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Advancing forensic RNA profiling

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Chapter 8

DNA and RNA profiling of excavated human remains with varying postmortem intervals

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Abstract

When postmortem intervals (PMIs) increase such as with longer burial times, human remains suffer increasingly from the taphonomic effects of decomposition processes such as autolysis and putrefaction. In this study, various DNA analysis techniques and a messenger RNA (mRNA) profiling method were applied to examine for trends in nucleic acid degradation and the postmortem interval. The DNA analysis techniques include highly sensitive DNA quantitation (with and without degradation index), standard and low template STR profiling, insertion and null alleles (INNUL) of retrotransposable elements typing and mitochondrial DNA profiling. The used mRNA profiling system targets genes with tissue specific expression for seven human organs as reported by Lindenbergh et al. (Int J Legal Med 127:891-900, [27]) and has been applied to forensic evidentiary traces but not to excavated tissues. The techniques were applied to a total of 81 brain, lung, liver, skeletal muscle, heart, kidney and skin samples obtained from 19 excavated graves with burial times ranging from 4 to 42 years. Results show that brain and heart are the organs in which both DNA and RNA remain remarkably stable, notwithstanding long PMIs. The other organ tissues either show poor overall profiling results or vary for DNA and RNA profiling success, with sometimes DNA and other times RNA profiling being more successful. No straightforward relations were observed between nucleic acid profiling results and the PMI. This study shows that not only DNA but also RNA molecules can be remarkably stable and used for profiling of long-buried human remains, which corroborate forensic applications. The insight that the brain and heart tissues tend to provide the best profiling results may change sampling policies in identification cases of degrading cadavers.

Introduction

The determination of a postmortem interval (PMI; also known as time since death (TSD)) is an important aspect in forensic death investigations. Traditionally, the PMI is estimated based on various physical and biochemical changes occurring shortly after death, such as rigor, algor and livor mortis [1, 2]. These factors can, however, only be used for estimating relatively short PMIs with a wide window of estimation [2, 3]. More recently, studies have been performed to investigate postmortem stability of nucleic acids and search for trends relating nucleic acid degradation and PMI [4–15]. The analysis of hard tissue such as the bone and dental tissues are described to be more favourable for DNA analysis due to the relatively higher stability of nucleic acids in these tissue types [11]. Processing of these types of samples, however, is time consuming and labour intensive, which makes the analysis of soft tissue favourable [11]. Especially soft postmortem tissues are affected by decomposition processes such as autolysis and putrefaction [2, 15]. The rate of decomposition is known to be influenced by external variables such as type of clothing, presence of a body bag and/or coffin, soil type, burial depth, water context, ambient temperature, weather conditions, air circulation, accessibility to insects, body mass index (BMI) of the deceased, agonal state and microbiome composition [5, 16–18]. Moreover, it is described that different tissue types may be affected differently by these degradation processes, as tissues are differentially shielded from the external factors or microbial sources and because enzymes tend to be more active in tissues such as the kidney and liver, resulting in putrefaction, thus early DNA degradation in these tissue cells [3].

DNA profiling of postmortem tissues mainly aims for genetic identification through short tandem repeat (STR) profiling [3–5, 10, 11, 14]. Besides, messenger RNA (mRNA) profiling has become increasingly utilized in forensics in the past few decades [19]. mRNA profiling uses markers targeting mRNA transcripts of gene combinations predominantly expressed in, and thus characteristic for, specific cells or conditions. This technique is commonly applied for the identification of body fluids [20–26], and more recently years also for the inference of organ tissue type [27]. Few studies have been performed to investigate the applicability of RNA profiling in postmortem human tissues to investigate RNA degradation as a possible indicator of the postmortem interval [18, 28–31] or to determine the cause of death [32]. In this current study, we apply various DNA analysis techniques (*i.e.* DNA quantification [33–35], STR profiling, InnoTyper profiling [35, 36], mitochondrial DNA (mtDNA) SNaPshot [37]) and mRNA profiling to search for trends relating nucleic acid degradation and PMI in 81 exhumed human organ tissues (*i.e.* brain, lung, liver, skeletal muscle, heart, kidney and skin samples) with burial times/PMIs ranging from 4 to over 42 years.

Materials and methods

Sample collection

The organ specimens used in this study were collected during exhumations at two cemeteries in The Netherlands. The reason for evacuation of the graves was redevelopment of the cemetery. In The Netherlands, a space for a grave is made available for 15 or 20 years. Exhumations were as in forensic setting, performed by a forensic anthropologist MD and a forensic archaeologist with necessary approvals from the required ethical examination commissions and the municipality. In total, 81 organ tissue samples comprising brain, lung, liver, skeletal muscle, heart, kidney and skin were collected from 19 exhumed human remains. Organ tissues were collected in plastic storage containers and stored at -80°C for 7.5 years until processed. Due to the varying states of decomposition of the bodies, it was not always possible to find and thus collect all organ types from all bodies. Burial times of the donors, ranged between 4 and over 42 years. Additional information regarding the different donors, such as burial times, sex and age, are provided in Table 1.

For three of the donors, the burial times are unknown, and it is expected that these samples range between 23 and 42 years, as this is the trend for the remaining donors buried at this cemetery. Table 1 additionally includes details regarding the state of decomposition according to the decomposition Staging Scale [38] and the condition of the human remains. The decomposition scale uses scores ranging from 1 to 10, in which 1 represents a complete fresh body and 10 represents a completely skeletonized body. The condition of the preservation of the skeleton is rated from 1 to 4, in which 1 represents whole and visually undamaged skeletal elements and 4 represents skeletal elements that are reduced to a powdery substance.

DNA/RNA extraction, DNA quantification

In preparation for DNA/RNA co-extraction, small tissue sections (approximate size 3 mm³) were excised from the centre of a tissue sample. These sections were finely cut and transferred to 2-mL tubes to improve cell lysis. The excised tissue sections were weighed prior to isolation in order to determine the DNA concentration per gram of crude tissue for each sample. After excision, the tissues immediately underwent cell lysis according to the protocol described in Ref. [20] by using 600 μL lysis binding buffer (Ambion), 40 μL proteinase K (20 mg/mL, QIAGEN) and incubating at 56°C for 2 h. After 30 min of incubation, an additional 40 μL proteinase K was added to samples that seemed not completely lysed. After cell lysis, lysates were stored at -80°C until further processing according to the DNA/RNA extraction as described in Ref. [20], in which residuals of tissue debris are removed by the use of QIAshredder columns.

Table 1 Information regarding burial and exhumation conditions for the 19 donors from whom samples were collected for this study.

										Tissues included								
Donor	Burial ground	Soil type	# years buried	Gender	Age at death	Stage of decomposition ^a		Condition skeleton	Depth interment (cm)	Additional information	Brain	Heart	Kidney	Lung	Skeletal muscle	Liver	Skin	
1	A	Sand	4y6m ^b	M	82y3m	7	I	80	Water in coffin	✓	✓	✓	✓	✓	✓	✓		
2			4y6m	M	83y10m	7	I	80	Water in coffin	✓	✓	✓	✓	✓	✓	✓		
3			4y10m	M	84y3m	7	I	80	Water in coffin	✓	✓	✓	✓	✓	✓	✓		
4			5y2m	M	83y10m	7	I	80	Water in coffin and mould growth on waterline	✓	✓	✓	✓	✓	✓	✓		
5			5y4m	M	88y2m	7	I	80	Water in coffin	✓	✓	✓	✓	✓	✓	✓		
6			5y6m	M	80y4m	7	I	80	Water in coffin	✓	✓	✓	✓	✓	✓	✓		
7			6y11m	M	79y5m	7	I	80	Water in coffin	✓	✓	✓	✓	✓				
8	B	Sandy loams with gravel inclusions	23y9m	M	-	8	2	110							✓		✓	
9			23y9m	F	-	9	2.5	220									✓	
10			26y6m	F	-	8	I	120		✓					✓			
11			30y4m	F	-	8	2	120	Underwent autopsy prior to burial	✓	✓		✓	✓			✓	
12			36y6m	M	-	8	I	100		✓	✓	✓	✓	✓	✓	✓	✓	
13			37y3m	M	-	8	I	180		✓			✓	✓	✓			
14			39y	-	-	8/9	2	180							✓			
15			40y4m	F	-	7/8	1/2	120							✓		✓	
16			42y4m	F	-	8	3	120									✓	
17			-	M	-	7/8	I	180		✓	✓	✓	✓	✓	✓	✓	✓	
18			-	F	-	7/8	I	80								✓		✓
19			-	F	-	7	I	80		✓	✓	✓			✓	✓	✓	✓
Number of samples per tissue type											13	11	10	11	17	10	9	

^aAccording to the Decomposition Staging Scale described in Ref. [38].^by/ representing the number of years, m/ representing the number of months.

Small adjustments were made to the protocol described in Ref. [20] regarding elution volumes, which were reduced to 50 μ L for DNA and 40 μ L for RNA extracts (compared to 100 and 60 μ L, respectively). DNase treatment and DNA quantification (*Alu* assay [33]) were performed, according to protocols described in Ref. [20]. Besides the *Alu* assay quantification system, an additional quantification was performed using the InnoQuant™ Kit (InnoGenomics). The InnoQuant system is one of the more novel quantification systems which, in addition to a total human DNA quantification, provides degradation and inhibition analysis [34, 35, 39]. The system can accurately measure the DNA quantity in a sample down to 1 pg/ μ L (compared to 0.5 pg/ μ L for the total DNA marker and 4 pg/ μ L for the male DNA indicator in the *Alu* assay, as determined in house), and DNA concentrations are determined by amplification of two separate high copy number nuclear DNA targets, i.e. a short (80 bp) *Alu* target and a long (207

bp) SVA target. A degradation index (DI) indicating the degree of DNA degradation in a sample is determined by calculating the ratio between the quantitative values of the short target and the long target ($DI = [\text{short}/\text{long}]$). A DI value of one implies no degradation in a sample; increased DI values imply degradation. A synthetic target is simultaneously amplified as internal positive control (IPC) to assess for the presence of PCR inhibitors. The IPC is an indicative of a PCR inhibitor when cycle threshold (Ct) values of the IPC in a sample exceed the mean IPC Ct value for the dilution standards by more than two units [34, 35]. InnoQuant quantification was performed according to manufacturer's instructions using 2 μL DNA extract per reaction during a 32-cycle PCR on a 7500 Real-Time PCR system (Applied Biosystems). Data analysis was performed using SDS v2.3 with a Ct set as described by manufacturer's instructions [34]. Maximal PCR efficiency (100 % efficiency equals a slope of -3.3) was acquired by applying empirically established baseline settings (3–11 for the short fragment, 3–12 for the long fragment and 3–15 for the IPC). When an indication for inhibition was obtained (Ct values above 34 for the *Alu* assay or based on IPC for the InnoQuant), tenfold dilutions were prepared and submitted to quantification.

STR profiling

STR amplification was performed on all 81 DNA extracts using the AmpF ℓ STR $^{\circ}$ NGM $^{\text{TM}}$ PCRAmplification Kit (Life Technologies) during a 29-cycle PCR and a maximum of 500 pg DNA input (or 10 μL) based on quantification results as described in [20]. For samples with low DNA concentrations (i.e. below 0.05 ng/ μL), the maximum input of 10 μL DNA extract per reaction was used. PCR products were separated according to standardized protocols [20] using a 3130XL Genetic Analyser (Life Technologies) with POP-4 (Life Technologies) separation matrix and 3 kV, 15 s injection settings. Profile analysis was performed using Genemapper ID-X version 1.1.1 (Life Technologies), a detection threshold of 50 relative fluorescence units (rfu) and locus-specific stutter ratio thresholds as described in Ref. [40]. Samples resulting in incomplete profiles with less than eight detected alleles were subjected to +5-cycle NGM PCR amplification [40]. Amplification products were separated and analysed as described above using 1.5 $\times$ stutter filter thresholds [40]. Samples resulting in profiles with less than eight detected alleles in the 29 + 5-cycle STR profiles were subjected to mtDNA analysis using a SNaPshot assay [37]. The percentage of detected alleles in the STR profiles was determined considering the 15 STR loci in the NGM kit (Amelogenin excluded). When no reference profile could be deduced from the profile set for a donor (for instance when none of the tissues presented a full profile), single allele calls were regarded as heterozygote alleles accompanied with a drop-out. Degradation rates of the STR profiles were calculated based on ski slope measurements by comparing the average peak height of long (>225 bp) versus short (<225 bp) alleles.

InnoTyper profiling

InnoTyper profiling was performed for all 81 DNA extracts using the InnoTyper[®] 21 Human DNA Analysis Kit (InnoGenomics) [35, 36]. A maximum of 500 pg or 16 µL DNA extract was used for amplification during a 31-cycle PCR according to manufacturer's instructions. PCR products were separated according to manufacturer's instructions using a 3130XL Genetic Analyser (Life Technologies) with POP-4 (Life Technologies) separation matrix and 3 kV, 15 s injection settings. Profile analysis was performed using Genemapper ID-X version 1.1.1 (Life Technologies) and a detection threshold of 50 rf. The percentage of detected alleles was determined considering the 20 bi-allelic loci (Amelogenin excluded).

mtDNA SNaPshot

Mitochondrial DNA SNaPshot was performed on 14 of the DNA extracts for which less than eight alleles were detected using enhanced (29 + 5-cycles) NGM profiling. Reference samples (DNA extract of different tissue type for the same donor for which STR profiling was successfully applied) were included in mtDNA SNaPshot analyses.

Amplification of mtDNA fragments was performed as described in Ref. [37] using the mini-mtDNA method [41, 42]. This method aims to amplify ten overlapping mini-amplicons covering the entire control region in two multiplex PCR assays (set 1 and set 2). Due to limited amounts for the DNA extracts, only multiplex set 1 was used for which 5 µL extract was amplified in a reaction volume of 50 µL [37]. Minor adjustments involved the increase of the number of PCR cycles by 1 and adjustments in primer concentrations. mtDNA product formation was checked using QIAxcel capillary electrophoresis [37]. Prior to performing the single-base extension (SBE) PCR, the mtDNA PCR products were purified by using ExoSAP-IT[®] (Affymetrix) according to manufacturer's protocol.

The SNaPshot PCR was performed as described in Ref. [37] by using SBE-primers in combination with fluorescently labelled ddNTPs to extend the SBE-primers at the single nucleotide polymorphisms (SNP) position. These 18 SBE-primers are equally divided over two assays (SBE set 1 and 2) that match the two mini-mtDNA PCR sets. However, as the two mini-mtDNA PCR multiplexes have overlapping amplicons, quite some SBE-primers selected for set 2 also function with set 1 PCR products and vice versa. Thus, when both SBE-primer sets are (separately) applied to mini-mtDNA set 1 PCR products, 15 of the 18 SNPs can be analysed (nine set one SNPs: 16270, 16278, 16519, 195, 16362, 185, 16294, 182 and 16311 and six set 2 SNPs: 16223, 16129, 16126, 150, 146 and 152). Unincorporated ddNTPs were removed by using Shrimp Alkaline Phosphatase (2U, Affymetrix) according to manufacturer's protocol. Fragments were separated and detected as described in Ref. [37] on a 3130XL Genetic

Analyser (Life Technologies) using POP-4 (Life Technologies) separation matrix and 1.2 kV, 15 s injection settings. Profile analysis was performed using Genemapper® v4.0 (Life Technologies) with a detection threshold of 50 rfu and an allele balance cut-off value of 0.3. The percentage of detected SNPs was determined considering a maximum of 15 SNPs.

RNA profiling

Organ type inference was performed on all 81 RNA extracts using an updated version for an in-house developed multiplex (van den Berge and Sijen, manuscript in preparation), which allows for the inference of brain, lung, liver, skeletal muscle, heart, kidney and skin tissues. All RNA extracts, regardless of DNA quantification results [33], were subjected to ethanol precipitation prior to reverse transcription to maximize profiling results (and using the knowledge that the reverse transcription reaction can take up to 2 µg RNA, which will not be reached with the small tissues sections used for extraction). Ethanol precipitation, reverse transcription, PCR amplification and product detection were performed according to standardized protocols [20]. After reverse transcription, a serial input of 0.5 and 5 µL cDNA was used in the PCR to determine the input providing an informative RNA profile. Supplementary PCRs were performed to obtain three informative PCR replicates per sample. PCR products were purified [20] prior to detection using a 3130XL Genetic Analyser (Life Technologies). Amplification products were analysed using POP-4 (Life Technologies) separation matrix and 3 kV, 10 s injection settings. Profile analysis was performed using Genemapper ID-X version 1.1.1 (Life Technologies) with a detection threshold of 150 rfu.

RNA data interpretation was performed according to the “ $x=n/2$ ” rule as described in [43]. This method compares the number of observed (x) to the number of theoretically possible peaks (n) in all replicates. A cell type is scored “observed” when at least half of the possible peaks are observed ($x \geq n/2$), denoted “sporadically observed” when less than half of the possible peaks are observed ($0 < x < n/2$) and scored “not observed” when no peaks are detected ($x=0$).

Results

DNA quantification

For this study, a total of 81 decomposed organ tissues were analysed comprising 13 brain, 11 lung, 10 liver, 17 skeletal muscle, 11 heart, 10 kidney and 9 skin samples. Two highly sensitive DNA quantitation methods were used: firstly, the *Alu* assay described in Ref. [33] that provides accurate quantification results down to 0.5 pg/µL total human

DNA (Table 2). For eight samples, quantification results below this detection threshold were obtained. These samples represent three skeletal muscle, two liver, two skin and one heart sample (indicated "«" in Table 2). Although these samples most likely do not carry human DNA, they were processed like the samples that have detectable human DNA concentrations. As each excised sample was weighed prior to isolation, DNA yields could be normalized and presented in nanograms DNA per gram of isolated tissue for each sample (Table 2). The first six donors presented in Table 2 all provided each organ type except skin tissue and had been buried for approximately similar times (4 years and 6 months–5 years and 6 months). When comparing DNA yields per gram crude tissue for the different organ tissues within one donor, none of the organs stands out to produce the highest yield, as large variations in yields are observed for all tissue types. When regarding DNA yields for samples with longer burial times, less samples with high yields are seen although various samples have good yields; thus, indicating that DNA yields do not necessarily decrease with increased burial times (Table 2). The *Alu* assay also carries a male DNA indicator, which is less sensitive than the total indicator as it is based on much less repetitive units [33]. Nevertheless, male gender was correctly indicated for all samples with a concentration above the detection threshold (4 pg/μL).

Secondly, a novel quantitation system named the InnoQuant™ kit by InnoGenomics [34, 35] was used. The InnoQuant kit provides quantitative analysis and additionally determines a degradation index (DI) for quality and an internal positive control (IPC) for integrity, which is highly beneficial for forensic samples that are often degraded [34, 35]. The *Alu* assay and InnoQuant results follow the same trend (with InnoQuant 10 samples remain under the detection level including the eight samples for which the *Alu* assay did not return a quantification result), although differences are seen, which can be explained by the use of different repetitive elements in both systems. Most added value of the InnoQuant lies in providing a DI and assessing for PCR inhibitors. The DI is determined based on the ratio of quantitative values for the long and short amplicons and are presented in Table 2. For only a few samples, no degradation is detected (14 %). For the majority of samples, the DI indicates moderate (DI 2.5–20; 56 %) to severe (DI >20; 9 %) degradation, while for the remaining 22 %, no DI could be determined due to drop-out of the long fragment (which indicates very severe degradation). The skeletal muscle appears to be the organ type resulting in the most degraded DNA, as for 41 % of the skeletal muscle samples, no DI could be determined, followed by liver, skin, brain, kidney and heart tissues. Based on IPC results, two samples showed indication of PCR inhibitors (brain donor I 1 and I 3, data not shown). For one of these samples, inhibition was also observed using the *Alu* assay (undetermined Ct value).

Table 2 Overview of the DNA and RNA profiling results obtained after analysing 81 postmortem organ tissues collected after varying burial times.

Donor	# years buried	Input NGM (pg)					% detected alleles NGM					STR profile degradation					InnoQuant degradation					% detected alleles InnoTyper					RNA														
		Brain	Heart	Kidney	Lung	Skeletal muscle	Liver	Skin	Brain	Heart	Kidney	Lung	Skeletal muscle	Liver	Skin	Brain	Heart	Kidney	Lung	Skeletal muscle	Liver	Skin	Brain	Heart	Kidney	Lung	Skeletal muscle	Liver	Skin												
1	4y6m																																								
2	4y6m																																								
3	4y10m																																								
4	5y2m																																								
5	5y4m																																								
6	5y6m																																								
7	6y11m																																								
8	23y9m																																								
9	23y9m																																								
10	26y6m																																								
11	30y4m																																								
12	36y6m																																								
13	37y3m																																								
14	39y																																								
15	40y4m																																								
16	42y4m																																								
17	-																																								
18	-																																								
19	-																																								
Average		392	269	296	238	120	167	84	91	84	83	54	55	42	0.6	0.6	0.5	0.6	0.5	0.3	0.4	6.8	7.4	6.5	11.9	3.6	34.2	4.8	97	91	95	93	65	75	54	54	45	20	41	20	11
Input NGM (pg)		≥500					499-250					249-50					<50					«					«					«					«				
% detected alleles NGM		≥90					89-30					29-5					<5					5					5					5					5				
STR profile degradation		>0.7					0.7-0.5					<0.5					n.d.					»					»					»					»				
InnoQuant degradation		<2.5					2.5-20					>20					n.d.					»					»					»					»				
% detected alleles InnoTyper		≥90					89-30					29-5					<5					n.d.					n.d.					n.d.					n.d.				
RNA		Obs.					Sporadically Obs.					Not Obs.					Not Obs.					Not Obs.					Not Obs.					Not Obs.					Not Obs.				

STR profiling

NGM profiling was performed for all 81 tissue samples. Not all DNA yields allowed for a full 500 pg DNA input, and Table 2 shows the range in pg DNA that was submitted to NGM profiling (either 500 pg DNA or 10 μ L DNA extract). For 78 % of the samples, informative profiles with eight alleles or more were obtained after performing the standard 29- cycle PCR. PCR products of the 18 samples that resulted in profiles with seven or less detected alleles underwent an additional 5-cycle amplification (indicated “5” in Table 2). These include the eight samples with quantification results below the detection threshold of the *Alu* assay (section “DNA quantification”). As a result of the additional cycles, the percentage of samples with informative profiles (eight alleles or more) increased to 83 %. Besides the final percentage of detected alleles for each sample, Table 2 shows the average percentage of detected alleles for each organ type, from which we infer that the brain is the most promising organ tissue to be used for DNA profiling followed by heart, lung and kidney, liver, skeletal muscle and lastly skin tissue. STR profiles were additionally used to determine degradation rates by comparing the average peak height of short (75 to 225 bp) versus long (225 to 375 bp) alleles. For 16 % of the samples, this ratio was above 0.75, which indicates no or little degradation. The majority of samples showed moderate (ratio 0.1–0.75, 70 %) or severe (“n.d.”, no long fragments detected, 14 %) degradation in the profiles. On average, brain, heart and lung samples show the least degraded profiles, followed by skeletal muscle and kidney, skin and lastly, liver samples.

InnoTyper profiling

For all 81 samples, additional autosomal profiling was applied through the InnoTyper™ 21 kit [35,36]. This kit targets genomic sites known to carry variation for the presence or absence of retrotransposable elements (RE) and amplifies either the insertion or the null (INNUL) allele. Primer design achieved small amplicons (60–124 bp) for both allelic states [35,36]. The InnoTyper kit is described to be highly sensitive, tolerant to degradation and inhibition and applicable to extremely degraded or low template samples. The kit accommodates 16 μ L extract as input, due to which numerous samples received a higher DNA input than with NGM profiling that accommodates 10 μ L input. The percentage of detected alleles was determined based on the 20 bi-allelic INNUL markers. An overview of the results can be found in Table 2. Full InnoTyper profiles were obtained for 65 % of the samples. The lowest input used to obtain a full profile was 47 pg. However, with this sample set that comprises many severely degraded samples, a certain DNA input will not guarantee a full profile, as for example, an allele detection percentage of 50 % was obtained for an input of 9 pg DNA and 53 % detected alleles for an input of 336 pg. Overall, higher average allele detection percentages were obtained compared to those obtained using NGM profiling (Table 2).

mtDNA SNaPshot

Furthermore, mtDNA analysis was performed. Due to the much higher copy number for mitochondrial DNA molecules than for genomic DNA (gDNA), mtDNA profiling is commonly applied to samples that lack or have severely degraded gDNA [44]. We used an mtDNA SNaPshot system described in Ref. [37] that uses SBE primers to target 18 SNPs with a high discriminatory power in a European Dutch population. The mtDNA SNaPshot was applied to 14 samples for which seven or less alleles were detected using enhanced NGM profiling (indicated “>” in Table 2). These samples are mainly skeletal muscle, liver and skin samples, including the eight samples with gDNA quantification results below the detection threshold of the quantification system (section “DNA quantification”). The percentage of detected SNPs was determined by comparing SNaPshot results of the 14 samples to corresponding reference samples. For all 14 samples, 100% of the expected SNPs could be typed (data not shown), and we infer that full mtDNA SNaPshot profiles would be achieved for all samples as these 14 samples represent the samples performing worst in autosomal profiling.

RNA profiling

mRNA profiling for the inference of seven organ tissue types was performed on all 81 RNA extracts, and the results are presented in Table 2. The brain appears the most successful organ to be sampled for mRNA tissue profiling, as samples were scored “observed” in 54 % of the brain tissue samples, followed by heart, skeletal muscle, kidney and liver, lung and lastly skin tissues. There is no clear relation between the burial time and the mRNA profiling results, as can be seen for example when regarding the heart tissue results. Overall, the skin appears to be the least successful tissue type to use for mRNA profiling, although only relatively old (23 years and up) samples were collected for this tissue type. Notwithstanding, a skin sample was scored observed which had been buried for over 40 years.

Discussion and concluding remarks

This study aims to search for trends in nucleic acid degradation of exhumed organ tissue samples with increased PMIs. For this purpose, DNA/RNA co-extractions were performed on 81 exhumed organ tissues with PMIs ranging from 4 to over 42 years. A summary of the outcome of various DNA and mRNA profiling techniques can be found in Table 3. Although kidney tissue presents the highest DNA yield on average, the organ does not always present the highest DNA yield when the various tissues for the same donor are regarded, which is consistent with results described in Ref. [5].

Table 3 DNA concentrations in ng per gram crude tissue.

Burial ground	Donor	# years buried	Brain	Heart	Kidney	Lung	Skeletal muscle	Liver	Skin
A	1	4y6m	3.74	267.51	0.36	6.24	0.69	0.11	
	2	4y6m	22.79	110.70	323.73	10.08	1.65	33.64	
	3	4y10m	139.83	7.47	24.71	145.98	0.82	2.06	
	4	5y2m	148.37	5.80	7.15	1.75	45.98	0.00	
	5	5y4m	22.99	36.40	1544.66	11.72	80.24	19.06	
	6	5y6m	71.95	134.39	746.36	0.29	6.11	9.84	
	7	6y11m	170.45	82.41	16.40	1354.27	15.11		
B	8	23y9m					0.21		32.15
	9	23y9m							4.83
	10	26y6m	6.80				0.97		
	11	30y4m	44.29	0.72		1.33	0.25		2.83
	12	36y6m	79.27	0.00	3.71	4.62	0.00	0.00	0.25
	13	37y3m	4.34			25.96	4.98	3.06	
	14	39y					0.00		
	15	40y4m					0.71		6.82
	16	42y4m							0.54
	17	-	345.71	8.61	5.48	177.09	0.68	0.89	0.00
	18	-					0.00		1.64
	19	-	66.57	7.17	5.98		12.16	6.50	0.00

Additionally, DNA yields did not always decrease with increased burial times, implying there is no straightforward relation between DNA concentration and the PMI. The degradation index provided by the InnoQuant kit and the peak heights at long and short amplicons in the STR profiles were used to determine the DNA degradation level in a sample.

As shown in Tables 2 and 4, varying degrees of degradation are observed for the different tissue types; such as a tendency for more degradation in liver and kidney and less

degradation in brain tissue. This variation can be explained by various factors such as the high activity of hydrolysis enzymes in liver tissue [14], the high degree of autolysis in kidney tissue [15], a low tissue turnover rate and poor source of digestive enzymes in brain tissue plus a well-protected anatomical location of the brain in the body [9, 14, 45]. This also explains why certain tissue types with high DNA inputs show degradation, while other samples with lower inputs show less degradation (e.g. Table 2 input and profiling results for liver and kidney versus brain tissue in donor 5). The InnoQuant kit additionally provides an IPC value to indicate the presence of PCR inhibitors. Inhibition was observed only in a few brain samples, which may be caused by increased levels of proteins and inhibiting substances present in cerebrospinal fluid [46, 47]. The inhibiting effect could be overcome by reanalysing these samples with less input.

The INNUL profiling system appears of added value for analysing severely degraded samples, as the kit slightly outperforms the NGM STR profiling kit based on the percentage of detected alleles. This seems to be due to the small amplicon sizes and the larger DNA extract volume that can be added as PCR input. However, the InnoTyper kit performed less well than the mtDNA SNaPshot assay that resulted in full profiles for all samples for which gDNA profiling was unsuccessful. There is a much higher copy number for mtDNA than gDNA [44] although the mtDNA/gDNA ratio

Table 4 Ranking of the different tissue types for the different analysis methods. Tissues are ranked from 1 (best) to 7 (worst).

	DNA concentration / g tissue	InnoQuant degradation	% detected alleles NGM	STR profile degradation	% detected alleles InnoType	RNA
Brain	3	1-3	1	1-3	1	1
Heart	2	1-3	2	1-3	4	2
Kidney	1	1-3	3-4	4-5	2	4-5
Lung	4	4	3-4	1-3	3	6-7
Skeletal muscle	5	7	6	4-5	6	3
Liver	6	6	5	7	5	4-5
Skin	7	5	7	6	7	6-7

can vary between tissues and is described to be specifically high in tissues with higher ATP requirements (e.g. skeletal and heart muscle) [44, 48] and in liver tissue, where hepatocytes are described to have high number of mitochondrial genomes [11].

It is stated that RNA is less stable than DNA because of the hydroxyl group at the 2' position of the ribose sugar, which makes RNA more prone to hydrolysis than DNA [49, 50]. This hydroxyl group and the fact that G-U base pairing occurs within RNA molecules allow RNA to form secondary and tertiary structures [49–51], which may explain the remarkable stability in the analysed postmortem tissues.

Stability can be transcript-dependent due to the structure at the 3' region [52, 53]. Furthermore, the presence of ribonucleases may vary in tissues, which affects overall RNA abundance. Correspondingly, the lowest RNA stability was found in lung and skin samples, which are known to be ribonuclease-rich organs [18]. Studies have previously described a correlation in transcript analysis in various tissue types and the PMI [18, 54] using (compared to this study) relatively short PMIs up to 11 h [18] and various factors are described to significantly affect mRNA expression levels, such as the agonal state [55], sex, or age at death [56], but these relations were not straightforwardly observed in this study.

The results of this study encourage the use of brain and heart tissue for postmortem DNA and RNA profiling. The association of DNA and/or RNA degradation and the postmortem interval is discouraged, as no trends were observed for the aspects regarded. Evidently, many factors both pre- and postmortem have a role in degradation and relations may >occur for factors not assessed in this study, such as the effect of taphonomic differences between the cemeteries and individuals, which may strongly influence determination of the PMI [16]. Furthermore, no relations were observed between DNA and RNA profiling success, which is in concordance with previous studies [57] and important knowledge for the interpretation of combined RNA and DNA profiling data.

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