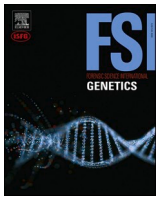
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Forensic DNA extraction methods for human hard tissue: A systematic literature review and meta-analysis of technologies and sample type

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ABSTRACT

DNA identification of human remains has a valuable role in the field of forensic science and wider. Although DNA is vital in identification of unknown human remains, post-mortem environmental factors can lead to poor molecular preservation. In this respect, focus has been placed on DNA extraction methodologies for hard tissue samples, as these are the longest surviving. Despite decades of research being conducted on DNA extraction methods for bone and teeth, little consensus has been reached as to the best performing. Therefore, the aim of this study was to conduct a thorough systematic literature review to identify potential DNA extraction technique (s) which perform optimally for forensic DNA profiling from hard tissue samples. PRISMA guidelines were used, by which a search strategy was developed. This included identifying databases and discipline specific journals, keywords, and exclusion and inclusion criteria. In total, 175 articles were identified that detailed over 50 different DNA extraction methodologies. Results of the meta-analysis conducted on 41 articles – meeting further inclusion criteria - showed that statistically significant higher DNA profiling success was associated with solid-phase magnetic bead/resin methods. In addition, incorporating a demineralisation pre-step resulted in significantly higher profiling successes. For hard tissue type, bone outperformed teeth, and even though dense cortical femur samples were more frequently used across the studies, profiling success was comparable, and in some cases, higher in cancellous bone samples. Notably, incomplete data sharing resulted in many studies being excluded, thus an emphasis for minimum reporting standards is made. In conclusion, this study identifies strategies that may improve success rates of forensic DNA profiling from hard tissue samples. Finally, continued improvements to current methods can ensure faster times to resolution and restoring the identity of those who died in obscurity.

1. Introduction

The importance of identifying human remains is not only required in forensic or medicolegal death investigations, but also in human rights' contexts, migrant deaths, and mass fatality incidents [1,2]. In many of these cases, the identification process of the deceased is led by a multidisciplinary team, which may include forensic pathologists, forensic odontologists, geneticists, forensic anthropologists, police staff, and other external agencies. In cases where human remains are visually unidentifiable, lack personal possessions, or where there is minimal background context – such as a suspected missing person – the procedure of identification may be laborious. Therefore, it is unsurprising that accuracy in identification is continually sought out in post-mortem contexts. One such method of positive identification that has garnered a highly valuable role within forensics, is DNA profiling.

Forensic DNA profiling via Short Tandem Repeat (STR) typing lies within the power of discriminating between individuals genetically. This relies heavily on comparative analyses with known-person profiles [3], which can be seen as a limitation in some instances. Even so, it is still seen as an identification method that is associated with the greatest certainty, especially when used alongside odontology and fingerprints [4-6]. Therefore, it has been the focus of many researchers to improve

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DNA techniques to increase the likelihood of generating a reportable forensic DNA profile. Specifically, DNA extraction methodologies have been extensively investigated, as this is the first important step within the forensic DNA workflow where the DNA molecule is preferentially isolated, and where contaminants or potential PCR inhibitors, are removed [3,7].

Although DNA is a powerful tool in the identification of human remains, certain post-mortem environments are suboptimal for DNA preservation, as described in past reviews [8,4,9]. In addition, as the decomposition process progresses, the selection of high-yielding DNA sources narrows, and often in these situations bone and teeth samples are favoured due to their longevity in a wide variety of post-mortem contexts.

Hard tissue samples – bones and teeth – undergo far more labour-intensive processes to extract the DNA molecule; thus research to improve and facilitate successful DNA extraction from these sample types is continuous and important considering, that many humanitarian and medicolegal cases involve human skeletal remains. However, despite optimal methods being researched for decades, there remains little consensus as to the "best" DNA extraction technique for human hard tissues in terms of efficiency and successful DNA profiling for downstream profiling. For example, for many years, organic extraction methods were the standard across molecular studies and based on the results of several studies, are still considered the most effective compared to magnetic bead or silica-based methods [10-15]. However, the phenol-chloroform reagents used in these methods are toxic and are associated with PCR inhibitors [16]. In addition, these protocols often require larger quantities of starting material [17].

Even prior to DNA extraction, published literature reports discrepant strategies in terms of hard tissue sample selection from the body [18,13,19]. Further inconsistent and varying results for DNA yields and profiling success of DNA extraction methods for hard tissues have created a large body of disparate literature. Variation in successful DNA extraction from bone has also been shown to differ greatly when compared to other samples such as teeth, muscle, and post-mortem blood, with the degree of decomposition also playing a major role [19]. Thus, selection of post-mortem samples should take this into consideration.

To date, no systematic literature review (SLR) has been undertaken to address whether or not there is an optimal DNA extraction method for hard tissue samples. Many prospective or case studies investigating this topic suffer from small sample size and low statistical strength. Therefore, the aim of this paper is to undertake a thorough and extensive SLR, in combination with a meta-analysis, to amalgamate the results of multiple studies that address DNA extraction techniques for hard tissue samples. Importantly, questions were not only formulated to address laboratory-based activities, but also those in forensic field practice in terms of sample selection. The following questions were thus addressed:

1. What DNA extraction protocols are most frequently used?
2. What hard tissue sample is most frequently used?
3. Are there any notable differences between liquid- and solid-phase DNA extraction in terms of STR profiling success?
4. Does the underlying DNA technology effect STR profiling success?
5. Does inclusion of a demineralisation pre-treatment step improve STR profiling success?
6. Are certain hard tissue types associated with an increase in STR profiling success?

2. Methods

The '*preferred reporting items for systematic literature reviews and meta-analyses*' (PRISMA) were used to guide the methodology in conducting the SLR [20,21].

2.1. Literature search strategy

A search strategy was implemented to encompass the main database repositories, which included: Web of Science™ (a meta-database), Cochrane (database holding systematic literature review publications), PubMed and Scopus. In addition to these databases, fourteen discipline specific journals for forensic science research were also searched to ensure data collection was saturated. These journals were: *Forensic Science International, Forensic Science International: Genetics* (including two supplement series and a special edition), *Forensic Science International: Synergy*, *Forensic Science International: Reports, Science & Justice, Journal of Forensic and Legal Medicine, Egyptian Journal of Forensic Science, Legal Medicine, Journal of Forensic Sciences, Australian Journal of Forensic Sciences,* and the *International Journal of Legal Medicine*.

Articles published between 1st January 2000 and 31st December 2021 were included to ensure comparison of similar technologies and inclusion of full publication years. Keywords were selected to build search terms and were adapted for each database and discipline specific journal accordingly (Table 1). Research articles retrieved from the databases and discipline specific journals searches were reviewed under inclusion and exclusion criteria (Table 2). In the initial stage, abstracts were screened for relevance and then articles that met the inclusion criteria were read in full. Reference lists of review articles were scanned to further ensure saturation of all relevant articles. In terms of quality, the articles were reviewed for scientific rigor, and where this was not achieved either due to poor communication, lack of sample information, or inconsistencies in reporting results, these articles were excluded. Language of articles was also limited to those written in English, or where a copy in English was available, due to limited translation capabilities. Commercially sensitive data where proprietary methods relating to DNA extraction was not disclosed, were also excluded. Grey literature (textbooks, theses, conference papers, reports and working papers) were also reviewed. Finally, 'hand-searching' was done whereby the reference lists of the included articles were searched and assessed against the same inclusion and criteria.

2.2. Data collection

For all articles included in the study the publication journal, authors, year of publication, and information pertinent to DNA extraction methods/techniques, and the results thereof, were collated in an excel spreadsheet (Microsoft Excel version 16.53). Excluded articles and the reasoning are also listed in the excel spreadsheet. A link to the digital excel spreadsheet of articles included in the SLR can be found within the supplementary data file. Finally, the studies that reported sufficient data to be included within the meta-analysis of DNA extraction success, such as full sample information, DNA yields and importantly sufficient reporting of forensic DNA profiles, were identified.

Table 1
Keywords for the systematic literature search. These were used to form search terms and search strings for the literature search on the various databases and discipline specific journals included.

Keywords	
DNA extraction/isolation/ purification	Sample preparation/sampling
Methodology/technique/workflow	Forensics
Bone OR skeleton OR skeletal	Victim identification
Teeth OR tooth or Dental	Unidentified remains
DNA quantity	DNA degradation
PCR	DNA profiling
Success	qPCR
Mass graves	Mass fatalities/disaster victim identification
Forensic genetics	Forensic anthropology
Recommendations	Guidelines

Table 2
Preliminary inclusion and exclusion criteria for systematic literature review search and assessment of research studies.

Inclusion	Exclusion
Studies reporting on DNA extraction techniques/methodology for hard tissue samples – bones and teeth	Ancient DNA studies Animal studies Studies where only mitochondrial DNA (mtDNA) targeted. Studies only targeting Y-STRs or X-STRs Studies only targeting single nucleotide polymorphisms (SNPs)
Case reports Original articles Short communications Recommendations and guidelines	Studies where it is not clear as to the DNA methodology used, or where details are lacking
Novel methods for DNA extraction for bones and teeth samples for forensics DNA identification	Samples that are dated/aged prior to 20th century or from ancient/ archaeological context i.e., post-mortem interval > 100 years
All bone and teeth samples exposed to different terrestrial post-mortem contexts (including damp)	Samples from submerged marine/ aquatic environments, those exposed to high temperatures, or exposed to strong acids/detergent, or any other physical/chemical manipulation that was "unnatural"

2.3. Meta-analysis

To statistically assess the collated results from studies, a meta-analysis was also conducted on the subset of articles that met additional criteria. These criteria included complete reporting of sample information and accessible raw data for quantification and DNA profiling success per sample. To ensure comparisons could be made between samples, only papers where DNA yield was quantified by real-time quantitative PCR (qPCR) were included and units were converted to nanograms per gram of sample (ng/g). Where this could not be achieved, papers were excluded for this component. In parallel to quantification, appropriate reporting for forensic STR profiling success was also used as eligibility criteria. Papers that reported the number of successful STR loci as a fraction of a total number of specified target loci were included. This fraction was then converted into a percentage for comparison across studies and samples. As per published guidelines, profiles were considered full where between 80% and 100% of loci were successfully amplified, whereas anything between 20% and 80% was considered partial, and below 20% was considered a failed profile [22]. In addition, age of sample, or post-mortem interval (PMI) was grouped broadly in to < 1 year; 1 – 10 years, 11 – 20 years, 21 – 50 years and > 51 years. This grouping was based on the studies included in the meta-analysis. More accurate PMIs could not be reported due to inconsistencies in reporting and low resolution of data for this variable.

Prior to data-analysis, the presence of potential outliers in terms of DNA yields was evaluated. This was initially done on IBM© SPSS© Statistic Version 28, using the anomaly function and later, an outlier formula which excluded any DNA yield values that were three standard deviations above the mean of the dataset. Papers that were identified from performing the anomaly function, and those where 50% of the samples were three standard deviations above the mean, were excluded. Individual sample assessment was also done, and samples reporting values three standard deviations above the mean of the dataset, were also excluded (Supplementary data file).

2.4. Data analysis

The first component of the data analysis was to report the overall results from the SLR. This included reporting the PRISMA criteria for the number of records retrieved from each database and other sources, and the exclusion of articles at each step. In addition, topics, publication years, journal titles, DNA extraction techniques, and types of skeletal material used, were also reported. For this, all figures and diagrams were created in Microsoft PowerPoint or Excel (version 16.53).

For the meta-analysis, all statistical analyses were performed on IBM© SPSS© Statistic Version 28. Statistical analyses were done in a stepwise approach to test the independent variables against the two main dependent variables, these being DNA yield reported as ng/g and STR profiling success reported as a percentage (as described above). The focus was on the DNA extraction protocols across studies, and these were categorized into liquid- and solid-phase, DNA extraction technology and automated versus manual. In addition, the influence of incorporating a demineralisation pre-step on successful STR profiling was also tested. Furthermore, the effect of different hard tissue types, body region, bone composition was also tested as potential co-factors for STR profiling success.

Error bars representing the 95% confidence interval and mean for DNA yields and profiling success were created for the different DNA extraction methodology variations to visually assess any patterns. To statistically test the effects of categorical variables on continuous variables, for example DNA extraction technology versus DNA yield and STR profiling success, univariate analysis of variances (ANOVAs) was performed. Where two or more categories were present, a pairwise comparison via post-hoc least significance difference tests (LSD) was done to identify where differences truly came from. For these, a p-value < 0.5 indicated significance. Finally, in order to analyse any associations between two continuous variables, such as DNA yield and profiling success, Pearson correlations were done, and scatter plots were generated to visually represent relationships.

3. Results

3.1. Systematic review

The PRISMA flow diagram representing the number of records identified in the search phase is presented in Fig. 1. The highest number of records were identified in PubMed (n = 268), followed by Web of Science (n = 227), Scopus (n = 66), and Cochrane (n = 1). An additional 278 records were identified through other sources, these being field specific journals (n = 252), shared sources such as textbooks and conference proceedings (n = 19), citation hand-searching (n = 6), and websites (n = 1) (Fig. 1).

By applying eligibility criteria, as detailed in the methods section, the final number of articles included in the systematic review equated to 175 records (Fig. 1). Full details of the records included in the systematic review can be found within the supplementary data file. A mean of 8.33 articles were published per year over the 21 years-time span, with 2019 being the year with the most published (n = 28), followed by 2020 (n = 23), and 2021 (n = 20) (Fig. 2 and supplementary Table 1). An upward trend was also noted, whereby more articles were published in later years (from 2007) compared to earlier years. In addition, articles that met criteria for meta-analysis inclusion were only from the year 2007 onwards (Fig. 2).

The distribution of journals where articles included in the systematic review were published varied, with 30 different journals being identified. Most articles were published in *Forensic Science International: Genetics supplement series* (27%), followed by *Forensic Science International: Genetics* (14%), and the *International Journal of Legal Medicine* (9%) (Supplementary Table 2). In addition, one article was published as a conference proceeding. However, of note is that articles published in *Forensic Science International: Genetics supplement series* are derived from the International Society of Forensic Genetics conferences, which do not undergo the same review process as full journal articles.

3.1.1. DNA extraction variables

With respect to article research focuses, these included disaster victim identification (DVI), forensic medicolegal cases, human rights,

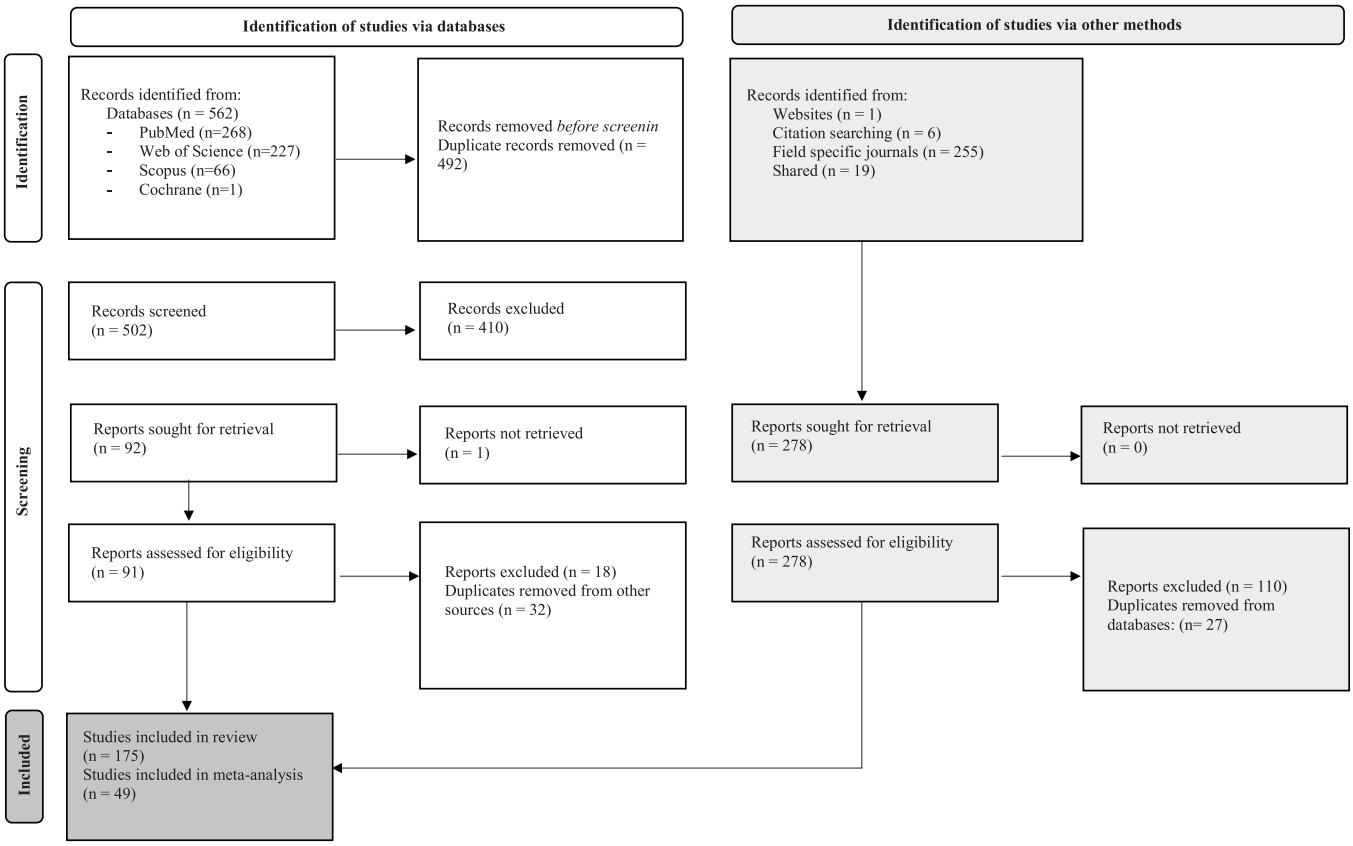


Fig. 1. Diagram detailing the number of records identified and retrieved in each critical step of the systematic literature review. PRISMA flow diagram adapted from [21].

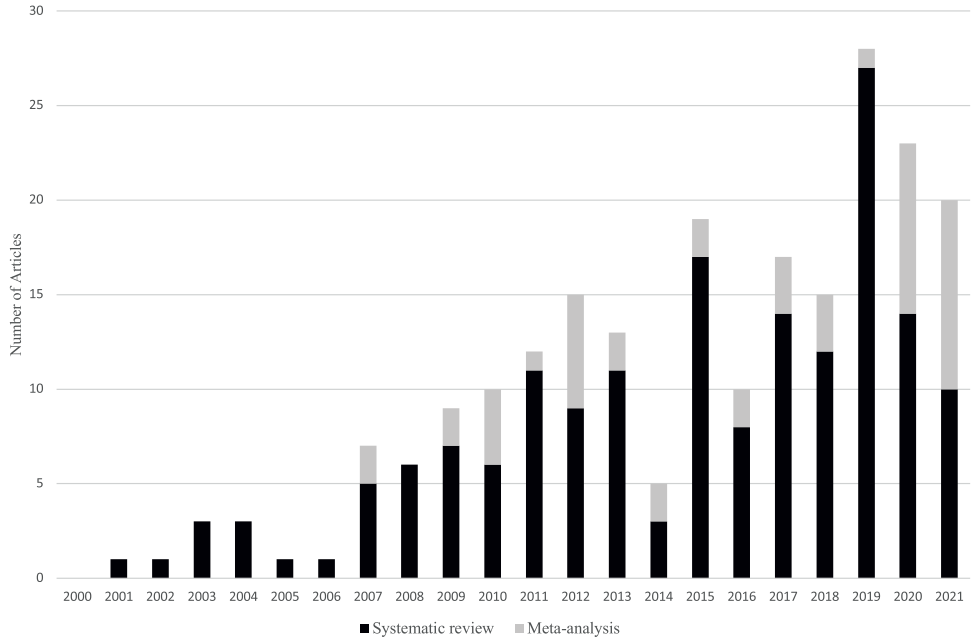


Fig. 2. Number of articles included in the systematic literature review (n = 175) and meta-analysis, published per year with a trendline. Publication details for each article can be found in the supplementary file.

and historical cases (mainly from World War 1 and World War 2). The majority of samples were obtained from unidentified human remains (71% of articles) from various contexts including, but not limited to, mass fatality incidents, forensic casework of missing persons, exhumations, and various past and contemporary conflicts (Table 3.).

In addition, almost half of the articles included used only bone samples for DNA sources (48%) compared to only teeth samples (16%), and 36% of articles used a combination of bone and teeth samples

Table 3
Table of the article focus (DVI, forensic, historic, human rights) and origin of samples (unidentified human remains, donors, and so forth) from articles included in the systematic review. (*Note: "Other" includes articles that did not fit within the broad topics or did not detail origin of samples in the protocol.*).

Article focus	Total Number of Articles	Sample origin	Number of Articles
Disaster victim identification	12	Mass fatality events	12
Forensics	105	Donors (patients, cadavers)	32
		Casework (missing persons, unidentified human remains, paternity, kinship)	49
		Exhumations (paternity, unidentified remains)	15
		Not detailed	9
Historic	3	Exhumations or unidentified human remains	3
Human rights	46	Unidentified human remains from Argentinian conflict	2
		Unidentified human remains from Yugoslav wars	7
		Unidentified remains from Guatemala Civil War	2
		Unidentified human remains from Iraq war	1
		Unidentified human remains from Korean war	3
		Unidentified American Service men (numerous conflicts)	1
		Unidentified human remains from Spanish Civil war	4
		Unidentified human remains from WW1 and WW2	26
Other	9		

(Supplementary Table 3). For teeth samples, and where reported, molars were more commonly used as the DNA source compared to incisors, canines, and pre-molars (Supplementary Table 4). With regard to articles where cranial samples were used and reported, the temporal and the petrous portions were selected more frequently (Supplementary Table 4). The most routinely used elements across all studies were femur and teeth samples, followed by tibia, humerus, cranial elements, and ribs. Less common sample types were ear ossicles (reported in only one study), the sacrum, carpals, and patellae (Supplementary Table 4).

In 74% of protocols reported in articles, pulverising of the bone or tooth sample was done prior to DNA extraction (Supplementary Table 5). This was mainly achieved by specialised equipment such as freezer mills, tissue lysers (bead beating), or hand-held drills. Furthermore, in most studies (64%) demineralisation was incorporated as an extra pre-treatment (Supplementary Table 6), with some studies using this as the main method of extracting DNA (Supplementary Table 7). Though demineralisation was a commonly used protocol, there were markedly more studies in later years that included this step compared to earlier years (Supplementary Fig. S1). Specifically, articles from 2016 to 2021 accounted for 54% of studies where demineralisation was incorporated.

Of all the variables collected, by far the most diverse across the studies was the DNA extraction method itself. There were over 50 different variations of DNA extraction and purification protocols reported in the 175 studies included (Supplementary Table 7). In terms of frequency of use, magnetic bead/resin technology was used more often, followed by organic, silica and inorganic methods (Fig. 3). Distribution between solid and liquid-phase extraction was near even; however, manual DNA extraction was used more frequently in studies compared to automated platforms (Supplementary Table 7).

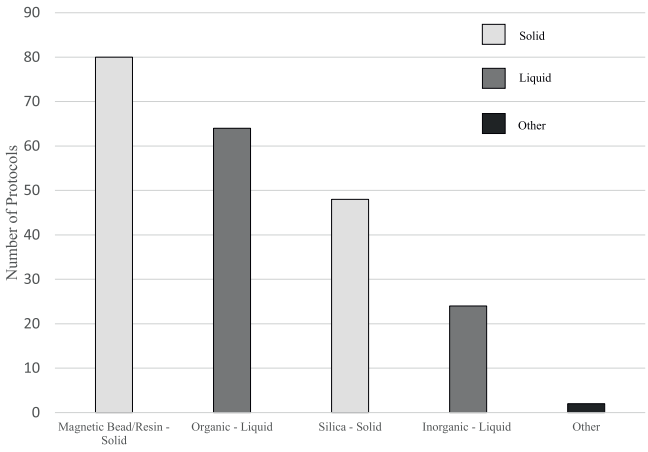


Fig. 3. DNA technologies used across all studies included in the systematic review. (*Note: some studies investigate/use more than one type of DNA extraction technology).*

3.2. Meta-analysis

Forty-nine (49) articles were identified as potentials to include in the meta-analysis. However, subsequent to anomaly testing, one article and several individual samples were removed (supplementary data file). Therefore, in total, 41 articles yielding 2 505 individual sample data points were taken forward for the meta-analysis component of the SLR (supplementary data file). The focus of the meta-analysis component of the systematic review was on the different DNA extraction protocols and hard tissue sample types and the effect of these variables on both DNA yield and STR profiling success.

3.2.1. DNA extraction method

Across the protocols, nine (9) distinct DNA extraction methods (not including any additional purification) were identified (Table 4). Of these, most samples were extracted using QIAGEN's EZ1 DNA Investigator kit (39%), followed by only demineralisation (29%) (non-distinct liquid-phase inorganic extraction), and phenol-chloroform methods (9%) (non-distinct liquid-phase organic extraction). With respect to underlying technology, magnetic bead methods exceeded all others in terms of use, with almost half of the samples undergoing DNA extraction with this type of technology (49%). This was followed by inorganic (29%) and silica (13%), with the least number of samples extracted via organic methods (9%) (Table 4). In addition, only three kits: EZ1 DNA Investigator (QIAGEN), QIAamp DNA Investigator (QIAGEN), and DNA IQ Casework Pro (Promega) were developmentally validated for forensic use according to ISO 17025 standards.

Both DNA yield and profiling success differed within and between studies included in the meta-analysis (Fig. 4). The mean DNA yield was 33.86 ng/g (standard deviation 92.10) and the mean number of successfully typed STR loci across all studies was 79% (standard deviation: 29.87). DNA yield (ng) was not consistent in predicting successful STR profiling, as evidenced by a weak correlation ($r = 0.57$; $p = 0.005$) (Supplementary Fig. S2). However, a stronger relationship was found between sample input weight and resultant total DNA yield (ng) ($r = 0.133$; $p < 0.001$) (Supplementary data Fig. S3), yet no correlation was found between sample input weight and STR profiling success ($r = 0.011$; $p = 0.291$).

In exploring the success of the different DNA extraction technologies, it was found that liquid-phase DNA extraction yielded significantly higher DNA quantities per gram of bone/tooth input sample compared to solid-phase extraction ($F\ (1, 2504) = 46.286$, $p < 0.001$) (Fig. 5A). However, when comparing STR profiling success, solid-phase DNA extraction resulted in significantly higher numbers of target STR loci being successfully amplified and detected, compared to liquid-phase

Table 4
List of the 9 DNA extraction methods ranked in their frequency of use per sample included in the meta-analysis (from 41 research articles).

DNA Extraction Method	Frequency	Solid/Liquid	Technology	Total	Platform
EZI DNA Investigator	967	Solid	Magnetic Bead	1227	Automated
PrepFiler BTA	201				Manual/Automated
DNA IQ Casework Pro	45				
Maxwell RSC Blood DNA	14				Automated
Only Demineralisation	727	Liquid	Inorganic	727	Manual/Automated
QIAGEN Blood Maxi	176	Solid	Silica	325	Manual
QIAamp DNA Investigator	100				Manual
QIAamp DNA Mini	49				Manual
Phenol-chloroform	226	Liquid	Organic	226	Manual

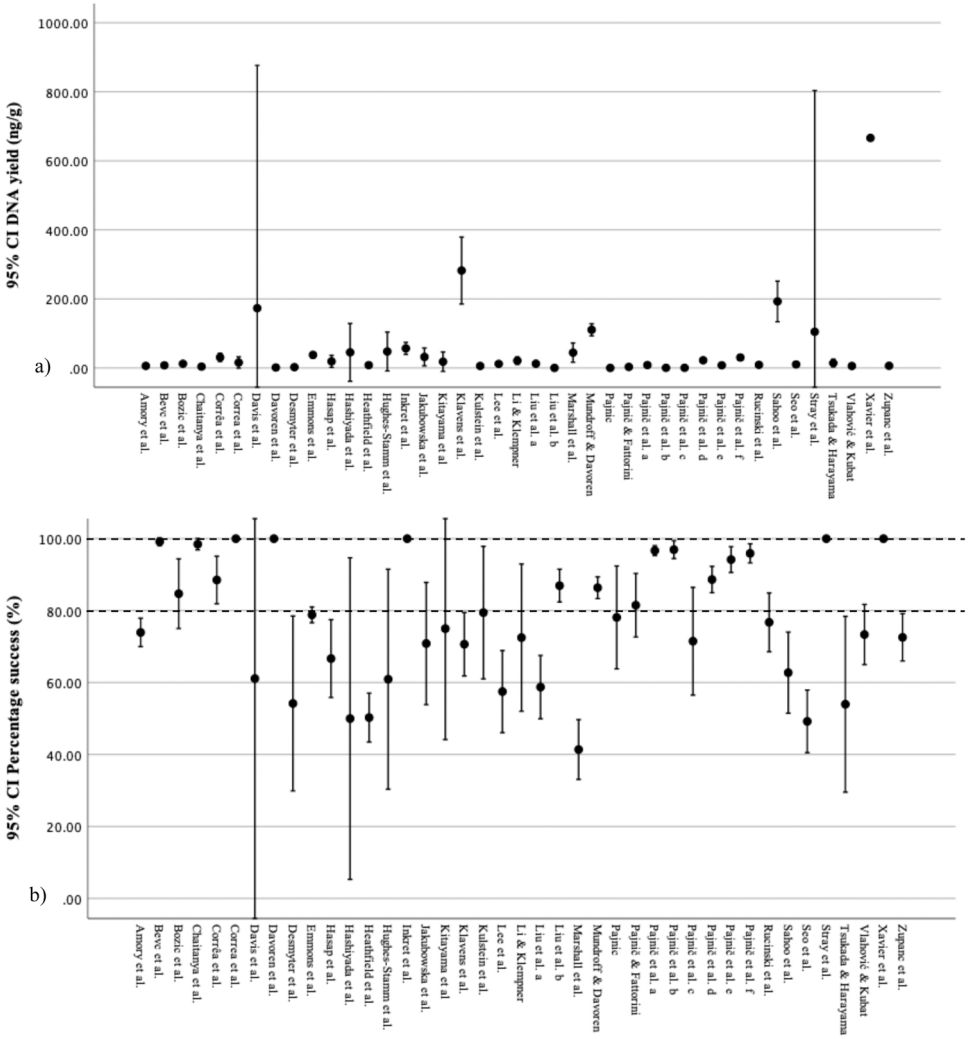


Fig. 4. Error bars displaying mean and 95% confidence interval for DNA yields per gram of sample (ng/g) (a) and STR profiling success (b) as a percentage of successfully typed STR across the 41 studies included in the meta-analysis. *CI = confidence interval. The dotted line in graph b is what would be considered as a full profile.*

extraction $F\ (1, 2504) = 23.066, p < 0.001$ (Fig. 5B).

In terms of DNA technology, inorganic methods yielded higher DNA quantities (ng/g) compared to magnetic bead (solid-phase), organic (liquid-phase), and silica methods (solid-phase) (Fig. 6A). Pairwise comparison via post-hoc LSD also showed that this was significant (*p's < 0.001*). Furthermore, magnetic bead techniques resulted in significantly higher DNA yield compared to silica methods (p = 0.008). Contrastingly, magnetic bead and inorganic methods resulted in higher STR profiling success rates compared to organic and silica methods (Fig. 6B). Also, the pairwise comparison via post-hoc showed that all extraction techniques significantly differed for STR profiling success (p's < 0.001), except for silica and organic methods ($p = 0.675$).

On comparing other aspects of DNA extraction, no significant differences were found in DNA yields (ng/g) when comparing automated versus manual platforms, type of samples input (slices/powdered), and where demineralisation was incorporated into the extraction process. However, DNA samples extracted via automated platforms had higher profiling success ($F\ (2, 2504) = 8.432$, $p < 0.001$), as did samples where the protocol incorporated a demineralisation pre-step ($F\ (1, 2504) = 10.019$, $p = 0.002$). There was no significant difference found in STR profiling success between samples that underwent powdering prior to DNA extraction compared to those that did not. For demineralisation,

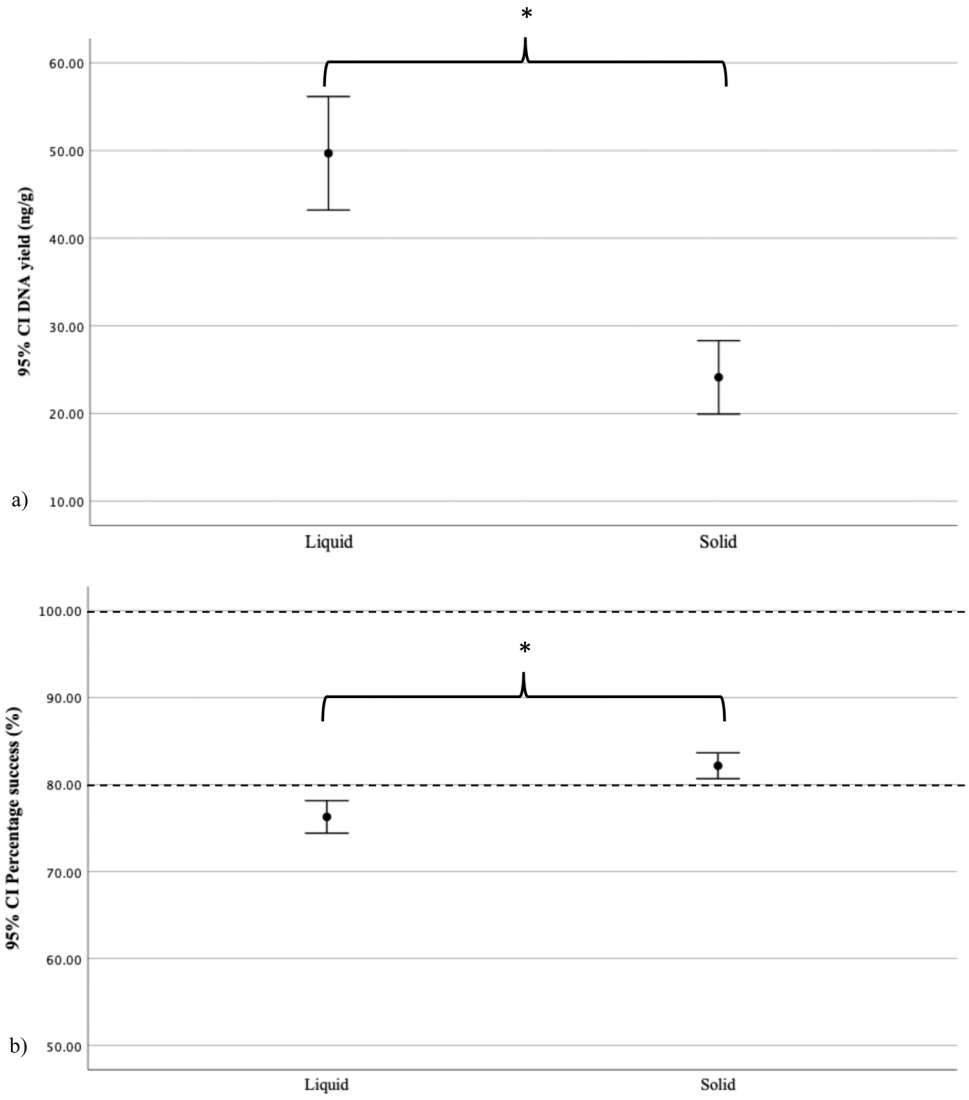


Fig. 5. Error bars displaying mean and 95% confidence interval for DNA yields per gram of sample (ng/g) (a) and STR profiling success (b) as a percentage of successfully typed STR loci for liquid- and solid-phase extraction. *CI = confidence interval. The dotted line in graph b is what would be considered as a full profile.*

concentration of EDTA was constant at 0.5 M across all studies, whereas length of demineralisation varied between hours to days (Fig. 7). The minimum was 0 h whereas the maximum of 120 h with a mean of 38.73 h, and longer demineralisation was not always associated with more full profiles (Fig. 7). In addition, the temperature of the demineralisation also differed between samples where the minimum was 23 °C and the maximum was 56 °C with a mean of 44.12 °C. As with time, higher temperature was not associated with more full profiles (Supplementary Fig. S4).

3.2.2. Hard tissue type

Even though DNA extraction was the focus of the meta-analysis, hard tissue type was also explored as a factor in STR profiling success. Out of the 25 samples, 89% (2230/2505) of DNA was extracted from bone samples compared to only 11% (275/2505) from teeth. In terms of DNA yield (ng/g) there was no significant difference between bone and teeth samples, but there was significant difference in STR profiling success where bone samples produced more full profiles ($F(1, 2504) = 13.419$, $p < 0.001$).

For bone samples, the skeletal element most commonly used across studies was the femur making up 38% (945/2505) of samples. This was followed by the humerus (4.6%; 115/2505), metacarpals (4.4%; 110/2505) and tarsals (4.4%; 109/2505) (Supplementary Table S8). The skeletal elements that yielded the most DNA per gram were tarsals, phalanges, carpals, metacarpals, metatarsals and patellae, where mean yields were above or equal to 50 ng/g (Fig. 8). In addition, carpals, metatarsals, metacarpals, tarsals and phalanges also produced more full profiles alongside vertebrae, pelvis (innominates), femora, and tibiae (Fig. 8). Lowest DNA yields and STR profiling success was noted in ulna, radius, and humerus samples (Fig. 8).

For skeletal elements that produced the highest profiling success, it was found that the majority of carpal, tarsal, metatarsal, phalanx, pelvis samples were extracted via an inorganic method (6/9; 86/109; 50/85; 40/52; 18/29), and metacarpal, vertebra, femur, and tibia samples via magnetic bead methods (65/110; 46/77; 670/945; 92/230) (Supplementary Table S9). Due to unreported information, tooth type could not be collected, however the mean DNA yield for teeth was 29.06 ng/g and mean STR profiling success was 73.71%. In addition, tooth samples were more frequently extracted via magnetic bead methods (Supplementary Table S9).

In exploring body region, it was found that no significant differences for DNA yields (ng/g) was found when comparing appendicular upper and lower extremities, the axial skeleton and teeth. However, there was significant differences for STR profiling success ($F(4,2504 = 41.062$, $p < 0.001$). Upon further inspection via post-hoc LSD, samples from the lower appendicular skeleton, which includes the pelvis, femur, tibia,

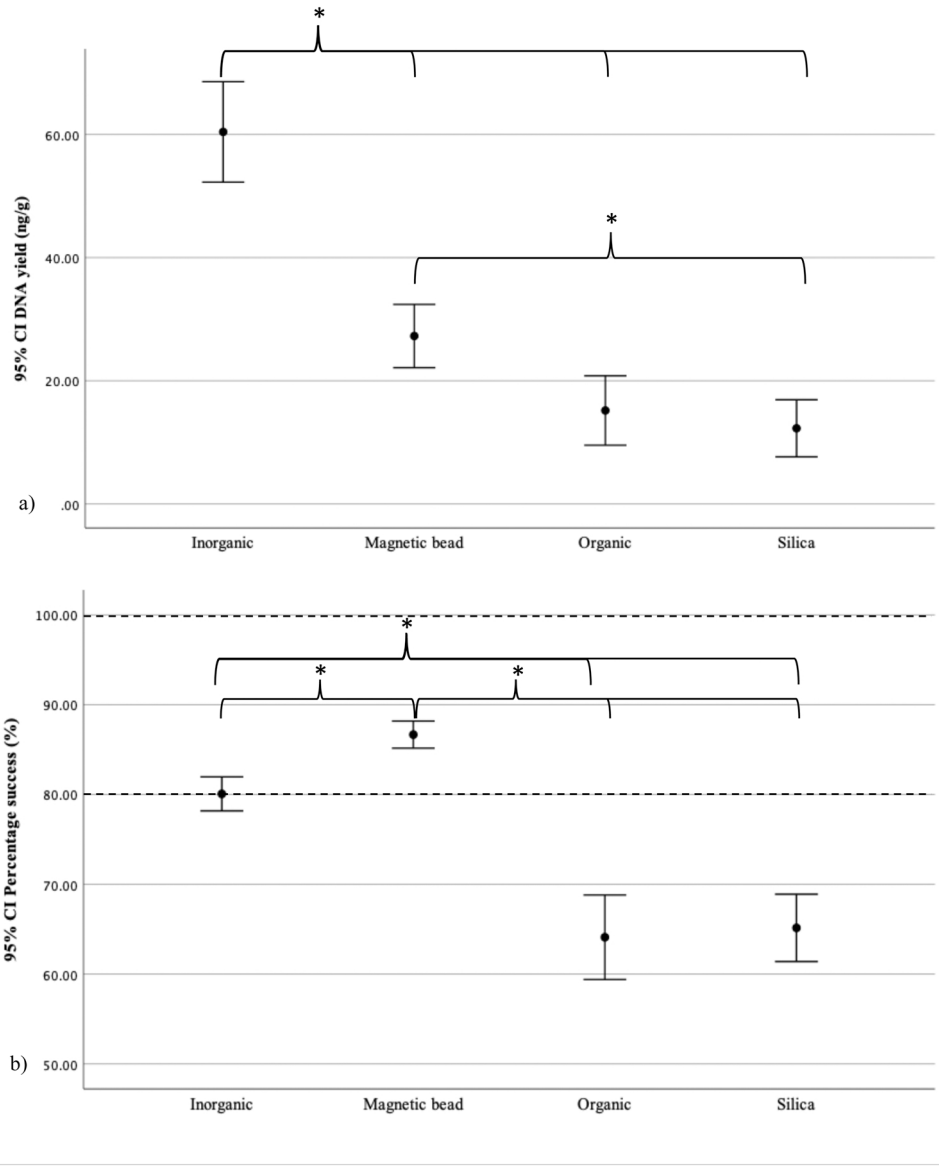


Fig. 6. Error bars displaying mean and 95% confidence interval for DNA yields per gram of sample (ng/g) (a) and STR profiling success (b) as a percentage of successfully typed STR loci for the four different DNA extraction technologies. *CI = confidence interval. The dotted line in graph b is what would be considered a full profile.*

fibula, and foot bones, produced significantly more full STR profiles (*p's < 0.01)*. In addition, samples from the axial skeleton had higher STR profiling success compared to appendicular upper and teeth samples (*p's < 0.008*) (Supplementary Fig. S5).

Cancellous bone also yielded significantly higher DNA yields per gram of sample compared to dense cortical and teeth samples regardless of whether a pre-treatment demineralisation step was incorporated (Post-hoc LSD, p's < 0.001). For profiling success, cancellous bone did produce significantly more full profiles compared to dense cortical or teeth without the incorporation of a demineralisation step. However, where demineralisation was included, cancellous bone did produce significantly more full STR profiles compared to dense cortical samples, but not compared to teeth (Supplementary Fig. S6). Skeletal elements that comprised a higher ratio of cancellous bone to cortical bone included: carpals, tarsals, metatarsals, metacarpals, and phalanges, whilst dense cortical samples were derived mainly from the long bones (humerus, radius, ulna, femur, tibia, fibula) (Supplementary Table S10).

Finally, an important consideration other than DNA extraction technology for the success of hard tissue samples, is age of the sample or post-mortem interval. In 105 samples (4%) this was not reported. Femora accounted for some of the oldest samples as more than half of these (62%; 588/945) had a PMI of over 51 years (Supplementary Table S13). In addition, metacarpals, metatarsals, phalanges, and teeth also came from aged samples. However, teeth also accounted for the majority of samples that had a PMI of < 1 year (69%; 105/152) (Supplementary Table S11).

4. Discussion

Given the discrepancies reported in the literature, the purpose of conducting this systematic review was to pool data from published articles meeting eligibility criteria to identify any trends in DNA extraction technologies, and related factors, on both DNA yield and importantly successful STR profiling. Considering the decades of study into the topic of DNA extraction methods for hard tissue samples, it was not unexpected that a high number of research papers exploring DNA extraction from hard tissue were identified through the search process. Once duplicates were removed and inclusion/exclusion criteria were

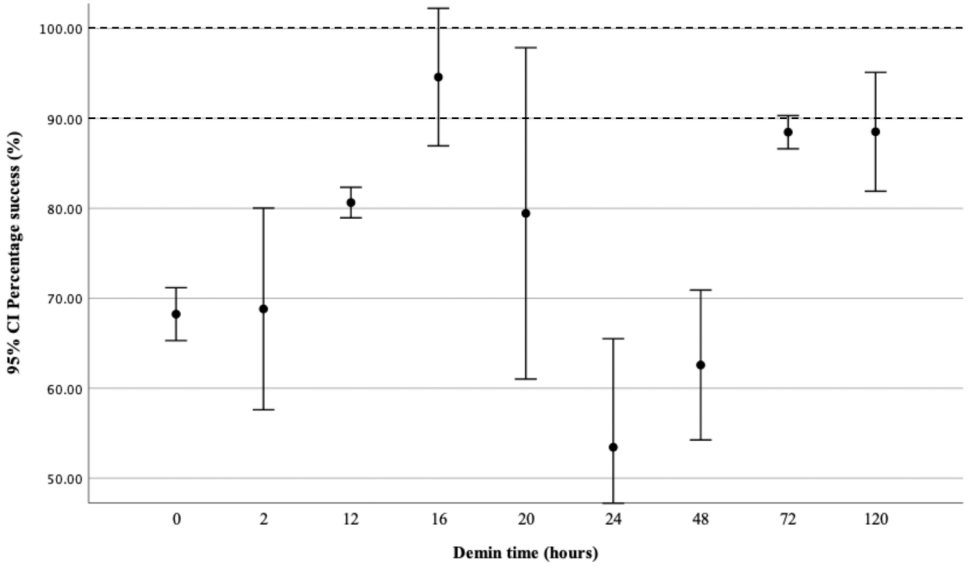


Fig. 7. Error bar graph of mean and 95% confidence interval for STR profiling success. *CI = confidence interval. The dotted line is what would be considered a full profile.*

implemented, the number of articles greatly reduced, and only 26% were included in the meta-analysis aspect of the study. Disregarding duplication and irrelevant records, the main reason for exclusion was lack of detail in DNA extraction methodology and/or inconsistent/non-standardised reporting of DNA quantification and profiling results. These problems may have contributed to the bias of records coming from recent years compared to earlier years, as reporting standards and dissemination of raw data has become a publication standard.

Despite the historical popularity of organic liquid-phase DNA extraction methods, magnetic bead/resin-based methods were more frequently used in the articles included in the SLR (magnetic bead/resin: 48% of article versus organic liquid-phase: 37% of articles). In addition, from the results of the exploratory meta-analysis, samples that underwent DNA extraction via magnetic bead methods were suggested to produce significantly more full profiles compared to samples extracted via organic methods, even though there was no significant difference in DNA yield. This also highlights that although DNA quantity is an important step within the DNA profiling workflow, it cannot conclusively predict that STR profiling will be successful. An observation that was noted throughout the meta-analysis, however DNA yields were still included since quantifying DNA via qPCR is an integral step within the forensic DNA workflow.

In terms of the specific technique, magnetic bead/resin DNA extraction methods outperformed not only silica-based methods for DNA profiling success but also both liquid-phase extraction methods. The greater success of magnetic bead/resin methods may be ascribed to the fact that this extraction technique mainly takes place within a liquid-phase substrate, where positively charged paramagnetic beads are placed within the solution. These beads attract the negatively charged DNA molecule, thus isolating it from other cellular components in a liquid-phase environment [3]. Even so, it should be noted that magnetic bead/resin methods were mainly performed on automated platforms, where a higher proportion had demineralisation incorporated into the protocol, both associated with improved forensic DNA profiling success. Automated platforms have also been reported to not only increase profiling success, but also reduce contamination, which impacts successful STR profiling [16,23].

In contrast, silica methods produced the lowest yields and STR profiling successes, with inorganic liquid-phase methods coming second best. The low performance of silica-based methods may be attributed to the fact that smaller DNA fragments can be lost after multiple wash steps through the silica-columns, which is linked to reduced DNA yield [24]. However, this does not explain the associated lower STR profiling success that was also noted and the fact these samples extracted via silica methods had a similar performance to organic methods, which do not traditionally include silica columns. A previous study suggested that there may be stochastic variation due to the powdering of hard tissue samples that can produce variation between DNA extractions and successful profiling. [12]. Another study suggests that choice of extraction method should be based on the sample, degradation state and the DNA profiling method [25]. Therefore, the observation of that silica methods were outperformed may be attributed to limitations in the dataset from the pooled studies and strict inclusion/exclusion criteria.

In assessing the success of DNA extraction, ancillary components to protocols and techniques had to be considered. Particularly the incorporation of a demineralisation step, as numerous studies have indicated that demineralisation greatly improves successful DNA extraction from hard tissue samples. The main mechanism of demineralisation is the use of EDTA to inactivate *DNAases* via chelating calcium and magnesium ions present in the mineral matrix of hard tissue, which also aids in breaking down the mineralised complexes [26]. The chelating of ions is important since calcium and magnesium can act as PCR inhibitors, which are known to affect successful amplification of STR targets [26,27].

From the results of the meta-analysis, demineralisation was found to improve STR profiling success, which highlights this step as potentially important, improving the amplifiability of DNA molecules by reducing inhibition. Further to this, it has also been reported that paired with solid-phase purification, or another extraction technology targeted at reducing inhibition, DNA yields could further be improved [24,28]. Demineralisation, however, did not always equate to more full STR profiles when investigating hard tissue structure of bone.

Preference of hard tissue type was clear in the results of the SLR, where most DNA extractions were performed on femur or tooth samples. Although reasons for this bias were not clear, it is suspected that the selection of these may have been due to ease of collection (in studies using patient or donor teeth samples), perceived success, or were the only samples that could be collected. Upon conducting the meta-analysis across the 2505 individual samples, DNA yields from femur and tooth samples were not as high compared to others, and for teeth, success of STR profiling was below 80%. Contrastingly, higher yields and profiling success were noted in tarsals, carpals, metacarpals, metatarsals, vertebrae and phalanges, even though these elements were not used as DNA sources as frequently across the studies.

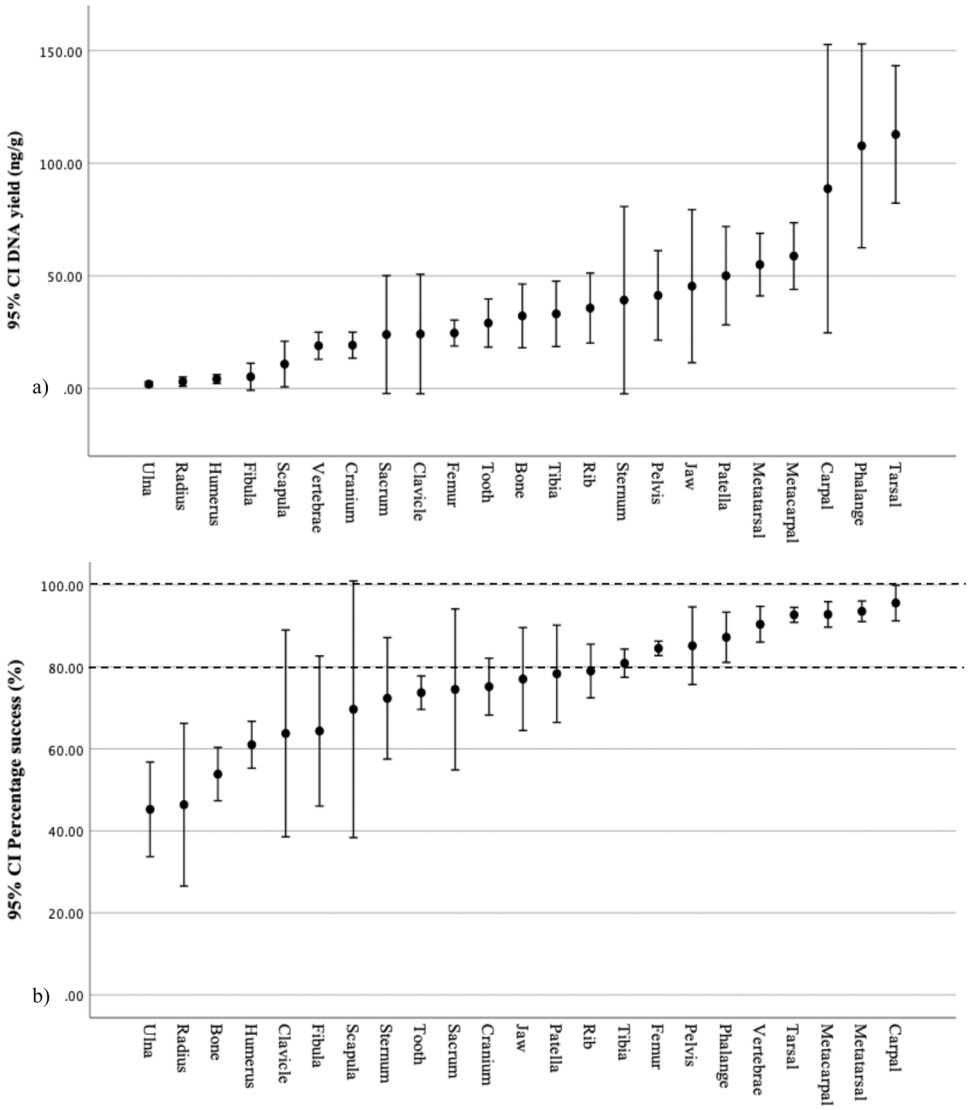


Fig. 8. Error bar graph of mean and 95% confidence interval for DNA yield (ng/g) (a) and STR profiling success (b) for hard tissue types. *CI = confidence interval. The dotted line is what would be considered a full profile.*

The main difference between skeletal elements are their anatomical structures, and for teeth, the cells targeted for DNA. Bone is considered one of the strongest materials [29], with enamel – part of the tooth crown - being the hardest tissue in the body with similar strength properties to steel [30]. However, enamel is largely avascular and is not considered a useful source of DNA [31]. Instead, DNA-rich regions include the vascularised pulp, cementum, and dentine, which house DNA containing odontoblasts, cementocytes and fibroblast cells [31,32]. In contrast, bone is a dynamic structure, continually remodelling to external forces and stresses, which is achieved by the many cellular bodies comprising this tissue – osteocytes, osteoblasts, and osteoclasts – that are interspersed within its mineral complex [29,33].

These anatomical and molecular contrasts between bone and teeth tissues may explain why across the studies in the meta-analysis, significantly higher success was associated with bone samples compared to teeth. However, due to failures in reporting types of teeth used and the regions of sampling, teeth samples were grouped into one category. This is of consequence since various studies have reported that targeted sampling of teeth improves DNA yield and DNA profiling success compared to pulverising whole teeth [34-39]. Unfortunately, the results of the meta-analysis were unable to provide evidence on the debate of "what tooth" is best. In addition, considering that targeted sampling of teeth often required specialist dental equipment and expertise [24,37,40,41], this may be better suited to a specialist review.

Returning to bone, anatomically these skeletal elements are classed into long, short, flat, or irregular bones; and on a micro-structural level, there are two basic components – dense cortical and cancellous bone (dense cortical is also referred to as compact and cancellous as spongy/trabecular bone) [29]. All bone contains both cancellous and cortical components, but the target for DNA is often restricted to one or the other, with only a few being an indistinct mixture of the two. For example, for samples derived from a femur, the target is often focused on the dense cortical regions that are nearer the surface of the external surface [42-44]. Moreover, based on nine studies that directly compared dense cortical to cancellous bone samples, five found better DNA success for dense cortical bone[45-47,43,48]. In contrast, stronger evidence was found to support cancellous bone in this meta-analysis, as these samples produced significantly higher DNA yields and profiling success compared to dense cortical bone. Importantly, this was also regardless of the inclusion of demineralisation pre-step.

Samples comprised of higher amounts of cancellous bone are smaller tubular bones such as metatarsals, metacarpals, and phalanges. These smaller bones do have some advantages since they are quicker to collect and safer to work with since there is no need for drilling or sawing

equipment, which also reduces potential contamination and heat production [49]. Cancellous bone is also four times more metabolically active compared to dense cortical bone, thus this higher cellular content may be associated with improved DNA results [50]. However, a disadvantage is that preservation of these elements can be sub-optimal since cancellous bone possesses lower structural strength compared to dense cortical bone, and in cases of co-mingling, it may be difficult to distinguish these smaller bones as belonging to a particular individual [49]. Therefore, in these cases larger bones such as the femur or tibia could be better, and given that these were found to produce nearly full STR profiles across samples despite being some of the oldest samples, support their continued use.

However, though this dataset pooled many studies across two decades and comprises of over 2000 individual data points, success in DNA extraction from bone varies greatly between and within samples. Again, this raises the question of stochastic effects in hard tissue sampling and inherent inter-individual variation – a factor that cannot be controlled for across experimental, retrospective, or review studies. Even so, the trends reported here provide a unique perspective on DNA extraction methodologies from hard tissue, however, further strength to the findings could have been gained if there were more standardised reporting.

Outside of the laboratory setting, and across all studies, there was a lack of reported standardisation surrounding pre-laboratory procedures to mitigate introduction of contamination. This relates, for example, to the type of personal protective equipment (PPE) a forensic archaeologist or anthropologist may need to wear during the exhumation of a mass grave in a humanitarian case, or in a forensic case. The lack of information pertaining to this may be a symptom of DNA laboratories working independently from external agencies and those conducting fieldwork. Or more concerning, an absence of understanding surrounding the potential impact of movement of samples from field to laboratory, or, as with other aspects – failure or an oversight in reporting. In addition, not all papers detailed the use of elimination databases or guidance with regards to minimum PPE, which is necessary for laboratory based forensic casework, but not well defined for forensic anthropologists and archaeologists working in the field. The presence of contamination can negatively impact profiling success, and recently microbial DNA has been found to greatly interfere with interpretation of forensic DNA profiles, which is of concern [51]. Thus, clearer recommendations surrounding these pre-laboratory steps require further field-to-laboratory research, communication, and discussion to guide quality assurance.

It is also important to note that no two papers implemented the exact same protocol, and these were often biased to reporting on "new" or "novel" methods for DNA extraction. Hence, only success or failure of these techniques were shared. The variables introduced by this type of reporting were innumerable and compounded by the inconsistencies in reporting of DNA yields and STR profiling success, collating and analysing results were challenging. Detail was also absent in the reporting of hard tissue sample type used, wherein some studies only divulged that "bone" or "teeth" samples were used. Additionally, incomplete information surrounding decontamination and sampling methods was noted, and in these instances, methods were essentially non-reproducible. This does raise the question of reporting standards in the literature. The full standard process of DNA identification is clearly outlined in numerous sources for forensic field practice [52,53], but it was clear through this SLR that these standards were not met for many research articles.

This SLR and meta-analysis provides a snapshot into a field that has developed greatly within the past two-decades. As downstream technologies become increasingly sensitive and other DNA targets, such as mtDNA or SNPs enter routine forensic DNA analyses, success of obtaining a DNA profile from degraded hard tissue samples will increase. However, it is clear from the 100′s of records evaluated in this review, there has not been a systematic approach to this type of research in the forensic community, and instead consists of many "trial and error" studies of small sample sizes with little to no statistical strength, thus this further supports the merging of samples via conducting SLRs.

The lack of a structured approach to tackling this research topic is staggering considering the first wide-spread systematic evaluation in the ancient DNA community took place in 2007, as detailed in [54]. Significantly, in this latter review the authors explain how demineralisation buffers are often discarded in forensic laboratories, yet they have been found to contain large amounts of DNA, which are regularly pooled with "pure" extracts in ancient DNA labs. In addition, [25] they recommend an ancient DNA protocol to improve the efficiency of retrieving shorter fragments of DNA when degradation is suspected to be high. Future research within forensic DNA analysis should be informed by robust systematic reviews that have the ability to hone in one specific question. In addition, it is recommended that this review be updated in five years' time to capture new developments in DNA technologies, with further attention being paid to teeth and post-mortem intervals for aged versus fresh samples.

5. Conclusion

In conclusion, this study represents the first SLR and meta-analysis conducted on DNA extraction methodologies for hard tissue samples used in forensic DNA identification. The aim was to collate the results of published studies that met specific inclusion criteria to answer questions surrounding DNA extraction protocols. From the results of the meta-analysis, it was found that higher DNA profiling success was associated with solid-phase magnetic bead/resin extraction technologies and incorporation of a demineralisation pre-step into the extraction workflow improved STR profiling success. Bone outperformed teeth, and even though femur samples were the most frequently used sample type across studies in the SLR, profiling success was poorer compared to lesser-used cancellous bone samples, such as metatarsals, metacarpals, and phalanges. A proposed reason for cancellous bone outperforming dense cortical bone is due to their higher metabolic activity, thus cellular content. This review offers a unique insight into two decades of research in this topic and elucidates on trends on DNA extraction methods for hard tissue samples. It is hoped that future studies will be guided by literature-driven evidence and transparent reporting of data, as well as increased communication between various stakeholders in addressing challenges faced with DNA identification methodologies for degraded samples. Importantly, the strategies identified could improve first-time success of forensic DNA profiling for hard tissue samples by guiding future research - critical for the improvement of fieldwork-to-report turnaround times and, therefore, case resolution timelines. Such will directly contribute towards the restoration of victims' identities and reuniting their remains with their families and communities.

CRediT authorship contribution statement

Chandra Finaughty – Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration. **Laura Jane Heathfield** – Conceptualization, Writing – review & editing, Visualization, Supervision. **Victoria Kemp** – Conceptualization, Writing – review & editing, Visualization, Supervision. **Nicholas Marquez-Grant** – Conceptualization, Writing – review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsigen.2022.102818.

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Table S11. Cross tabulaiton of hard tissue type and age samples - PMI (years).

Count							
	Age samples - PMI (years)	Total					
	not reported	<1	>51	1 to 10	11 to 20	21-50	Total
Bone	57	12	35	9	0	0	113
Carpal	0	0	3	6	0	0	9
Clavicle	1	0	3	9	0	0	13
Cranium	0	0	42	42	7	0	91
Femur	16	13	588	195	128	5	945
Fibula	0	0	5	10	0	0	15
Humerus	0	12	4	27	72	0	115
Jaw	2	0	8	14	5	0	29
Metacarpal	0	0	65	39	3	3	110
Metatarsal	0	0	35	44	3	3	85
Patella	0	0	3	15	3	3	24
Pelvis	0	0	9	20	0	0	29
Phalange	0	0	12	36	3	1	52
Radius	0	0	4	11	2	0	17
Rib	0	0	49	32	3	5	89
Sacrum	0	0	3	7	0	0	10
Scapula	0	0	3	6	0	0	9
Sternum	0	10	3	9	0	0	22
Tarsal	0	0	21	77	5	6	109
Tibia	22	0	71	74	60	3	230
Tooth	7	105	101	44	5	13	275
Ulna	0	0	7	9	17	0	33
Unknown	0	0	0	4	0	0	4
Vertebrae	0	0	44	27	3	3	77
Total	105	152	1118	766	319	45	2505



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