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Studying the Role of Soil Factors on Degree of Degradation of DNA of Human Remains

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Abstract Generating a DNA profile using autosomal Short Tandem Repeat (STR) DNA allele from skeletal remains is a crucial step in the identification process for mass disaster and unidentified person situations. The capacity to extract substantial amounts of relevant DNA from bones, which were highly durable biological components that can withstand soil elements and climatic conditions over long periods of time, would be advantageous. 70 femur male bone samples were collected from two different environmental mass graves (Al-Mahaweel massgrave) in Babil governorate and (Al-Saqlawiya massgrave) in Al-Anbar governorate in Iraq. The DNA from bone samples extracts were assessed by capillary electrophoresis and a standardized set of short tandem repeat (STR) loci are analyzed using Mutiplex 21 STR loci System to allow human identification by matching the profile of the dead individuals with their relatives. From these two different sites; soil samples were collected to analyze and study the different environmental factors and find a relation between the degree of preservation or degradation with these factors like temperature, humidity, salinity, PH and texture of soil and find how these factors can affect on pattern of degradation of DNA profiles. The average of the percentage of the detected alleles in each locus in the two sites; were 87% for Al-Mahaweel site and 32% for Al- Saqlawiya site. we found that clay soil, low salt concentration, PH slightly alkaline and low humidity for Al-Mahaweel site; all participate in DNA preservation while the chalky and very high salt concentration of Al-Saqlawiya site soil; all participate in DNA degradation. Predicting the effect of these factors on the quality of the DNA profiles can be helpful in finding better solutions for many complications that may face the process of analyzing samples and finding sufficient informative profiles.

Key Words skeletal remains, mass grave, DNA degradation, STR loci, soil factors

1. Introduction

Forensic anthropology utilizes the methods of physical anthropology to examine skeletal remains, especially in cases where the remains are in a state of decomposition or their identity is unknown. DNA fingerprinting is useful in determining relationships between individuals [1], [2] It can establish paternity, confirm the parents of adopted children [3], [4], resolve family disputes [5], and even settle inheritance cases [6]. STR typing involves the general steps for DNA profiling in the following order: isolation of DNA by a process called DNA extraction, quantification of the DNA in the sample, amplification of STR loci, separation of the PCR amplicons on a genetic analyzer using bioinformatics to analyze the resulting data and comparing the data from one specimen to databases housing previously generated STR sets [7]. Repetitive DNA sequences with varying numbers of repeats, referred to as STR loci, are amplified using primers with differently colored fluorophores and these amplicons are distinguished by both size and color [8]. These STR loci are widely used in forensic DNA analysis due to their high polymorphism [9], [10]. The Mass Graves Department in the Iraqi Medico-Legal directorate that was established in 2010 work to identifying the missing individuals and it has developed its capacity in forensic anthropology and forensic genetics to recover and identify more individuals [5]. In international law, the term "mass grave" is not officially defined, but it generally refers to a site where multiple human remains are buried. In Iraqi national law, a "mass grave" is defined as a land or location that contains the remains of more than one victim, who were buried or hidden. Identifying individuals from mass graves can be complicated due to different factors that affect the process [11]. Mass grave identification is challenging due to decomposition, soil activity, and lack of witnesses [12]. One limitation is the difficulty in obtaining

a DNA profile from bones that have undergone extensive diagenesis. The destructive burial environment can prevent the production of a usable DNA profile [13]

2. Material and Methods

70 femur bone samples were taken from remains of adult males' bodies stored in deep freeze (-80 C) in Iraqi Medico-Legal directorate lab, collected in 2011 after being buried for 20 years since they were killed by Saddam Hussein's regime in 1991.

These samples were recovered from two different environmental, soil type mass graves. 35 samples were taken from the Al-Mahaweel mass grave in Babil Governorate and 35 samples from the Al-Saqlawiya mass grave in Anbar Governorate.

Soil samples were taken from each mass grave, analyzed and studied at the National Center for Laboratories and Structural Research in Baghdad, which has the national accreditation ISO/IEC.TL009 according to standard specifications. The present study was carried out in Medico legal directorate in Baghdad in Iraq.

The skeletal samples displayed high levels of degradation and so they were impossible to get them identified by personal effects and other secondary methods of identification. The degradation processes rendered all the bodies in a deformed shape.

From these two different sites, soil samples were collected to analyze and study the soil environmental factors that affect the quality of DNA profiles and the intense of preservation or degradation of the DNA of these samples and the pattern of the profiles.

Many steps were performed according to the protocol used in DNA Labrotory for identification of mass graves victims in Medico legal directorate to extract DNA from bone samples.

Firstly, steps of preparing samples were performed including weighting between 0.5 and 1.0 g of bone powder taken from washed, purified and grinding bone [14]. Secondly extraction process was done in many steps involving using digestion buffer and Proteinase K to precipitate any bone debris by the centrifugation and taking the supernatant then Amicon Ultra were used to recover DNA by washing with ethanol, any residual ethanol was removed by centrifuging samples for 1 min at 13,000 rpm to elute the DNA.

Quantification of extracted DNA performed by using 7500HID Real Time PCR from Applied Biosystems and Quantifiler Human DNA quantification kit. The PCR is carried out using the Applied Bio systems 9700 thermo cycler as shown in Figure 1 under the recommended conditions provided by the manufacturer.

The Multiplex 21 STR loci System is utilized for various human identification applications, such as forensic analysis, relationship testing, and research. This system allows for the simultaneous amplification and detection of 21 loci using four-color fluorescent detection, as described in Table 1 and illustrated in Figure 2.

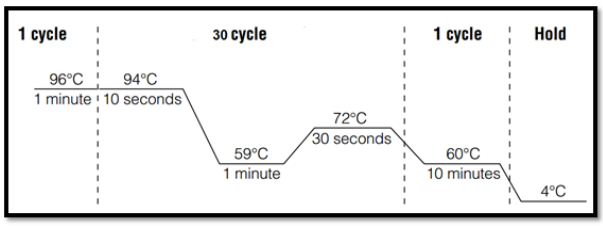

Figure 1: The thermal cycling protocol for the GeneAmp® PCR System 9700 thermal cycler for extracted DNA [15]

No.	STR Loci	Fluorescent Dye
1	D3S1358, D1S1656, D6S1043, D13S317, Penta E	Blue
2	D16S539,D18S51, D2S1338, CSF1PO, Penta D	Green
3	TH01, vWA, D21S11, D7S820, D5S818, TPOX	Yellow
4	D8S1179, D12S39, D19S433, FGA	Red

Table 1: Four-color fluorescent detection of 21 loci

The capillary electrophoresis procedure was conducted using a 3500XL Genetic Analyzer (Applied Biosystems, Foster City, USA) following the instructions provided by the manufacturer. An internal size standard called ILS-500 (Promega) was utilized for PowerPlex21 STR kit, the MLD uses the manufacturer's recommended volumes of reagents with Injection Voltage 1.2kV for 24 seconds, Performance optimizing polymer (pop4), Capillary Length 36cm and HID36_POP4 module run on an Applied Biosystems' 3500 xl Genetic Analyzer. The genotyping process was conducted using the GeneMapper ID-X v1.4 software developed by Applied Biosystems. Alleles were assigned based on the established naming system. If the off ladders fell within a specific range and the overall profile was considered to be of high quality, they were considered valid. Alleles that were not in a bin or virtual bin were re-run at least once before designation [15].

Profiles are considered reportable if results are obtained from a minimum of 18 STR loci and Amelogenin; homozygotes are called with a minimum Relative Fluorescent Unit (RFU) threshold of 300, and heterozygotes are called with a minimum RFU threshold of 50 and each allele should be confirmed by double amplification [16].

3. Results and Discussion

A. Case History

International organizations estimate that more than 400,000 Iraqis were killed by regime security forces during Saddam's rule, but some estimates are as high as 1 million. At least 270

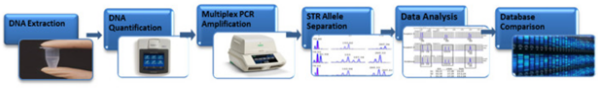

Figure 2: Work flow of STR typing

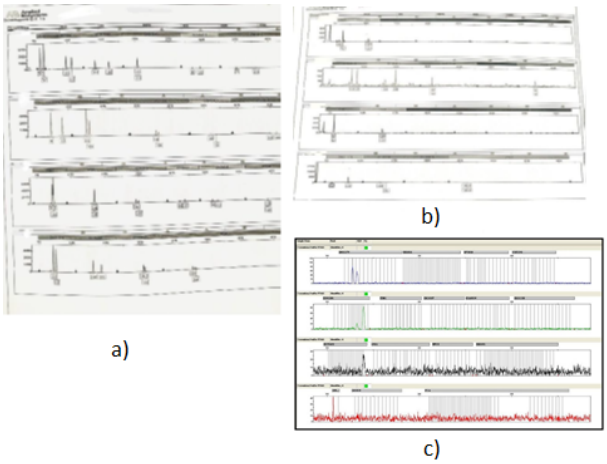

Figure 3: a-Represent complete profile. b- partial profile. c-failure profile

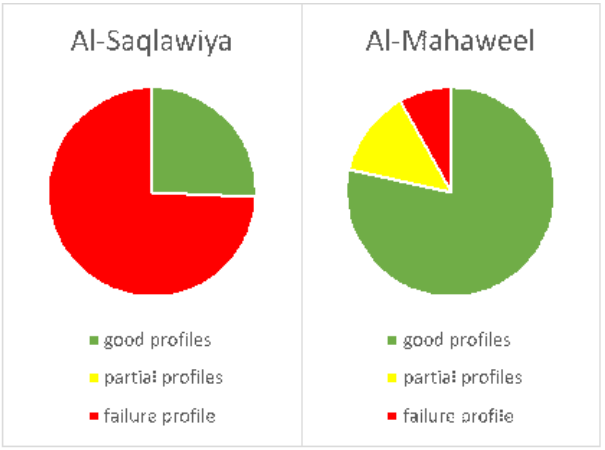

Figure 4: Represent the quality of DNA profiles that were recovered from bone samples from the two sites

mass graves have been reported by Iraqis and U.S. [17].

70 femur bone samples were taken from remains of adult males' bodies from two different sites mass graves to recover their DNA profiles and make a comparison between degree of degradation between the two sites which reflex the effect of soil factors on these profiles.

B. DNA Profiling

Bone samples that were taken from remains were received by DNA lab. Collection of samples, DNA Extraction, quantification, amplification and capillary electrophoresis, all these processes were done according to the standard operating procedure of DNA Laboratory for identification of missing persons in medico legal directorate MLD in Bagdad Iraq.

After getting DNA profiles for these bone samples, the tests of the soil samples, which included measuring soil salinity, acidity, soil texture and temperature, were compared with the pattern of the genetic fingerprints for each mass graves.

After categorizing these results of the profiles into 3 group; complete (or missing one or two locus), partial (missing more than 3 locus) and failed profiles (Figure 3 are examples for the three types of profiles) We conclude; The data from Al-Mahaweel were 29 complete (or missing one or two locus) DNA profiles obtained from different bone remains samples, 3 Partial (missing more than 3 locus) DNA profiles obtained from different bone samples, 3 bone samples failed to obtained any DNA profiles because they were highly degraded. The data from Al-Saqlawiya mass graves profiles: 9 Complete (or missing one or two locus) DNA profiles obtained from different bone samples. The rest 26 bone samples failed to obtained any DNA profiles because they were highly degraded. These results represented in (Figure 4).

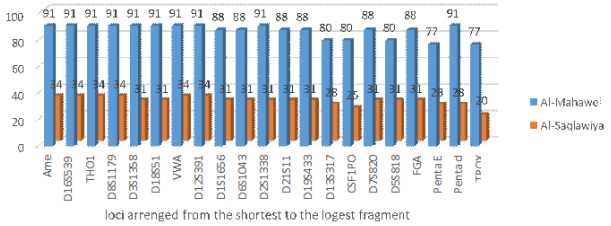

Figure 5: A Comparison of Percentage of alleles detection of each locus between Al- Mahaweel site samples and Al-Saqlawiya site samples

C. Statistical Analysis

Once profiles are obtained, statistical calculations are conducted on the data to evaluate which type of soil and environmental factors were more effective to cause degradation to the DNA of bone samples. After calculation the number of profiles succeeded to detect alleles in the mentioned locus in all the 35 profiles in each one of the mass graves sites, the percentage of each locus that succeeded to detect alleles can be calculated by application the following equation; the results are shown in Table 3.

$$= \frac{No.of profiles succeeded to detect alleles in the mentioned locus}{35(number of profiles taken from each site)}$$

After calculation the number of profiles that succeed to detect alleles in each mentioned locus in all the 35 profiles of each mass graves site, and calculating the percentage of each locus that succeeded to detect alleles: the results are shown in Table 2 and Table 3:

The data that were detailed in Table 3 were represented in Figure 5 gave simple understanding to the quality of profiles in each site reflecting the degradation and preservation degree and the effect of soil environmental factors on the percentage of detection of alleles of each locus. After calculation of the average of the percentage of the detected alleles in each locus in the two sites; were 87% for Al-Mahaweel site and 32% for Al- Saqlawiya site. This illustrate that the environmental

Locus	Number of DNA profiles that succeed to detect alleles of the mentioned locus in Al- mahaweel site	Number of DNA profiles that succeed to detect alleles of the mentioned locus in Al- saqulawiya site
AMEL	Detected in 32 profiles	Detected in 12 profiles
D3S1358	Detected in 32 profiles	Detected in 11 profiles
D1S1656	Detected in 31 profiles	Detected in 11 profiles
D6S1043	Detected in 31 profiles	Detected in 11 profiles
D13S317	Detected in 28 profiles	Detected in 10 profiles
Penta E	Detected in 27 profiles	Detected in 10 profiles
D16S539	Detected in 32 profiles	Detected in 12 profiles
D18S51	Detected in 32 profiles	Detected in 11 profiles
D2S1338	Detected in 32 profiles	Detected in 11 profiles
CSF1PO	Detected in 28 profiles	Detected in 9 profiles
Penta D	Detected in 32 profiles	Detected in 10 profiles
TH01	Detected in 32 profiles	Detected in 12 profiles
VWA	Detected in 32 profiles	Detected in 12 profiles
D21S11	Detected in 31 profiles	Detected in 11 profiles
D7S820	Detected in 31 profiles	Detected in 11 profiles
D5S818	Detected in 28 profiles	Detected in 11 profiles
TPOX	Detected in 27 profiles	Detected in 7 profiles
D8S1179	Detected in 32 profiles	Detected in 12 profiles
D12S391	Detected in 32 profiles	Detected in 12 profiles
D19S433	Detected in 31 profiles	Detected in 11 profiles
FGA	Detected in 31 profiles	Detected in 11 profiles

Table 2: Number of profiles that succeed to detect alleles in each mentioned locus in Al-Mahaweel and Al-Saqulawiya site

Locus	Percentage of the mentioned locus that succeeded to detect alleles in Al-Mahaweel site samples	Percentage of the mentioned locus that succeeded to detect alleles in Al-Saqulawiya site samples
AMEL	91%	34%
D3S1358	91%	31%
D1S1656	88%	31%
D6S1043	88%	31%
D13S317	80%	28%
Penta E	77%	28%
D16S539	91%	34%
D18S51	91%	31%
D2S1338	91%	31%
CSF1PO	80%	25%
Penta D	91%	28%
TH01	91%	34%
VWA	91%	34%
D21S11	88%	31%
D7S820	88%	31%
D5S818	80%	31%
TPOX	77%	20%
D8S1179	91%	34%
D12S391	91%	34%
D19S433	88%	31%
FGA	88%	31%

Table 3: Percentage of alleles detection of each locus in both sites

factor in A-Mahaweel site give more preserving for the DNA, while the environmental factors in Al-Saqlawiya site were more degrading for DNA.

D. Soil test results

Soil test results shows for Al-Mahaweel soil sample that the pH was 8.2, Humidity was 7.5%, the total soluble salts was 1.9% and the soil texture was clay Figure 6.

For Al-Saqlawiya soil the pH was 7.7, the humidity was 1.6%, the total soluble salts was 73% and the soil type was sandy chalk soil Figure 7.

For Al-Mahaweel site, soil test results show PH was 8.2 which consider slightly alkaline so it gives good preservation for DNA comparing to Al-saqlawiya site soil PH which was 7.7. Although DNA is less prone to damage in neutral or near neutral environments [18], some researches illustrate ; bones are stable in samples with a pH level higher than 8.1 [19].

The Humidity was low so it preserves the DNA because low humidity does not support growing the microorganism that play an important role in the degradation of bone material [20], also this low humidity could lower the high temperature because high temperature could increase bone degradation [21] during hot summer in Iraq which reach to 53 C. The total soluble salts of AL- Mahaweel which were 1.9% was low comparing with Al-Saqlawyia site, which was 73%,this gave more preserving to DNA in AL- Mahaweel because soil high in salt content can be highly destructive to bone [22].

Figure 6: Soil foe testing

Figure 7: Sample of tested soil

Soil texture which was clay in AL- Mahaweel site was preserving factor because clay soils that retain moisture as a result of their small particle size will generally retard decomposition rates to a greater extent than sand soils in Al-Saqlawiya site [23] as the soil permeability affects water permeability and air exchange within the soil, this occurs because the concentration of oxygen in water is much lower than in air, and oxygen diffusion through water is very slow [24]. In addition, because of its permeable nature, chalky soil of Al-Saqlawiya caused bones to become both fragile and eroded this made completely clean the bones is a too vigorous process, so this led damage, or loss of, information from the bone [22], [25].

The longer pieces have a greater probability of degradation compared to the shorter fragments. One possible explanation for this phenomenon is that as the length of the repeat sequence increases, the likelihood of a breaking occurring between the places where the primer binds also increases. As a result, signals from the longer alleles are often absent. The occurrence is referred to as allelic drop-out when only one of the two alleles at a certain genetic location is absent. The absence of any observed outcome in a specific genetic location is referred to as locus drop-out [26]. Further studies are planned to get more information about types of micro-organism that contributed in degradation process in each site.

4. Conclusion

This study is the first of its kind in Iraq. After study the assessment of the factors of soil type and other environmental factor that affect on DNA degradation or preservation and profile of STR pattern we found that clay soil, low salt concentration, PH slightly alkaline and low humidity all participate in DNA preservation while the chalky and very high salt concentration soil participate in DNA degradation.

Conflict of interest

The authors declare no conflict of interests. All authors read and approved final version of the paper.

Authors Contribution

All authors contributed equally in this paper.

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