aim and approach

Despite great advances in STR genotyping, decreasing DNA stability, tissue-specific differences and restrictions in technologies still present challenges in identifying altered human remains. Thus, the high number of studies concerning genotyping success rates in post-mortem material indicates the relevance and necessity of addressing those limitations in forensic casework. However, the high variability in study approaches and partly contradicting recommendations on the optimal tissue types revealed a gap in terms of standardized guidelines. Furthermore, majority of the studies lack statistical analyses and only a few focus on the analyses of body types such as burnt corpses or bodies found in water. Therefore, the overarching aim of this thesis was to improve DNA-based ID success rates of altered human remains by providing guidance and recommendations for optimal tissue sampling strategies. In order to achieve this aim, the following three projects were conducted.

Project I: The first aim of the thesis was to evaluate the current situation of ID processes in forensics and to address the question of which tissue is best suited for a DNA-based ID. For this, a retrospective study was conducted on the ID success of 402 altered remains over seven years to reveal the most commonly used methods at the Institute of Forensic Medicine Basel and to demonstrate the challenges in genetic analyses of degraded and inhibited samples. Each of the corpse's condition was visually scored to classify alteration processes and the sampled tissues, used DNA extraction methods and the resulting STR typing success were evaluated. For each analyzed tissue, the number of parallel and successive PCR amplifications was compared to identify tissue- and condition-specific differences.

Project II: The ongoing development of novel NGS technologies advance the resolution of forensic samples and provide new possibilities for human ID. Especially, the potential to predict a person´s phenotype and biogeographic ancestry enables the assessment of infor-mation that can assist in identifying a corpse within a given legal framework. Therefore, the first aim of *project II* was to compare NGS with CE-based genotyping and to verify the reliability of the MiSeq FGx system for challenging casework samples. Furthermore, due to the lack of studies evaluating the sequencing success of samples from altered remains, the second aim was to optimize the workflow and to define recommendations for improving sequencing results.

Project III: Based on the outcomes of *projects I* and *II*, the overall aim of project III was to present recommendations on the optimal tissue type for a DNA-based ID according to the corpse's condition. By performing a systematic approach on analyzing a wide range of tissues, classifying the corpse's alteration, considering impacts of DNA extraction methods and evaluating the applicability of NGS technologies, guidelines were concluded to enable ID success at first attempt. DNA yields and integrity, and CE- as well as NGS-based STR- and SNP typing success rates were predicted in an incomparable number of 1698 extracts from 949 tissues of altered corpses. Furthermore, with the cooperation of associated Institutes of Forensic Medicine, the influence of institute-specific DNA extraction methods and the reproducibility in prediction of the deceased's phenotype and ancestry was ascertained.

3. Results

3.1 Publication: *Project I*

Which tissue to take? A retrospective study of the identification success of altered human remains

Alina Senst[1], Eva Scheurer[1], Kathrin Gerlach[1], Iris Schulz[1]

[1]Institute of Forensic Medicine, University Basel, Pestalozzistrasse 22, 4056, Basel, Switzerland

Status: Published

Journal: Journal of Forensic and Legal Medicine 84 (2021) 102271

Received: July 2021

Accepted: October 2021

DOI: https://doi.org/10.1016/j.jflm.2021.10

Journal of Forensic and Legal Medicine 84 (2021) 102271

Contents lists available at ScienceDirect

Journal of Forensic and Legal Medicine

journal homepage: www.elsevier.com/locate/yjflm

Research Paper

Which tissue to take? A retrospective study of the identification success of altered human remains

Alina Senst, Eva Scheurer, Kathrin Gerlach, Iris Schulz *

Institute of Forensic Medicine, University Basel, Pestalozzistrasse 22, 4056, Basel, Switzerland

ARTICLE INFO

Keywords:
Identification
Altered human remains
Retrospective study
DNA analysis
Decomposition

ABSTRACT

In forensic medicine, deceased are usually identified by comparing ante- and post-mortem dental or radiological features. However, in severe putrefaction, burning or absent reference data, the remaining tool for identifying human remains is DNA genotyping. But even a DNA-based identification can be challenging when confronted with a high post-mortem interval or heat impacts because it can lead to undesirable degradation of the DNA that varies among tissue types.

This retrospective study investigated the identification success in 402 altered human corpses over seven years by comparing the examined tissue types from decomposed, skeletonised and burnt corpses as well as bodies found in water. For each tissue, the STR genotyping results and the number of additional or parallel genetic analyses were evaluated. By comparing the amplification success in samples from altered and unaltered remains, condition-based and tissue-specific differences were observed. With a mean number of 1.6 additional amplifications in cases with well-preserved corpses and 4.5 in altered corpses, the results showed significantly more DNA analyses for altered remains. In 83% of the cases, extra amplifications were performed to identify the corpse. The tissue-specific differences revealed an uncertainty in choosing suitable material from altered corpses for a successful DNA profile. Especially for bone and muscle samples, the genotyping success was the most unpredictable. Furthermore, comparing the retrospective outcome with other research findings, a remarkable variety of recommendations for the "best tissue choice" exists in the forensic community. Thus, our survey highlights the advantages of a broader and systematic approach on hard and soft tissues for successful DNA-based identification of altered human remains at first attempt.

1. Introduction

As a forensic medicine task, clarifying the deceased's identity is crucial for social matters and legal proceedings.[1,2] If a deceased person's identity is unknown or uncertain, an investigation is commissioned by the Law Enforcement Agency. The identification (ID) method depends on the available ante-mortem reference material, the corpse's condition, and the institute's methods and experience. Deceased are usually identified through a process consisting of two main steps: 1) collecting information, determining missing people, and developing a hypothesis about the person in question, and 2) comparing ante- and post-mortem data such as fingerprints or computed tomography (CT) scans to ascertain the dental status, implants or distinct osseous structures.[3] Yet, in cases where no such data is available, the analysis based on deoxyribonucleic acid (DNA), as a primary identifier,[2,4] is the most reliable and possibly the ultimate method for identifying human remains.[1]

Forensic molecular analysis is performed under the Federal Council's statutes of a given country and the regulations of the country-specific society for forensic medicine. For profiling, two independent extractions from the collected sample are (depending on the relevant legislation) required or recommended for subsequent amplifications, yielding one DNA profile per extraction. Both profiles are checked for concordance to validate the short tandem repeat (STR) genotype, followed by comparing the unknown deceased to the reference available.[5] Normally, minimal requirements for identification cases include the analysis of 16 STR loci containing the European Standard Set (ESS). Statistical evaluation is based on an evidence value, often calculated by a likelihood ratio (LR) for the probability of alternative hypotheses, with H0: the deceased is the assumed person, versus H1: the deceased is an unknown and non-related person to the assumed one. In general, an LR of 1000 or more for H0 is considered to be sufficient to identify a deceased person.[6]

Genomic DNA can be extracted from any tissue containing cells with

* Corresponding author. Institute of Forensic Medicine, University Basel, Health Department Basel-Stadt, Pestalozzistrasse 22, 4056, Basel, Switzerland.
E-mail address: iris.schulz@bs.ch (I. Schulz).

https://doi.org/10.1016/j.jflm.2021.102271
Received 2 July 2021; Received in revised form 21 October 2021; Accepted 24 October 2021
Available online 26 October 2021

a nucleus. Usually, blood samples or cheek swabs with mucosal epithelium translate into a (complete) profile of 16 STRs and are therefore a preferred DNA analysis source. However, with a high post-mortem interval (PMI), advanced decomposition, or other severe body alterations, DNA integrity cannot be ensured. Thus, the quality of STR analyses can be negatively affected.[1,3,7] This degradation varies among tissue types and depends on the tissue's protection strength against autolysis and putrefaction.[8,9] Degradation can lead to DNA disintegration, with longer genomic strands more affected than shorter ones. The fragmentation usually coincides with dropouts of either one or several alleles or loci, leading to partial or no STR profile.[8–10] Incomplete profiles can be misleading, and, in absent profiles, no comparison to a relative, direct material (e.g., toothbrush) or DNA database profile can be achieved.[7,11] Thus, the up-front selection of suitable tissue is essential for successful DNA-based identification.

To our knowledge, there are no standardised guidelines for a systematic DNA-based ID concerning the ideal material from the variety of hard and soft tissues for the different corpse alteration types such as burnt, decomposed, or skeletonised bodies and their varying degradation degrees. Several studies, however, describe the genotyping success rate from different materials, presenting their individual best ID method for altered corpses. Unfortunately, the guidance differs considerably from, for example, toe- and fingernails,[9,12] bladder swabs[4,7] to soft tissues like the Achilles tendon, muscles or organs,[8,13–16] not to mention personal experiences and verbally recommended materials from forensic experts.

The diverging recommendations may not be surprising when considering the highly diverse degradation degrees of each deceased, also within one body, the variety of available hard and soft tissues, the corpse alteration types, and the different laboratory processes. However, the published variety of suggestions for a potentially optimal tissue imply that the sample taken is more intuitive or the "best guess".

As an initial step to finding the optimal material for efficient DNA-based identification at first try, a retrospective survey was performed, providing data of medical and genetic ID methods from 402 altered corpses. The aim was to correlate applied ID methods to the genotyping success rate and evaluate the number of time- and cost-intense serial or parallel amplifications performed and the tissue-specific variances.

2. Materials and methods

The retrospective survey included 402 cases from 2014 to 2020 involving the identification of decomposed, burnt and skeletonised corpses and bodies found in water. Deceased who died from carbon monoxide poisoning without signs of burning and bodies found in water with no signs of decomposition were excluded from the study. These corpses were judged as unaltered.

The study involved the evaluation of both medical and genetic reports. Relevant data from medical expertise included the PMI, the person's age, the location where the body was found, the condition of the corpse, and the ID method used. To specify the corpse's alteration, decomposition and burn processes were scored using a visual classification scheme (Table 1). Depending on the corpse's condition, human bodies were categorised into four or five degrees, respectively. Description of the corpse condition for each degree of decomposition and skeletonization was based on the classification of Megyesi et al.[17] and the decomposition scoring method developed by Gelderman et al.[18] Post-mortem alterations of bodies found in water were described according to observations by Reh[19] and the aquatic decomposition score (ADS) developed by van Daalen et al.[20] For scaling the extent of fire indicated destruction, the Crow-Glassman scale[21] was applied. Since the retrospective evaluation of the alteration of the bodies was performed using photographs not including the relevant information for the entire body in all cases, an indexation like a Total Body Score[17] could not be applied.

For the DNA-based identification of decayed corpses, the information of the analysed tissue, the DNA extraction method, the commercial amplification kits, and the resulting STR profiles were recorded and compared with the results of unaltered human remains. In each case, a minimum of 16 autosomal STR-systems was analysed, at least in duplicates. For cases of partial profiles, the required LR of at least 1000 was targeted, undoubtedly identifying the corpse's identity. For the study, polymerase chain reactions (PCRs) were counted as additional when the two mandatory amplifications were exceeded, including autosomal and gonosomal kits. An amplification repetition was observed to have been carried out either successively or in parallel with different DNA dilutions from the outset. Therefore, replicates include poor profile quality and serially performed PCRs, plus high profile quality, amplified in parallel dilutions. Potential repetitions of the capillary electrophoresis were not recorded. The approaches were further compared between different tissue types to detect tissue-specific variances. The distributions of the data were analysed by using the Shapiro-Wilk normality test and the Chi-squared test. The non-parametric Wilcoxon rank-sum test was applied to assess statistical differences in the number of additional analyses between tissues of altered and unaltered human bodies based on the calculated Poisson distributions. Significance was defined at $p < 0.05$, and all hypothesis tests were performed with RStudio Version February 1, 1335.

Table 1
Categorisation of human remains into degrees of alteration. The categorisation of decomposed corpses was based on Megyesi et al.[17] and the scoring method developed by Gelderman et al.[18] The classification of bodies found in water was based on the description by Reh[19] and the aquatic decomposition score (ADS) by van Daalen.[20] The classification of burnt bodies was carried out according to the Crow-Glassman scale.[21]

Condition	Degree	Classification	Description
Decomposed	1	Initiating	Autolysis, beginning of discolouration of the head and/or abdomen
	2	Advanced	Resounding blood vessels, formation of putrefactive blisters, grey to green discoloration, skin slippage
	3	Highly	Extensive brown/green discolouration, abdominal bloating
	4	Profoundly	Loss of organic/inorganic substances, partial skeletonization
Skeletonised	–	–	Dry bones, complete loss of soft tissues
Found in water	1	Initiating	Marbling, wrinkling of skin on hands, slight pink discolouration
	2	Advanced	Yellow/green discolouration of the abdomen, skin slippage
	3	Highly	Black/green discolouration, bloating of abdomen and/or genitals
	4	Profoundly	Loss of organic/inorganic substances, partial skeletonization
Burnt	1	–	Blistering of upper dermal layer
	2	–	Damage of dermis, coagulation necrosis
	3	–	Dermis/subcutaneous fat tissue completely burnt
	4	–	Heat-induced bursting of the cranium
	5	–	Loss of soft tissue, partial skeletonization

3. Results

From 2014 to 2020, a total of 3943 cases of deceased were examined, with a mean of 563 corpses per year. 402 of these cases (10.2%) were altered, i.e., burnt, decomposed, skeletonised or putrefied bodies found in water (Fig. 1). Any unknown or uncertain corpse identity was successfully clarified.

The majority of the identified bodies (89.8%) were putrefied with different decomposition degrees (Fig. 2), with most of them found inside a domicile. Only 19 of the 361 corpses with signs of putrefaction were discovered outside, such as in a forest. The deceased persons' age ranged

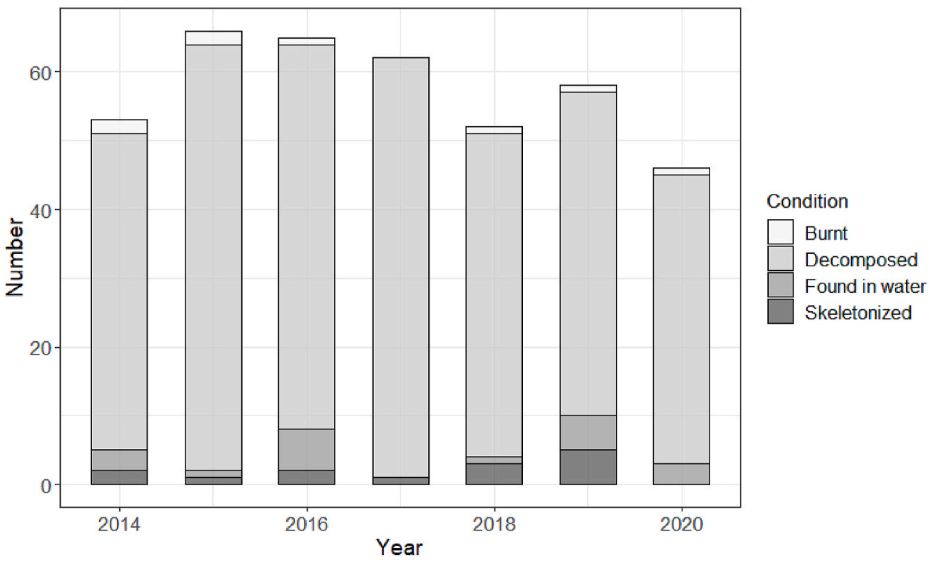

Fig. 1. Absolute number of examined cases and the corresponding distribution of the corpse condition from 2014 to 2020.

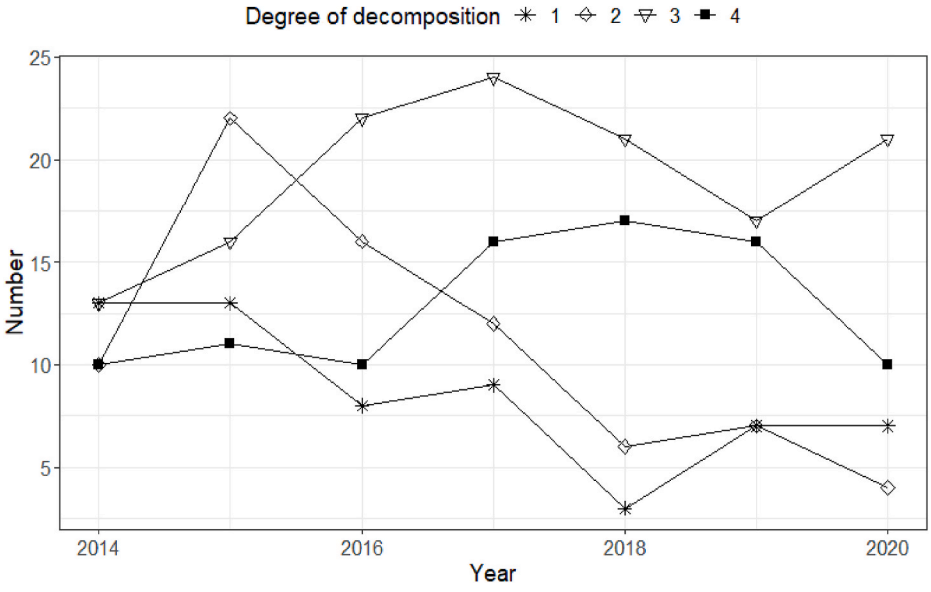

Fig. 2. Total number of decomposed corpses classified according to the body's condition into four degrees of decomposition (degree 1 to 4).

from 18 to 93 years, with a mean age of 63 years. Concerning the estimated putrefaction degree, the ratio of the deceased over and less than 50 years old was 5:1 per category (degree 1 to 4). While the number of corpses with initiating and advanced alterations decreased over time, the number of bodies with more substantial decomposition increased, correlating with greater age (data not shown).

Corpses found in water, as well as burnt and skeletonised bodies, represented 10.2% (Fig. 1). Within the selected period, only eight burnt bodies were examined. Five showed heat-related damage of the epidermis and dermis (degree 2), and three corpses exhibited stronger heat impacts (degree 4). Corpses found in water with varying degrees of decomposition (n = 19) represented 4.5% of the cases and had either been found in rivers or bathtubs. Most of them showed extensive discolouration and inflation (degree 3) while also degree 1 (n = 4), degree 2 (n = 4) and degree 4 (n = 2) were represented. Entirely skeletonised human remains (n = 14) made up for 3.5% of the investigated cases and showed PMIs of up to 100 years. In these cases, only single bones from varying anatomical regions were found.

A total of five methods were applied to identify altered human remains, i.e., comparing CT scans, fingerprints, or individual characteristics to ante-mortem data, DNA genotyping and visual ID by relatives or the police (Fig. 3).

With a mean of 49.1%, the comparison of ante-mortem and post-mortem CT scans was the most common ID method (Fig. 3). In most CT cases, the dental status was compared (n = 146). In 18.5% of the observed cases, relatives or the police identified the deceased person by viewing the remains or examining personal items like an ID card. This visual ID method was only used in cases with bodies showing only initial or advanced signs of decomposition (degree 1 and 2). In 10.2% of the investigated cases, corpses were identified by fingerprints. Identifications based on specific individual characteristics like conspicuous scars, tattoos, piercings, or amputations were only rarely performed (n = 4).

A total of 161 unknown human bodies with or without signs of alteration were identified by matching STR profiles. In 54% corpses were altered (n = 87). Most of them showed signs of decomposition (83.1%), while there were only a few cases with signs of burning (n = 3),

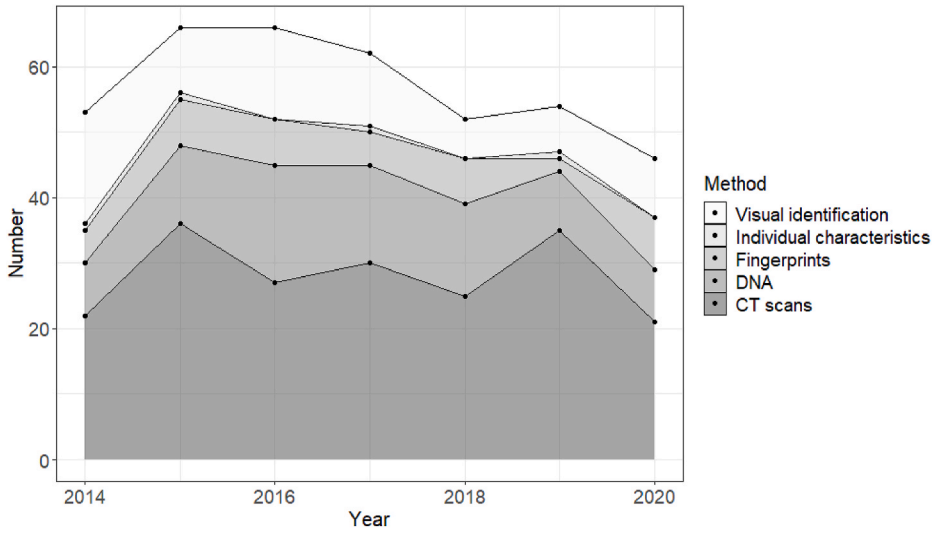

Fig. 3. Identification methods applied for altered human remains from 2014 to 2020, with the numeric value of each method stacked on the previous one, contributing all to the total number of identification methods.

skeletonization (n = 7), or were found in water (n = 8).

In almost all investigated cases (98.1%), a cheek swab with mucosal epithelium from close relatives like a putative child, parent, or sibling was used for relationship analysis. In only three cases, ante-mortem DNA samples from personal material like a toothbrush and comb or an ante-mortem skin biopsy were analysed.

The DNA extraction methods changed during the examined period from the GEN-IAL First All-Tissue DNA-Kit (Gen-ial GmbH, Troisdorf, Germany) to SwabSolution (Promega, Madison, WI, USA) and Maxwell purification (Promega). A potential correlation between DNA quantity and the extraction methods could not be evaluated because, in most cases, DNA quantification of material from the deceased was not performed. Over time, the used kits evolved, going hand in hand with the manufacturers' technical progress. On Veriti™ 96-well thermal cyclers (Thermo Fisher Scientific, Carlsbad, CA, USA), the following PCR amplification kits were performed: PowerPlex® 16 HS, PowerPlex® ESX 17 and ESI 17, PowerPlex® ESX 17 Fast and ESI 17 Fast, PowerPlex® Fusion 6C Systems (all Promega) and AmpFLSTR™ NGM SElect™ and NGM Detect™ PCR Amplification Kit (all Thermo Fisher Scientific). Capillary electrophoresis was performed using the ABI 3500 Genetic Analyzer and the Gene Mapper ID software (both Thermo Fisher Scientific), with a peak height detection threshold set to 50 rfu. In all cases, PCR set-up, cycling, and capillary electrophoreses conditions were according to the manufacturer's instructions. For profiling, mainly the consensus approach was used, i.e., an allele was only accounted for if present in at least two independent amplifications.[22] However, following the International Society of Forensic Genetics (ISFG) recommendation for Disaster Victim Identification (DVI), a composite interpretation was also approached for challenging samples. Here, kits with STRs of different lengths were analysed to complement allelic information from as many loci as possible and to strive LR 1000 or above .[23] For those and some exceptional cases, the gonosomal PCR amplification kits PowerPlex® Y23 System (Promega), AmpFLSTR™ Yfiler™ PCR Amplification Kit (Thermo Fisher Scientific) and Investigator Argus X-12 QS (QIAGEN) were additionally used.

During autopsies or identifications, seven tissue types were collected, preserved and provided for downstream DNA analysis. The majority of the samples (n = 55) were muscle tissue from the *musculus (M.) rectus femoris* (Fig. 4).

In 57% of the 74 unaltered human bodies, more than the required duplicate PCRs were performed. On average, 1.6 additional analyses (parallel and additional PCR amplifications) were performed to obtain STR profiles with a sufficient number of detected alleles (LR > 1000) to assure the identification of the corpse. DNA analyses in altered bodies showed a significantly higher rate of serial or parallel approaches (W = 4352.5, p-value < 0.01). More than two amplifications were performed in 86% of these cases, with a mean of 4.5 (n = 83, range = 1–28, see Fig. 5). In the group of putrefied bodies, consecutive PCRs were performed in 81% of the cases with up to 25 additional PCRs (n = 69, standard deviation (SD) = 4.6). For burnt human remains (n = 3),

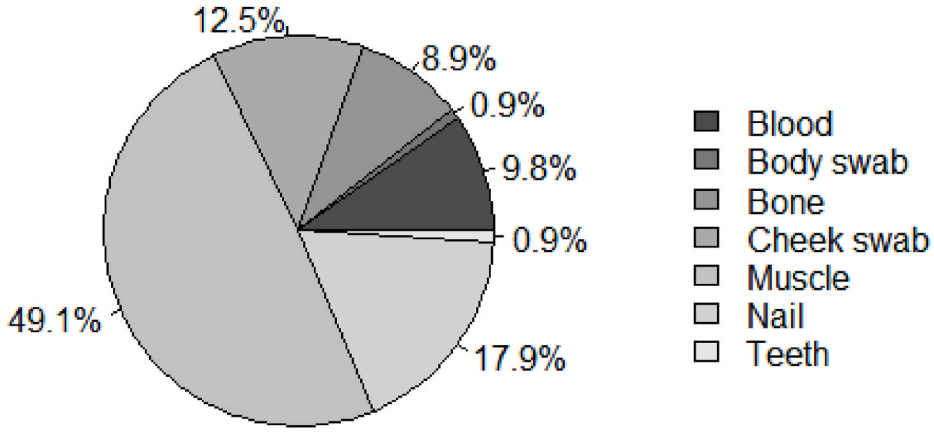

Fig. 4. Percentage distribution of tissue samples collected from altered corpses during forensic autopsies (2014–2020) for DNA-based identifications.

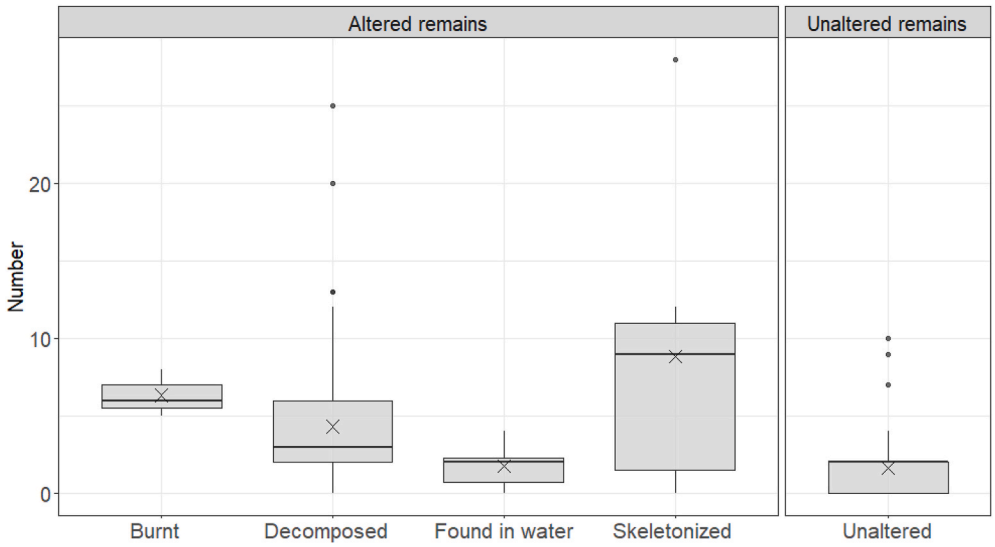

Fig. 5. Boxplot of the absolute number of additional (parallel or serial) PCRs for altered (n = 83, with corpse type categorisation) and unaltered human remains (n = 74). The boxes indicate the 25th to 75th percentile of the data. The ranges are shown by bars. Demonstrated in each plot is the mean (x) and median (–). Dots indicate outliers.

generally, more PCRs were necessary, and for bodies found in water, the number of additional PCRs was comparable low (n = 8, SD = 1.4). The analyses of bone samples from skeletonised human bodies displayed the highest number of additional analyses and the greatest SD (n = 7, SD = 9.7).

Besides the variation in the number of additional PCRs between altered and unaltered remains, differences between analysed tissue types were observed (Fig. 6). For each tissue, the mean and median of the extra PCRs were lower in bodies without signs of decomposition or burning. With a mean difference of 2.4 additional analyses, muscle samples revealed the highest distinction between altered (n = 55) and unaltered remains (n = 37). The divergence between the mean values was also high for nails, with a mean of 2.2 additional analyses needed (n altered = 11, n unaltered = 3). In contrast, only 1.4 additional analyses were performed for blood samples (n altered = 8, n unaltered = 6). With a difference of 1.2, the most minor deviation was seen for cheek swabs. Variances were also observed comparing the classified condition of the corpse (Table 1). However, a correlation between advanced decomposition and increased number of extra PCRs was only seen for muscle samples from decomposed human bodies, degree 1 to 4. Bone samples displayed the highest standard deviation (SD = 10.0), followed by muscle samples (SD = 4.0). Bones were, however, exclusively analysed in completely skeletonised remains and could, therefore, not be compared with unaffected remains. A statistical analysis of the comparison of all tissue types was not possible due to the small number of samples per category.

4. Discussion

Advanced decomposition, burning, or skeletonization can influence

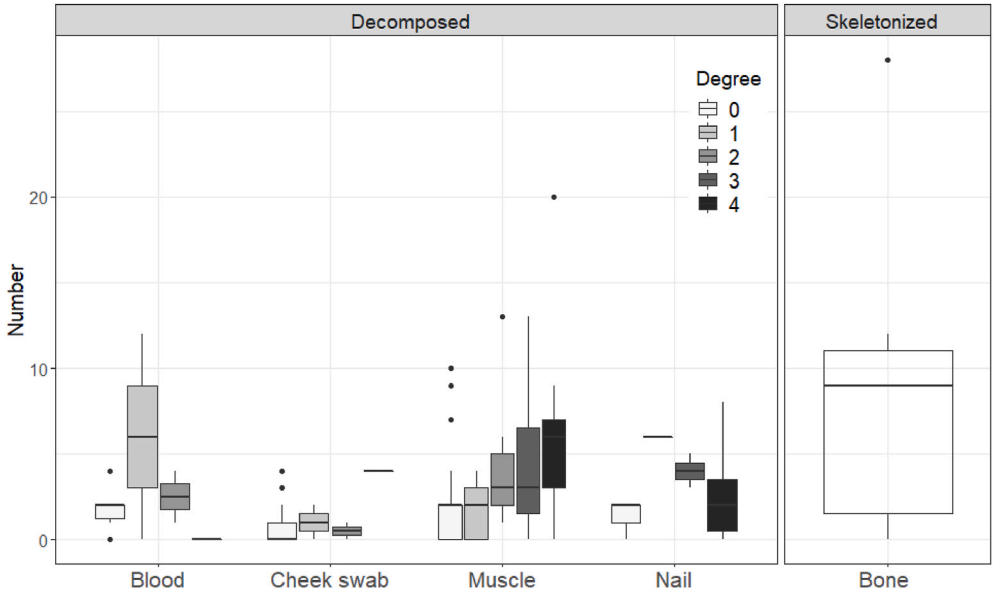

Fig. 6. Boxplot of the absolute number of additional PCRs performed on decomposed and skeletonised corpses and separated into tissue type and degree of decay (0 = unaltered, 4 = highly decomposed). The boxes indicate the 25th to 75th percentile of the data, and the ranges are shown by bars. Demonstrated in each plot is the median (–). Skeletonised bodies were not categorised. Dots indicate outliers.

and impede the identification success of human remains in forensic casework. Conventional methods are applied for well-preserved corpses, but in cases of severe alteration and the absence of reference data, the DNA-based analysis may be the ultimate tool to identify a human body.[12] As expected, most cases were decomposed corpses with various degrees of putrefaction, while cases with other alterations made up only about 10%.

The distribution of the decomposition degrees showed an increased occurrence of highly and profoundly putrefied bodies in the years 2017–2020 compared to the earlier years. The advanced PMI at the time of discovery correlated with the greater age of the deceased. As in the retrospective study by Birngruber et al., most of the deceased were above 50 years old and were found inside a domicile.[24] This observation could be explained by an increase in the number of people living alone in old age. According to country-specific statistical offices, most people over 65 years live on their own, with an increasing tendency.[25] This shift will reasonably lead to rising demands in forensic matters, highlighting the advantage for a systematic and, thus, effective approach for DNA identification methods.

In a high proportion of cases, a comparison of ante- and post-mortem CT scans was the chosen ID method, which is likely due to the growing number of radiological examinations in clinical diagnostics during the last decades. The number of IDs performed using fingerprints or relatives viewing the remains was low. This observation is possibly connected to the lack of ante-mortem fingerprint data and the increased awareness of the error-proneness of witness-based ID, particularly for higher putrefaction degrees, respectively.

The significant difference of PCR repetitions between well-preserved and altered remains was not unexpected as stability and degradation of DNA extracted from putrefied or burnt tissue samples decrease substantially.[26] For these samples, the genotyping success is hard to predict, leaving an uncertainty of how much and which corpse material should be taken. However, as a rapid and successful ID process is vital for social reasons and legal authorities, genetic analyses were apparently often set up in parallel from the beginning, associated with larger expenses.

Regarding the specific tissue types, a direct correlation between the degree of decomposition and the number of additional PCRs was only found for muscle tissue. With an advancing degree in alteration, the mean number increased proportionally. For the other tissue types, no correlation was observed, possibly due to the relatively small sample sizes. Another reason could be an inadequate category's assessment, describing the overall putrefaction status of the body rather than the single tissue sample.

In medico-legal routine, the corpse's condition is assessed but not scored, and the collected and preferred tissue type principally depends on the institute's routines and personal experience. Not surprisingly, other studies analysing tissue materials from altered human remains provide various recommendations regarding the "best material". One study described that the DNA stability in biological material exposed to fire depends on its tissue type, thickness and time of exposure.[26] Their results revealed that DNA degrades to a higher degree in hard tissues like bones and teeth than in soft tissues (e.g., muscle). The significantly higher water content in soft tissue was mentioned as a possible cause to protect the DNA and lead to its improved stability. Therefore, they concluded that choosing thick soft tissues provides the best chance to identify burnt remains successfully.

For decomposed bodies, several studies state that DNA stability is higher in hard than in soft tissues, concluding that bones and teeth are the safer choices when genetically identifying highly decomposed bodies.[1,23,27] This conclusion is confirmed by the ISFG's recommendations for the ID of decomposed corpses DVI scenarios.[23] Yet, other studies advise analysing soft tissues because analysing hard tissues like teeth and bones is more time and cost-consuming.[15,16] According to some authors, bladder swabs present an excellent source for the DNA-based ID of decomposed and burnt human bodies.[7,28] Birngruber et al. discovered blood as the most commonly used material for the ID of altered remains at their institute. At the same time, muscle tissue samples displayed only a small part of the analysed post-mortem material. Due to their STR profiling results from toe- and fingernails, their preferred biological material were toenails if blood was not available.[24] Also, other authors proposed to use toe- or fingernails.[9,29]

In conclusion, the question which is the optimal tissue for the DNA-based ID of altered corpses at first attempt remains unanswered. These retrospective findings confirm that the number of additional amplifications in altered remains is influenced by post-mortem alteration and differs between tissue types. The need for additional analyses was significantly higher in altered than in unaltered bodies and was generally observed for all tissue types. The genotyping success for bone and muscle samples was the most unpredictable, as reflected by a high standard deviation. This observation is also supported by different and partly even oppositional literature recommendations. The retrospective study outcomes emphasise the uncertainty regarding the best hard or soft tissue choice from the various corpses and conditions. The tendency of an increased number of profoundly altered remains is likely to proceed, driven by the changes occurring in demographics and social life, and with it the challenge to identify the deceased effectively. Thus, a systematic investigation of the great variety of hard and soft tissues from corpse types with their diverse degrees of degradation would be needed to identify the best-suited tissue and improve the DNA-based ID success at first attempt.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sector.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Code availability

Not applicable.

Ethics approval

This study was conducted retrospectively from data obtained for routine forensic purposes. No formal consent was required for this type of study.

Consent to participate

No formal consent was required for this type of study.

Consent for publication

No formal consent was required for this type of study.

Declaration of competing interest

The authors have no competing interest to declare that are relevant to the content of this article.

Acknowledgements

The authors would like to thank Katja Anslinger for the valuable comments on the paper.

References

1. Schwark T, Modrow J-H, Steinmeier E, et al. The auditory ossicles as a DNA source for genetic identification of highly putrefied cadavers. *Int J Leg Med*. 2015;129(3):457–462. https://doi.org/10.1007/s00414-015-1177-3.
2. Ketterer T, Wittig H, Schön C, et al. *Forensische Verfahren zur Identifizierung unbekannter Personen*. Schweizerische Gesellschaft für Rechtsmedizin; 2017.
3. Dettmeyer R, Verhoff MA, Schütz H. *Rechtsmedizin*. Springer; 2011. https://doi.org/10.1007/978-3-662-58658-7.
4. Brito F, Prata D, Martha S, et al. Evaluation of the urinary bladder swabs as a source of DNA for human identification using two different extraction methods. *Foren. Sci Int Genet*. 2015;5:e484–e486. https://doi.org/10.1016/j.fsigss.2015.09.192.
5. *Bundesgesetz über die Verwendung von DNA-Profilen im Strafverfahren und zur Identifizierung von unbekannten oder vermissten Personen*; 2016, 1. Oktober https://www.fedlex.admin.ch/eli/cc/2004/811/de.
6. Butler JM. *Advanced Topics in Forensic DNA Typing: Interpretation*. first ed. Academic Press; 2014.
7. Brito FC, Nunes MR, Prata DR, et al. DNA extraction of urinary bladder swabs collected from carbonized and decomposing corpses: possible application in disaster victim identification. *Leg Med*. 2019;37:15–17. https://doi.org/10.1016/j.legalmed.2018.12.002.
8. Roeper A, Reichert W, Mattern R. The Achilles tendon as a DNA source for STR typing of highly decayed corpses. *Forensic Sci Int*. 2007;173(2-3):103–106. https://doi.org/10.1016/j.forsciint.2007.02.004.
9. Piccinini A, Cucurachi N, Betti F, et al. Forensic DNA typing of human nails at various stages of decomposition. *Int Congr*. 2006;1288:586–588. https://doi.org/10.1016/j.ics.2005.08.029.
10. Gouveia N, Brito P, Bogas V, et al. THE effect of different levels of degradation and DNA concentrations on the quality of genetic profiles. *Foren. Sci Int Genet*. 2017;6:e428–e429. https://doi.org/10.1016/j.fsigss.2017.09.151.
11. Egger S, Wand D, Scheurer E, et al. Fehlerhafte Geschlechtsbestimmung aufgrund partieller Deletion des Y-Chromosoms. *Rechtsmedizin*. 2020;30(2):94–100. https://doi.org/10.1007/s00194-020-00373-5.
12. Allouche M, Hamdoum M, Mangin P, et al. Genetic identification of decomposed cadavers using nails as DNA source. *Foren. Sci Int Genet*. 2008;3(1):46–49. https://doi.org/10.1016/j.fsigen.2008.07.008.
13. Sato Y, Hayakawa M, Nakajima T, et al. HLA typing of aortic tissues from unidentified bodies using hot start polymerase chain reaction–sequence specific primers. *Int J Leg Med*. 2003;5:191–193. https://doi.org/10.1016/s1344-6223(02)00108-6.
14. Hansen J, Lesnikova I, Funder AMD, et al. DNA and RNA analysis of blood and muscle from bodies with variable postmortem intervals. *Forensic Sci Med Pathol*. 2014;10(3):322–328. https://doi.org/10.1007/s12024-014-9567-2.
15. Schwark T, Heinrich A, von Wurmb-Schwark N. Genetic identification of highly putrefied bodies using DNA from soft tissues. *Int J Leg Med*. 2011;125(6):891–894. https://doi.org/10.1007/s00414-010-0537-2.
16. Courts C, Sauer E, Hofmann Y, et al. Assessment of STR typing success rate in soft tissues from putrefied bodies based on a quantitative grading system for putrefaction. *J Forensic Sci*. 2015;60(4):1016–1021. https://doi.org/10.1111/1556-4029.12746.
17. Megyesi MS, Nawrocki SP, Haskell NH. Using accumulated degree-days to estimate the postmortem interval from decomposed human remains. *J Forensic Sci*. 2005;50(3):1–9.
18. Gelderman H, Boer L, Naujocks T, et al. The development of a post-mortem interval estimation for human remains found on land in The Netherlands. *Int J Leg Med*. 2018;132(3):863–873. https://doi.org/10.1007/s00414-017-1700-9.
19. Reh H. *Diagnostik des Ertrinkungstodes und Bestimmung der Wasserzeit*. first ed. Triltsch; 1969.
20. van Daalen MA, de Kat DS, Oude Grotebevelsborg BF, et al. An aquatic decomposition scoring method to potentially predict the postmortem submersion interval of bodies recovered from the North Sea. *J Forensic Sci*. 2017;62(2):369–373. https://doi.org/10.1111/1556-4029.13258.
21. Glassman D, Crow R. Standardization model for describing the extent of burn injury to human remains. *J Forensic Sci*. 1996;41(1):152–154.
22. Benschop C, Haned H, Sijen T. Consensus and pool profiles to assist in the analysis and interpretation of complex low template DNA mixtures. *Int J Leg Med*. 2013;127(1):11–23. https://doi.org/10.1007/s00414-011-0647-5.
23. Prinz M, Carracedo A, Mayr W, et al. DNA Commission of the International Society for Forensic Genetics (ISFG): recommendations regarding the role of forensic genetics for disaster victim identification (DVI). *Foren. Sci Int Genet*. 2007;1(1):3–12. https://doi.org/10.1016/j.fsigen.2006.10.003.
24. Birngruber C, Felske-Zech H, Heidorn F, et al. Molekulargenetische identifizierungen unbekannter leichen. *Rechtsmedizin*. 2018;28(2):123–128. https://doi.org/10.1007/s00194-017-0218-2.
25. *Statistik SEBf. Die Wohnverhältnisse der älteren Menschen in der Schweiz 2016. Bau- und Wohnungswesen*. 2018.
26. Maciejewska A, Wlodarczyk R, Pawlowski R. The influence of high temperature on the possibility of DNA typing in various human tissues. *Folia Histochem Cytobiol*. 2015;53(4):322–332. https://doi.org/10.5603/fhc.a2015.0029.
27. Staiti N, Di Martino D, Saravo L. A novel approach in personal identification from tissue samples undergone different processes through STR typing. *Forensic Sci Int*. 2004;146:171–173. https://doi.org/10.1016/j.forsciint.2004.09.052.
28. Owen R, Bedford P, Leditschke J, et al. Post mortem sampling of the bladder for the identification of victims of fire related deaths. *Forensic Sci Int*. 2013;233(1-3):14–20. https://doi.org/10.1016/j.forsciint.2013.07.018.
29. Schlenker A, Grimble K, Azim A, et al. Toenails as an alternative source material for the extraction of DNA from decomposed human remains. *Forensic Sci Int*. 2016;258:1–10. https://doi.org/10.1016/j.forsciint.2015.10.025.

3.2 Publication: *Project II*

Validation and beyond: Next generation sequencing of forensic casework samples including challenging tissue samples from altered human corpses using the MiSeq FGx system

Alina Senst[1], Amke Caliebe[2], Eva Scheurer[1], Iris Schulz[1]

[1]Institute of Forensic Medicine, University Basel, Pestalozzistrasse 22, 4056, Basel, Switzerland
[2]Institute of Medical Informatics and Statistics, Kiel University and University-Hospital Schleswig-Holstein, Kiel, Germany

Status: Published

Journal: Journal of Forensic Sciences (2022) 35318655

Received: January 2022

Accepted: March 2022

DOI: https://doi.org/10.1111/1556-4029.15028

Received: 18 January 2022 | Revised: 15 February 2022 | Accepted: 1 March 2022

DOI: 10.1111/1556-4029.15028

JOURNAL OF FORENSIC SCIENCES AAFS

PAPER

CRIMINALISTICS

Validation and beyond: Next generation sequencing of forensic casework samples including challenging tissue samples from altered human corpses using the MiSeq FGx system

Alina Senst MSc[1] | Amke Caliebe PhD[2] | Eva Scheurer MD[1] | Iris Schulz PhD[1]

[1]Institute of Forensic Medicine, Basel, Switzerland

[2]Institute of Medical Informatics and Statistics, Kiel University and University-Hospital Schleswig-Holstein, Kiel, Germany

Correspondence
Iris Schulz, Institute of Forensic Medicine, University Basel, Health Department Basel-Stadt, Pestalozzistrasse 22, 4056 Basel, Switzerland.
Email: iris.schulz@bs.ch

Abstract

The proceeding developments in next generation sequencing (NGS) technologies enable increasing discrimination power for short tandem repeat (STR) analyses and provide new possibilities for human identification. Therefore, the growing relevance and demand in forensic casework display the need for reliable validation studies and experiences with challenging DNA samples. The presented validation of the MiSeq FGx system and the ForenSeq™ DNA Signature Prep Kit (1) investigated sensitivity, repeatability, reproducibility, concordance, pooling variations, DNA extraction method variances, DNA mixtures, degraded, and casework samples and (2) optimized the sequencing workflow for challenging samples from human corpses by testing additional PCR purification, pooling adjustments, and adapter volume reductions. Overall results indicate the system's reliability in concordance to traditional capillary electrophoresis (CE)-based genotyping and reproducibility of sequencing data. Genotyping success rates of 100% were obtained down to 62.5 pg DNA input concentrations. Autosomal STR (aSTR) profiles of artificially degraded samples revealed significantly lower numbers of locus and allelic dropouts than CE. However, it was observed that the system still exposed drawbacks when sequencing highly degraded and inhibited samples from human remains. Due to the lack of studies evaluating the sequencing success of samples from decomposed or skeletonised corpses, the presented optimisation studies provide valuable recommendations such as an additional PCR purification, an increase in library pooling volumes, and a reduction of adapter volumes for samples with concentrations ≥31.2 pg. Thus, this research highlights the importance of all-encompassing validation studies for implementing novel technologies in forensic casework and presents recommendations for challenging samples.

KEYWORDS
altered human corpses, DNA degradation, ForenSeq™ DNA Signature Prep Kit, Illumina MiSeq FGx system, next generation sequencing, NGS, validation

Presented in part at SGRM Sommertagung 2021, September 4, 2021, in Arlesheim, Switzerland.

Highlights

- Due to a growing demand in forensics, validation of next generation sequencing methods is needed.
- The results show repeatable sequencing data and prove the MiSeq FGx to be robust and reliable.
- The MiSeq FGx shows drawbacks in performance when sequencing degraded and inhibited samples.
- The study presents valuable recommendations and experiences in sequencing challenging samples.
- Additional PCR purification and pooling adjustments are recommended for challenging samples.

1 | INTRODUCTION

Forensic DNA analysts are often confronted with low DNA quantities, mixtures of multiple contributors, and DNA degradation. Especially tissue samples from highly altered human corpses can be challenging in terms of low DNA quantity and quality [1]. For DNA profiling, current capillary electrophoresis (CE)-based short tandem repeat (STR) genotyping has been the gold standard for several years [2–5]. Despite its widespread use, CE displays limitations regarding the required amplicon size and the inability to detect sequence variations in PCR fragments [4,6–10]. Additionally, the number of multiplexed loci is restricted due to the required labelling of similar-sized DNA fragments with different fluorescent dyes [7,8,11,12], leading to a maximal marker capacity of 25–30 loci [3,6,9]. DNA samples from traces or biological material often undergo fragmentation induced by environmental influences like pH value, humidity, temperature, acidic soil, or enzymatic activity [13,14]. Due to the resulting DNA disintegration, larger loci are less likely to be amplified than shorter ones [15–17]. With a common polymerase chain reaction (PCR) fragment range of 80–500 base pairs (bp) and a current spectral overlap of six fluorescent dyes, allele typing based on amplicon size displays a distinct limitation for degraded DNA samples [1,8].

The development of high-throughput DNA sequencing technologies offers promising approaches to advance the resolution of forensic casework samples [18]. Over the past years, massive parallel sequencing (MPS) methods, also known as next generation sequencing (NGS), have expanded the spectrum of DNA analyses providing new opportunities for sequencing the entire human genome or sequences of interest [6,18]. As demonstrated by numerous studies, STR and single-nucleotide polymorphism (SNP) genotyping with NGS reveal a high potential and growing relevance in forensic casework [1–7,11]. In contrast to CE, base-by-base sequencing detects variants in the repeat and flanking region, enhancing the discrimination power [8]. Furthermore, the possibility to multiplex autosomal, X- and Y-STRs, identity informative SNPs (iiSNPs), ancestry informative SNPs (aiSNPs), and phenotype informative SNPs (piSNPs) in a single assay is a major advantage compared with CE [6,7,19,20]. Especially the potential to predict a person's phenotype and ancestry can aid investigative authorities within a given legal framework [11,21]. Moreover, since the DNA fragments do not have to be separated by size, the amplicon length can be reduced, which benefits the analysis of degraded samples [1,19]. Additionally, the sequencing of SNPs can provide valuable information if CE-based STR typing fails [4]. With the ForenSeq™ DNA Signature Prep Kit (Verogen) and the MiSeq FGx system (Verogen), target PCR amplification and parallel sequencing of up to 231 STR and SNP markers with most amplicon sizes of less than 200 bp were introduced [1,6,22]. The assay contains two PCR primer sets: (1) DNA Primer Mix A (DPMA) aiming for noncoding regions and (2) DNA Primer Mix B (DPMB) predicting, in addition, an individual's phenotype and biogeographic ancestry [6]. However, NGS still provides drawbacks regarding the labour-intensive workflow and costs per sample. Despite many studies concerning the applicability of sequencing technologies in forensic casework, NGS is still not implemented in many forensic laboratories [23]. Commuting the current CE-dominated technology to NGS requires time, qualified staff, and internal validation [23,24]. As stated by the Scientific Working Group on DNA Analysis Methods (SWGDAM), NGS-specific studies should address the limits of detection and the quantity and quality of libraries pooled in the sequencing runs [24]. The limited sample input volume (5 μL) [25] is a considerable restriction for the analyses, in particular of low-concentrated samples. An insufficient amount of DNA provokes an increase in the formation of adapter dimers, which can negatively impact the sequencing quality [26,27]. Especially DNA from postmortem tissue samples of altered corpses can be highly degraded and inhibited due to the decomposition process [18,28]. Identifying human remains with high postmortem intervals is a common task in forensic medicine, yet NGS technologies for STR and SNP analysis of altered tissue samples has not been widely evaluated [4]. Thus, further validation and optimisation of the workflow is necessary, especially for challenging samples. In this study, the capability of the ForenSeq™ DNA Signature Prep Kit (Verogen) on the MiSeq FGx system (Verogen) was thoroughly investigated for forensic samples following the Revised SWGDAM Validation Guidelines [24].

For investigating sensitivity, repeatability, reproducibility, and mixtures, several validation studies used human samples instead of artificial positive controls [2,5,7,9,11,21–23,29–31]. The latter builds a more solid basis for statistical analysis. However, it does not reflect the crime-scene traces of poor quality or quantity, often found in an associated criminal delict. Thus, the presented internal validation adds valuable information using human blood, saliva, and casework samples. Optimisations of the library preparation were evaluated to improve the results of challenging samples from altered corpses. Due to the higher possibility of adapter dimer formation, additional PCR purifications and, for the first time, a reduction of adapter volumes were tested to minimize the occurrence of PCR artifacts. Furthermore, varying pooling volumes were explored to increase the DNA input of low-concentrated samples and improve genotyping success. The study aimed to verify the reliability of the MiSeq FGx system with human materials, identify its limits, and beyond validation to optimize the sequencing workflow, in particular for degraded and inhibited DNA samples from altered human corpses.

2 | MATERIAL AND METHODS

2.1 | Sample collection, DNA extraction, quantification, and capillary electrophoresis

Buccal swabs were taken from four volunteers (male *n = 2*, female *n = 2*) with informed consent, with one of each gender also providing whole blood samples. Five GEDNAP (German DNA Profiling Group) proficiency test samples simulated case-type samples with known DNA results. During autopsies, samples were taken from the *musculus (M.) rectus femoris* of unaltered corpses (postmortem interval [PMI] < 24 h, *n* = 2). From altered remains (*n* = 9), *M. rectus femoris*, *M. pectoralis major*, heart, aorta, liver and lung, buccal swabs, rib fragments, *pars petrosa*, vertebra, femur, and toenails were sampled. The PMI ranged from a few days to several weeks, showing varying degrees of decomposition and skeletonisation. The remains' sampling was approved by the regional Ethical Review Board (No. 2019–02211).

Before extracting DNA from bones with the Bone DNA Extraction Kit (Promega), osseous matters were processed with a modified protocol adapted from Pajnic [32]. Genomic DNA was extracted from blood and tissue samples (100 mg each) and buccal swabs using the Maxwell® FSC DNA IQ™ Casework Kit (Promega) on the Maxwell® RSC instrument (Promega) according to the manufacturer's protocols for solid and liquid samples. For the variance study, DNA from buccal swabs, blood, and tissue samples from unaltered human remains was additionally extracted using the SwabSolution™ Kit (Promega). DNA quantification of all samples was performed on the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher) using either the PowerQuant® System (Promega) [33] or the Plexor® HY System (Promega) according to the manufacturer's protocol. If stated, the samples were additionally amplified using the Investigator 24Plex Kit (Qiagen), followed by a fragment length analysis on the ABI Prism3500 xL Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol. All analyses included required positive and negative controls.

2.2 | Library preparation and sequencing

DNA libraries were prepared using the ForenSeq™ DNA Signature Prep Kit (Verogen) according to the ForenSeq™ DNA Signature Prep Reference Guide [25] unless otherwise noted. For target amplification, samples were amplified either in duplicates or triplicates using the DPMA (27 autosomal STRs, 24 Y-STRs, 7 X-STRs, 94 identity SNPs) and the DPMB (22 phenotypic SNPs, 56 biogeographical ancestry SNPs, and the DPMA loci), each in a reaction volume of 15 μL. Since the DPMB primer mix also contains the DPMA primers and additionally provides a prediction of the phenotype and ancestry, only DPMB was used for the reproducibility, repeatability, mixed samples, degradation, and optimisation tests. If not otherwise specified, 1-ng DNA input concentrations were used as a template. Target enrichment, library purification, normalization, pooling, and denaturation of libraries were performed as stated in the manufacturer's protocol or with specified adjustments for the optimisation studies. Quality control of purified DNA libraries prior to sequencing was ascertained with the BioAnalyzer 2100 (Agilent) and the High Sensitivity DNA kit (Agilent). Normalized libraries were sequenced on the MiSeq FGx system using MiSeq FGx™ micro flow cells. Unless otherwise noted, the recommended maximal number of 12 pooled samples for DPMB and 36 samples for DPMA was not exceeded. Every sequencing run comprised fully loaded flow cells, including 2800 M Control DNA as positive and nuclease-free water as negative amplification controls.

2.3 | Data analysis

ForenSeq Universal Analysis Software (UAS) was used to analyze sequencing data with a default interpretation threshold of 4.5% of sequencing reads (except for DYS635 with a default interpretation threshold of 10%, DYS389II with 15%, and DYS448 with 10%). The default for the analytical threshold was at 1.5% of sequencing reads except for DYS635 (default 3.3%), DYS389II (default 5%), and DYS448 (default 3.3%). Marker coverage below the analytical threshold was considered as locus dropout (LD) or allelic dropout (AD). STR alleles between the analytical and interpretation threshold were manually called. SNPs below the interpretation threshold were called when coverage was ≥20. Biogeographical ancestry prediction was obtained from the principal component analysis provided by the UAS. Statistical analyses of each run's quality metrics and sequencing data were performed using R version 4.1.1 [34] and R studio version 2021.09.0 [35]. Data distribution

was evaluated with the Shapiro–Wilk normality test, density, and Q-Q plots using the *dplyr* [36] and *ggpubr* [37] packages. For normally distributed data, linear regression models, and analysis of variances (ANOVA) and the post hoc Tukey's HSD (honestly significant difference) test were performed using the package *lpSolve* [38] and the function *aov* and *TukeyHD*. Significance was defined at $P < 0.05$, and all tests were two-sided. The total number of reads of the sensitivity study were log2 transformed and used for the regression and ANOVA models. To measure reproducibility and repeatability, the intraclass correlation coefficient (ICC) was calculated with two-way random-effects and absolute agreement. Data visualization was carried out using the *ggplot2* [39] and *BlandAltmanLeh* [40] packages. Regression lines were plotted using the function *stat_smooth* and 95% confidence bands. CE data analyses were carried out using the GeneMapper ID-X v.1.6 Software (Applied Biosystems). A threshold of 50 relative fluorescence units (rfu) was used for allele typing.

2.4 | Sensitivity

The DNA extract of a male blood sample was quantified and serially diluted with nuclease-free water to quantities ranging from 1000 pg, 500 pg, 250 pg, 125 pg, 62 pg, 31 pg, 15 pg to 7.8 pg. Each dilution was quantified with the Plexor® HY System (Promega) for a second time to confirm the desired input concentration. Amplification was performed using DPMA and DPMB according to the manufacturer's protocol for purified lysates. The eight dilutions were sequenced in triplicates. For concordance, diluted samples were in addition CE genotyped.

2.5 | Variances, reproducibility, and repeatability

For evaluating variances between tissue types and extraction methods, DNA was extracted from two muscle samples of unaltered corpses, two buccal swabs, and two blood samples using both extraction methods. Each sample was amplified with DPMA and DPMB in duplicates with input concentrations of 1 ng using the manufacturer's protocol for purified (Maxwell® FSC DNA IQ™ Casework Kit extracts) and crude lysates (SwabSolution™ Kit extracts).

To measure the method's strength in repeatability, the same analyst sequenced DNA libraries from the study of variances for a second time. Both sequencing runs were performed within 1 week and under the same laboratory conditions. A second analyst reprocessed the same samples within a week and under the same conditions to determine reproducibility.

2.6 | Pooling variations, mixed samples, and concordance

To test the impact of varying pooling quantities of DNA libraries, replicate batches of 31, 36, and 41 samples amplified with DPMA were pooled and sequenced on three separate flow cells. Replicate batches of 7, 12, and 17 samples were amplified with DPMB and sequenced on three flow cells. Each pool consisted of a corresponding number of replicates of libraries from the same library preparation, including DNA from two buccal swabs and two whole blood samples (Table S1).

Mixtures of quantified DNA samples from buccal swabs were prepared as follows: Trial 1 (male and female sample ratios of 1:1, 5:1, 10:1, 1:5, and 1:10), Trial 2 (male ratios of 1:1, 5:1, and 10:1), and Trial 3 (female ratios of 1:1, 5:1, and 10:1). DPMB was used for amplification, and every mixture was sequenced in duplicates.

Concordance was assessed by comparing sequencing with CE-based genotyping results. Here, DNA was extracted from two whole blood samples, two buccal swabs, and two fresh muscle samples. Each sample was amplified with DPMA and sequenced according to the reference guide [25].

2.7 | Degraded samples

For assessing the system's stability, artificially degraded samples were prepared to simulate DNA damage. Degradation was induced by exposing extracted DNA from a whole blood sample to UV light. Light exposure was conducted in intervals of 0, 10, 15, 20, and 30 min (T_0 to T_{30}) using a UV bank. After quantifying the samples with the PowerQuant Kit (Promega) and measuring the degradation index, samples were amplified with DPMB and sequenced in duplicates according to the Reference Guide [25]. Additionally, each sample was CE-based genotype. Internal quality sensors of the Investigator 24plex QS Kit were used to assess the DNA degradation.

2.8 | Workflow adjustments for challenging samples

The ForenSeq™ DNA Signature Prep Reference Guide [25] includes a library purification step using sample purification beads (SPB). For evaluating the impact of further PCR purifications, the purification was repeated at different steps of the library preparation, and an additional method from Qiagen was used. DNA extracts from a heart sample, toenail, and *pars petrosa* from decomposed human corpses were sequenced without additional purification (RE), with additional purification after target amplification (adjustment 1), with a repetition of the manufacturer's recommended purification step (adjustment 2) and with a subsequent extrapurification using the MinElute Kit (Qiagen) after the protocol's library purification step (adjustment 3). Each sample was amplified using DPMB and sequenced in duplicates. Additionally, tissue samples (*M. rectus femoris*, *M. pectoralis major*, heart, aorta, liver and lung, buccal swabs, rib fragments, *pars petrosa*, and toenails) from decomposed corpses were tested for varying amounts of pooling volumes and their impact on sequencing coverage. A low-concentrated sample (0.05 ng/μL) from the *M. pectoralis major* was pooled in volumes of 5, 10, and 15 μL, with the volumes of the remaining nine tissue samples, each kept at 5 μL.

Further, the effect of varying amounts of indexed adapters was assessed by reducing the recommended input amount from each 4 μL of index 1 (i7) and index 2 (i5), to 3 and 2 μL, respectively. DNA extract from a male blood sample was diluted 8-fold from 1000 pg to 7.8 pg. DPMB amplicons were enriched using the three different volumes for both index adapters.

To validate the results for inhibited and degraded tissue samples from altered human corpses, three bone samples with input concentrations lower than the recommended (vertebra: 581.23 pg, femur: 127.78 pg, *pars petrosa*: 754.21 pg) were each sequenced with 4, 3, and 2 μL of index 1 and index 2 adapters.

3 | RESULTS

Genotype data of 353 samples from varying tissue types were generated. Each run passed the required quality metrics and showed a mean cluster density of 1165 K/mm^2 (462–1501 K/mm^2). From these runs, on average 92.15% (80.33%–98.59%) passed the chastity filter. Phasing and prephasing rates were below the recommended threshold (≤0.25% and ≤0.15%) and showed mean values of 0.15% (0.11%–0.25%) and 0.05% (0.01%–0.09%), respectively. In each run, the overall intensity of the human sequencing control (HSC) passed the minimum intensity level and genotype concordance. Unless otherwise noted, predicted phenotype and biogeographic ancestry were consistent with the individuals' descriptions, regardless of potential single allelic dropouts.

3.1 | Sensitivity

Sensitivity samples amplified with both DPMA and DPMB revealed decreasing total read intensities and increasing LDs with declining DNA input concentrations (Figure 1, Figure 2). Mean read intensities of samples amplified with DPMB ranged from 522,419 (1000 pg input DNA) to 32,330 (7.8 pg). Coverage below the recommended sample read count (85,000 [41]) was obtained from input concentrations ≤15.6 pg. A linear regression model was created with log2-transformed total read intensities (Figure 1). Comparison of log2-transformed read intensities' mean values showed a significant difference by both DNA input concentration ($p < 0.001$) and primer mix ($p < 0.001$). The interaction of both factors was also significant ($p < 0.001$, all *P*-values from two-way ANOVA). Concordant and 100% complete autosomal STR (aSTR) profiles were obtained with input concentrations down to 62.5 pg (Figure 2). Only one triplicate (62.5 pg) displayed a read count of one allele at CSF1PO below the interpretation threshold of 4.5%. With 31.2 pg, the first AD was observed at D18S51, and at a DNA level of 7.8 pg, Amelogenin, TPOX, and FGA dropped out. Complete Y-chromosomal STR profiles were obtained with input concentrations down to 62.5 pg. For X-chromosomal STRs, the first LDs occurred at 15.6 pg (DXS10135 and DXS10103).

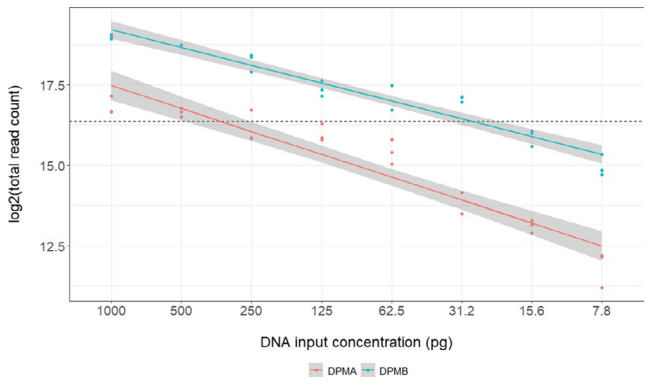

FIGURE 1 Sensitivity study. Total number of reads for DNA input concentrations ranging from 1000 to 7.8 pg amplified with DPMA and DPMB. The total number of reads is log2-transformed. The dotted line indicates the manufacturer's read count threshold of 85,000 (log2-transformed) [41]. Regression lines are plotted with 95% confidence bands (gray)

Compared to STR loci, sensitivity results for iiSNPs displayed an LD of rs2920816 at 125 pg. Even though the number of *alleles below the interpretation threshold* (ABITs) and imbalanced alleles decreased below 250 pg input concentrations, genotyping success rates of ≥93% were still observed down to 15.6 pg. Except for one amplification with 31.2 pg and the loss of rs310644, piSNPs and aiSNPs exhibited initial LDs and ADs at 15.6 pg. To the latter DNA input level, the subject's phenotype was assessable, except for one amplification, in which no phenotype estimation was possible due to the LD of rs683. Ancestry was predictable for all dilutions, with a distance to nearest centroids for ancestry estimation ranging from 1.34 to 2.88 provided by the UAS.

Sensitivity results for DPMA amplicons revealed lower total read intensities than DPMB for the investigated DNA range (Figure 1). Mean read intensity for DNA input concentration of 1000 pg was 117,557, and 3863 for 7.8 pg, respectively, resulting in coverages already dropping below the recommended sample read count at 250 pg. The DPMA genotype success rate of 100% for aSTRs was obtained down to 62.5 pg, except for no read counts for CSF1PO in one amplification (data not shown). First ADs occurred with 31.2 pg input, and ABITs were already observed at 250-pg input. Y- and X-chromosomal STRs genotyping success rates were ≥88% and ≥71%, respectively, down to 15.6 pg. For iiSNPs, allele loss was already detected for input concentrations of 125 pg. Concordant CE-based analyses yielded similar results compared with NGS-based genotyping, with success rates of 100% down to 62.5 pg. The kit-specific quality sensors showed expected peak heights and confirmed successful amplification.

3.2 | Variances

For evaluating possible variances, the influence of different tissue types and extraction methods on the total read intensities were calculated. The outcome was significantly different between tissue types ($p < 0.001$, for DPMA and DPMB), whereas the extraction

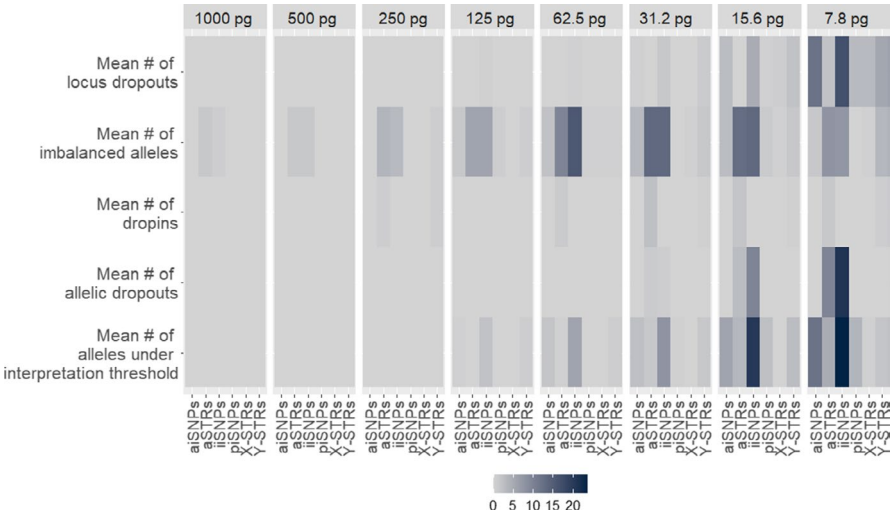

FIGURE 2 Sensitivity study for DPMB. Mean amount of locus dropouts, imbalanced alleles, dropins, allelic dropouts, and alleles under the interpretation threshold for DNA input concentrations ranging from 1000 pg to 7.8 pg. For each DNA concentration, profile quality is separated in aiSNPs, aSTRs, iiSNPs, piSNPs, X-STRs, and Y-STRs

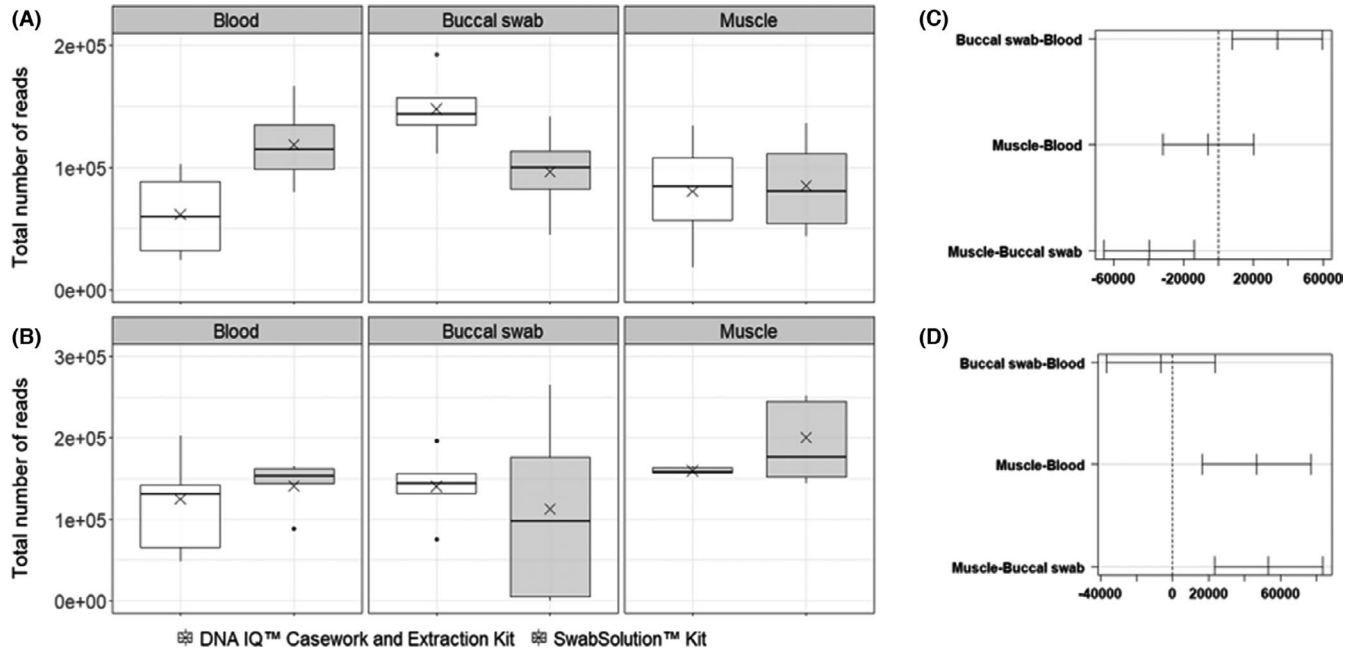

FIGURE 3 Variance study. Total read intensities for DNA extracted with the DNA IQ Casework and Extraction Kit and the SwabSolution Kit from buccal swabs, blood, and muscle samples amplified with DPMA (A) and DPMB (B). Displayed in each plot is the mean (x) and median (–). For both extraction methods, multiple pairwise comparisons of the mean difference (Tukey's honestly significant difference test) were plotted for DPMA (C) and DPMB (D). Confidence intervals for the mean value between the groups crossing the zero line indicate significant differences between groups

method showed no significant influence (DPMA: $p = 0.753$; DPMB: $p = 0.364$). For both primer mixes, a significant interaction between the two factors was found (DPMA: $p < 0.001$; DPMB: $p = 0.023$, all p-values two-way ANOVA). Therefore, each DNA extraction method and sequencing protocol for target amplification demonstrated effective removal of PCR inhibitors. DNA extracted with the SwabSolution™ Kit and amplified with DPMB had the greatest interquartile range (IQR) for DNA extracted from buccal swabs (Figure 3B). Statistically significant differences between each tissue obtained with multiple pairwise comparisons of the mean difference (Tukey's range test, data from both extraction methods were included) are shown in Figure 3C, D. For DPMA, the confidence intervals for the mean value between the tissue groups do not cross the zero line when comparing buccal swabs with blood and muscle with buccal swabs, showing significant differences between the tissues ($p < 0.001$, $p < 0.001$, respectively). For DPMB, a comparison of muscle and blood, and muscle and buccal swabs, shows significant differences ($p < 0.001$, $p < 0.001$, respectively). Genotyping success rate and concordance of autosomal, X- and Y-chromosomal STRs amplified with DPMA was 100% for all sequenced tissue types. Profile completeness of iiSNPs obtained from blood and buccal swabs extracted with the Maxwell® FSC DNA IQ™ Casework Kit reached

98% and 97%, respectively. For the same tissue types extracted with the SwabSolution™ Kit, 99% of iiSNP loci were typed successfully (LD of rs1736442 and rs1031825, respectively). No significant differences were observed when comparing the number of ABITs. Samples amplified with DPMB showed a 100% genotyping success rate of STRs and SNPs only for blood and muscle samples extracted with the Maxwell® FSC DNA IQ™ Casework Kit. DNA samples from buccal swabs extracted with the SwabSolution™ Kit generated no or partial profiles. Due to the high number of piSNP LDs, no phenotype estimation was possible from two buccal swabs. Otherwise, phenotype and biogeographic predictions showed no differences between extraction methods.

3.3 | Repeatability and reproducibility

Agreement between both repeatability runs was measured by plotting the mean and the difference between both runs' total read intensities in a Bland–Altman plot (Figure 4A). The 95% limits of agreement were 109,330.10 reads and −328,235.10 reads, indicating low agreement. In most cases, the difference was negative, with a mean of −109,452.51 reads, showing that read intensities were higher in the repeated run. Additionally, the ICC was calculated with a two-way random-effects model to estimate the strength of agreement. The repeatability results revealed an ICC of −0.24, representing poor agreement. With differences of up to 300,000 reads, no repeatability of read intensities is given. For both runs, genotyping success rate was 100% for compared aSTRs, X- and Y-chromosomal STRs, iiSNPs, piSNPs, and aiSNPs. All targeted loci were concordant and yielded profile completeness. With no significant difference in the number of ABITs, repeatability in the profile completeness is given.

The agreement was also assessed for the reproducibility analysis by plotting mean total read intensities and the difference between the two runs (Figure 4B). The 95% limits of agreement were −63,016.89 and −425,581.31, also indicating low agreement. In all cases, the difference was negative with a mean of −244,299.12, showing that read intensities were higher in the reproduced run. The measured ICC of 0.03 also indicates no agreement. Differences of up to 400,000 reads revealed no reproducibility in read intensities. Furthermore, genotyping, phenotype, and ancestry predictions were successful for every sample.

3.4 | Pooling variations

The manufacturer recommends a maximal number of 12 pooled samples for DPMB and 36 samples for DPMA on micro flow cells [42]. With an increased number of pooled samples, both primer mixes show decreasing total read intensities per sample (Figure 5). Significant differences were observed between the batches for DPMA ($p = 0.132$, one-way ANOVA) and DPMB ($p < 0.001$, one-way ANOVA). The Tukey's HSD p-values for significant differences between the runs' mean values are displayed in Figure 5. Differences between the batches were also observed when comparing the average read intensities of STRs and SNPs separately (Figure 6). There was no distinct decline in read intensities from the lowest to the highest number of pooled samples, except for iiSNPs. Instead, pooling 36 samples amplified with DPMA showed the highest average numbers. The most remarkable differences between the recommended number of pooled samples and the variations were observed for X-STRs. In DPMB amplified samples, the highest average numbers were obtained by pooling seven samples, with distinct differences detected for iiSNPs. However, even though the total number of reads decreased with increasing numbers of pooled samples, the genotyping success was not affected. For $n = 31$ (DPMA), only one LD (DYS389II) was observed in a sample obtained from a buccal swab. The batch of $n = 36$ revealed no LD, and $n = 41$ showed two LDs (rs1736442 and rs1031825). No ADs were detected, and with an increasing number of pooled samples, the total number of ABITs increased only slightly. All samples in batches amplified with DPMB revealed genotyping success rates of 100% with no LD, AD, and ABITs. Therefore, no impact on the phenotype and biogeographic ancestry estimation was observed.

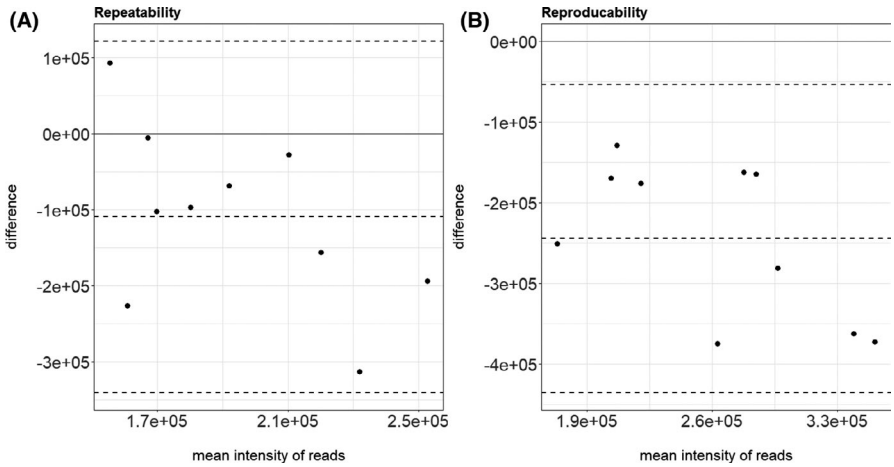

FIGURE 4 Repeatability and reproducibility study for DPMB. Bland–Altman plots for assessing repeatability and reproducibility. Shown are the mean of the total intensity of reads of both runs (x-axis) and the difference between both values (y-axis). The middle dotted line indicates the bias, and the upper and lower dotted lines indicate 95% limits of agreement

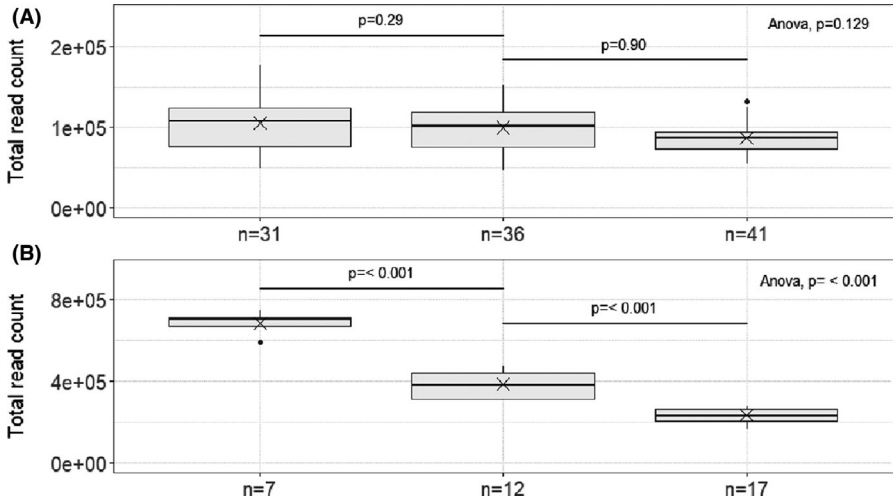

FIGURE 5 Pooling variation study for DPMB. Total number of reads for batches of 31, 36, and 41 pooled libraries (DPMA, A) and 7, 12, and 17 pooled libraries (DPMB, B). Shown are the p-values of the ANOVA analysis and pairwise p-values obtained with the Tukey's post hoc test. Displayed in each plot are the mean (x) and median (–)

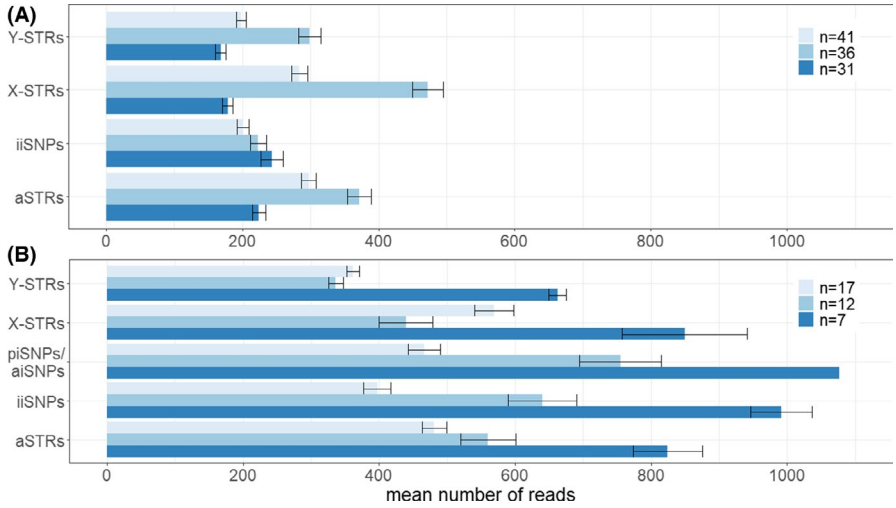

FIGURE 6 Pooling variation study. Mean number of reads for batches of 31, 36, and 41 pooled libraries (DPMA, A) and 7, 12, and 17 pooled libraries (DPMB, B) separated in Y-STRs, X-STRs, iiSNPs, aSTRs, and piSNPs/aiSNPs. Error bars indicate the standard error

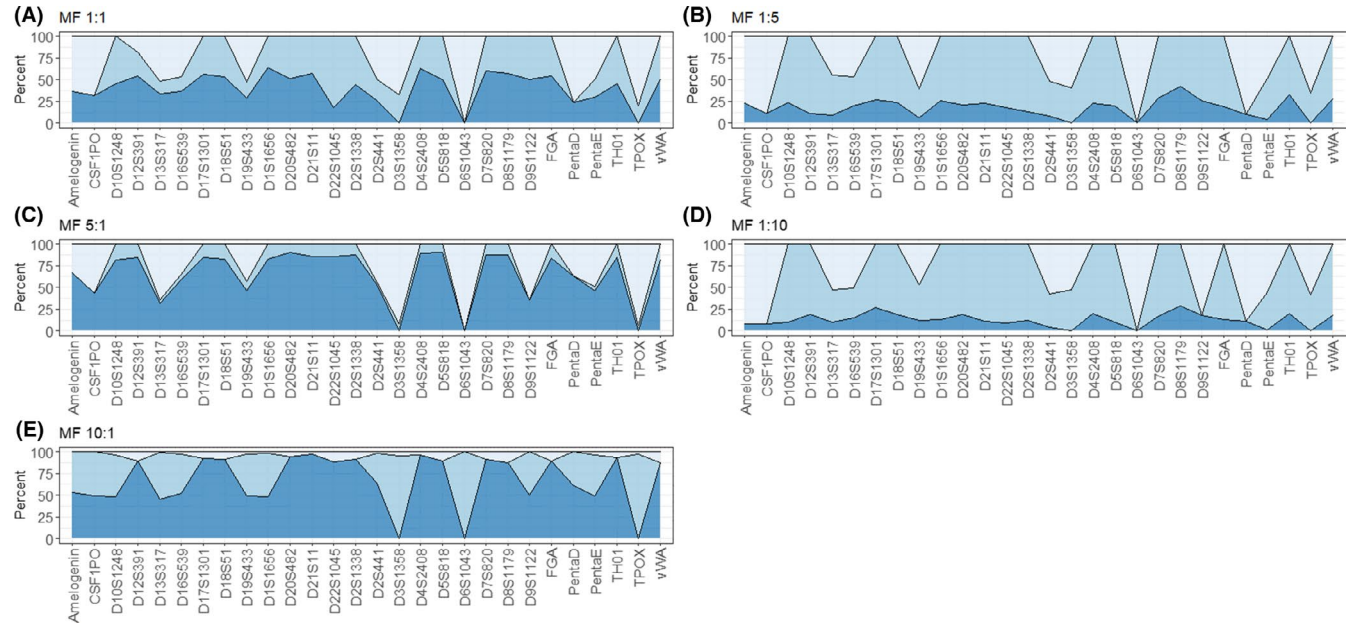

FIGURE 7 Mixture study for DPMB. Percentage of female (F) and male (M) read intensities per marker for different ratios of a male and female (MF) sample (A–E). Shared alleles were summarized as female/male

TABLE 1 Concordance study

			CE		NGS (DPMA)	
Sample	Gender	Extraction method	Profile completeness (%) CE (aSTRs)	Quality sensors (QS)	Profile completeness (%) NGS (aSTRs)	Avg. no. of reads (aSTRs)
Buccal swab	Male	Maxwell® FSC DNA IQ™	100	Present	100	506
		SwabSolution™ Kit	100	Present	100	304
	Female	Maxwell® FSC DNA IQ™	100	Present	100	513
		SwabSolution™ Kit	100	Present	100	304
Blood	Male	Maxwell® FSC DNA IQ™	100	Present	100	334
		SwabSolution™ Kit	100	present	100	402
	Female	Maxwell® FSC DNA IQ™	100	Present	100	188
		SwabSolution™ Kit	100	Present	100	403
M. rect. Femoris	Male	Maxwell® FSC DNA IQ™	100	Present	100	336
		SwabSolution™ Kit	43	QS2 absent	100	403
	Female	Maxwell® FSC DNA IQ™	100	Present	100	483
		SwabSolution™ Kit	0	QS1 and QS2 absent	100	259

Note: Genotyping success rates separated for NGS and CE showed in profile completeness (%) for buccal swabs, blood samples, and samples from the *M. rectus femoris*.

3.5 | Mixed samples

For male–female mixtures (MF), read intensity was above 85,000 for each mixture ratio, and concordant results were obtained from duplicates. Compared to known single-source reference profiles, only one AD at D1S1656 was observed at a ratio of 10:1. However, aSTRs of the minor contributor could be differentiated in every ratio and marker (Figure 7). As expected, the read intensities of the minor contributor decreased with a reduction of the input volume. Even in ratios of 10:1, the male minor contributor showed a total read count of 9881 (mean = 253), compared to the female major contributor (read count = 46,683, mean = 1556). Compared to the results of the female–male mixture, the male–male mixture (MM) revealed a higher number of ADs. Each read intensity was above 85,000. However, the ratio of 5:1 revealed ADs of the minor contributor at D2S441 and vWA, and a complete LD of the Y-STR DYS481. Consequently, the ratio of 10:1 also showed ADs of the minor contributor at CSF1PO, vWA, PentaE, D21S11, and PentaD. In contrast to a total read count of 83,186 for the major contributor (mean = 1698), the minor's was only 5451 (mean = 130). For the female–female mixture (FF), no ADs of aSTRs and X-STRs were observed for a ratio of 1:1, and two ADs of D1S1656 and D21S11 for a ratio of 5:1. With a mean read count of 226, the read intensity of the minor contributor was slightly lower than for the major contributor (mean = 347). The ratio of 10:1 revealed an AD of D5S818 and mean read counts of 585 and 160, respectively.

3.6 | Casework samples

All five GEDNAP samples revealed concordant aSTR results of mixtures or single-source samples with no LDs or ADs. The intensity of reads was above 85,000 for both mixtures (with a mean of 91,751 and 106,893, respectively). For three samples, one per duplicate fell below the threshold of 85,000 (83,529, 60,726, and 80,895, respectively). However, no decline in data quality was observed.

3.7 | Concordance

For the buccal swabs and blood samples, all aSTR genotypes were concordant between NGS- and CE-based genotyping and complete with regard to each method-specific marker set (Table 1). For STRs analyzed with CE, both low-concentrated DNA samples from muscle tissues (0.02 ng and 0.01 ng) extracted with the SwabSolution™ Kit showed a lower genotyping success rate for male (43%) and female profiles (0%), respectively. For these samples, one or both PCR quality sensors were missing. Unexpectedly, the same NGS-based STR typing revealed complete aSTR profiles. On average, four loci with imbalanced alleles were observed.

3.8 | Degradation

Quantification of artificially degraded DNA samples showed a decrease in autosomal DNA concentration and an increased ratio of the autosomal target relative to the degradation target ([Auto]/[Deg]). Each sample exposed to UV light displayed no internal positive control quantification threshold (IPC Cq) shift but values exceeding the manufacture's threshold of two, indicating the presence of degraded DNA [43] (Table 2). The comparison of read intensities and genotyping success revealed a significant decrease with increasing minutes of UV light exposure ($p < 0.001$, one-way ANOVA) (Figure 8). First

TABLE 2 Degradation study

Sample	UV light exposure (Tmin)	ng [Auto]	ng [Deg]	ng [Y]	[Auto]/[Deg]	[Auto]/[Deg] threshold
Blood	T0	14.11	17.93	14.96	0.79	Below
Blood	T10	7.90	0.26	4.21	**30.25**	Above
Blood	T15	5.92	0.08	2.77	**72.05**	Above
Blood	T20	4.53	0.03	1.97	**155.09**	Above
Blood	T30	3.33	0.01	1.27	**302.95**	Above

Note: Quantification results were obtained with the PowerQuant Kit (Promega) for a blood sample exposed to UV light. [Auto]/[Deg] ratio greater than the threshold of two is marked in bold.

LD of rs354439 (iiSNP) and alleles below the interpretation threshold were shown for UV light exposures of 10 min. For longer UV light exposure times, a frequent LD and AD of PentaE were observed. Genotyping success rate for iiSNPs ranged from 100% (0 and 10 min) to 99% (15 and 20 min) and 93% (30 min). Phenotype and ancestry prediction were possible for all samples, except for one duplicate exposed to UV light for 30 min with a loss of rs683. Each DNA profile of degraded samples obtained with CE exhibited a distinct "ski-slope effect" [44] with a loss of the larger loci D2S1338, D21S11 D5S818, D7S820, D8S1179, FGA, and vWA. Evaluation of the influence of UV light exposure and analysis system on the number of dropouts revealed significantly higher numbers in samples analyzed with CE ($p = 0.019$, Figure 9). The factor UV light exposure showed no significant influence ($p = 0.255$). No significant interaction between the two factors was found ($p = 0.633$, all p-values from two-way ANOVA). UV light exposure times of 10 min led to first dropouts in CE-based genotyping (D2S1338, D7S820), compared with one AD at UV T_{15} for NGS-based genotyping (PentaE). For UV T_{30}, seven times more LD were observed for CE-STRs.

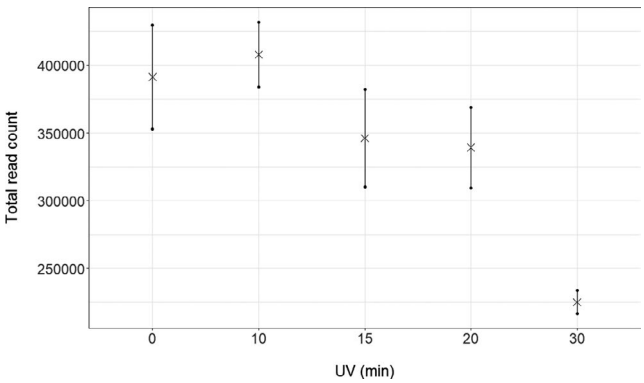

FIGURE 8 Degradation study for DPMB. Total number of reads from a DNA extract exposed to UV light for 0, 10, 15, 20, and 30 min. Displayed in each plot is the mean (x) of both samples

3.9 | Additional sample purification for challenging samples

Comparison of total read intensities revealed a distinct decrease after purification adjustment 1 (Figure 10). Consequently, the NGS genotyping success was relatively low (Table 3). The purification adjustment 2 showed the highest intensities and genotyping success rates for each tissue type compared with the reference. Samples from the *pars petrosa* showed comparable low read numbers due to low DNA input concentration (0.015 ng/μL). The predicted phenotypes from purification adjustments 2 and 3 corresponded to the reference and the person`s visual phenotype. With regard to ancestry prediction, samples clustered between European and ad-mixed American ancestry in a principal component analysis. No estimations were possible for samples with purification adjustment 1.

3.10 | Pooling adjustments for challenging samples

Increasing the input volume of a low-concentrated tissue sample from a human corpse within the pool led to an increase in its total read intensities (Figure 11). However, even with an input volume of 15 μL, the recommended maximal threshold of 85,000 reads [41] was not reached. However, the NGS genotyping success increased, with an associated LDs and ADs decrease (Figure 12). Profile NGS completeness for autosomal and gonosomal STRs as well as SNPs was 79% for 5 μL, 84% for 10 μL, and 96% for 15 μL. Increasing the low-concentrated sample volume showed no considerable influence on the read count and genotyping success of the other samples within the pool. However, even though the read numbers of the low-concentrated sample increased, no phenotype prediction was possible due to an LD of rs1805009 in each run. Despite LDs of aiSNPs, accurate European ancestry was predicted for every sample.

3.11 | Reduction of adapters for challenging samples

A regression model was used to determine the influence of adapter reduction on the resulting read intensities of various input concentrations (Figure 13). With reduced adapter amounts, read intensities dropped below the recommendation of 85,000 [41] from samples with input concentrations ≤15.6 pg. The factor *DNA input concentration* showed significant influence ($p < 0.001$), whereas the factor *volume of adapters* displayed no significant influence on the read count ($p = 0.399$). Additionally, a significant interaction

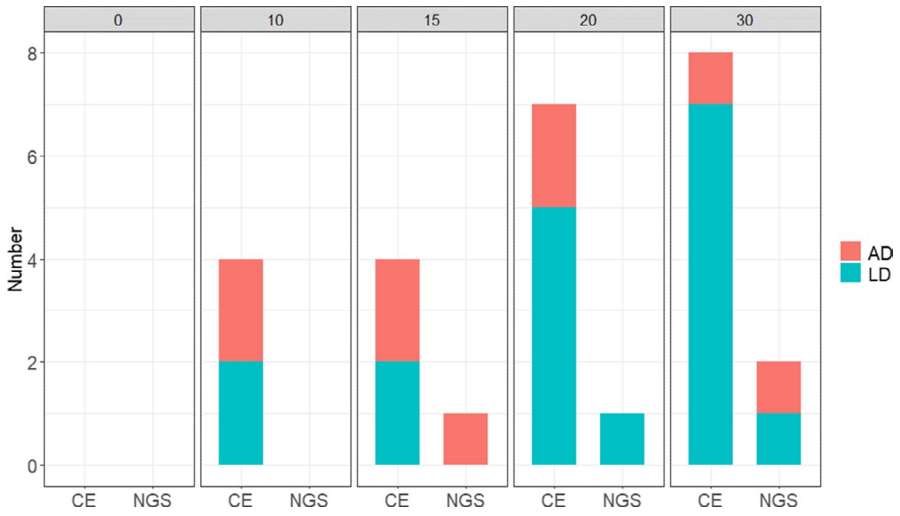

FIGURE 9 Degradation study for DPMB. Profile quality comparison of NGS and CE. Number of locus dropouts (LD) and allelic dropouts (AD) obtained from a DNA extract exposed to UV light for 0, 10, 20, and 30 min

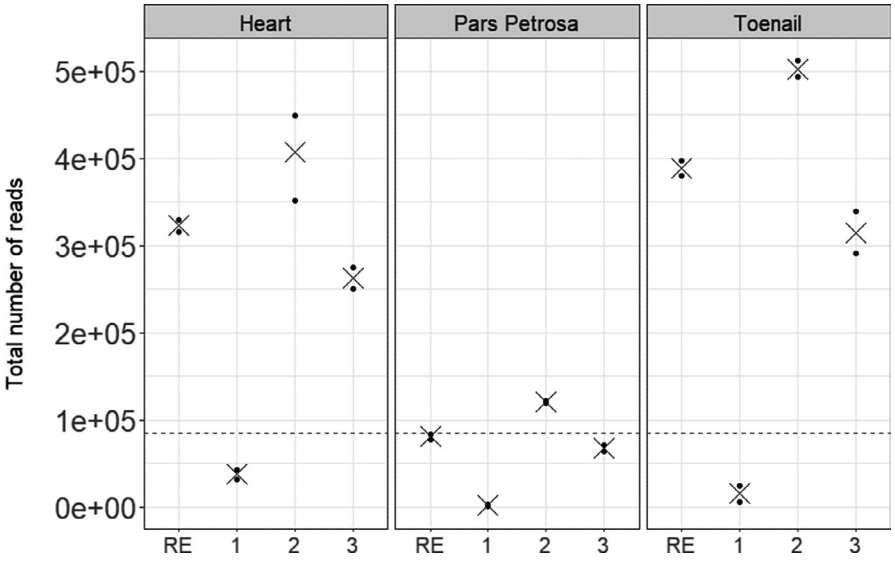

FIGURE 10 Optimisation study: Additional PCR purification for DPMB. Total number of reads obtained for samples from heart, toenail, and *pars petrosa* without additional purification (RE), with additional purification after target amplification (adjustment 1), with a repetition of the manufacturer's recommended purification step (adjustment 2) and with an extra purification using the MinElute kit (Qiagen) after the protocols library purification step (adjustment 3). Displayed in each plot is the mean (x), and the dotted line marks the threshold of 85,000 reads

between the two factors was found ($p < 0.001$, all p-values two-way ANOVA).

As expected, the amount of LDs, ADs, ABITs, dropins, and imbalanced alleles increased with decreasing DNA input concentrations (Figure 14). Concentrations down to 31.2 pg showed no distinct differences between adapter volumes. For an input concentration of 15.6 pg, the ADs increased from 31 (4 μL) to 119 (2 μL), with most dropouts observed for iiSNPs and aiSNPs. The most apparent differences were observed when comparing ADs of input concentrations of 7.8 pg. Samples with 4 μL of each index showed mostly dropouts of iiSNPs, while 2 μL in addition led to dropouts of aiSNPs. No phenotype prediction was possible for 7.8 pg and each tested adapter volume. Despite dropouts of aiSNPs, estimation of European ancestry was predicted for all adapter volume variations. Concordant genotype successes were obtained for each adapter volume and the vertebra and *pars petrosa* sample. No decrease in NGS-STR and iiSNP genotyping success rates was observed. For the femur sample, typing success of STRs slightly decreased from 98% (4 μL) to 97% (2 μL) and profile completeness of iiSNP decreased from 98% (4 μL) to 95% (2 μL). Quality control of the purified libraries conducted with the BioAnalyzer revealed large peaks at about 170 bp, which is the length for the adapter dimers, indicating their presence in each sample. The range of the ForenSeq target fragments was between the expected 200–600 bp [45]. When comparing adapter input volumes, decreases were observed for adapter dimer concentrations. For the femur samples, peak height was reduced from 693 fluorescence units (FU) (4 μL) to 400 FU (2 μL), for the vertebra sample from 249 FU (4 μL) to 208 FU (2 μL) and the *pars petrosa* sample from 971 FU (4 μL) to 423 FU (2 μL).

4 | DISCUSSION

Over the past years, NGS moved more and more in the focus of forensic genetics, providing new opportunities for forensic DNA

TABLE 3 Optimization study: Additional PCR purification for DPMB

	Genotype success rate (aSTRs) (%)	Genotype success rate (Y-STRs) (%)	Genotype success rate (X-STRs) (%)	Genotype success rate (iiSNPs) (%)	Genotype success rate (ai SNPs) (%)	Genotype success rate (piSNPs) (%)	Phenotype concordance with reference (%)	Ancestry concordance with reference (%)
Reference	100	100	100	100	100	100	100	100
Purification after target amplification (1)	≥27	≥71	≥43	≥1	≥1	≥3	NA	NA
Repetition of purification step (2)	100	100	100	≥93	100	100	100	100
MinElute kit (Qiagen) (3)	≥95	100	100	≥92	≥99	100	100	100

Note: Genotyping success rates (%) obtained for aSTRs, Y-STRs, X-STRs, iiSNPs, aiSNPs, and piSNPs. Samples from heart, toenail, and pars petrosa with additional purification after target amplification (1), with a repetition of the manufacturer's recommended purification step (2), and with an extra purification using the MinElute kit (Qiagen) after the protocols' library purification step (3).

FIGURE 11 Optimisation study: Pooling adjustment for DPMB. Intensity of reads obtained from a low-concentrated DNA sample with input volumes of 5, 10, and 15 µL and same input volumes (5 µL) for the other samples within each pool

FIGURE 12 Optimization study: Pooling adjustment for DPMB. Number of locus dropouts, allelic dropouts, imbalanced alleles, and alleles under threshold for a sample pooled in volumes of 5, 10, and 15 µL

analyses. Its advantages of parallel sequencing of many autosomal and gonosomal STRs as well as SNPs, and detecting intravariations of STRs result in an increase in discrimination power compared with CE-based genotyping. However, prior to using novel systems or methods, internal validation studies must evaluate their potential power and limits within the forensic environment [24], especially for challenging samples like DNA mixtures of multiple persons and low-concentrated, degraded, or inhibited samples. Here, the extensive internal validation and optimisation study demonstrated the limitations and reliability of the MiSeq FGx system and the ForenSeq™ DNA Signature Prep Kit.

4.1 | Sensitivity

In agreement with published work, the sensitivity results demonstrated the possibility to generate complete DNA profiles with less than the recommended input concentration of 1 ng [6,7,11,31]. As shown in Jäger et al., it was possible to obtain 100% genotype success rates for concentrations down to 62.5 pg [6]. The total read numbers of samples amplified with DPMA dropped below the recommended threshold much earlier than those amplified with DPMB, which could be explained by the higher number of markers included in the primer mix B. Compared with STRs, piSNPs, and aiSNPs, the mean percent of LDs (6.3%), ADs (17.0%), and ABITs (16.0%) was the highest in iiSNPs, probably because of its highest number of markers in the ForenSeq™ DNA Signature Prep Kit. Additionally, the marker DXS10103 underperformed at lower concentrations as previously reported by Hollard et al. and Köcher et al., among others [2,6,11,22,23,31,46]. However, in contrast to these studies, no artificial DNA sample, but a real blood sample was used for dilution.

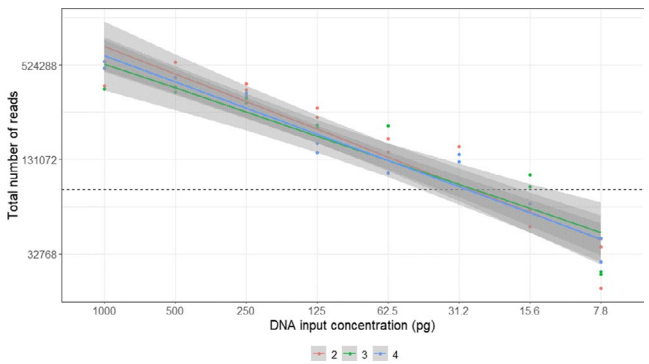

FIGURE 13 Optimisation study: Reduction of adapter for DPMB. Total number of reads for a serial dilution from 1000 to 7.8 pg DNA and adapter volumes of 2, 3, and 4 µL. Regression line for each adapter volume is plotted with 95% confidence bands. The dotted line indicates the manufacturer's read count threshold of 85,000

4.2 | Variances

The variance study analyzed the impact of purified and crude lysates from different body fluids and tissue types. No significant differences were assessed between DNA extraction methods, indicating the protocols' reliability of target amplification regardless of the extraction method. Hence, even not purified DNA samples revealed robust sequencing results when using the protocol for crude lysates. Despite equal input material and fresh samples, tissue-specific differences were observed, potentially due to deviations during the complex and manual library preparation. In particular, buccal swabs extracted with the SwabSolution™ Kit and amplified with DPMB showed the most unpredictable number of reads and the lowest genotyping success. Such a deviation was also seen in a previous work [28], but the underlying samples were from altered and degraded human materials, for which divergent results are expected. Potentially different sequencing efficiencies could cause a general poor agreement in read intensities despite equal DNA input from fresh samples, which were found in the repetition and reproduction studies. However, due to the small sample size, further validation is necessary.

4.3 | Repeatability and reproducibility

With respect to read intensities, both repeatability and reproducibility studies showed poor agreement between the sequencing runs. Differences of up to 300,000 reads were observed between two runs, repeated by the same analyst. This could be explained by divergences during the complex library preparation and/or deviating efficiencies during cluster generation. According to Hollard et al., the automation of all library preparation steps with the Hamilton ID STARlet robotic platform led to repeatable results in terms of depth of coverage (DoC) [23]. Additionally, in contrast to other studies [2,6], no artificial reference control like the 2800 M DNA was used, but DNA from human buccal swabs, blood, and muscle samples to

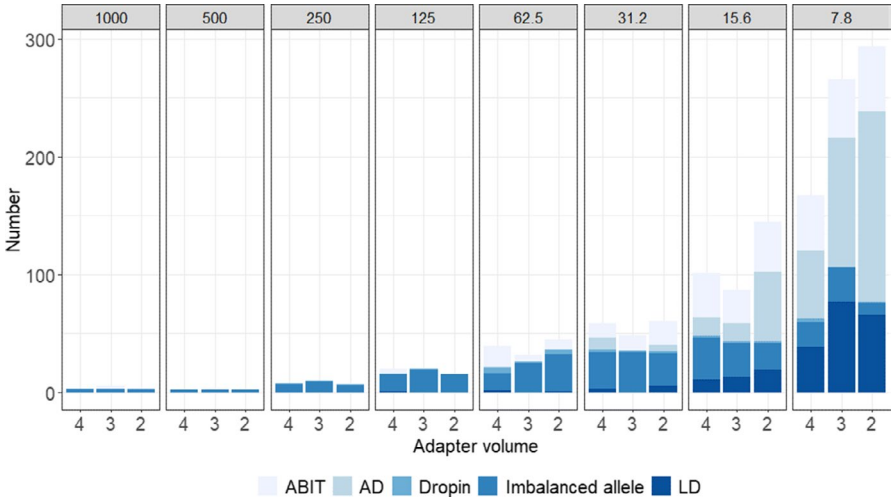

FIGURE 14 Optimisation study: Reduction of adapter for DPMB. Number of alleles below the interpretation threshold (ABITs), allelic dropouts (ADs), dropins, imbalanced alleles, and locus dropouts (LDs) for a serial dilution from 1000 to 7.8 pg DNA and adapter volumes of 2, 3, and 4 µL

represent real casework samples. However, regarding the genotyping success, profile completeness was completely reproducible and repeatable despite significant differences in read intensities. Even though only one run was repeated by the same analyst and reproduced by a second analyst, the genotyping success rates correspond to the results of comparable studies [2,7,31,47]. For SNP genotypes, biogeographic ancestry and phenotype prognosis, accurate, and concordant results were obtained for each evaluated run, regardless of the analyst. The results demonstrate reproducibility and repeatability and were also achieved in the study by Frégeau [21].

4.4 | Pooling variations

Adjusting the manufacturer's recommended number of pooled samples resulted in significant differences between total read intensities. With higher numbers of samples, the decrease was more distinct in samples amplified with DPMB, which could be linked to the greater number of markers included in the primer mix. Furthermore, in forensic casework, not only the sample's total intensity is crucial but also the performance of STRs and SNPs separately, as shown in Figure 6. Average intensities vary the most for SNPs amplified with DPMB, demonstrating that lower numbers of pooled samples should be preferred to achieve higher read numbers. However, for DPMB, no negative impact on the data quality and, for DPMA, only minor decreases in genotyping success rates were determined. Even though standard MiSeq FGx™ flow cells were evaluated, Moreno et al. also observed no decrease in profile quality when pooling 24, 32, and 40 samples with DPMB [47]. Nevertheless, as noted by Just et al., exceeding the number of pooled libraries by almost twice the recommended amount leads to decreasing numbers of recovered loci [30]. Thus, overclustering has a negative impact on sequencing performance, likely due to the difficulty of image analyses, including loss of focus [48].

4.5 | Concordance

The concordance of both methods is essential for implementing NGS into the currently CE-dominated routine work of forensic genetics. The aSTRs amplified with the ForenSeq™ DNA Signature Prep Kit and the Investigator 24plex QS Kit (Qiagen) showed concordant genotypes for buccal swabs and blood samples. Surprisingly, for muscle samples from corpses, aSTR markers enclosed in both kits revealed higher typing rates when sequenced with NGS. DNA extracted with the SwabSolution™ Kit surprisingly only led to 43% partial or zero profiles with CE but to complete profiles with NGS. In our laboratory routine casework, the SwabSolution™ Kit is validated and demonstrates sufficient results even for tissue samples. However, the ForenSeq protocol's purification step obviously removed inhibitors efficiently when compared to the nonpurified SwabSolution extracts.

4.6 | Degradation

As expected, with higher degrees of degradation, the total number of reads and NGS genotyping success decreased. Yet, 87% of iiSNPs was still typed with UV exposure times of 30 minutes, and the only aSTR marker dropping out was PentaE. LD of PentaE was probably due to its second-longest amplicon length (392 bp) within the multiplex. The intensity of the longest amplicon (DXS8378, 450 bp) was just above the analytical threshold. In agreement with Jäger et al. [6] and Fattorini et al. [18], SNP markers showed a mean genotyping success of 98% and were about as stable as the STR markers (97%). The slight difference in the typing rate could be associated with the smaller amplicon size of SNPs.[18,27] Especially for degraded samples, sizes of ≤125 bp increase the chance to obtain sufficient genotypes [1,25]. Most likely, the reduced amplicon size is also the reason for the highly diverging profile completeness results obtained with the traditional CE method. Equivalent to the results of the concordance study, DNA profiles obtained with NGS revealed significantly higher genotype success rates than CE. This enhanced potential and clear advantage were also observed by Almohammed et al., who demonstrated significant differences in obtaining sufficient profiles from degraded bone samples analyzed with NGS and CE (GlobalFiler™ kit) [49].

4.7 | Additional sample purification for challenging samples

The MiSeq FGx system can prematurely abort a sequencing run when too many low-quality samples are pooled, as experienced for internal sequencing runs with highly degraded and inhibited samples from altered human remains (data not shown). Comprised input material can increase the formation of adapter dimers that might remain in the solution after purification. Due to their short size, such dimers have a higher amplification efficiency than DNA libraries and can interfere with cluster generation. Consequently, excessive cluster formation of dimers can result in underclustering of actual libraries [27,45,50]. Additionally, as experienced in sequencing runs (data not shown) and observed by Guo et al., highly inhibited samples can not only affect their genotyping success but also influence the cluster generation of the initially not inhibited samples on the same flow cell [22]. This potential contamination led to the decision against using artificially inhibited samples in this study.

For the aforementioned aborted runs, pooling several highly inhibited and degraded samples resulted in a total loss of sequencing data. To improve the sequencing of such challenging samples, three purification tests were conducted with highly inhibited and degraded samples from decomposed corpses. By repeating the manufacturer's purification workflow, including the magnetic beads' step, the removal of PCR artifacts was improved, and the sequencing results were enhanced. The total read intensities increased with a decrease in adapter dimers. The purification with additional spin

columns showed only slightly less improved genotyping success rates. Since the *spin column method* deviates from the actual workflow, repeating the manufacturer's purification workflow is more efficient and recommended.

4.8 | Pooling adjustments for challenging samples

By increasing the pooling volume of a low-concentrated muscle sample from a decomposed corpse to 15 µL, the genotyping success rate increased by 17% to an almost complete profile. In addition, the volume-wise excess of low-concentrated DNA does not affect the remaining samples on the same flow cell, at least if they contain sufficient DNA amounts. Therefore, when sequencing highly degraded and inhibited samples, we recommend an additional PCR purification, an adjusted pooling volume, and adding higher quality samples to the library pool.

4.9 | Reduction of adapters for challenging samples

Furthermore, to minimize the formation of adapter dimers, a reduction of adapter volumes was tested down to 2 µL for the entire DNA range. Library quality results of tissue samples from altered remains showed a decrease in adapter dimer concentration and concordant genotyping success rates for the vertebra and *pars petrosa* sample and each adapter volume. Although the opposite results were expected, the profile completeness of the femur sample and DNA input concentrations ≤15.6 pg decreased with reducing adapter volumes, indicating an insufficient amount of adapter. Therefore, a reduction of adapter is only recommended for samples with concentrations ≥31.2 pg and expected high-adapter dimer occurrence.

5 | CONCLUSION

The presented study comprised extensive analysis of the MiSeq FGx system's and ForenSeq™ DNA Signature Prep Kit's sensitivity, repeatability, reproducibility, concordance to CE, evaluated pooling variations, and validated different DNA extraction methods and casework, degraded, and mixed samples. Overall results showed the system to be reliable, robust, and implementable for forensic casework samples. In agreement with previous validation studies, the sequencing data are accurate and reproducible. Compared with CE, NGS revealed clear advantages in terms of marker multiplexing and concordant or even improved genotyping results. Especially for degraded samples, the reduced amplicon sizes lead to enhanced amplification efficiencies and typing rates.

However, the restricted amount of DNA input for target amplification and interference of cluster generation by adapter dimers is still a main drawback for forensic applications. Particularly highly degraded and inhibited tissue samples from altered corpses expose the system's limits. According to the presented results of the optimisation studies, adjustments of library preparation prior to sequencing are recommended. An additional PCR purification step should be added, and the pooling volumes for low-concentrated DNA samples should be increased to prevent run failures. Therefore, further studies are required to improve the genotyping success of challenging tissue samples. Nevertheless, the MiSeq FGx has been successfully validated internally, and the results can be used as a basis for further implementation of NGS in forensic laboratories.

ACKNOWLEDGMENTS

The authors would like to thank Katja Anslinger for the valuable comments on the paper. Open access funding provided by Universitat Basel. WOA Institution: Universitat Basel Blended DEAL: CSAL.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article.

RESEARCH INVOLVING HUMAN PARTICIPANTS AND/OR ANIMALS, HUMAN AND/OR ANIMAL SUBJECTS

This study involved samples from voluntary participants and human corpses obtained for forensic purposes. No formal consent was required for this study, which was approved by the regional Ethical Review Board (No. 2019-02211). All procedures were performed in compliance with the relevant laws and institutional guidelines.

ORCID

Iris Schulz https://orcid.org/0000-0002-7933-2563

REFERENCES

1. Carrasco P, Inostroza C, Didier M, Godoy M, Holt CL, Tabak J, et al. Optimizing DNA recovery and forensic typing of degraded blood and dental remains using a specialized extraction method, comprehensive qPCR sample characterization, and massively parallel sequencing. Int J Leg Med. 2020;134(1):79–91. https://doi.org/10.1007/s00414-019-02124-y
2. Köcher S, Müller P, Berger B, Bodner M, Parson W, Roewer L, et al. Inter-laboratory validation study of the ForenSeq™ DNA Signature Prep Kit. Forensic Sci Int Genet. 2018;36:77–85. https://doi.org/10.1016/j.fsigen.2018.05.007
3. Wu J, Li J-L, Wang M-L, Li J-P, Zhao Z-C, Wang Q, et al. Evaluation of the MiSeq FGx system for use in forensic casework. Int J Leg Med. 2019;133(3):689–97. https://doi.org/10.1007/s00414-018-01987-x
4. Hwa H-L, Wu M-Y, Chung W-C, Ko T-M, Lin C-P, Yin H-I, et al. Massively parallel sequencing analysis of nondegraded and degraded DNA mixtures using the ForenSeq™ system in combination with EuroForMix software. Int J Leg Med. 2019;133(1):25–37. https://doi.org/10.1007/s00414-018-1961-y
5. Van Neste C, Van Nieuwerburgh F, Van Hoofstat D, Deforce D. Forensic STR analysis using massive parallel sequencing. Forensic Sci Int Genet. 2012;6(6):810–8. https://doi.org/10.1016/j.fsigen.2012.03.004
6. Jäger AC, Alvarez ML, Davis CP, Guzmán E, Han Y, Way L, et al. Developmental validation of the MiSeq FGx forensic genomics system for targeted next generation sequencing in forensic DNA casework and database laboratories. Forensic Sci Int

Genet. 2017;28:52–70. https://doi.org/10.1016/j.fsigen.2017.01.011
7. Xavier C, Parson W. Evaluation of the Illumina ForenSeq™ DNA Signature Prep Kit–MPS forensic application for the MiSeq FGx™ benchtop sequencer. Forensic Sci Int Genet. 2017;28:188–94. https://doi.org/10.1016/j.fsigen.2017.02.018
8. van der Gaag KJ, de Leeuw RH, Hoogenboom J, Patel J, Storts DR, Laros JF, et al. Massively parallel sequencing of short tandem repeats—Population data and mixture analysis results for the PowerSeq™ system. Forensic Sci Int Genet. 2016;24:86–96. https://doi.org/10.1016/j.fsigen.2016.05.016
9. Zeng X, King JL, Stoljarova M, Warshauer DH, LaRue BL, Sajantila A, et al. High sensitivity multiplex short tandem repeat loci analyses with massively parallel sequencing. Forensic Sci Int Genet. 2015;16:38–47. https://doi.org/10.1016/j.fsigen.2014.11.022
10. Børsting C, Morling N. Next generation sequencing and its applications in forensic genetics. Forensic Sci Int Genet. 2015;18:78–89. https://doi.org/10.1016/j.fsigen.2015.02.002
11. Churchill JD, Schmedes SE, King JL, Budowle B. Evaluation of the Illumina® beta version ForenSeq™ DNA signature prep kit for use in genetic profiling. Forensic Sci Int Genet. 2016;20:20–9. https://doi.org/10.1016/j.fsigen.2015.09.009
12. Butler JM. Fundamentals of forensic DNA typing. Burlington, MA: Academic Press; 2009.p. 191–5.
13. Diegoli TM, Farr M, Cromartie C, Coble MD, Bille TW. An optimized protocol for forensic application of the PreCR™ Repair Mix to multiplex STR amplification of UV-damaged DNA. Forensic Sci Int Genet. 2012;6(4):498–503. https://doi.org/10.1016/j.fsigen.2011.09.003
14. Burger J, Hummel S, Herrmann B, Henke W. DNA preservation: A microsatellite-DNA study on ancient skeletal remains. Electrophoresis. 1999;20(8):1722–8.
15. Roeper A, Reichert W, Mattern R. The Achilles tendon as a DNA source for STR typing of highly decayed corpses. Forensic Sci Int. 2007;173(2–3):103–6. https://doi.org/10.1016/j.forsciint.2007.02.004
16. Piccinini A, Cucurachi N, Betti F, Capra M, Coco S, D'Avila F, et al. Forensic DNA typing of human nails at various stages of decomposition. Int Congr Ser. 2006;1288:586–8. https://doi.org/10.1016/j.ics.2005.08.029
17. McCord B, Opel K, Funes M, Zoppis S, Meadows JL. An investigation of the effect of DNA degradation and inhibition on PCR amplification of single source and mixed forensic samples. National Criminal Justice References Report 236692 2011. https://www.ojp.gov/pdffiles1/nij/grants/236692.pdf. Accessed March 1, 2022.
18. Fattorini P, Previderé C, Carboni I, Marrubini G, Sorçaburu-Cigliero S, Grignani P, et al. Performance of the ForenSeq™ DNA Signature Prep kit on highly degraded samples. Electrophoresis. 2017;38(8):1163–74. https://doi.org/10.1002/elps.201600290
19. Carboni I, Fattorini P, Previderè C, Ciglieri SS, Iozzi S, Nutini A, et al. Evaluation of the reliability of the data generated by Next Generation Sequencing from artificially degraded DNA samples. Forensic Sci Int Genet Suppl Ser. 2015;5:e83–e5. https://doi.org/10.1016/j.fsigss.2015.09.034
20. Iozzi S, Carboni I, Contini E, Pescucci C, Frusconi S, Nutini A, et al. Forensic genetics in NGS era: New frontiers for massively parallel typing. Forensic Sci Int Genet Suppl Ser. 2015;5:e418–9. https://doi.org/10.1016/j.fsigss.2015.09.166
21. Frégeau CJ. Validation of the Verogen ForenSeq™ DNA Signature Prep kit/Primer Mix B for phenotypic and biogeographical ancestry predictions using the Micro MiSeq® Flow Cells. Forensic Sci Int Genet. 2021;53:102533. https://doi.org/10.1016/j.fsigen.2021.102533
22. Guo F, Yu J, Zhang L, Li J. Massively parallel sequencing of forensic STRs and SNPs using the Illumina® ForenSeq™ DNA signature prep kit on the MiSeq FGx™ forensic genomics system. Forensic Sci Int Genet. 2017;31:135–48. https://doi.org/10.1016/j.fsigen.2017.09.003
23. Hollard C, Ausset L, Chantrel Y, Jullien S, Clot M, Faivre M, et al. Automation and developmental validation of the ForenSeq™ DNA Signature Preparation kit for high-throughput analysis in forensic laboratories. Forensic Sci Int Genet. 2019;40:37–45. https://doi.org/10.1016/j.fsigen.2019.01.010
24. Scientific Working Group on DNA Analysis Methods (SWGDAM). Validation guidelines for DNA analysis methods. 2016. https://www.swgdam.org/. .
25. MiSeq FGx™ Forensic Genomics System. Solve more cases and generate more leads with the power and accuracy of Illumina next-generation sequencing. System Specification Sheet: Forensic Genomics. Illumina. Pub. No. 1470–2014-004. San Diego, CA: illumina; 2016 April.
26. Bronner IF, Quail MA, Turner DJ, Swerdlow H. Improved protocols for illumina sequencing. Curr Protoc Hum Genet. 2013;79(1):18.2.1–18.2.42. https://doi.org/10.1002/0471142905.hg1802s79
27. England R, Harbison S. A review of the method and validation of the MiSeq FGx™ Forensic Genomics Solution. Wiley Interdisciplinary Rev Forensic Sci. 2020;2(1):e1351. https://doi.org/10.1002/wfs2.1351
28. Senst A, Scheurer E, Gerlach K, Schulz I. Which tissue to take? A retrospective study of the identification success of altered human remains. J Forensic Leg Med. 2021;84:102271. https://doi.org/10.1016/j.jflm.2021.102271
29. Hussing C, Huber C, Bytyci R, Mogensen HS, Morling N, Børsting C. Sequencing of 231 forensic genetic markers using the MiSeq FGx™ forensic genomics system–An evaluation of the assay and software. Forensic Sci Res. 2018;3(2):111–23. https://doi.org/10.1080/20961790.2018.1446672
30. Just RS, Moreno LI, Smerick JB, Irwin JA. Performance and concordance of the ForenSeq™ system for autosomal and Y chromosome short tandem repeat sequencing of reference-type specimens. Forensic Sci Int Genet. 2017;28:1–9. https://doi.org/10.1016/j.fsigen.2017.01.001
31. Almalki N, Chow HY, Sharma V, Hart K, Siegel D, Wurmbach E. Systematic assessment of the performance of illumina's MiSeq FGx™ forensic genomics system. Electrophoresis. 2017;38(6):846–54. https://doi.org/10.1002/elps.201600511
32. Pajnič IZ. Extraction of DNA from human skeletal material. Forensic DNA typing protocols. Cham, Switzerland: Springer Nature; 2016. p. 89–108.
33. Promega Corporation. PowerQuant® System Technical Manual #TMD047, Rev. 01/20. Madison, WI: Promega Corporation; 2020.
34. Team R. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2021.
35. Team R. RStudio: Integrated development for R. Boston, MA: RStudio, PBC; 2020.
36. Wickham H, François R, Henry L, Müller K. dplyr: A grammar of data manipulation. R package version 1.0.7. 2021 2021. https://CRAN.R-project.org/package=dplyr. .
37. Kassambara A. ggpubr: 'ggplot2' based publication ready plots. R package version 0.4.0. 2020. https://CRAN.R-project.org/package=ggpubr. .
38. Berkelaar M. lpSolve: Interface to 'Lp_solve' v. 5.5 to solve linear/integer programs. R package version 5.6.15. 2020. https://CRAN.R-project.org/package=lpSolve. .
39. Wickham H. ggplot2: Elegant graphics for data analysis. New York, NY: Springer-Verlag; 2016.
40. Lehnert B. BlandAltmanLeh: Plots (slightly extended). Bland-Altman Plots. R package version 0.3.1. 2015. https://CRAN.R-project.org/package=BlandAltmanLeh.
41. Verogen. ForenSeq™ Universal Analysis Software guide. Sample and run results. Verogen. Document # VD2018007. Rev. A. June 2018. San Diego, CA: Verogen; 2018.

42. Verogen. ForenSeq™ DNA Signature Prep reference guide. Document # VD2018005 Rev. C. August 2020. San Diego, CA: Verogen; 2020.
43. Kraemer M, Prochnow A, Bussmann M, Scherer M, Peist R, Steffen C. Developmental validation of QIAGEN Investigator® 24plex QS Kit and Investigator® 24plex GO! Kit: Two 6-dye multiplex assays for the extended CODIS core loci. Forensic Sci Int Genet. 2017;29:9–20. https://doi.org/10.1016/j.fsigen.2017.03.012
44. Butler JM. Advanced topics in forensic DNA typing: Interpretation. San Diego, CA: Academic Press; 2014.p. 109–10.
45. England R, Nancollis G, Stacey J, Sarman A, Min J, Harbison S. Compatibility of the ForenSeq™ DNA Signature Prep Kit with laser microdissected cells: An exploration of issues that arise with samples containing low cell numbers. Forensic Sci Int Genet. 2020;47:102278. https://doi.org/10.1016/j.fsigen.2020.102278
46. Silvia AL, Shugarts N, Smith J. A preliminary assessment of the ForenSeq™ FGx System: next generation sequencing of an STR and SNP multiplex. Int J Leg Med. 2017;131(1):73–86. https://doi.org/10.1007/s00414-016-1457-6
47. Moreno LI, Galusha MB, Just R. A closer look at Verogen's Forenseq™ DNA Signature Prep kit autosomal and Y-STR data for streamlined analysis of routine reference samples. Electrophoresis. 2018;39(21):2685–93. https://doi.org/10.1002/elps.201800087
48. Optimizing cluster density on Illumina sequencing systems. Understanding cluster density limitations and strategies for preventing under- and overclustering. Illumina. Pub. No. 770-2014-038. San Diego, CA: Illumina. 2016 April. p. 1–12.
49. Almohammed E, Zgonjanin D, Iyengar A, Ballard D, Devesse L, Sibte H. A study of degraded skeletal samples using ForenSeq™ DNA Signature Kit. Forensic Sci Int Genet Suppl Ser. 2017;6:e410–2. https://doi.org/10.1016/j.fsigss.2017.09.158
50. Head SR, Komori HK, LaMere SA, Whisenant T, Van Nieuwerburgh F, Salomon DR, et al. Library construction for next-generation sequencing: overviews and challenges. Biotechniques. 2014;56(2):61–77. https://doi.org/10.2144/000114133

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Senst A, Caliebe A, Scheurer E, Schulz I. Validation and beyond: Next generation sequencing of forensic casework samples including challenging tissue samples from altered human corpses using the MiSeq FGx system. J Forensic Sci. 2022;00:1–17. https://doi.org/10.1111/1556-4029.15028

Project II: Supplementary material

Table S1: Pooling variations. Replicate batches of pooled samples from buccal swabs and blood samples. For samples amplified with DPMB, pooled samples were sequenced on flow cell 1 (7 replicates), 2 (12 replicates) and 3 (17 replicates) and samples amplified with DPMA on flow cell 4 (31 replicates), 5 (36 replicates) and 6 (41 replicates). To each flow cell, the corresponding number of positive controls (PC) and negative controls (NC) was added.

Flow Cell 1 n = 7 DPMB		Flow Cell 2 n = 12 DPMB		Flow Cell 3 n = 17 DPMB		Flow Cell 4 n = 31 DPMA		Flow Cell 5 n = 36 DPMA		Flow Cell 6 n = 41 DPMA	
Sample	Replicates	Sample	Replicates	Sample	Replicates	Sample	Replicates	Sample	Replicates	Sample	Replicates
Buccal swab	0	Buccal swab	2	Buccal swab	3	Buccal swab	7	Buccal swab	8	Buccal swab	9
Buccal swab	0	Buccal swab	2	Buccal swab	3	Buccal swab	7	Buccal swab	8	Buccal swab	9
Blood	0	Blood	2	Blood	3	Blood	7	Blood	8	Blood	9
Blood	0	Blood	2	Blood	3	Blood	7	Blood	8	Blood	9
PC	2	PC	3	PC	4	PC	2	PC	3	PC	4
NC	1	NC	1	NC	1	NC	1	NC	1	NC	1

3.3 Manuscript: *Project III*

Novel recommendations on the successful identification of altered human remains using standard and forthcoming technologies: results of a systematic approach

Alina Senst[1], Amke Caliebe[2], Matthias Drum[3], Christian Cossu[3], Martin Zieger[4], Eva Scheurer[1], Iris Schulz[1]

[1]Institute of Forensic Medicine, University Basel, Pestalozzistrasse 22, 4056 Basel, Switzerland
[2]Institute of Medical Informatics and Statistics, Kiel University and University-Hospital Schleswig-Holstein, Brunswiker Str. 10, 24105 Kiel, Germany
[3] Institute of Forensic Medicine, Kantonsspital St. Gallen, Rorschacher Strasse 95, 9007 St. Gallen, Switzerland
[4]Institute of Forensic Medicine, University Bern, Murtenstrasse 26, 3008 Bern, Switzerland

Status: Submitted

Journal: Journal of Forensic Science International: Genetics

Manuscript number: FSIGEN-D-22-00099

Submitted: 29.04.2022

Novel recommendations on the successful identification of altered human remains using standard and forthcoming technologies: results of a systematic approach

Alina Senst[1], Amke Caliebe[2], Matthias Drum[3], Christian Cossu[3], Martin Zieger[4], Eva Scheurer[1], Iris Schulz[1]

[1]Institute of Forensic Medicine, University Basel, Pestalozzistrasse 22, 4056 Basel, Switzerland
[2]Institute of Medical Informatics and Statistics, Kiel University and University-Hospital Schleswig-Holstein, Brunswiker Str. 10, 24105 Kiel, Germany
[3] Institute of Forensic Medicine, Kantonsspital St. Gallen, Rorschacher Strasse 95, 9007 St. Gallen, Switzerland
[4]Institute of Forensic Medicine, University Bern, Murtenstrasse 26, 3008 Bern, Switzerland

Abstract

The DNA-based identification success of altered human remains relies on the condition of the corpses and varies between tissue types. The aim of this prospective multicenter study was to generate evidence based recommendations for successful identification of altered human remains. For this purpose, 19 commonly used soft and hard tissues from 102 altered human bodies were investigated. The corpses' condition was categorized in three anatomical regions using a practical scoring system. Besides other data, DNA yields, degradation indices, and short tandem repeat (STR) profile completeness were determined in 949 tissue samples. Additionally, varying degrees of alteration and tissue-specific differences were evaluated with the Next Generation Sequencing (NGS) platform MiSeq FGx. Selected challenging samples were in parallel sequenced with the Ion S5™ platform to assess platform-specific performances in prediction of the deceased's phenotype and biogeographic ancestry.

Differences between tissue types and DNA extraction methods were found, revealing, for example, lowest degradation for vertebral disc samples from corpses with initiating, advanced and high degrees of decomposition. With respect to STR profile completeness, blood samples outperformed all other tissues, including even profoundly degraded corpses. NGS results revealed higher profile completeness compared with standard capillary electrophoresis (CE) genotyping. Per sample, material and degradation degree, a probability for its genotyping success, including the "extended" European Standard Set (ESS) loci, is provided for the forensic community. Based on the observations, recommendations for the alteration-specific optimal tissue types are made to improve the first-attempt identification success of altered human remains for forensic casework.

Introduction

Short tandem repeat (STR) genotyping is essential for identifying altered human remains and particularly important when no reference data like computed tomography scans or fingerprints are available [2]. Yet, the DNA-based identification (ID) success relies on the quantity and quality of the extracted DNA [103]. Extended post-mortem intervals (PMI) with associated decomposition processes and exposure to high temperatures, for example, can detract DNA integrity, reducing overall amplification success [13, 96, 99, 103]. Also, proceeding microbial growth augments DNA degradation [74], and polymerase chain reaction (PCR) inhibitors like humic compounds, produced during decay processes, or Ca^{2+} Ions, from for example dry bones, can impair the polymerase activity during PCR [84, 124]. Since the decomposition degree depends on several factors like environmental conditions, which impact the human body unevenly, the extent of DNA degradation can strongly vary among tissue types [9, 85, 86, 95, 103]. Therefore, the question of the right sampling material arises, directly affecting STR genotyping and the desired ID success of altered human remains [13, 85, 86, 125].

To our knowledge, until now, a systematic approach and recommendations for identification of the large variety of soft and hard tissues from decomposed and burnt corpses and bodies found in water with their diverse degrees of putrefaction or burning are still missing. Comparison of previous studies revealed mostly small sample sizes and partly contradicting recommendations [126]. In the field of disaster victim identification, for example, recommendations and strategies exist, that provide valuable guidance for sample collection and prioritize bone samples of decomposed corpses [97, 102]. Accordingly, long, compact bones, healthy teeth and/or other available bones should be the first choice from decomposed corpses, and DNA stability is considered higher in hard than in soft tissues. However, bone samples are sensibly circumvented if soft tissues are still available since the osseous preparation is more time-consuming, tedious and requires well-trained staff [9, 10, 127]. Therefore, multiple studies describe alternative sample material like bladder swabs [95, 96], nails [6, 86, 108] or soft tissues like intervertebral discs [99], organs [9, 10] or the Achilles tendon [85]. Those diverging recommendations and observations from a previous study [126] highlight an uncertainty in the choice of the best-suited tissue according to its degree of decomposition. Varying DNA extraction protocols and other laboratory-specific processes render the study results less comparable and impede reproducibility. Furthermore, the corpse's condition is usually not or not optimally scored due to challenges in categorizing the alteration processes even within one body.

Most studies focus on capillary electrophoresis (CE) analysis, which is considered as gold standard in forensic genetics [53, 55-57, 128]. However, the proceeding development of high throughput DNA sequencing technologies leads to their growing relevance in forensic casework and provides promising approaches for the analysis of altered remains by expanding the spectrum of forensic DNA investigations [53, 57, 114, 120, 129]. Next Generation Sequencing (NGS) methods allow multiplexing autosomal and gonosomal STRs, as well as SNPs on a much larger scale. The reduction of amplicon length is of benefit for degraded samples and displays an additional advantage compared to CE [120, 129, 130]. As a previous study has shown, NGS genotyping revealed significantly lower numbers of allelic dropouts compared to CE when analyzing autosomal STR profiles from artificially degraded blood samples [130]. Furthermore,

the potential to predict a deceased`s phenotype and biogeographic ancestry within a given legal framework can add valuable information about the person's identity and assist investigative leads [116, 117].

Using a systematic approach, the aim of this multicenter study was to establish recommendations on the optimal tissue types for a DNA-based ID of altered human remains according to the respective degree of alteration at first attempt. By evaluating a broad variety of different soft as well as hard tissues and comparing DNA extraction methods of the Institutes of Forensic Medicine Basel and Bern, corpse material with the highest probability of STR genotyping success were determined. Besides standard CE analysis, NGS was completed for suited samples to explore potential better performance on degraded and inhibited samples [129, 130]. Next, using the leading site's MiSeq FGx technology and the Ion S5™ system of the Institute of Forensic Medicine St. Gallen, selected challenging samples were sequenced to assess platform-specific prediction power on the phenotype and the biogeographic ancestry of the deceased. Finally, recommendations are represented that are fast and easy to implement in routine forensic casework to standardize the choice of the optimal tissue type for an improved first-attempt identification success of altered human remains.

Material and methods

Sample collection

Over a period of three years, 949 samples from soft tissues (each about 500 mg of heart, lung, spleen, kidney, liver, *M. rectus femoris*, *M. pectoralis major*, aorta), hard tissues (rib, *pars petrosa*, vertebra, femur, humerus, toenails, fingernails) and body fluids (blood, buccal swabs, bladder swabs) were collected from human bodies during medico-legal autopsies or identification at the Institutes of Forensic Medicine Basel and Bern. Decomposed corpses (n = 91, Basel, n=4, Bern), bodies found in water (n = 5, Basel) and burnt bodies (n = 2, Basel) showed varying signs of decomposition or burning (Tab.1) and post-mortem intervals (PMI) from < 24 hours to several years. Tissue samples from unaltered corpses (n = 5, Basel) were collected as control group, leading to a total of 107 human bodies. The study design and sampling were approved by the regional Ethical Review Board (No. 2019-02211).

Scoring method for measuring the degree of decomposition and burning

For grading the extent of decomposition and burning, human remains were categorized according to the body's condition using five degrees from no (D_0) to severe degradation (D_5), prior to the medico-legal autopsy or identification (Tab.1). Due to possibly deviating states of alteration throughout the body, the parameters were - in contrast to other categorization methods – scored independently for the three anatomical segments: 1) the head (including the neck), 2) the trunk (thorax, abdomen and pelvis), and 3) the limbs (arms and legs). The stages of decomposition were classified using partly modified methods and categorizations [20, 35, 131, 132]

[31, 133] as outlined in Tab. 1. The three anatomical regions' scores were not summed to form a Total Body Score (TBS) [131] since bodies with the same TBS but with different decomposition patterns per region were observed.

DNA extraction, quantification, amplification and capillary electrophoresis (CE)

Genomic DNA was extracted from blood (15 µl), soft tissue samples (100 mg each), nails (1 mm^2 from the nail bed) and swabs (ThermoFisher Scientific) using two extraction methods, eluted in 50 µl each: 1) the Maxwell® FSC DNA IQ™ Casework Kit (MWK, Promega) on the Maxwell RSC instrument (Promega) and 2) the SwabSolution™ Kit (SSK, Promega) according to the manufacturer`s protocols. Osseous samples were processed with a modified protocol adapted from Pajnic [134]. Subsequent to removing the remaining tissue with scalpels, the bones were manually cleaned with distilled water (Qiagen), 5% Alconox and ethanol to eliminate adherent contaminants. The bone surface was polished with a sanding tool (Dremel) under a fume hood (Erlab) and then dried at 50°C for 2h in an incubator (Labnet International). To ensure the removal of contaminations, the wash and drying steps were repeated. Following the fragmentation of the bone in a DNA-free bag using a hammer, the shattered pieces were pulverized using a tube mill (Tracomme) and 100 mg of the gained bone powder was used for DNA extraction with the Bone DNA Extraction Kit (Promega).

Tissue samples from the decomposed corpses collected at the Institute of Forensic Medicine Bern were extracted with the institute-specific extraction method to determine the influence of DNA extraction methods. For soft tissues, the iPrep™ Forensic Kit (IPK, ThermoFisher Scientific) and for bone samples, the PrepFiler Express BTA™ Kit (BTA, ThermoFisher Scientific) were used as described in [135] with the following modifications: in the cell lysis step, the volume for PrepFiler Express BTA™ was doubled, directly added to 100 mg bone sample and overnight incubated at 56°C. Both kits were not analyzed separately and are thus referred to as IPK/BTA method.

DNA quantification of all 1698 extractions was performed on the 7500 Real time PCR System (Applied Biosystems) in a total reaction volume of 25 µl using the Plexor HY System (Promega) according to the manufacturers' protocols. For samples extracted with the SSK method, 5X AmpSolution™ Reagent (Promega) was added. Subsequent to amplification with the Investigator 24Plex QS Kit (further referred to as 24Plex, Qiagen), a fragment length analysis was performed on the ABI Prism 3500 xL Genetic Analyzer (Applied Biosystems). The amplification kit includes the European Standard Set (ESS): FGA, TH01, VWA, D1S1656, D2S441, D3S1358, D8S1179, D10S1248, D12S391, D18S51, D21S11, D22S1045, the additional loci D2S1338, D16S539, D19S433, SE33 (further referred to as *extended ESS (eESS) loci*), as well as the STR loci TPOX, DYS391, CSF1PO, D5S818, D7S820, D13S317 plus Amelogenin [136-138]. All analyses included the required positive and negative controls.

Table 1: Categorization of the corpses' degrees of alteration. Decomposed corpses were classified according to Megyesi et al. [131] and Gelderman et al. [20] and bodies found in water according to van Daalen [132], Heaton [35] and Reh [31]. Degrees of burning were classified according to the Crow-Glassman scale [133], Dettmeyer et al. [2] and Symes et. al. [39].

Condition	Degree	Classification	Description		
			Head	Trunk	Limbs
Decomposed	D_0	Unaltered	No visible alteration	No visible alteration	No visible alteration
	D_1	Initiating	Livor mortis, rigor mortis, drying of nose, lips and ears	Livor mortis, rigor mortis, skin appears pink-white	Livor mortis, rigor mortis, drying of finger and toes
	D_2	Advanced	Bloating of the face, formation of putrefactive blisters, grey to green discoloration, skin slippage	Resounding blood vessels, formation of putrefactive blisters, grey to green discoloration, skin slippage	Resounding blood vessels, formation of putrefactive blisters, grey to green discoloration, skin slippage
	D_3	Highly	Extensive green to black discoloration, moist and detachable skin, purging of putrefaction fluids	Abdominal bloating, extensive green to black discoloration, moist and detachable skin, purging of putrefaction fluids	Extensive green to black discoloration, moist and detachable skin, purging of putrefaction fluids
	D_4	Profoundly	Partial skeletonization, loss of organic/inorganic substances, caving in of the flesh and tissue of eyes	Partial skeletonization, loss of organic/inorganic substances, caving in on the abdominal cavity	Partial skeletonization, loss of organic/inorganic substances, joints still articulated
	D_5	Skeletonized	Complete skeletonization	Complete skeletonization	Complete skeletonization
Found in water	D_0	Unaltered	No visible alteration	No visible alteration	No visible alteration
	D_1	Initiating	Livor mortis, rigor mortis, darkened lips, slight pink discoloration	Livor mortis, rigor mortis, slight pink discoloration	Livor mortis, rigor mortis, wrinkling of skin on hands and feet
	D_2	Advanced	Bloating of the face, formation of putrefactive blisters, grey to green discoloration, skin slippage	Resounding blood vessels, formation of putrefactive blisters, grey to green discoloration, skin slippage	Resounding blood vessels, formation of putrefactive blisters, grey to green discoloration, degloving and/or absence of nails
	D_3	Highly	Extensive green to black discoloration, moist and detachable skin, purging of putrefaction fluids	Abdominal bloating, extensive green to black discoloration, moist and detachable skin, purging of putrefaction fluids	Extensive green to black discoloration, moist and detachable skin, purging of putrefaction fluids
	D_4	Profoundly	Partial skeletonization, loss of organic/inorganic substances, caving in of the flesh and tissue of eyes	Partial skeletonization, loss of organic/inorganic substances, caving in on the abdominal cavity	Partial skeletonization, loss of organic/inorganic substances, joints still articulated
	D_5	Skeletonized	Complete skeletonization	Complete skeletonization	Complete skeletonization
Burnt	D_0	Unaltered	No visible alteration	No visible alteration	No visible alteration
	D_1	Level 1	Blistering of upper dermal layer, red skin	Blistering of upper dermal layer, red skin	Blistering of upper dermal layer, red skin
	D_2	Level 2	Damage of dermis, coagulation necrosis	Damage of dermis, coagulation necrosis	Damage of dermis, coagulation necrosis, pugilistic posture
	D_3	Level 3	Dermis/subcutaneous fat tissue completely burnt	Dermis/subcutaneous fat tissue completely burnt	Dermis/subcutaneous fat tissue completely burnt, parts of arms and/or legs missing
	D_4	Level 4	Extensive burn destruction, heat-induced bursting of the cranium	Extensive burn destruction	Extensive burn destruction
	D_5	Level 5	Cremation with little or no tissue left	Cremation with little or no tissue left	Cremation with little or no tissue left

Next Generation Sequencing with the MiSeq FGx and the Ion S5™ system

A total of 155 tissue samples with varying degrees of decomposition were sequenced using the ForenSeq DNA Signature Prep Kit on the MiSeq FGx System (both Verogen). Target amplification was performed with DNA Primer Mix A (DPMA: 27 autosomal STRs, 24 Y-STRs, 7 X-STRs, 94 identity informative (ii) SNPs) and DNA Primer Mix B (DPMB: 22 phenotypic informative (pi) SNPs, 56 biogeographical ancestry informative (ai) SNPs and the DPMA loci) in reaction volumes of 15 µl. Target enrichment, library purification, normalisation, pooling, and denaturation of libraries were conducted according to the manufacturer`s protocol [139]. To assure the libraries' quality prior to sequencing on MiSeq FGx™ micro flow cells (Verogen), the High Sensitivity DNA Kit on the BioAnalyzer 2100 (both Agilent) was used as quality control. Each sequencing run included 2800 M Control DNA (Promega) as positive control and nuclease-free water (Qiagen) as negative control.

For possible device-depending deviations in predicting the deceased's phenotype and biogeographic ancestry, a subset of 20 tissue types D_2 to D_4 were additionally sequenced using the Ion S5™ (ThermoFisher Scientific) at the Institute of Forensic Medicine St Gallen. For this, the leading site provided quantified DNA extracts from heart, Achilles tendon, aorta, vertebral disc, *M. rectus femoris* and lung samples as well as teeth, rib, blood samples, toenails and bladder swabs. For each sample, the Precision ID Ancestry Panel (Applied Biosystems™) and HIrisPlex-S Panel (AmpliSeq Designer Panel) were analyzed together. Library preparation was performed on the Ion Chef™ (ThermoFisher Scientific) using the Precision ID DL8 Kit (Applied Biosystems™). With the exception of the mixture of both primer panels, which is described in a ThermoFisher Technical Note [140], the library preparation was performed according to the manufacturer`s protocols. Sequencing was performed on the Ion S5™ using the Ion S5™ Precision ID Chef & Sequencing Kit (Applied Biosystems™) according to the manufacturer`s protocol on an Ion 520™ Chip (Ion Torrent™).

Data analysis

Quantification was performed using the Plexor Analysis Software (Promega, version 1.5.6.7) according to the manufacturer's instructions. CE data was analyzed by using the GeneMapper ID-X v.1.6 Software (Applied Biosystems) with default stutter filters and a threshold of validated 50 relative fluorescence units (RFU). For each corpse, a reference STR profile was generated by combining reportable alleles derived from all of the corpse's analyzed tissue samples (composite profile) or by using previous STR information from the respective case. Profile completeness was calculated separately for 1) successful typing of all 24Plex kit included 22 STR loci and 2) successful typing of the 16 eESS loci. Peak heights below the analytical threshold were interpreted as dropouts. Profile completeness in percentage was calculated by dividing the reportable alleles by the number of alleles from the corpse's reference profile. Additionally, the probability of genotyping success was calculated separately for 22 and 16 loci (further referred to as probability of genotyping success).

For average peak height, all allele heights were summarized and divided by the number of alleles. The 24Plex kit-specific quality sensors QS1 (74 bp) and QS2 (435 bp) were used to assess the presence of PCR inhibitors and confirm DNA degradation [141]. The ratio of the sensors, calculated by dividing the peak height of QS1 by the peak height of QS2, indicates inhibited DNA in case of decreasing peak heights for QS2 [141]. Besides the interpretation of the artificial quality sensors, an additional degradation index (DI) was calculated to assess the true extent of DNA degradation by using the genetic material itself with the following equation:

$$DI = \left(\frac{peak\ height\ D21S11}{peak\ height\ THO1} + \frac{peak\ height\ SE33}{peak\ heigth\ TPOX} + \frac{peak\ height\ D2S1338}{peak\ height\ D10S1248} + \frac{peak\ height\ FGA}{peak\ height\ D2S441} + \frac{peak\ height\ D7S820}{peak\ height\ D16S539} \right) / 5$$

The resulting DI ranged between 1 (no degradation) and 0 (complete degradation) [142].

MiSeg FGx sequencing data was analyzed using the ForenSeq Universal Analysis Software (UAS, Verogen) with default interpretation and analytical threshold settings. Coverage below the analytical threshold of 1.5% was considered as allelic dropout. For each corpse, the reference profile generated by the CE based STR (CE STR) was used. Completeness between CE and NGS profiles was compared using the overlapping STR loci Amelogenin, TPOX, FGA, TH01, VWA, D1S1656, D2S1338, D2S441, D3S1358, D5S818, D7S820, D8S1179, D10S1248, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11, DYS391, D22S1045 and CSF1PO. The polymorphic STR locus SE33 (ACTBP2) is not included [143]. Estimation of biogeographic ancestry and prediction of phenotype was provided by the UAS. Sequencing data by the Ion S5™ were analyzed using the Converge™ Software (ThermoFisher Scientific) with default interpretation and analytical threshold settings. DNA phenotyping was performed using the HIrisPlex-S online prediction tool (https://hirisplex.erasmusmc.nl/)[144-146] and estimation of biogeographic ancestry was obtained from Converge™ Software. For the comparison of the platform-specific predictions on phenotype and ancestry, a NGS profile was defined as complete when all loci were reportable per MiSeq (24 piSNPs, 54 aiSNPs) and Ion S5™ kit (42 piSNPs, 165 aiSNPs), respectively.

Statistical analyses were performed using R version 4.1.1 [147] and R studio version 2021.09.0 [148]). Distribution of the data was assessed with the Shapiro-Wilk normality test, density and Q-Q plots using the *dplyr* [149] and *ggpubr* [150] packages. Normality could be assumed for the outcomes DNA quantity and RFU peak heights. Then, one-, two- and three-way factor analysis of variances (ANOVA) was used to determine statistical significance for the influence variables *degrees of alteration*, *tissue types* and *DNA extraction method* (with and without interaction). For this, the package *lpsolve* [151] as well as the function *aov* and *TukeyHD* were used and significance was defined as $p<0.05$. Since no normal distribution could be assumed for outcomes DI, QS and profile completeness (in percent), the nonparametric Kruskal-Wallis test was used in these cases to determine significant differences for either *degree of decomposition, tissue type* or the *extraction methods* MWK and SSK. The attainment of a complete STR profile is a dichotomous outcome and was thus analyzed by a logistic regression with influence variables *tissue type, degree of alteration* and *extraction method*. Comparison of profile

completeness between NGS and CE as well as profile completeness for the piSNPs and aiSNPs for phenotype and ancestry prediction between the MiSeq FGx and Ion S5^{TM} systems was assessed by using the paired Wilcoxon signed-rank test. Data visualization was performed using the *ggplot2* package [152].

Results

DNA quantification

DNA quantities of samples from decomposed corpses differ significantly between *tissue types* ($p<0.001$) and *degrees of decomposition* ($p<0.001$) but not for the *extraction method* ($p=0.632$). Significant interactions between the factor *extraction method* and *degree* ($p<0.001$) and between *extraction method* and *tissue type* ($p<0.001$) were found (three-way ANOVA). Stratified analyses for the decomposition degrees showed significant influence in DNA *extraction method* only in unaltered human remains (D0, $p<0.001$) and significant influence in *tissue type* for degree D_0-D_3 (all $p<0.001$). Interaction were significant for D_1, D_2 and D_3 (two-way ANOVA).

For DNA extracted with the MWK method, yields ranged from 3 pg/µl to 748 ng/µl and showed the highest mean quantities in kidney and spleen samples from unaltered remains (222 ng/µl, and 211 ng/µl, respectively) (Fig.1). As expected, for most tissue types, the DNA yield decreased with advanced decomposition processes. Lowest DNA yields were measured for teeth samples in each degree of putrefaction. Wide confidence intervals indicated high variations even within the same tissue type and degree of decomposition and showed the greatest range for liver samples D_1 (3 pg/µl to 314 ng/µl). In contrast, confidence intervals of samples from the vertebral disc, aorta and blood were smaller and more consistent between degrees of decomposition, indicating low variances. DNA extracted with the SSK method displayed a higher concentration range from 4 pg/µl to 1374 ng/ µl. Spleen sample D_1 yielded the highest mean DNA concentrations (530 ng/µl) and samples from the Achilles tendon the lowest within each degree of decomposition (Fig.S1). For comparing the efficiency of DNA extraction methods, 57 tissue D_3 or D_4 samples were extracted with the IPK/BTA method showed highest mean DNA yields for spleen samples D_4 and fingernails D_3 (158 ng/µl, 104 ng/µl, respectively). With the small sample size (n=4), no samples D_0, D_1, D_2 and D_5 could be collected at the Institute of Forensic Medicine Bern.

For bodies found in water (n=5), only tissue samples D_0, D_1 and D_3 could be obtained. As for decomposed bodies, DNA yields decreased with advanced signs of decomposition. With mean amounts of 692 ng/µl highest yields were observed for spleen samples D_1 and lowest yields for buccal swabs D_3 (0.83 ng/µl), both extracted with the SSK method. DNA yields of burnt human remains (n=2) ranged from 4 pg/ µl to 514 ng/µl and showed the lowest yields of DNA from the *M. rectus femoris* D_3 (5 pg/µl MWK) and highest from spleen samples D_3 (166 ng/µl MWK).

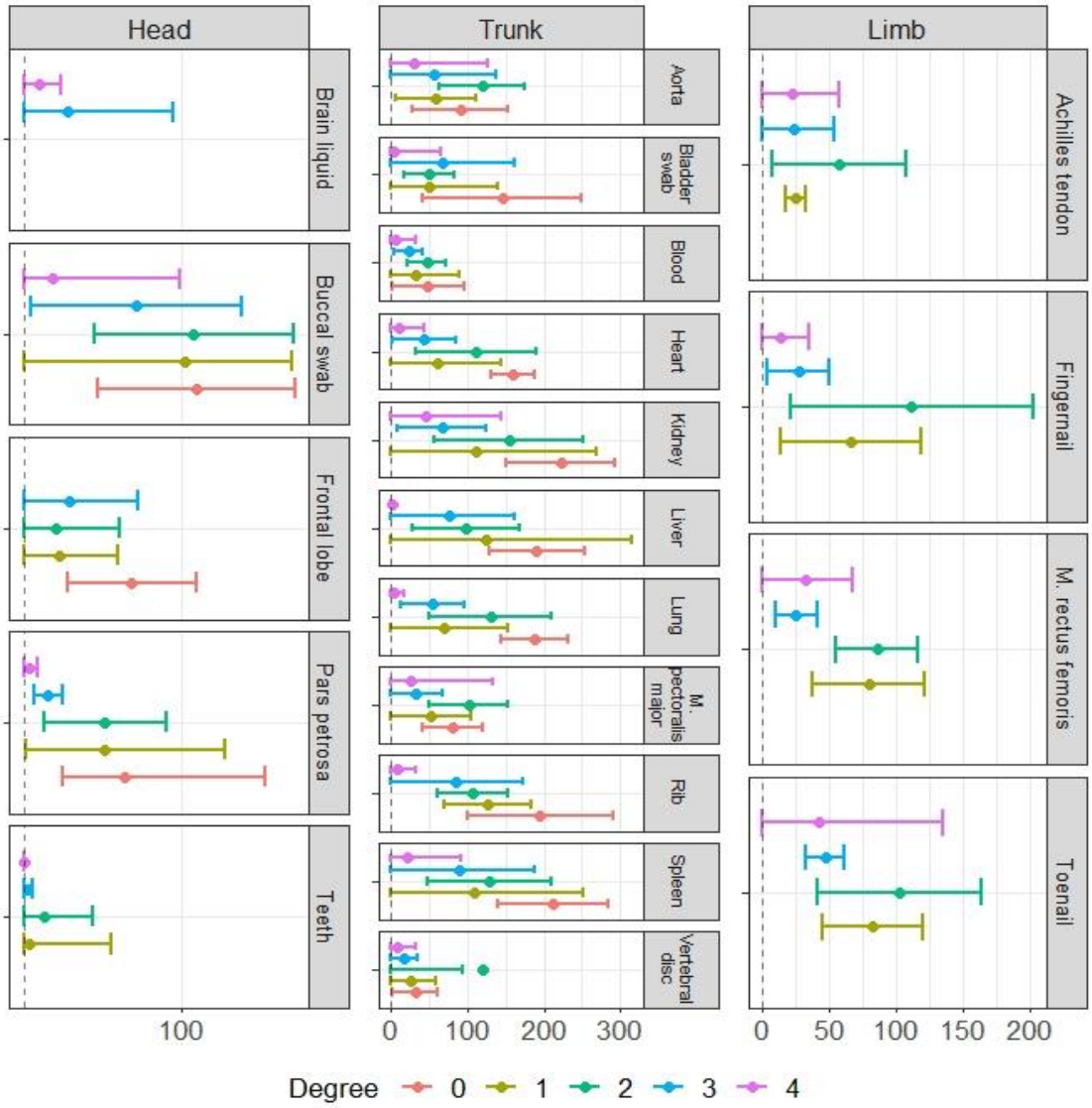

Fig. 1: Quantification results (ng/µl) of DNA extracted with the Maxwell® FSC DNA IQ™ Casework Kit from tissue samples of decomposed corpses. Presented is the confidence interval of the mean. Samples are separated according to the anatomical regions and the corpse's degrees of decomposition ranging from 0 (unaltered) to 4 (profoundly). Due to the small number of samples, D_5 results are not shown. The other missing data represent not available sample material.

DNA integrity

For decomposed corpses, the calculated degradation indices differed prominently by *tissue types* ($p<0.001$ MWK, $p<0.001$ SSK), *degrees of putrefaction* ($p<0.001$ MWK, $p<0.001$ SSK) and extraction method ($p<0.001$). Stratified analyses showed significant difference in *extraction method* for D_2 ($p=0.001$), D_3 ($p<0.001$) and D_4 ($p<0.001$) and *tissue type* for D_0-D_3 ($p<0.001$, all p-values Kruskal-Wallis test). For DNA extracted with the MWK method, low DI of bladder swabs D_4 (median = 0.19), buccal swabs D_4 (median = 0.07), kidney samples D_4 (median = 0.07), and *pars petrosa* samples D_5 (median = 0.01) indicated a strong "ski-slope

effect" [51] and therefore highly degraded DNA (Fig.2A). With the exception of D_4, vertebral disc samples revealed the highest DI of > 0.80 for each degree of decomposition and, thus, low DNA degradation. For the SSK method, samples from the liver and fingernail D_4 revealed the lowest DI. Indices of kidney and liver samples were < 0.15 for each degree of decomposition, representing highly degraded DNA (Fig.2B). For tissue samples extracted with the IPK/BTA method, the DI were comparably low for each analyzed tissue type. The lowest DI was observed for Achilles tendon D_4 (median = 0) and the highest for samples from blood D_4 (median = 0.93) and ribs D_3 (median = 0.78).

Bodies found in water also showed lower DI with advanced signs of putrefaction. For D_3, DNA from liver samples and buccal swabs displayed the lowest median DI for both MWK and SSK extraction methods (0.09, and 0.11, respectively). DNA from burnt corpses was most degraded in muscle samples (*M. rectus femoris* and *M. pectoralis major*) and showed DI of > 0.001 even for D_1. In contrast, buccal swabs D_3 showed the highest DI median indices (0.98) with the MWK method.

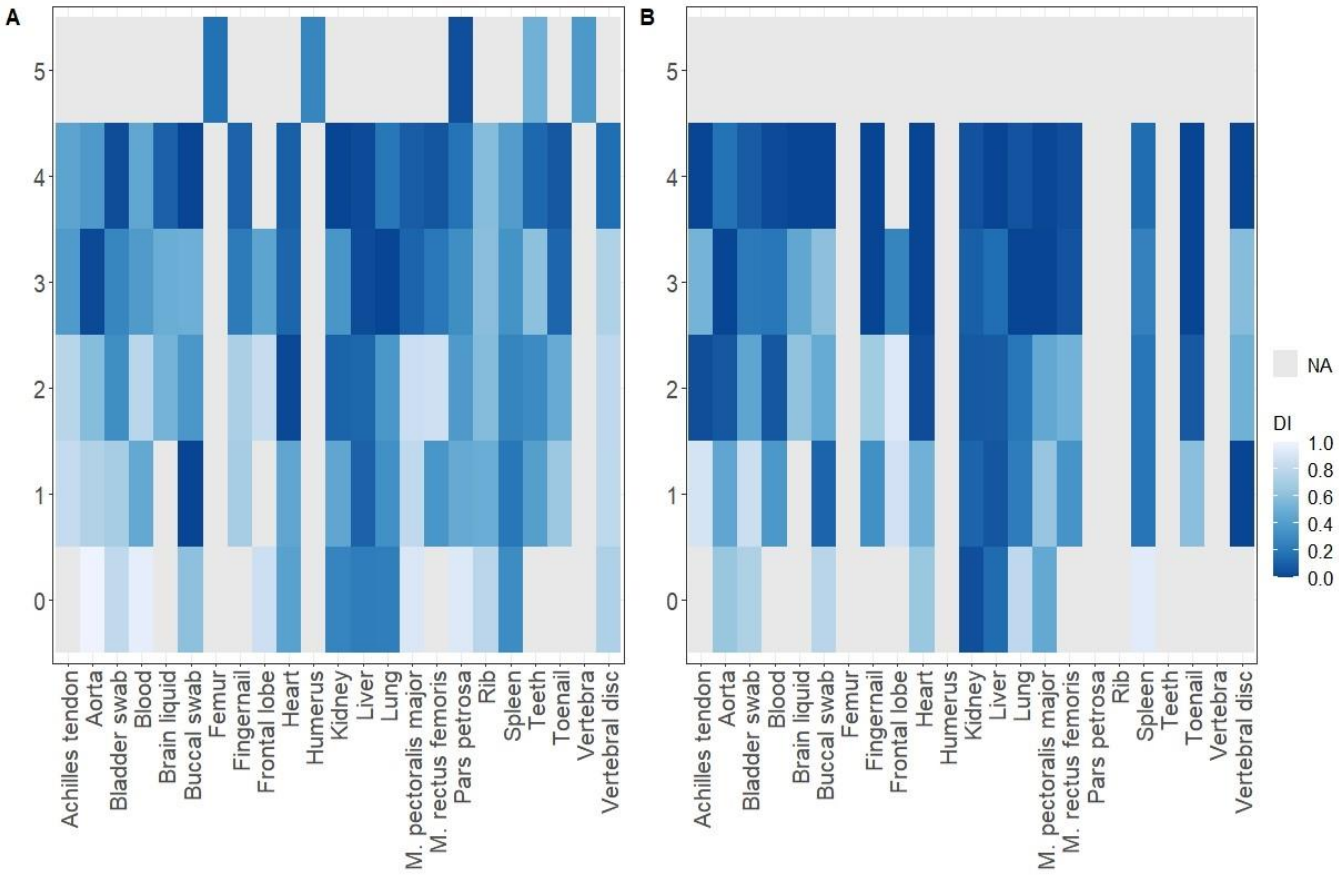

Fig. 2: Heatmap of degradation indices (DI) of DNA extracted with the Maxwell® FSC DNA IQ™ Casework Kit (A) and the SwabSolution™ Kit (B). Tissue samples were collected from putrefied corpses classified in degrees of decomposition ranging from 0 (unaltered) to 5 (skeletonized). Missing data represents not available sample material.

The ratio of peak heights of internal quality sensors ranged from 0 to 2.13 (MWK) and 0 to 1.59 (SSK) for decomposed corpses (Fig. S2). DNA extracted with the MWK method showed no significant differences between *tissue types* ($p=0.968$) but between *degrees of decomposition* ($p<0.01$, all p-values Kruskal-Wallis test). For D_0, a median of 0.95 indicated balanced peak heights and no presence of inhibitors. With advanced degree of decomposition, the median only slightly increased from 1.03 (D_1) to 1.09 (D_5), which reflects absence of inhibitors and an efficient purification of the DNA extracts. For three samples the larger quality sensor QS2 dropped-out, implying the presence of inhibitors. For samples extracted with the SSK method, the ratios differed significantly between *tissue types* ($p=0.011$) and *degrees of decomposition* ($p<0.001$, all p-values Kruskal-Wallis test). With median of 0.91 (D_0) and 0.85 (D_4), a decrease in QS2 peak heights was observed, indicating advanced degrees of decomposition. Compared to the MWK method, more samples were below 0. In comparison, DNA extracted with the IPK/BTA method exposed less ratios below 0, with median of 1.12 (D_3) and 1.37 (D_4).

For bodies found in water, results were comparable to decomposed bodies and showed medians of 1.01 (D_1) to 1.11 (D_3) for samples extracted with MWK and 0.94 (D_1) to 0.87 (D_3) for SSK. Medians of burnt human remains ranged from 1.09 (D_1) to 1.21 (D_3) for samples extracted with MWK and 1.34 (D_1) to 1.0 (D_3) for samples extracted with SSK.

STR genotyping with CE (CE STR)

RFU peak heights

RFU peak heights of samples from decomposed corpses differ significantly between *tissue types* ($p<0.001$) and *degrees of decomposition* ($p<0.001$) and *extraction method* ($p<0.001$). Significant interactions between the factor extraction method and degree ($p<0.001$) were found (three-way ANOVA). Stratified analyses for the decomposition degrees showed significant influence in DNA *extraction method* only in D_2 ($p<0.001$) and D_0 ($p<0.001$) and significant influence in *tissue type* for degree D_1-D_4 (all $p<0.001$). Interaction was significant for D_2 (two-way ANOVA).

Highest mean peak heights were noted in MWK extracted samples from the frontal lobe D_1 and D_3 (7279 RFU, 6497 RFU, respectively) as well as heart D_3 (4061 RFU) (Tab.S1). In comparison, mean peak heights of liver samples were below 2966 RFU for each degree of decomposition. For SSK extracts, greater variations in each degree of decomposition were observed, with the highest mean heights for heart samples D_1 (3002 RFU) and the lowest for lung samples D_4 (540 RFU). For tissue IPK/BTA extracted samples, no great differences were shown for samples D_3 and D_4. Mean heights of blood samples D_4 (6457 RFU) and samples from the frontal lobe D_3 (4562 RFU) were the largest and *pars petrosa* samples D_4 revealed the lowest peak heights (875 RFU).

Mean peak heights of samples from bodies found in water also varied between *degree of putrefaction* as well as *tissue types* and ranged from 564 to 9695 RFU (MWK) and 55 to 26042 RFU (SSK). Comparable to samples from decomposed remains, DNA extracted with the SSK method revealed a greater variation within each degree and greatest mean heights for samples from the *M. pectoralis major* D_3 (6416 RFU SSK) and the frontal lobe D_1 (5312 RFU MWK). The low number of samples from burnt human remains showed high peak heights variations within both extraction methods and the greatest heights in samples from the vertebral disc D_3 (8406 RFU MWK) and fingernails D_3 (14820 RFU SSK) as well as lowest in samples from the liver D_3 (1996 RFU MWK) and *M. rectus femoris* D_3 (109 RFU SSK).

Profile completeness

Statistical differences in profile completeness of the 22 STRs were observed for *tissue types* ($p<0.001$ MWK, $p<0.001$ SSK) as well the *degree of decomposition* ($p<0.001$ MWK, $p<0.001$ SSK) (Fig.3). Although the DNA extraction methods differed statistically ($p<0.001$, all p-values Kruskal-Wallis test), differences were not significant in the subgroups D_0, D_1 and D_2. As expected, profile completeness decreased with advanced signs of decomposition. Median profile completeness of MWK extracted heart samples revealed a constant decline from 99 % (D_0), 99 % (D_1), 98 % (D_2), 91 % (D_3) to 86% (D_4) (Fig. 3A). However, DNA from blood samples showed no decrease and a median profile completeness of 100% for each degree. As comparison, profile completeness of each SSK extracted tissue displayed significantly more allele dropouts for D_3 and D_4 (Fig. 3B). Due to greater variances within each tissue and degree, median profile completeness of heart and vertebral disc samples D_4 decreased to 29% and 28%, respectively.

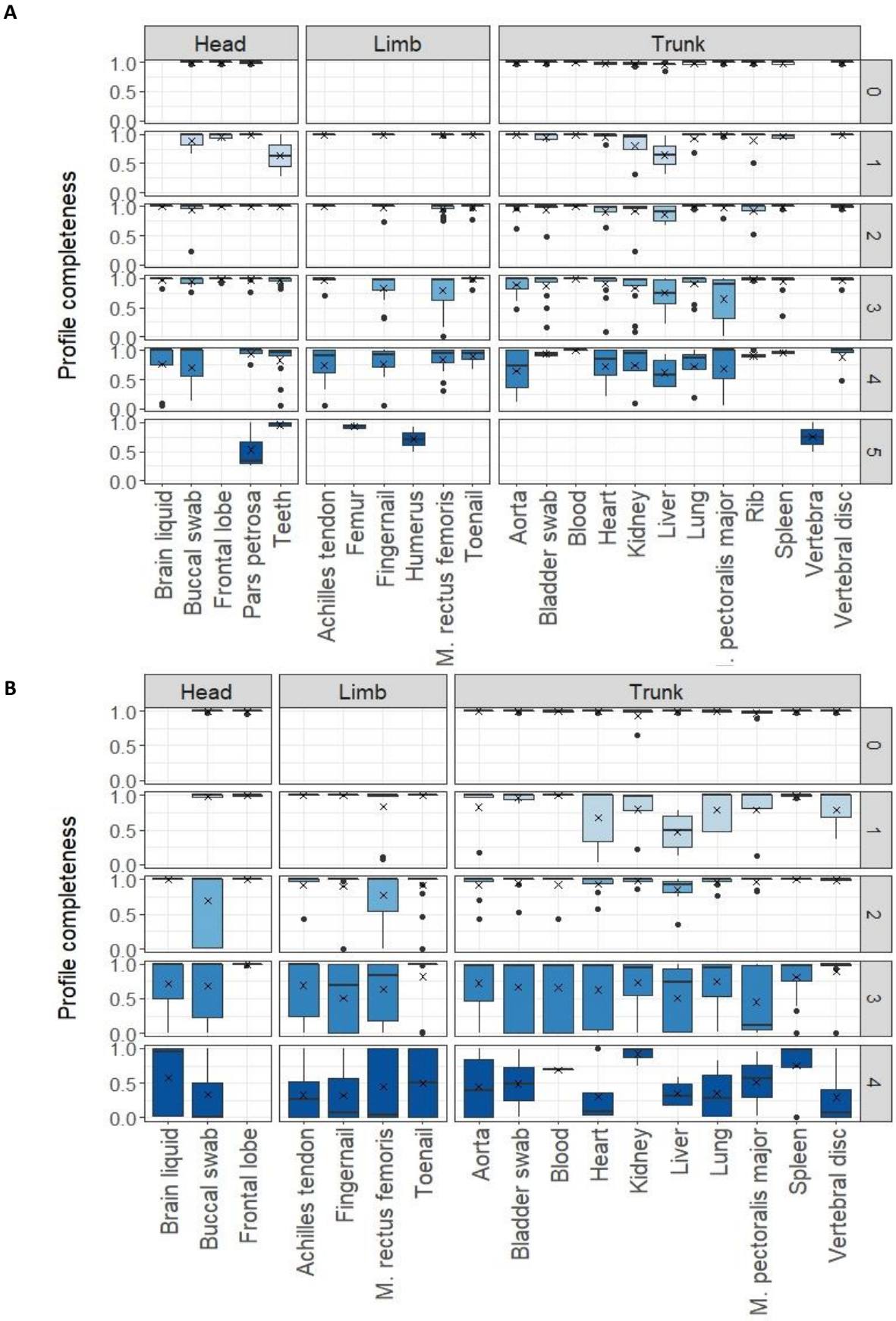

Fig. 3: Profile completeness (%) of 22 STRs separated according to DNA extracted with the Maxwell® FSC DNA IQ™ Casework Kit (A) and SwabSolution™ Kit (B) from tissue samples of decomposed corpses. Samples are separated according to the corpse's degrees of decomposition from 0 (unaltered) to 5 (skeletonized) and the three anatomical regions. Missing data represent not available sample material.

For predicting the genotyping success with respect to all 22 loci, significant differences between *degree of decomposition* ($p<0.001$), *tissue types* ($p<0.001$) and *extraction method* ($p<0.001$, all p-values logistical regression) were detected. The prediction of typing success for each tissue type and degree of decomposition is summarized in table 3 and exposed blood samples (MWK) with the highest probability of complete CE STR profiles for each degree. As comparison, the probability of typing success for the 16 eESS loci is opposed (Tab.3). DNA extracted with the IPK/BTA method showed genotyping success rates of 100% for samples from the brain, blood, rib, vertebral disc and femur (D_3 and D_4). The highest number of allelic dropouts was observed in a sample from the Achilles tendon D_4.

The genotyping success of samples from bodies found in water was similar to that of decomposed remains with > 95% of the 24Plex loci for each MWK extracted D_1 tissue, except for kidney samples (mean = 56%). For liver, lung and spleen D_3 samples, mean completeness decreased to < 78 %. As for decomposed bodies, number of allelic dropouts increased with the SSK method and mean profile completeness of liver and fingernail samples were 85 % and 75 %, respectively. DNA from burnt human remains only showed a decrease for D_3 samples from *M. rectus femoris* and *M. pectoralis major* (56 % and 50 %, MWK) and D_3 liver samples (95 %, SSK).

Table 3: Probability of obtaining complete CE STR profiles of DNA samples from decomposed corpses (D_0-D_5), extracted with the Maxwell® FSC DNA IQ™ Casework and the SwabSolution™ kits. Separated are probabilities for all 24Plex kit included STR loci and the 16 eESS loci with respect to each tissue and anatomical region

Extraction method	Anatomical region	Tissue	Probability of success for 24 loci						Probability of success for eESS 16 loci					
			D_0	D_1	D_2	D_3	D_4	D_5	D_0	D_1	D_2	D_3	D_4	D_5
MWK	Head	*Pars petrosa*	0.75	1.00	1.00	0.77	0.64	0.34	0.75	1.00	1.00	0.77	0.64	0.34
		Buccal swab	0.80	0.67	0.62	0.64	0.67	NA	0.80	0.67	0.62	0.64	0.67	NA
		Frontal lobe	0.80	0.67	0.67	0.80	NA	NA	0.80	0.67	1.00	0.80	NA	NA
		Brain liquid	NA	NA	1.00	0.86	0.62	NA	NA	NA	1.00	0.86	0.57	NA
		Teeth	NA	1.00	1.00	0.61	0.46	0.50	NA	0.50	1.00	0.61	0.46	0.50
	Trunk	Aorta	0.80	1.00	0.82	0.53	0.50	NA	0.80	1.00	0.82	0.53	0.50	NA
		Bladder swab	0.80	0.67	0.70	0.62	0.50	NA	0.80	0.67	0.80	0.62	0.50	NA
		Blood	1.00	1.00	1.00	1.00	1.00	NA	1.00	1.00	1.00	1.00	1.00	NA
		Heart	0.40	0.60	0.50	0.69	0.50	NA	0.40	0.60	0.50	0.75	0.50	NA
		Kidney	0.40	0.50	0.50	0.50	0.40	NA	0.40	0.50	0.60	0.57	0.40	NA
		Liver	0.20	0.00	0.34	0.24	0.00	NA	0.20	0.00	0.34	0.29	0.00	NA
		Lung	0.60	0.80	0.80	0.53	0.25	NA	0.60	0.80	0.90	0.60	0.25	NA
		Spleen	0.60	0.50	0.75	0.69	0.25	NA	0.60	0.50	0.78	0.69	0.25	NA
		M. pectoralis major	0.80	0.80	0.91	0.24	0.67	NA	0.80	0.80	0.91	0.24	0.67	NA
		Vertebral disc	1.00	1.00	0.67	0.50	0.60	NA	0.80	1.00	0.67	0.70	0.60	NA
		Rib	0.80	0.80	0.70	0.73	0.25	NA	0.80	0.80	0.70	0.80	0.75	NA
	Limb	Achilles tendon	NA	1.00	1.00	0.70	0.43	NA	NA	1.00	1.00	0.80	0.43	NA
		Fingernail	NA	1.00	0.90	0.46	0.34	NA	NA	1.00	0.90	0.54	0.29	NA
		M. rectus femoris	NA	0.91	0.65	0.39	0.46	NA	NA	0.91	0.65	0.39	0.46	NA
		Toenail	NA	1.00	0.90	0.91	0.50	NA	NA	1.00	0.90	0.91	0.50	NA
SSK	Head	Buccal swab	0.80	0.67	0.62	0.64	0.34	NA	0.80	0.67	0.62	0.64	0.33	NA
		Frontal lobe	0.80	0.67	1.00	0.80	NA	NA	0.80	1.00	1.00	0.80	NA	NA
		Brain liquid	NA	NA	1.00	0.71	0.43	NA	NA	1.00	0.70	0.71	0.50	NA
	Trunk	Aorta	1.00	0.60	0.73	0.40	0.25	NA	1.00	0.80	0.73	0.40	0.25	NA
		Bladder swab	0.80	0.67	0.80	0.31	0.00	NA	0.80	0.67	0.80	0.38	0.00	NA
		Blood	0.60	1.00	0.88	0.44	0.50	NA	0.60	1.00	0.88	0.44	0.50	NA
		Heart	0.80	0.60	0.60	0.44	0.25	NA	0.80	0.60	0.70	0.38	0.25	NA
		Kidney	0.60	0.50	0.60	0.29	0.40	NA	0.60	0.50	0.60	0.29	0.40	NA
		Liver	0.80	0.00	0.11	0.06	0.00	NA	0.80	0.00	0.11	0.06	0.00	NA
		Lung	0.60	0.60	0.70	0.34	0.00	NA	0.60	0.60	0.80	0.33	0.00	NA
		Spleen	0.20	0.75	1.00	0.38	0.50	NA	0.80	0.75	1.00	0.38	0.50	NA
		M. pectoralis major	0.40	0.60	0.82	0.18	0.00	NA	0.60	0.60	0.82	0.17	0.00	NA
		Vertebral disc	0.80	0.67	0.67	0.60	0.20	NA	0.80	0.67	0.67	0.70	0.80	NA
	Limb	Achilles tendon	NA	1.00	0.62	0.60	0.14	NA	NA	1.00	0.75	0.60	0.14	NA
		Fingernail	NA	1.00	0.80	0.38	0.29	NA	NA	1.00	0.80	0.38	0.29	NA
		M. rectus femoris	NA	0.73	0.65	0.26	0.38	NA	NA	0.82	0.65	0.32	0.38	NA
		Toenail	NA	1.00	0.81	0.77	0.50	NA	NA	1.00	0.89	0.77	0.50	NA

NGS with the MiSeq FGx system

Read count

DNA of 155 tissue samples from decomposed corpses extracted with the MWK method was sequenced with the MiSeq FGx system and revealed decreasing read count for most tissue types with advanced signs of decomposition (Fig.4). For D_1, mean number of reads below the recommended threshold of 85,000 [153] were only obtained from liver samples (29,207). Greater alteration of the body (D_3 and D_4) showed increased influence on soft tissues from the trunk and mean read counts below 85,000 for, among others, samples from the liver D_3 and aorta D_4 (38,086, and 2,779, respectively). Read counts were the lowest for dry bone samples D_5 from the *humerus* and *pars petrosa* (13,037, and 4,866, respectively).

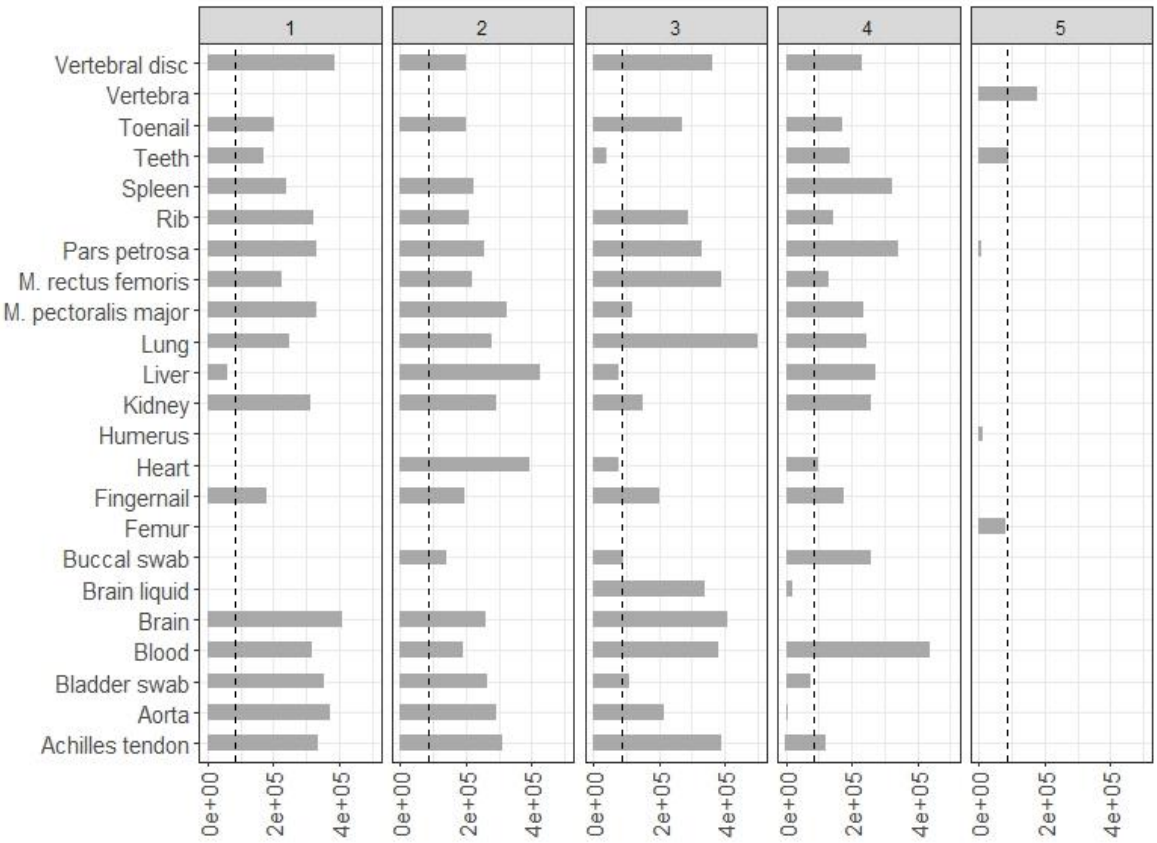

Fig. 4: Read counts of samples extracted with the Maxwell® FSC DNA IQ™ Casework Kit of decomposed corpses. Samples are separated according to the corpse's degrees of decomposition and range from 0 (unaltered) to 5 (skeletonized). Dotted line represents manufacturers' threshold of 85,000. Missing data represent not available sample material.

Concordance of profile completeness between CE and NGS

Statistical analysis revealed significant differences ($p<0.001$, paired Wilcoxon test) between profile completeness of overlapping loci from CE and NGS. On average, more samples (MWK) showed lower numbers of allelic dropouts with NGS (Fig.5). However, comparison of each decomposition degree showed significant differences only for D_4 ($p<0.001$, paired Wilcoxon test). Samples from the heart and liquid brain samples had the greatest deviations with median profile completeness of 93 %, 70% respectively for NGS and 73 %, 48% for CE. Blood and brain samples each exposed genotyping success rates of 100% for both methods.

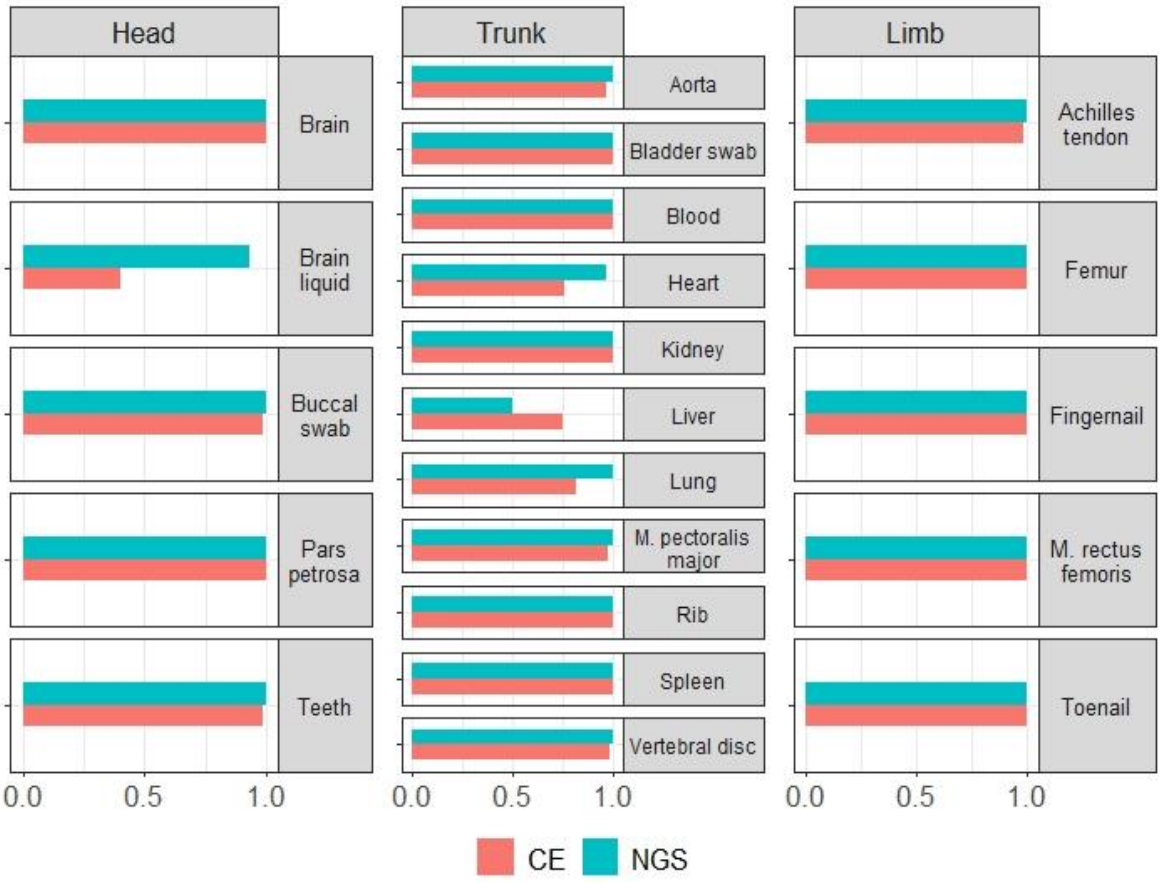

Fig. 5: Profile completeness (%) of tissue samples of decomposed corpses extracted with the Maxwell® FSC DNA IQ™ Casework Kit. The results for the different degrees were summed up and the median is presented. Compared are STR profile completeness of the overlapping loci obtained with CE and NGS.

Phenotype and ancestry prediction with the MiSeq FGx and the Ion S5™ systems

Performance comparison of the MiSeq FGx and the Ion S5™ systems revealed no significant differences between completeness of each sequenced SNP panel (piSNPs: p=0.141, aiSNPs: p=0.753, all p-values paired Wilcoxon test, Fig.6). For piSNPs, a total of 14 samples from Achilles tendons, blood, a lung, *M. rectus femoris*, ribs, a vertebral disc and toenails revealed genotyping success rates of 100% for both technologies. Only one Achilles tendon sample D_4 showed distinct lower profile completeness with MiSeq FGx (18% with MiSeq FGx, 43% with Ion S5™). For the tendon, similar results were observed when comparing profile completeness of aiSNP panels (30% with MiSeq FGx, 35% with Ion S5™). Predictions of the corpses' phenotypes revealed similar tendencies for both platforms and, with the exception of one sample, concordant genotypes. For one aorta sample, genotypes differed in rs1042602, rs4959270, rs1393350, rs28777 and rs12913832. However, no deviations in hair or eye color were observed between tissue types, indicating the devices' reproducibility. Except for one deceased, the estimation of biogeographic ancestry was also concordant for both systems, and predicted European ancestry. The exception, a highly decomposed corpse, revealed African ancestry in three out of four sequenced samples. The fourth sequence from the aorta led to a switch in ancestry estimation due to the high number of dropouts, indicating ad mixed American ancestry with the MiSeq FGx system and European ancestry with the Ion S5™ system.

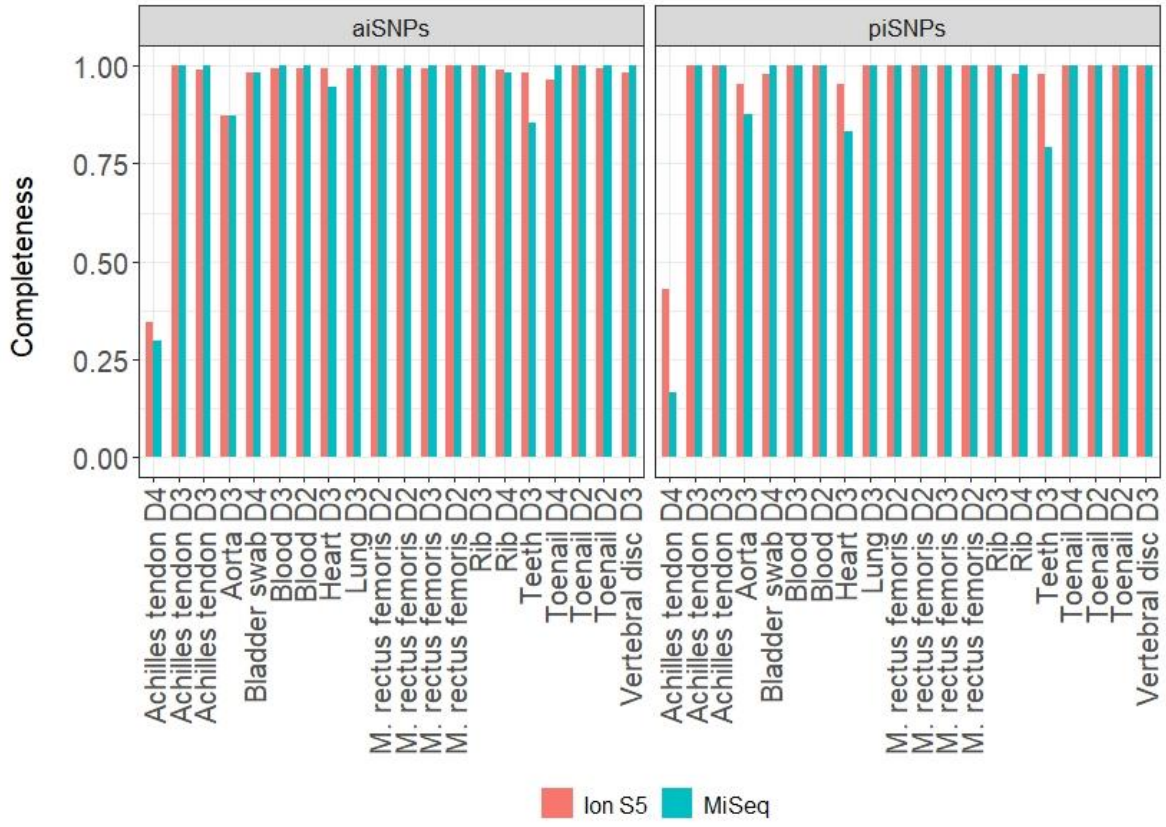

Fig. 6: Profile completeness (%) of tissue samples (D_2 to D_4) of decomposed corpses, extracted with the Maxwell® FSC DNA IQ™ Casework Kit. Compared are aiSNP and piSNP genotyping success rates of loci obtained with the Ion S5™ and the MiSeq FGx systems. D_1 and D_5 tissues were not available for comparison.

Discussion and Recommendations

The choice of sampling material can influence STR genotyping and is essential for a successful DNA-based identification of altered human remains. Just recently, a retrospective study has shown an uncertainty on the right sample selection, which was accompanied by parallel or sequential extra analyses, elevating time and costs [126]. Also, the large number of studies confirms the need to detect the most promising ID material for genetic profiling [85, 97, 102 9, 10, 127 6, 86, 108 95, 96 99]. Whereas the prognosis for unaltered human corpses seems not to be so demanding, material from highly degraded bodies is much more challenging or even unpredictable concerning successful STR profiling [126]. To minimize this gap, an unprecedented variety of soft and hard tissues, including the most common ones for ID processes, were systematically investigated within this multicenter study. Known impact factors, such as the DNA extraction methods, were covered analyzing the influences of non-purifying and purifying systems on the ID success. As a forthcoming technology in forensic ID processes, NGS was included in the study, showing its valuable advantages but also limitations when compared to standard CE analysis [130]. The comparison of those currently used technologies confirms the similar power in phenotype and ancestry prognosis of human deceased as it was also proven for mock samples in fairly new NGS proficiency tests [154].

The following recommendations were established for improving the first-attempt identification success of altered remains on the basis of our systematic approach and its results. Prior to sample collection, visual classification of the corpses' condition is essential. Appearances like skin discolorations, blisters or partial skeletonization can be easily identified, yet, due to highly variable environmental influences and processes of alteration within a corpse, categorizing the whole body in one degree of decomposition or burning is not precise enough in most cases. Also, a TBS [131] was not calculated due to too high deviations observed within one score. The TBS is relevant for quantifying the body's total progress of decomposition and estimating the PMI, however, it is not suitable for categorizing the degree of decomposition of single tissue types. Therefore, the bodies' analyzed tissues were separated in three anatomical regions and were each assigned an independent degree. This allows a faster, simple and more precise description of possible and often diverse degradation degrees within one body.

According to the DNA quantification results, no significant differences were observed between the MWK and SSK extraction method for decomposed remains, with a tendency to higher DNA yields for SSK extractions, probably due to the capacity restriction of the magnetic beads with the MWK method [111]. Although the SSK method was developed specifically for DNA extractions from buccal swabs, it results in sufficient DNA yields even with challenging samples from altered remains. Accordingly, the extraction represents a cost-efficient and fast alternative in case of time shortage or financial constraints [89]. While liver and spleen samples displayed high DNA yields for both extraction methods, samples from the vertebral disc, aorta and blood revealed similar confidence intervals and higher consistency between degrees of decomposition, indicating little influence of deviations from the subjective classification of the corpse's alteration.

Besides DNA quantity, the decision of the best-suited tissue type for STR genotyping depends mainly on the quality of the extracted DNA. Allelic dropouts, imbalanced alleles and a "ski slope effect" [43]caused by DNA degradation complicate the differentiation between homo- and heterozygotes and impede the interpretation of genotyping results. Calculation of DI and the evaluation of quality sensor ratios was used to evaluate DNA integrity and revealed, concordant to the findings of Uerlings et al. [89], highest degradation values for soft tissues extracted with the SSK method. Compared to the MWK method, DI were significantly lower in DNA extracted with the SSK method, thus the DNA was more subject to degradation. Since the sampling material was identical, lower DI observed in SSK extracted samples are likely the result of the non-purified extraction method with inhibitors still being present. Differences between DI of tissue types were even observed for unaltered and presumably unproblematic remains (D_0), highlighting the importance of choosing the best-suited tissue for ID purposes. As expected, the ratio of the artificial sensors QS1 and QS2 showed a higher tendency of inhibitors in DNA extracted with the SSK method, which can be explained by the absence of extract purification. However, the wide ranges of quality sensor ratios and imbalances between peak heights impede the interpretation whether inhibitors are present or not. Since no significant differences were observed between tissue types, no proposition could be made as to which tissue is more susceptible to PCR inhibition.

Due to the importance of typing as many loci as possible for identification purposes of unknown human remains, profile completeness and prognoses on STR genotyping success was presented for all 24Plex kit included loci. The detected differences between tissue types and degree of decomposition emphasize the collection of sampling material according to the corpses' condition with respect to the three anatomical regions. For MWK extracted samples, D_1 samples of the trunk revealed lower profile completeness compared to D_2, which could be explained by differing sample sizes or the subjective categorization of the corpses. Interestingly, although DNA extracts were not purified with the SSK method, sufficient profiles were obtained from tissue types up to D_2 for identification purposes, indicating the robustness of the method.

The transnational 16 *extended loci* of the *European Standard Set* are important to report profiles for an effective search in nations' databases. Since no distinct difference in probabilities of genotyping success was observed compared to 22 loci, the following recommendations focus on the completeness of the 16 *eESS* loci, extracted with MWK method, since the majority of forensic laboratories purify expected challenging samples. Due to the smaller number of bodies found in water and burnt corpses our recommendations address only decomposed corpses. Since the sample size of bone samples D_5 was not sufficient, no guidance can be given for dry bones. However, for these materials, the valuable DVI recommendations [97] should be consulted.

Recommendation #1: *Collect a buccal swab (D_0, D_4), a sample from the frontal lobe (D_0, D_2, D_3), a sample from the pars petrosa (D_1, D_2) or teeth (D_2) from the head of decomposed corpses.*

Comparison of genotyping success of tissues collected from decomposed heads revealed brain samples with the highest probabilities of complete STR profiles for various degrees of putrefaction. Those findings were also observed in the study of Uerlings et al., in which 16 out of 20 DNA samples from brain tissue extracted with the DNeasy Kit revealed complete STR profiles [89]. This could be explained by the location in the skull and the enclosure of the *dura mater*, which provides a longer protection against bacterial and insect infestation compared to soft tissues of the trunk [94]. Furthermore, according to Huang et al., the chromatin structure is properly preserved for at least 30 hours after death [90]. Study results also revealed bone powder from the *pars petrosa* as reliable sample material from decomposed corpses. Since the petrous bone is one of the most compact and dense bone in the human body, DNA preservation is greater compared to cancellous bones [125] and high DNA yields as well as low degradation can be expected. Although teeth samples yielded low DNA quantities, the probability of complete STR profiles was the highest in teeth for D_2 samples, which could also be explained by the teeth's density and the enamel providing environmental protection [120]. Unexpectedly, despite high degradation in DNA extracted from buccal swabs D_4, STR genotyping revealed the highest probability (67%) for generating complete profiles. Since the buccal collection is fast and easy and there is no damage to the body, the sampling represents a potential source for further molecular analyses.

Recommendation #2: *Collect a blood sample (D_0-D_4), a sample from the aorta or vertebral disc (D_1) from the trunk of decomposed corpses.*

For sampling material from the trunk, blood samples are the optimal sources for STR genotyping and are recommended for unaltered and decomposed corpses ranging from D_1 to D_4. Despite advanced decomposition, small amounts of blood were still available in the heart. Even though DNA yields were comparably low, high DNA degradation indices and genotyping success rates were observed, indicating high DNA preservation and stability. Those findings are also supported by the study of Bär et al. [73]. Additionally, best-suited tissue samples from decomposed corpses D_1 are samples from the aorta and vertebral disc. Concordant to the results of Sato et al. [93], aorta tissue represents a promising source for STR genotyping. This could be explained by the resilient and elastic structure of the aortic wall, making it more resistant to decomposition processes [94]. Furthermore, as also shown in the study of Becker et al. [99], samples from the vertebral disc revealed high quality DNA profiles, which could be explained by the cells being embedded in an extracellular matrix, making them less susceptible to decomposition processes. Compared to other soft tissues from the trunk, liver samples strongly underperformed for each degree of decomposition and are therefore not recommended for DNA based identification. In line with the findings of Uerlings et al. [89], Schwark et al. [10] and Helm et al. [94], liver samples revealed high DNA degradation and low profile completeness, which could be explained by the great number of lysosomes facilitating post-mortem destruction of the cell membrane [89].

Recommendation #3: *Collect a sample from the Achilles tendon (D_1, D_2), fingernail (D_1), or toenail (D_1, D_3, D_4) for limbs from decomposed corpses.*

Comparison of tissue types from limbs revealed the Achilles tendon as optimal sampling material. Supported by the study results of Roeper et al. [85], high STR genotyping success is observed, indicating profound DNA stability and protection against autolysis and putrefaction. Furthermore, nails are recommended as best-suited sampling material from corpses classified D_1, D_3 and D_4. Finger- and toenails are robust, available from bodies with wide ranges of alteration and can be easily removed when limbs are not mummified or dried [85]. Since no opening of the body is necessary, nail samples can be collected even if no autopsy is ordered or a manipulation of the body should be avoided due to religious reasons.

Recommendation #4: *Combination of recommended tissue types*

As the provided classification is divided into three anatomical regions of the corpse, up to three different degrees of decomposition or burning are possible. For example, a decomposed corpse categorized D_1 (head), D_3 (trunk) and D_4 (limbs) would lead to a sampling recommendation of *pars petrosa,* blood and toenail samples. In order to collect the best-suited material, the individual case and the availability of sampling material (in case of missing body parts) has to be considered. Also, the integrity of the body has to be considered, especially if no autopsy is ordered or not feasible due to religious reasons, the body cannot (or should not) be opened and only minimal invasive alteration of the body can be executed. Considering the probability of the genotyping success, table 3 provides guidance on the most promising material for human ID at the first attempt. For example, *pars petrosa* from the D_1 head, blood from a D_3 trunk and toenails from D_4 limbs regions, will lead to 100% and 50% profiling success. To keep it simple, the most promising region and method are trunk and blood, regardless of the decomposition degree.

Recommendation #5: *Consider NGS for identification*

The growing demands in human identification requires constant improvements in analyses methods, as shown for the late NGS application. Here, the study results display the sequencing technology as a reliable and promising method for improving the ID success of altered human remain, exceeding CE STR genotyping as demonstrated in a recent study [120, 129, 130]. Assessment of tissue-specific differences revealed higher SNP profile completeness with NGS for samples from the heart, vertebral disc and Achilles tendon, which could be explained by the advantage of smaller amplicon size. Lowest read counts for dry bone samples D_5 could be explained by lower DNA yields and small sample sizes. Observed genotyping success rates of 100% in blood and brain samples for both CE- and NGS-based genotyping could also be explained by the location of the brain in the skull and the higher preservation and stability. For countries who currently undergo law revisions (or might do in the future) with respect to phenotype and biogeographic ancestry prognoses, the evaluation of reliable analysis methods are increasingly important. Our research results show a similar performance power in profile completeness and

correctness of SNP panel, with both the MiSeq FGx and the Ion S5^{TM} systems. However, caution is advised when interpreting sequencing data of degraded DNA samples. As observed for one sample of the aorta, high numbers of allelic dropouts can lead to a switch in estimation of biogeographic ancestry for both sequencing technologies.

Project III: Supplementary material

Table S1: Mean RFU peak heights samples extracted with the Maxwell® FSC DNA IQ™ Casework and the SwabSolution™ Kits from putrefied corpses classified in degrees of decomposition ranging from 0 (unaltered) to 5 (skeletonized). Standard deviation (SD) and confidence interval (CI) are listed for each degree and extraction method.

Extraction method	Degree	n	Mean (RFU)	SD (RFU)	Median (RFU)	95% CI (RFU)
MWK	D_0	69	2660	1927	2390	2198, 3124
	D_1	90	3708	2650	3132	3153, 4263
	D_2	195	2829	2450	2279	2483, 3175
	D_3	285	3162	3119	2103	2799, 3526
	D_4	112	2006	1661	1590	1695, 2317
	D_5	13	1204	1613	576	229, 2179
SSK	D_0	60	6059	3922	5895	5046, 7072
	D_1	80	4535	3769	3516	3696, 5374
	D_2	171	3928	3619	2959	3382, 4474
	D_3	206	3474	4445	2231	2863, 4084
	D_4	58	2585	3673	1374	1619, 3551

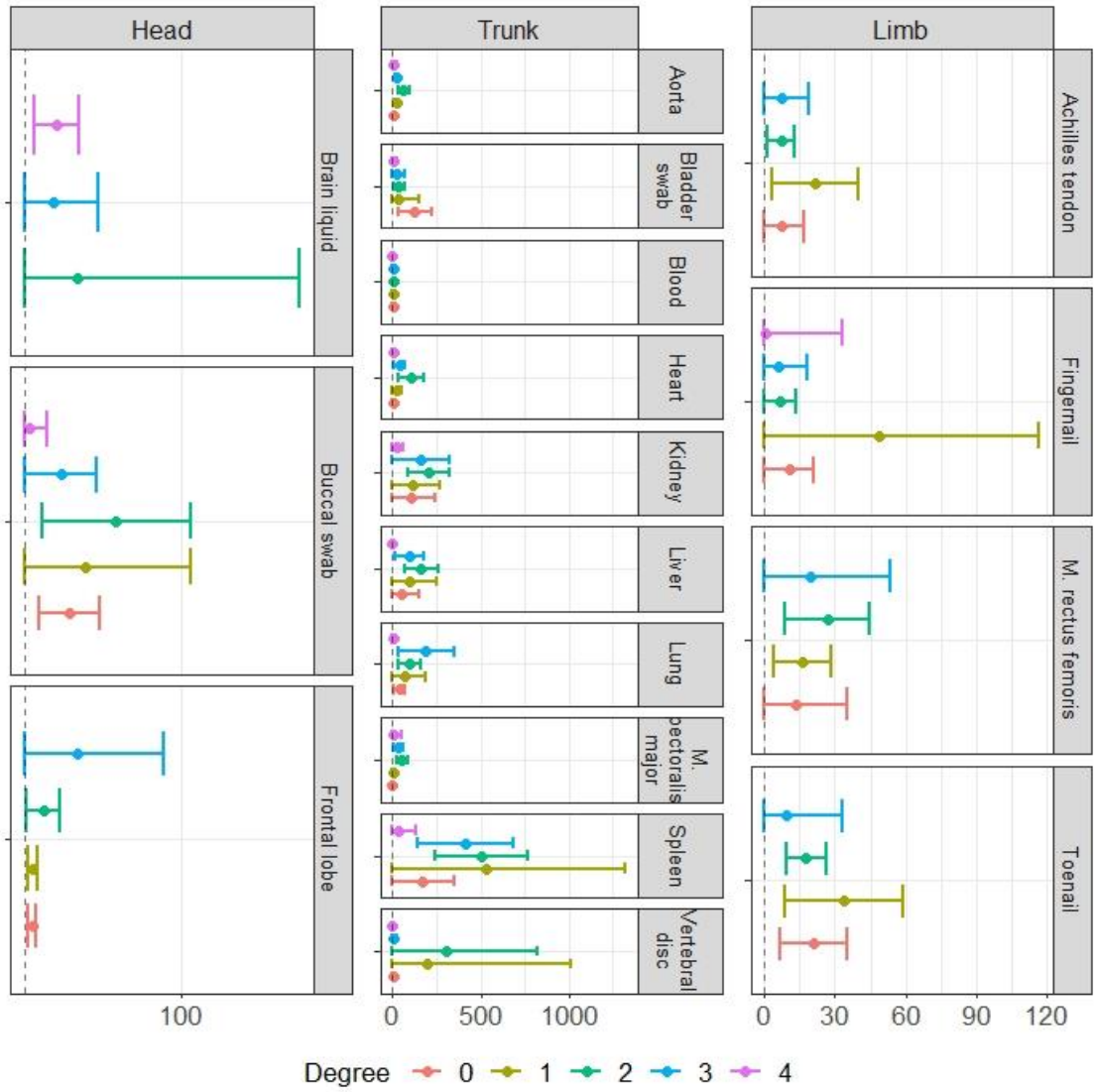

Fig. S1: Quantification results (ng/µl) of DNA extracted with the SwabSolution™ Kit from tissue samples of decomposed corpses. Presented is the confidence interval of the mean. Samples are separated according to the anatomical regions and the corpse's degrees of decomposition ranging from 0 (unaltered) to 4 (profoundly). Since the kit is not suitable for bone samples, D_5 results are not available. The other missing data represent not available sample material.

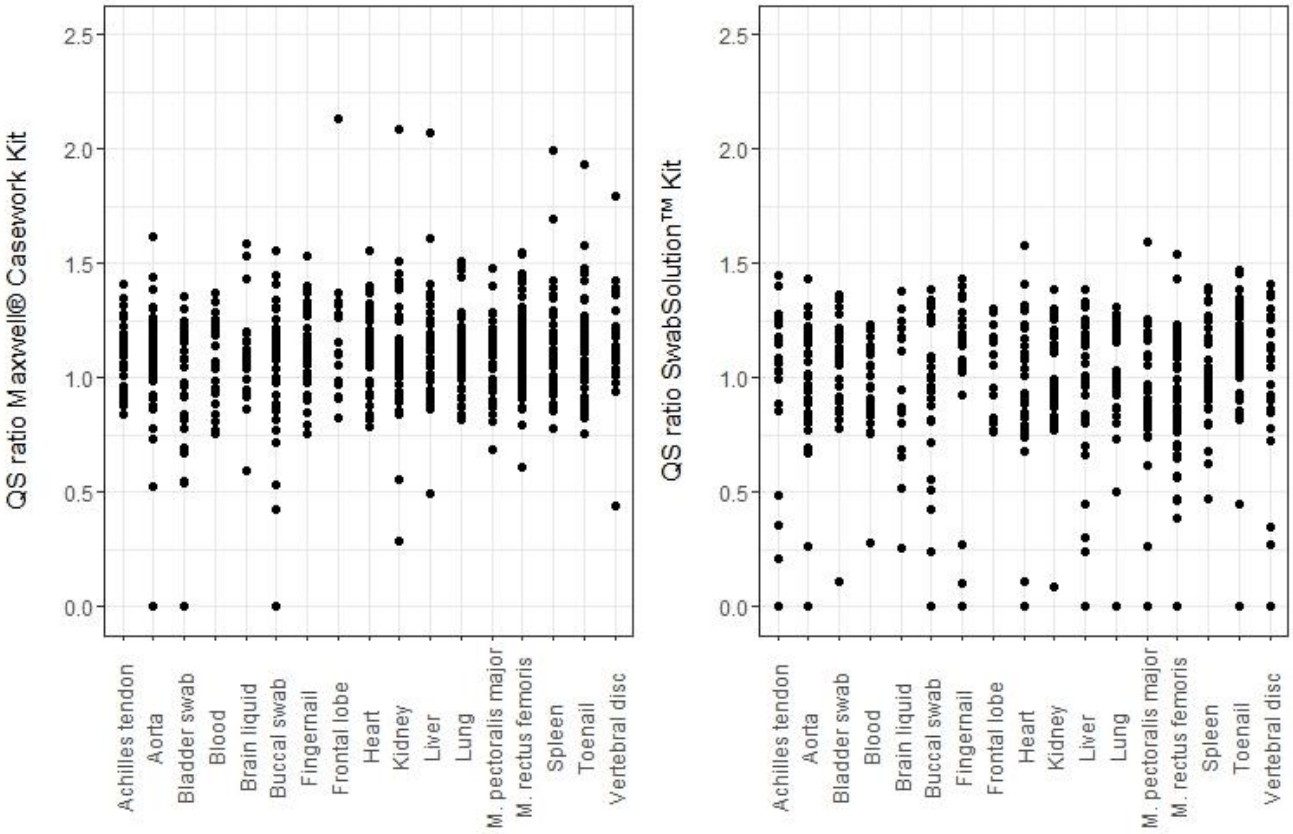

Fig. S2: Quality sensor (QS) ratio of DNA extracted with the Maxwell® Casework Kit and SwabSolution™ Kit from tissue samples of decomposed corpses. The results for the different degrees were summed up.

4. Discussion

4.1 Forensic implications and relevance in routine casework

The herein presented recommendations for improving the DNA-based ID success of altered human remains are of significance for forensic casework and should be considered when selecting and analyzing challenging samples. As the outcomes of *project I* confirm, genotyping success rates are influenced by post-mortem alteration and tissue type. Although previous IDs were successful, significantly more cost- and time-intensive DNA analyses were observed for altered corpses. Additionally, the comparison of published studies showed a distinct variety in outcomes concerning DNA stability in post-mortem tissues and optimal sampling material, indicating a great uncertainty in sampling strategies. Since the actual routine casework is reflected, this observation emphasizes the relevance of the systematic approach conducted in this study and the necessity of novel guidelines in forensic casework. The thesis outcomes are directly applicable at the intersection of forensic medicine and forensic genetics, improving the communication between both forensic fields. Although the interaction is crucial for ensuring a rapid DNA-based ID success of corpses, it can be impeded by the in *project I* demonstrated and personally observed insecurities. Therefore, the specific recommendations on optimal sampling material according to the condition of the corpse presented in *project III* will overcome those issues with both traditional and novel technologies as shown in *project II*.

Forensic medicine

As the first step in DNA-based ID of corpses, tissue samples have to be collected within forensic medicine casework. However, since the categorization of the corpse's condition is crucial for genotyping success, the results of *project III* provide a novel scoring system that can be implemented prior to sample collection during medico-legal autopsies or ID processes. Usually, the alteration of decomposed or burnt corpses is assessed but not scored. Hence, the presented classification is based on visual post-mortem characteristics including skin discoloration or blisters, which can be easily identified and described by the examiner. By scoring separate anatomical regions of the body, three tissue types are recommended as optimal sampling material according to the decomposition degree. As the resulting probabilities of *project III* revealed, blood is the optimal sample for a wide range of decomposition degrees (D_0-D_4) and could even be collected from the heart of corpses D_4. However, since several factors such as potentially missing body parts can limit the selection, multiple tissues are presented as preferable sources for molecular analyses. Hence, in cases no autopsy is ordered, non- or minimal-invasive sample alternatives are provided. Especially the recommended sampling of nails from decomposed corpses D_1, D_3 and D_4 represent a suitable tissue if the body is not opened. In most cases, skin slippage (particularly the processes of washerwoman's skin) facilitates detachment and enables a non-invasive, rapid and easy removal. In general, since manipulation of the body has to be avoided, sampling should be considered for each case individually. Therefore, table 3 of *project III* in chapter 3.3 represents a first-time overview of

a wide range of tissue types with descending probabilities of generating complete STR profiles, which can be used as further guidance.

Forensic genetics

For further analyses in forensic genetic laboratories, the standardized and systematic approach of comparing a broad variety of tissue types, including the impact of DNA extraction methods, can reduce time and cost in STR genotyping of challenging samples. As shown in *project I*, sampling of less optimal tissue types can lead to PCR failure and associated high numbers of successive or parallel PCR amplifications. Especially in bone and muscle tissue, the unexpectedly high numbers of additional DNA analyses highlight the impact of insecurities in forensic casework. In over 80 % of cases concerning the ID of decomposed corpses, more than the two required amplifications were conducted. Therefore, by predicting DNA integrity and the probability of typing success prior to sample analyses, parallel and repeated approaches can be avoided.

As the results of *project III* demonstrate, the success of DNA profiling can be predicted based on scoring characteristic post-mortem alterations of the corpse. Since the necessary number of analyzed loci depends on the case assignments, probabilities of obtaining complete DNA profiles were opposed for all 22 loci included in the 24Plex kit and the 16 loci of the eESS. For the ID of corpses, successfully typing of as many loci as possible is important and beneficial for comparison of, i.e. ante- and post-mortem samples. Thereby, the generated DNA profile, independent of typing specific loci, can be compared with DNA profiles of possible relatives or personal items [43]. Yet, in a majority of European countries, typing of the 16 eESS loci recommended by the ENFSI is required for reporting profiles to a database. This standardization and selection of transnational loci enhances the communication across forensic institutes and facilitates possible cooperation [136]. However, no distinct difference in probabilities of genotyping success was observed compared to 22 loci. Neither the degree of decomposition nor the extraction method revealed distinct deviations in optimal tissue types.

Beside prognoses of successful genotyping in each tissue, the comparison of purifying- and non-purifying DNA extraction methods provides valuable information for routine casework. Usually, purifying methods such as the Maxwell® FSC DNA IQ™ Casework Kit (Promega) or iPrep™ Forensic Kit (ThermoFisher Scientific) are used for the analyses of challenging samples. However, the methods are time- and cost-intensive. Contrary to previous expectations, it was possible to generate sufficient profiles of corpses up to D_2 with the non-purifying SwabSolution™ Kit (Promega). Although the method was initially intended for buccal swabs with high quality DNA [112], the results of *project III* demonstrated no significant differences compared to DNA yields of the purifying Maxwell method. Therefore, the method represents a suitable alternative in case of time shortage and financial restrictions.

Furthermore, beside standard CE-based genotyping, *project II* and *project III* evaluated novel NGS technologies for potential better performances of degraded and inhibited samples. The presented results provide valuable recommendations, which can be implemented into forensic casework to improve sequencing success. Since concordant loci are used and the outcomes of *project II* demonstrate higher profile completeness for degraded samples compared to CE, NGS

can already be used for enhanced DNA profiling of today's casework samples. However, although NGS is a novel technology for forensic purposes and is not yet applied in a majority of forensic laboratories, the extensive and all-encompassing validation study described in *project II* demonstrates valuable guidance for further implementations and handling of samples from altered corpses. Therefore, the provided data is a valuable basis in case of a potential future superstition of CE methods by NGS technologies.

4.2 Limitations of the project

The progress of the study was defined by three successive projects comprising standard and forthcoming technologies, which lead to novel and valuable recommendations for first-attempt identification of altered corpses. With 949 collected tissue samples resulting in 1698 DNA extracts, an incomparable high number of tissue types was analyzed and compared. Although representing an exceptional broad approach on predicting STR genotyping success including cooperation with associated institutes, the study had to face some limitations and challenges. In the following sections, drawbacks in sample size and challenges in classifying the corpse's alterations as well as laboratory-specific deviations are discussed.

4.2.1 Sample size

When analyzing tissue samples from deceased, a major limitation is the restriction of sample sizes. Especially the collection of material from altered human remains is highly dependent on available and suitable cases. Since forensic research relies on the analyses of practice-oriented and realistic references, no artificial or modified samples could have been considered for the projects within this thesis. Therefore, the sample size demonstrated in *project III* represents the casework material that was available during this study period. Due to the expected low numbers of altered remains, a cooperation with the Institute of Forensic Medicine Bern was established to increase the number of samples. Nevertheless, despite the inclusion of material collected at a further institute, the distribution of body types and alteration degrees is uneven. As the results of *project I* revealed, the most common type is decomposed corpses D_3. Since cases involving the examination of bodies found in water and burnt corpses were less frequent, not each of the classified degrees was available for the study and no recommendation on optimal sample material could be provided. In particular, dry bone samples of completely skeletonized human remains were rare. Yet, a larger number would have been preferred since bone samples represent several challenges in molecular genetic analyses and further information on intra- and inter-specific variations in DNA stability would have been advantageous. Although the study would have benefited from a more evenly distributed sample size, it reflects the current situation in forensic casework. Since decomposed corpses are the most frequent body types, the provided recommendations are feasible and valuable for future routine work.

4.2.2 Challenges in categorizations: Why not classify each tissue?

Although decomposition is a continuous process, the progress and extent is subject to numerous intrinsic and extrinsic factors, leading to highly variable alterations that impede a standardized categorization of stages [27]. This might be the reason why a majority of previously published studies about tissue-specific differences in DNA stability did not classify the corpses' condition or only used an approximate description of the overall alteration process. Since the sample size was relatively small in most studies, each corpses' condition was described individually, and the results were not subdivided and characterized into degrees but generalized for each analyzed tissue. Although the progress of decomposition displays a great influence on optimal sampling strategies, the categorization into degrees, as conducted here in *project I*, was rarely done. Therefore, a classification scheme was developed and presented based on previous observations and methods [20, 31, 131-133]. However, results during the course of this thesis revealed that this scaling of the whole body is not sufficient. Since the extent of decomposition can differ throughout the body, a more specific classification of three separate anatomical regions was chosen during *project III*. Usually, the assigning of decomposition stages is used for estimating the post-mortem interval (PMI) in forensic anthropology. Megyesi et al.[131] presented a point-based system based on scoring post-mortem characteristics of the head, trunk, and limbs, which are summed for a total body score (TBS). During *project III*, a modification of such a scoring system, without a TBS, was developed separately for decomposed and burnt corpses and bodies found in water. Due to too high deviations observed within one score, the calculation of a summed up TBS was rejected. The TBS can be used to quantify the progress of decomposition of the whole body, but it is not suitable for single tissue types. Therefore, the further presentation of recommendations for three anatomical regions and each degree provides a significantly more precise and accurate strategy for predicting the optimal sampling material.
However, the question might arise why the alteration of the corpse is classified according to visible characteristics of three anatomical regions and not of each analyzed tissue type. Since the corpses' condition is assessed but not scored during medico-legal routine, a rapid and practical scoring method is required that can be easily implemented in routine casework. Due to limited time and resources in examinations of corpses, a classification of each tissue would be too time- and labor-intensive. Therefore, a simple and rapid scoring into the three major anatomical regions was performed. As an alternative to visual classification, biomarkers analyzed for estimating the time since death might be used to assess alterations directly in the sampled tissue. In forensic toxicology, a large variety of biomarkers could be detected by using the approach of metabolomics to find potential markers for autolysis and putrefaction using e.g. nuclear magnetic resonance or mass spectrometry methods. As many biochemical changes occur after death, metabolic deterioration seems to be suitable for determining decomposition [155, 156]. However, the changes cannot be used to quantify the extent of decomposition and a single biochemical marker is not accurate enough. Furthermore, the methods have relatively low sensitivity, are labor-intensive and are not widely adopted in forensic settings [156]. Accordingly, such an extensive analysis prior to sample selection for DNA profiling is not suitable and implementable in forensic casework. Therefore, classification based on visible and easily identifiable post-mortem characteristics represents the most efficient and practical method for rapidly predicting the suitability of tissue types for further genetic analyses so far.

4.2.3 Laboratory-specific methods

In forensic genetic casework, laboratory-specific instrumentation and methods display a limitation and challenge in standardizing processes and workflows. Usually, the used technologies and standards depend on available resources, qualifications of the staff, and the preferences of the examiner. Since especially the choice of DNA extraction methods differs among forensic laboratories, *project II* and *project III* assessed the possible impact on further DNA profiling by comparing two in-house methods and evaluating a further method of the cooperating Institute of Forensic Medicine Bern. Thus, recommendations on optimal sampling material are provided for purifying and non-purifying methods. Therefore, the respective laboratory has the choice of selecting a method dependent on the case and possible time or financial restrictions.
Additionally, an increasing number of different commercially available PCR amplification kits exits in the forensic community that differ in included STR markers, robustness, sensitivity and primer sequences for the same loci [43, 49, 157]. The unique primer positions lead to different amplicon sizes and therefore variable orders of the same marker in the resulting electropherogram. Since larger markers tend to dropout first on degraded samples, a comparison of direct markers performance in different PCR kits is impeded. Furthermore, the number of multiplexed loci has an impact on amplification rates. An increased number of loci can lead to lower detection sensitivity, a competition of primer for target sequences and reagents or a lack of detection of a single target sequence [157, 158]. Therefore, for standardizing the interpretations, the performance of specific markers was not evaluated within this study. However, even though not each available PCR amplification kit can be assessed, the eESS loci and more were included in this study, which is sufficient for comparison with DNA profiles of potential relatives or a search in databases.

4.3 New trend in Forensic Genetics: Next Generation Sequencing

With ongoing developments in technologies, the emerging availabilities of novel DNA sequencing methods do not bypass the field of forensic genetics. Constantly growing demands and expectations in DNA analyses lead to the forensic scientist being faced with progressively more challenging casework including highly degraded samples, complex kinship testing or missing person ID[159]. Since currently used CE methods display several technological limitations, the trend of molecular genetic analyses is progressively shifting towards DNA sequencing methods [53, 57, 69]. Especially the application of the NGS platforms MiSeq FGx (Verogen) and Ion S5™ (ThermoFisher Scientific) is constantly increasing in forensic research [67, 160, 161]. Due to numerous advantages compared to current CE-based genotyping including the potential to predict a person's phenotype and ancestry, NGS technologies might become the future gold standard in forensic routine casework [67]. In the following section, particularly the emerging possibilities for degraded samples of altered remains will be discussed. However, the methods are still primarily used in research and are not implemented in many laboratories yet. Especially the increased research in extended DNA analyses comprising the investigation of

coding DNA regions raised concerns regarding privacy as well as ethical issues and initiated a public debate about changes in legislation that are further discussed in section 4.3.2.

4.3.1 Emerging possibilities for degraded DNA samples

Obtaining reliable genotyping results in degraded DNA samples of post-mortem tissues is challenging and demanding. Although degraded samples may have sufficient DNA quantities, the fragmentation impedes amplification of required target sites [8]. Since degradation is usually coincidental with locus or allele dropouts, the resulting partial or missing STR profiles impede interpretation or might even prevent a DNA-based ID [84, 129]. Comparably, PCR inhibitors present a minor challenge since today's commercially available PCR amplification kits are becoming increasingly resistant and robust or are designed particularly for challenging samples[81, 162]. Hence, the focus lies on improving current methods or developing new technologies for increasing genotyping success rates of degraded DNA. The emerging possibilities of NGS show promising approaches to overcome the current technological limitations. Especially the DNA-based ID of altered human remains can benefit from reduced amplicon sizes, detection of sequence variations in repeat or flanking regions of STRs and the potential to multiplex large numbers or STRs and SNPs in a single assay [53, 114, 121, 161]. The outcomes of *projects II* and *III* demonstrate the relevance, feasibility and applicability of advanced NGS technologies in forensic casework. As shown in *project II*, STR typing rates were higher in artificially degraded blood samples analyzed with the MiSeq FGx compared to CE. Furthermore, the parallel sequencing of iiSNPs reveals great potential for fragmented DNA. Beyond presented enhancements in STR typing, the non-traditional SNP markers might display a greater role in future DNA profiling [119]. As the name indicates, the target of genotyping is a single base, which allows a significant decrease in amplicon size and associated lower probabilities of dropouts. Although the discrimination power is lower compared to STRs and no comparison with databases is possible yet, additional SNP genotyping might be helpful in analyzing degraded samples that lead to low typing success of STR loci [57]. However, despite validating the robustness and reliability in *project II*, the MiSeq FGx still presents restrictions in DNA input volumes and drawbacks in sequencing highly inhibited and degraded samples. In particular, the potential formation of adapter dimers can negatively impact the sequencing quality [69, 163]. By optimizing the library preparation, the outcomes of *project II* present valuable recommendations for improving the sequencing success of samples from altered human remains. Experiments with additional PCR amplification steps, increased pooling volumes and reduction of adapter volumes for DNA input concentrations ≥ 31.2 pg revealed an increase in genotyping success as well as minimized formation of adapter dimers. Therefore, even though the technology still has room for improvement, NGS demonstrates great potential in analyzing degraded DNA samples. Current developments in enhancing the PCR1 buffer system by the manufacturer demonstrate promising approaches to overcome the presented limitations in future sequencing of challenging samples [164].

4.3.2 Prospective relevance and challenges of NGS

Emerging technologies of NGS display increasing relevance in forensic casework by providing new applications and advanced resolution. The in *project II* validated Signature Prep Kit (Verogen) enables the possibility to multiplex over 200 STR and SNP markers. Especially the potential to sequence aiSNPs and piSNPs for the prediction of phenotype and ancestry presents a substantial advantage that might assist in future corpse ID or criminal investigations. Thereby, in case the STR profile of an unidentified corpse might not lead to a match after a search in databases, the estimation of eye and hair color or ancestry could provide further direction and increase the informational yield for following investigations [117, 165]. By evaluating sensitivity, reproducibility and robustness, among others, in *project II*, the outcomes demonstrate the applicability even in challenging samples. Furthermore, performance testing of the two most commonly used NGS devices in forensic research settings was enabled by cooperating with the Institute of Forensic Medicine St. Gallen. A comparison of the MiSeq FGx (Verogen) and the Ion S5™ (ThermoFisher Scientific) in *project III* revealed, with the exception of one highly degraded sample, concordant phenotype and ancestry tendencies. Reliability and accuracy of predictions in forensic samples were also stated and validated by Frégau [117].

However, those SNPs are located in coding DNA regions, whose analyses are currently not legalized in Switzerland [166]. After many years of research, including the outstanding achievements of the Visible Attributes Through Genomics (VISAGE) consortium [167], a public debate about legalization arose. The emerging potential in forensic phenotyping, including the prediction of skin color, lead to the development of several concern regarding the invasion of privacy, conflicts with data protection and exaggerated expectations from the public [136, 165]. Furthermore, the grouping of persons according to their biogeographic ancestry might be misleading and could encourage police attention to a specific population subgroup that might lead to discrimination against minorities [168]. Therefore, particularly the communication with investigative authorities and the reporting of resulting predictions are crucial. Since extended DNA analyses are not part of routine casework yet, further training of forensic experts is necessary to ensure correct data interpretation and documentation so that the results will be transmitted in a comprehensible manner and proper use is ensured [165]. It should always be considered that phenotyping itself cannot identify a person and the sequencing results so far are only predictions that may not always have high probative value. Additionally, artificial changes and alterations of appearance cannot be considered that might cause a mislead in investigations [168]. For this reason, amendments in law are discussed and debated in several countries. In Switzerland, legalization is currently considered and the German parliament and federal council approved a change in law in 2019. In comparison, the use of extended DNA analyses for specific cases is permitted by law in the Netherlands and Slovakia since 2019 and practiced in compliance with existing laws in the United Kingdom, Poland, the Czech Republic, Sweden, Hungary, Austria, and Spain. [165].

However, the Signature Prep Kit provides two separate DNA primer sets containing 1) exclusively non-coding regions (DPMA) and 2) including additional coding regions for the prediction of phenotype and ancestry (DPMB) [53]. Thus, the respective laboratory can select the assay according to the current legal situation. NGS technologies can therefore also be used for standard STR genotyping if extended DNA analyses are not permitted. Though, NGS represents

novel and comprehensive DNA sequencing methods, which are not yet translated into routine casework. The technology still has drawbacks in terms of high costs as well as labor and time intensity of library preparations. Implementing NGS requires comprehensive validation, qualified staff, additional training and appropriate infrastructure including computer capacities and storage [65]. In particular, the extensive amount of data requires sufficient management, expertise in bioinformatics and accuracy in interpretation. This might be the reason why some institutes still hesitate and remain with current CE methods. Additionally, further research is necessary for improving the sequencing success of challenging DNA samples. However, although a potential swift from standard CE-genotyping to the use of NGS technologies takes time and effort, DNA sequencing might supersede current methods in the future. Therefore, the outcomes of *project II* and *III* present a solid basis for further research studies and provide recommendations for current DNA-based ID of altered remains as well as for a potential transition from CE to NGS technologies in the future.

5. Conclusion and outlook

In conclusion, the thesis aim was reached by providing novel and valuable prognoses on STR genotyping success rates in an incomparable high number of soft and hard tissue types and presenting recommendations on alteration-specific optimal sampling strategies leading to first-attempt DNA-based ID of unidentified human remains.

The three projects performed within this thesis started with *project I* highlighting the challenges regarding tissue-specific differences in DNA stability and revealing insecurities and inconsistencies in tissue selection existing in forensic casework. By addressing limitations in current CE-based genotyping and advantages of novel DNA sequencing technologies, the following *project II* provides a valuable basis for implementing novel NGS technologies in routine casework and improves the sequencing success of challenging samples from altered remains. Finally, the exceptional systematic approach of comparing DNA yields, DNA integrity and profile completeness in 1698 DNA extracts of 19 tissue types and five alteration degrees presented in *project III* provide guidance and recommendations that overcome the presented insecurities in optimal sampling material. Based on the project outcomes, the corpse's condition should be classified according to the provided scoring system in three anatomical regions prior to sample collection. The further presentation of prognoses on genotyping success separated for 22 loci and the 16 eESS loci as well as for a purifying and non-purifying DNA extraction method allows the examiner to select the optimal tissue types depending on the complexity of the case.

Those outcomes provide not only valuable data for future corpse ID in forensic casework, but also for further disciplines including forensic anthropology and analyses of ancient DNA as well as disaster victim identification scenarios with high numbers of altered human remains. In particular, by not only covering current gold standard CE-based genotyping but also validating and implementing novel NGS methods in *project II* and *III*, the thesis provides future-oriented recommendations on DNA sequencing success of challenging samples. As the future will most likely tend towards DNA sequencing, the presented guidance for human ID paved the way for upcoming implementations and a wider use of NGS in forensic casework. Despite concerns in data interpretation or invasion of privacy, forthcoming amendments in law might enable a wider use of extended DNA analyses in routine work including phenotyping, ancestry predictions, age estimations or facial reconstructions. However, the study results display NGS as a reliable and promising method that should not only be considered for improving ID of altered human remains in the future, but also in today's casework. The possibility to sequence exclusively non-coding STR loci, when further extended DNA analyses are not permitted by law, enables an enhanced resolution in samples for not only the ID of corpses, but also for further forensic applications including the analyses of degraded or low copy numbered trace samples or kinship testings.

Therefore, based on the thesis outcomes, upcoming research studies can further progress the prognoses on STR genotyping success rates in tissue samples of altered human remains by increasing the number of sequenced samples and including further extended DNA analyses.

6. References

1. Hochmeister M, Grassberger M, Stimpfl T. Todesermittlung: Befundaufnahme und Spurensicherung. Ein praktischer Leitfaden für Polizei, Juristen und Ärzte. Springer. pages 67-80, 2009.

2. Dettmeyer R, Verhoff MA, Schütz H. Rechtsmedizin. Springer. pages 6-19, 89-107, 239-47, 2011.

3. Eckert WG. Introduction to forensic sciences. CRC press. pages 9-11, 1996.

4. Caplova Z, Obertova Z, Gibelli DM, et al. Personal identification of deceased persons: An overview of the current methods based on physical appearance. *J Forensic Sci*; 63(3):662-71, 2018.

5. Schwark T, Modrow J-H, Steinmeier E, et al. The auditory ossicles as a DNA source for genetic identification of highly putrefied cadavers. *Int J Leg Med*; 129(3):457-62, 2015.

6. Allouche M, Hamdoum M, Mangin P, et al. Genetic identification of decomposed cadavers using nails as DNA source. *Forensic Sci Int Genet*; 3(1):46-9, 2008.

7. R Hughes-Stamm S, J Ashton K, Van Daal A. Assessment of DNA degradation and the genotyping success of highly degraded samples. *Intl J Leg Med*; 125(3):341-8, 2011.

8. Gouveia N, Brito P, Bogas V, et al. THE effect of different levels of degradation and DNA concentrations on the quality of genetic profiles. *Forensic Sci Int Genet Suppl Ser*; 6:e428-e9, 2017.

9. Courts C, Sauer E, Hofmann Y, et al. Assessment of STR typing success rate in soft tissues from putrefied bodies based on a quantitative grading system for putrefaction. *J Forensic Sci*; 60(4):1016-21, 2015.

10. Schwark T, Heinrich A, von Wurmb-Schwark N. Genetic identification of highly putrefied bodies using DNA from soft tissues. *Int J Legal Med*; 125(6):891-4, 2011.

11. Yorulmaz C, Arican N, Afacan I, et al. Pleural effusion in bodies recovered from water. *Forensic SciInt*; 136(1-3):16-21, 2003.

12. Dettmeyer RB, Verhoff MA, Schütz HF. Forensic medicine: fundamentals and perspectives. Springer Science & Business Media. pages 33-57, 191-213, 43-60, 2013.

13. Maciejewska A, Wlodarczyk R, Pawlowski R. The influence of high temperature on the possibility of DNA typing in various human tissues. *Folia Histochem Cytobiol*; 53(4):322-32, 2015.

14. Vass AA. Beyond the grave-understanding human decomposition. *Microbiology today*; 28:190-3, 2001.

15. Damann FE, Carter DO. Human decomposition ecology and postmortem microbiology. *Manual of forensic taphonomy*:37-49, 2013.

16. Zhou C, Byard RW. Factors and processes causing accelerated decomposition in human cadavers–an overview. *J Forensic Leg Med*; 18(1):6-9, 2011.

17. Janaway RC, Percival SL, Wilson AS. Decomposition of human remains. Microbiology and aging: Springer. p. 313-34, 2009.

18. Hedges RE. Bone diagenesis: an overview of processes. *Archaeometry*; 44(3):319-28, 2002.

19. Ross AH, Hale AR. Decomposition of juvenile-sized remains: a macro-and microscopic perspective. *Forensic Sciences Research*; 3(4):310-9, 2018.

20. Gelderman H, Boer L, Naujocks T, et al. The development of a post-mortem interval estimation for human remains found on land in the Netherlands. *Int J Leg Med*; 132(3):863-73, 2018.

21. Gill-King H. Chemical and ultrastructural aspects of decomposition. In: Forensic taphonomy: the postmortem fate of human remains. p. 93-108, 1997.

22. Clark MA, Worrell MB, Pless JE. Postmortem changes in soft tissues. In: Forensic taphonomy: the postmortem fate of human remains. p. 151-64, 1997.

23. Rodriguez W. Decomposition of buried and submerged bodies. In: Forensic taphonomy: the postmortem fate of human remains. p. 459-68, 1997.

24. Byard RW, Farrell E, Simpson E. Diagnostic yield and characteristic features in a series of decomposed bodies subject to coronial autopsy. *Forensic science, medicine, and pathology*; 4(1):9-14, 2008.

25. Alaeddini R, Walsh SJ, Abbas A. Forensic implications of genetic analyses from degraded DNA: A review. *Forensic Sci Int Genet*; 4(3):148-57, 2010.

26. Nysten PH. Mémoire sur la roideur cadavérique qui survient aux corps de l'homme et des animaux après la mort. On cadaverous rigidity that occurs in the body of man and animals after death. Paris. JA Brosson. 1816.

27. Amendt J, Goff ML, Campobasso CP, et al. Current concepts in forensic entomology. Springer. pages 1-24, 2010.

28. Fink AR. Estimating postmortem intervals of human remains recovered in Mid-Western waterways: a test of terrestrial and aquatic body scoring methods. Thesis 2017.

29. Dix J, Graham M. Time of death, decomposition and identification: an atlas. CRC press. pages 1-81, 1999.

30. Cockle DL, Bell LS. Human decomposition and the reliability of a 'Universal'model for post mortem interval estimations. *Forensic SciInt*; 253:136. e1-. e9, 2015.

31. Reh H. Diagnostik des Ertrinkungstodes und Bestimmung der Wasserzeit. Triltsch. pages 127-44, 1969.

32. De Donno A, Campobasso CP, Santoro V, et al. Bodies in sequestered and non-sequestered aquatic environments: a comparative taphonomic study using decompositional scoring system. *Science & Justice*; 54(6):439-46, 2014.

33. Dix J. Asphyxia and drowning: an atlas. CRC Press. pages 1-21, 2017.

34. Petrik M, Hobischak N, Anderson G. Examination of factors surrounding human decomposition in freshwater: a review of body recoveries and coroner cases in British Columbia. *Canadian Society of Forensic Science Journal*; 37(1):9-17, 2004.

35. Heaton V, Lagden A, Moffatt C, et al. Predicting the postmortem submersion interval for human remains recovered from UK waterways. *J Forensic Sci*; 55:302-7, 2010.

36. Boyle S, Galloway A, Mason RT. Human aquatic taphonomy in the Monterey Bay area. In: Forensic taphonomy: the postmortem fate of human remains. p. 605-13, 1997.

37. Fiedler S, Graw M. Decomposition of buried corpses, with special reference to the formation of adipocere. *Naturwissenschaften*; 90(7):291-300, 2003.

38. Schwark T, Heinrich A, Preuße-Prange A, et al. Reliable genetic identification of burnt human remains. *Forensic Sci Int Gen*; 5(5):393-9, 2011.

39. Symes SA, Chapman EN, Dirkmaat DC, et al. Recovery and interpretation of burned human remains. Document No.: 237966: BiblioGov Project; 2012.

40. Becker J. The Position of a Human Body Burnt, but not Completely Destroyed, by Fire. *British Medical Journal*; 1(1746):1297, 1894.

41. Jeffreys AJ, Neumann R, Wilson V. Repeat unit sequence variation in minisatellites: a novel source of DNA polymorphism for studying variation and mutation by single molecule analysis. *Cell*; 60(3):473-85, 1990.

42. International Committee of the Red Cross: Missing people, DNA analysis and identification of human remains: A guide to best practice in armed conflicts and other situations of armed violence.3-42, 2009.

43. Butler JM. Fundamentals of forensic DNA typing. Academic press. 2009.

44. Thompson R, Zoppis S, McCord B. An overview of DNA typing methods for human identification: past, present, and future. *DNA Electrophoresis Protocols for Forensic Genetics*:3-16, 2012.

45. Ballard D, Winkler-Galicki J, Wesoły J. Massive parallel sequencing in forensics: advantages, issues, technicalities, and prospects. *Int J Leg Med*; 134(4):1291-303, 2020.

46. Butler JM. Advanced Topics in Forensic DNA Typing: Methodology. pages 1-369, 2012.

47. Budowle B, Van Daal A. Forensically relevant SNP classes. *Biotechniques*; 44(5):603-10, 2008.

48. Thomas PJ, Weedn VW. Preservation and recovery of DNA in postmortem specimens and trace samples. In: Forensic taphonomy: the postmortem fate of human remains. p. 109-38, 1997.

49. Goodwin W, Linacre A, Hadi S. An introduction to forensic genetics. John Wiley & Sons. pages 1-154, 2011.

50. Schneider PM. Expansion of the Europian standard set of DNA database loci-the current situation. *Profiles in DNA*; 12:6-7, 2009.

51. Butler JM. Advanced Topics in Forensic DNA Typing: Interpretation. Academic Press. pages 1-371, 2014.

52. Daniel R, Walsh SJ. The continuing evolution of forensic DNA profiling-from STRS to SNPS. *Australian Journal of Forensic Sciences*; 38(2):59-74, 2006.

53. Jäger AC, Alvarez ML, Davis CP, et al. Developmental validation of the MiSeq FGx forensic genomics system for targeted next generation sequencing in forensic DNA casework and database laboratories. *Forensic Sci Int Genet*; 28:52-70, 2017.

54. Van Oorschot RA, Ballantyne KN, Mitchell RJ. Forensic trace DNA: a review. *Investigative genetics*; 1(1):1-17, 2010.

55. Köcher S, Müller P, Berger B, et al. Inter-laboratory validation study of the ForenSeq™ DNA Signature Prep Kit. *Forensic Sci Int Genet*; 36:77-85, 2018.

56. Wu J, Li J-L, Wang M-L, et al. Evaluation of the MiSeq FGx system for use in forensic casework. *Int J Leg Med*; 133(3):689-97, 2019.

57. Hwa H-L, Wu M-Y, Chung W-C, et al. Massively parallel sequencing analysis of nondegraded and degraded DNA mixtures using the ForenSeq™ system in combination with EuroForMix software. *Int J Leg Med*; 133(1):25-37, 2019.

58. Butler JM. Fundamentals of forensic DNA typing. Academic Press/Elsevier. pages 1-438, 2010.

59. Die Bundesversammlung der Schweizerischen Eidgenossenschaft. Bundesgesetz über die Verwendung von DNA-Profilen im Strafverfahren und zur Identifizierung von unbekannten oder vermissten Personen (DNA-Profil-Gesetz) vom 20. Juni 2003

60. Bruijns B, Tiggelaar R, Gardeniers H. Massively parallel sequencing techniques for forensics: A review. *Electrophoresis*; 39(21):2642-54, 2018.

61. Fakruddin M, Chowdhury A. Pyrosequencing an alternative to traditional Sanger sequencing. *American Journal of Biochemistry and Biotechnology*; 8(1):14-20, 2012.

62. Alvarez-Cubero MJ, Saiz M, Martínez-García B, et al. Next generation sequencing: an application in forensic sciences? *Annals of human biology*; 44(7):581-92, 2017.

63. Børsting C, Morling N. Next generation sequencing and its applications in forensic genetics. *Forensic Sci Int Genet*; 18:78-89, 2015.

64. Liu Y, Liao J, Lu Q. Laboratory Methods in Epigenetics. Epigenetics and Dermatology: Elsevier. p. 7-35, 2015.

65. Behjati S, Tarpey PS. What is next generation sequencing? *Archives of Disease in Childhood-Education and Practice*; 98(6):236-8, 2013.

66. Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of next-generation sequencing technologies. *Nature Reviews Genetics*; 17(6):333-51, 2016.

67. Caratti S, Turrina S, Ferrian M, et al. MiSeq FGx sequencing system: a new platform for forensic genetics. *Forensic Sci Int Gen Suppl Ser*; 5:e98-e100, 2015.

68. Moorthie S, Mattocks CJ, Wright CF. Review of massively parallel DNA sequencing technologies. *The HUGO journal*; 5(1):1-12, 2011.

69. England R, Harbison S. A review of the method and validation of the MiSeq FGx™ Forensic Genomics Solution. *Wiley Interdisciplinary Reviews: Forensic Science*; 2(1):e1351, 2020.

70. Liu L, Li Y, Li S, et al. Comparison of next-generation sequencing systems. *Journal of Biomedicine and Biotechnology*; 2012, 2012.

71. Lu Y, Shen Y, Warren W, et al. Next generation sequencing in aquatic models. *Next Generation Sequencing-Advances, Applications and Challenges*; 1:13, 2016.

72. Chan K, Sterling JF, Roberts SA, et al. Base damage within single-strand DNA underlies in vivo hypermutability induced by a ubiquitous environmental agent. *PLoS genetics*; 8(12):e1003149, 2012.

73. Bär W, Kratzer A, Mächler M, et al. Postmortem stability of DNA. *Forensic SciInt*; 39(1):59-70, 1988.

74. Dash HR, Das S. Microbial degradation of forensic samples of biological origin: potential threat to human DNA typing. *Molecular biotechnology*; 60(2):141-53, 2018.

75. Shapiro R. Damage to DNA caused by hydrolysis. Chromosome damage and repair: Springer. p. 3-18, 1981.

76. Lindahl T. Instability and decay of the primary structure of DNA. *nature*; 362(6422):709-15, 1993.

77. Poinar HN. The top 10 list: criteria of authenticity for DNA from ancient and forensic samples. *International congress series*; 1239:575-9, 2003.

78. Karni M, Zidon D, Polak P, et al. Thermal degradation of DNA. *DNA and CELL biology*; 32(6):298-301, 2013.

79. Imaizumi K. Forensic investigation of burnt human remains. *Research and Reports in Forensic Medical Science*; 5:67, 2015.

80. Bright J-A, Taylor D, Curran JM, et al. Degradation of forensic DNA profiles. *Australian Journal of Forensic Sciences*; 45(4):445-9, 2013.

81. Schrader C, Schielke A, Ellerbroek L, et al. PCR inhibitors–occurrence, properties and removal. *Journal of applied microbiology*; 113(5):1014-26, 2012.

82. Bessetti J. An introduction to PCR inhibitors. *J Microbiol Methods*; 28:159-67, 2007.

83. von Wurmb-Schwark N, Schwark T, Harbeck M, et al. A simple duplex-PCR to evaluate the DNA quality of anthropological and forensic samples prior short tandem repeat typing. *Legal Medicine*; 6(2):80-8, 2004.

84. Alaeddini R. Forensic implications of PCR inhibition—a review. *Forensic Sci Int Genet*; 6(3):297-305, 2012.

85. Roeper A, Reichert W, Mattern R. The Achilles tendon as a DNA source for STR typing of highly decayed corpses. *Forensic Sci Int*; 173(2-3):103-6, 2007.

86. Piccinini A, Cucurachi N, Betti F, et al. Forensic DNA typing of human nails at various stages of decomposition. *International Congress Series 1288*:586-8, 2006.

87. Shintani-Ishida K, Harada K, Nakajima M, et al. Usefulness of blood vessels as a DNA source for PCR-based genotyping based on two cases of corpse dismemberment. *Legal Medicine*; 12(1):8-12, 2010.

88. Watherston J, McNevin D, Gahan ME, et al. Current and emerging tools for the recovery of genetic information from post mortem samples: New directions for disaster victim identification. *Forensic Sci Int Gen*; 37:270-82, 2018.

89. Uerlings S, Welter V, Madea B, et al. Comparative analysis of DNA extraction processes for DNA-based identification from putrefied bodies in forensic routine work. *Forensic SciInt*; 320:110707, 2021.

90. Huang H-S, Matevossian A, Jiang Y, et al. Chromatin immunoprecipitation in postmortem brain. *Journal of neuroscience methods*; 156(1-2):284-92, 2006.

91. van den Berge M, Wiskerke D, Gerretsen R, et al. DNA and RNA profiling of excavated human remains with varying postmortem intervals. *Int J Leg Med*; 130(6):1471-80, 2016.

92. Hansen J, Lesnikova I, Funder AMD, et al. DNA and RNA analysis of blood and muscle from bodies with variable postmortem intervals. *Forensic science, medicine, and pathology*; 10(3):322-8, 2014.

93. Sato Y, Hayakawa M, Nakajima T, et al. HLA typing of aortic tissues from unidentified bodies using hot start polymerase chain reaction–sequence specific primers. *Int J Legal Med*; 5:191-3, 2003.

94. Helm K, Matzenauer C, Neuhuber F, et al. Suitability of specific soft tissue swabs for the forensic identification of highly decomposed bodies. *Int J Leg Med*; 135(4):1319-27, 2021.

95. Brito F, Prata D, Martha S, et al. Evaluation of the urinary bladder swabs as a source of DNA for human identification using two different extraction methods. *Forensic science international Genetics*; 5:e484-e6, 2015.

96. Brito FC, Nunes MR, Prata DR, et al. DNA extraction of urinary bladder swabs collected from carbonized and decomposing corpses: Possible application in disaster victim identification. *Int J Leg Med*; 37:15-7, 2019.

97. Prinz M, Carracedo A, Mayr W, et al. DNA Commission of the International Society for Forensic Genetics (ISFG): recommendations regarding the role of forensic genetics for disaster victim identification (DVI). *Forensic Sci Int Genet*; 1(1):3-12, 2007.

98. Owen R, Bedford P, Leditschke J, et al. Post mortem sampling of the bladder for the identification of victims of fire related deaths. *Forensic Sci Int*; 233(1-3):14-20, 2013.

99. Becker J, Mahlke NS, Ritz-Timme S, et al. The human intervertebral disc as a source of DNA for molecular identification. *Forensic Science, Medicine and Pathology*; 17(4):660-4, 2021.

100. Kulstein G. Next generation of forensic body fluid/tissue identification: Determination of forensic relevant body fluids of challenging forensic samples by molecular RNA-based analysis. Thesis 2017.

101. Edson SM. Getting ahead: Extraction of DNA from skeletonized cranial material and teeth. *Journal of forensic sciences*; 64(6):1646-57, 2019.

102. INTERPOL, Disaster Victim Identification Guide 2009, https://www.interpol.int/How-we-work/Forensics/Disaster-Victim-Identification-DVI?msclkid=fbe55a65bc1411ec9d4a6821bfaeab6f. Retrieved 25.03.2022.

103. Emmons AL, Davoren J, DeBruyn JM, et al. Inter and intra-individual variation in skeletal DNA preservation in buried remains. *Forensic Sci Int Gen*; 44:102193, 2020.

104. Staiti N, Di Martino D, Saravo L. A novel approach in personal identification from tissue samples undergone different processes through STR typing. *Forensic Sci Int*; 146:171-3, 2004.

105. Pinhasi R, Fernandes D, Sirak K, et al. Optimal ancient DNA yields from the inner ear part of the human petrous bone. *PloS one*; 10(6):e0129102, 2015.

106. Hansen HB, Damgaard PB, Margaryan A, et al. Comparing ancient DNA preservation in petrous bone and tooth cementum. *PloS one*; 12(1):e0170940, 2017.

107. Pilli E, Vai S, Caruso MG, et al. Neither femur nor tooth: petrous bone for identifying archaeological bone samples via forensic approach. *Forensic SciInt*; 283:144-9, 2018.

108. Schlenker A, Grimble K, Azim A, et al. Toenails as an alternative source material for the extraction of DNA from decomposed human remains. *Forensic Sci Int*; 258:1-10, 2016.

109. Jakubowska J, Maciejewska A, Pawłowski R. Comparison of three methods of DNA extraction from human bones with different degrees of degradation. *Int J Leg Med*; 126(1):173-8, 2012.

110. Lassen C, Hummel S, Herrmann B. Comparison of DNA extraction and amplification from ancient human bone and mummified soft tissue. *Int J Leg Med*; 107(3):152-5, 1994.

111. Maxwell® RSC Instrument Operating Manual. Promega. TM411. Revised 12/21. Catalog Number AS4500.

112. SwabSolution™ Kit. Technical manual. Instructions for Use of Product DC8271. Promega. TMD037. Revised 4/21.

113. Zeng X, King JL, Stoljarova M, et al. High sensitivity multiplex short tandem repeat loci analyses with massively parallel sequencing. *Forensic Sci Int Genet*; 16:38-47, 2015.

114. Xavier C, Parson W. Evaluation of the Illumina ForenSeq™ DNA Signature Prep Kit–MPS forensic application for the MiSeq FGx™ benchtop sequencer. *Forensic Sci Int Genet*; 28:188-94, 2017.

115. van der Gaag KJ, de Leeuw RH, Hoogenboom J, et al. Massively parallel sequencing of short tandem repeats—population data and mixture analysis results for the PowerSeq™ system. *Forensic Sci Int Genet*; 24:86-96, 2016.

116. Churchill JD, Schmedes SE, King JL, et al. Evaluation of the Illumina® beta version ForenSeq™ DNA signature prep kit for use in genetic profiling. *Forensic Sci Int Genet*; 20:20-9, 2016.

117. Frégeau CJ. Validation of the Verogen ForenSeq™ DNA Signature Prep kit/Primer Mix B for phenotypic and biogeographical ancestry predictions using the Micro MiSeq® Flow Cells. *Forensic Sci Int Genet*; 53:102533, 2021.

118. Breslin K, Wills B, Ralf A, et al. HIrisPlex-S system for eye, hair, and skin color prediction from DNA: Massively parallel sequencing solutions for two common forensically used platforms. *Forensic Sci Int Gen*; 43:102152, 2019.

119. Gettings KB, Kiesler KM, Vallone PM. Performance of a next generation sequencing SNP assay on degraded DNA. *Forensic Sci Int Gen*; 19:1-9, 2015.

120. Carrasco P, Inostroza C, Didier M, et al. Optimizing DNA recovery and forensic typing of degraded blood and dental remains using a specialized extraction method, comprehensive qPCR sample characterization, and massively parallel sequencing. *Int J Leg Med*; 134(1):79-91, 2020.

121. Carboni I, Fattorini P, Previderè C, et al. Evaluation of the reliability of the data generated by Next Generation Sequencing from artificially degraded DNA samples. *Forensic Sci Int Genet Suppl Ser*; 5:e83-e5, 2015.

122. Hollard C, Ausset L, Chantrel Y, et al. Automation and developmental validation of the ForenSeq™ DNA Signature Preparation kit for high-throughput analysis in forensic laboratories. *Forensic Sci Int Genet*; 40:37-45, 2019.

123. Illumina. MiSeq FGx™ Forensic Genomics System. System Specification Sheet: Forensic Genomics. 2016. .

124. Sidstedt M, Rådström P, Hedman J. PCR inhibition in qPCR, dPCR and MPS—mechanisms and solutions. *Analytical and Bioanalytical Chemistry*; 412(9):2009-23, 2020.

125. Kulstein G, Hadrys T, Wiegand P. As solid as a rock—comparison of CE-and MPS-based analyses of the petrosal bone as a source of DNA for forensic identification of challenging cranial bones. *Int J Leg Med*; 132(1):13-24, 2018.

126. Senst A, Scheurer E, Gerlach K, et al. Which tissue to take? A retrospective study of the identification success of altered human remains. *J Forensic Leg Med*; 84:102271, 2021.

127. Schwark T, Modrow J-H, Steinmeier E, et al. The auditory ossicles as a DNA source for genetic identification of highly putrefied cadavers. *Int J Leg Med*; 129(3):457-62, 2015.

128. Van Neste C, Van Nieuwerburgh F, Van Hoofstat D, et al. Forensic STR analysis using massive parallel sequencing. *Forensic Sci Int Genet*; 6(6):810-8, 2012.

129. Fattorini P, Previderé C, Carboni I, et al. Performance of the ForenSeqTM DNA Signature Prep kit on highly degraded samples. *Electrophoresis*; 38(8):1163-74, 2017.

130. Senst A, Caliebe A, Scheurer E, et al. Validation and beyond: Next generation sequencing of forensic casework samples including challenging tissue samples from altered human corpses using the MiSeq FGx system. *J Forensic Sci*, 2022.

131. Megyesi MS, Nawrocki SP, Haskell NH. Using accumulated degree-days to estimate the postmortem interval from decomposed human remains. *J Forensic Sci*; 50(3):1-9, 2005.

132. van Daalen MA, de Kat DS, Oude Grotebevelsborg BF, et al. An aquatic decomposition scoring method to potentially predict the postmortem submersion interval of bodies recovered from the North Sea. *J Forensic Sci*; 62(2):369-73, 2017.

133. Glassman D, Crow R. Standardization model for describing the extent of burn injury to human remains. *J Forensic Sci*; 41(1):152-4, 1996.

134. Pajnič IZ. Extraction of DNA from human skeletal material. Forensic DNA typing protocols: Springer. p. 89-108, 2016.

135. Stoop B, Defaux PM, Utz S, et al. Touch DNA sampling with SceneSafe Fast™ minitapes. *Legal medicine*; 29:68-71, 2017.

136. Butler JM. The future of forensic DNA analysis. *Philosophical Transactions of the Royal Society B: Biological Sciences*; 370(1674):20140252, 2015.

137. Gill P, Fereday L, Morling N, et al. The evolution of DNA databases—recommendations for new European STR loci. *Forensic SciInt*; 156(2-3):242-4, 2006.

138. NIST. National Institute of Standards and Technology. U.S Departement of Commerce. STRBase (SRD-130). Retrieved from https://strbase.nist.gov/coreSTRs.htm 20.04.2022.

139. ForenSeq™ DNA Signature Prep Reference Guide. Verogen. Document # VD2018005 Rev. C. August 2020.

140. Precision ID SNP Panels with the HID Ion S5™/HID Ion GeneStudio™ S5 System. Appliction guide. ThermoFisher Scientific. Catalog Numbers A25643, A25642. Publication Number MAN0017767. Revision C.0.

141. Elwick K, Mayes C, Hughes-Stamm S. Comparative sensitivity and inhibitor tolerance of GlobalFiler(R) PCR Amplification and Investigator(R) 24plex QS kits for challenging samples. *Leg Med (Tokyo)*; 32:31-6, 2018.

142. Comte J, Baechler S, Gervaix J, et al. Touch DNA collection–Performance of four different swabs. *Forensic Sci Int Genet*; 43:102113, 2019.

143. Butler JM, Hill CR, Kline MC, et al. The single most polymorphic STR Locus: SE33 performance in US populations. *Forensic Sci Int Genet Suppl Ser*; 2(1):23-4, 2009.

144. Chaitanya L, Breslin K, Zuñiga S, et al. The HIrisPlex-S system for eye, hair and skin colour prediction from DNA: Introduction and forensic developmental validation. *Forensic Sci Int Genet*; 35:123-35, 2018.

145. Walsh S, Chaitanya L, Breslin K, et al. Global skin colour prediction from DNA. *Human genetics*; 136(7):847-63, 2017.

146. Walsh S, Chaitanya L, Clarisse L, et al. Developmental validation of the HIrisPlex system: DNA-based eye and hair colour prediction for forensic and anthropological usage. *Forensic Sci Int Genet*; 9:150-61, 2014.

147. Team R. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2021.

148. Team R. RStudio: Integrated Development for R. RStudio, PBC, Boston, MA 2020.

149. Wickham H, François R, Henry L, et al. dplyr: A Grammar of Data Manipulation. R package version 1.0.7., 2021.

150. Kassambara A. ggpubr: 'ggplot2' Based Publication Ready Plots. R package version 0.4.0., 2020.

151. Berkelaar M. lpSolve: Interface to 'Lp_solve' v. 5.5 to Solve Linear/Integer Programs. R package version 5.6.15., 2020.

152. Wickham H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York. 2016.

153. ForenSeq™ Universal Analysis Software Guide. Sample and Run results. Verogen. Document # VD2018007. Rev. A. June 2018.

154. GEDNAP. German DNA Profiling. Retrieved from https://www.gednap.org/de/ 24.04.2022.

155. Szeremeta M, Pietrowska K, Niemcunowicz-Janica A, et al. Applications of metabolomics in forensic toxicology and forensic medicine. *International Journal of Molecular Sciences*; 22(6):3010, 2021.

156. Donaldson AE, Lamont IL. Estimation of post-mortem interval using biochemical markers. *Australian Journal of Forensic Sciences*; 46(1):8-26, 2014.

157. Harder M, Renneberg R, Meyer P, et al. STR-typing of ancient skeletal remains: which multiplex-PCR kit is the best? *Croatian medical journal*; 53(5):416-22, 2012.

158. Cunha MdLRdSd. Molecular Biology in Microbiological Analysis. *Food Science*, 2019.

159. Guo F, Yu J, Zhang L, et al. Massively parallel sequencing of forensic STRs and SNPs using the Illumina® ForenSeq™ DNA signature prep kit on the MiSeq FGx™ forensic genomics system. *Forensic Sci Int Genet*; 31:135-48, 2017.

160. Elwick K, Bus MM, King JL, et al. Utility of the Ion S5™ and MiSeq FGx™ sequencing platforms to characterize challenging human remains. *Legal Medicine*; 41:101623, 2019.

161. Iozzi S, Carboni I, Contini E, et al. Forensic genetics in NGS era: New frontiers for massively parallel typing. *Forensic Sci Int Genet Suppl Ser*; 5:e418-e9, 2015.

162. Parys-Proszek A, Wróbel M, Marcińska M, et al. Dual amplification strategy for improved efficiency of forensic DNA analysis using NGM Detect™, NGM™ or Globalfiler™ kits. *Forensic Sci Int Gen*; 35:46-9, 2018.

163. Bronner IF, Quail MA, Turner DJ, et al. Improved protocols for illumina sequencing. *Current protocols in human genetics*; 79(1):18.2. 1-.2. 42, 2013.

164. ForenSeq DNA Signature Prep Kit with ForenSeq Enhanced PCR1 Buffer System. Reference guide. Verogen. Document # VD2021046 Rev. A. January 2022.

165. Schneider PM, Prainsack B, Kayser M. The use of forensic DNA phenotyping in predicting appearance and biogeographic ancestry. *Deutsches Ärzteblatt International*; 116(51-52):873, 2019.

166. Jansen S. Erweiterte DNA-Analyse in der Strafverfolgung. *Forensic Sci Int Gen*; 7(28):36, 2013.

167. Xavier C, de la Puente M, Mosquera-Miguel A, et al. Development and validation of the VISAGE AmpliSeq basic tool to predict appearance and ancestry from DNA. *Forensic Sci Int Gen*; 48:102336, 2020.

168. Amankwaa A, McCartney C, Tidball-Binz M, et al. Opinion on draft amendments to the DNA profiles act of Switzerland. Warsaw, 03 August 2021. Opinion-Nr.: CRIM-CHE/418/2021 [AT]. 2021.