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유골에서 STR 분석 및 위여성 사례

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STR analysis of bone samples recovered from cemetery and false female phenomenon

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Abstract - Extraction of DNA from damaged bone sample is of great importance in the identification of human remains, but is particularly difficult. A novel technique based on phenol-chloroform technique, with a further DNA purification step using the QIAquick system, combined with 10ul is described. The presence of DNA in the extract being confirmed by amplification of STR using the PCR. The method was applied to forensic femur samples (n = 67) from 11 month to 10 years old of burial time with a variety of postmortem histories. Parallel extractions using the QIAamp mini kit were performed on all samples in order to compare the efficiency of the two methods. In contrast with no satisfied results by QIAamp mini kit method, the success rate for the STR typing of bones was very high by novel method. The analysis of allele peak heights using the ABI Identifiler kit was as follows: strong signal (>150 rfu); 65.2%, low signal (150-30 rfu); 22.1%, and very low or no signal (< 30rfu) 12.7%. False female phenomena were observed in 3 cases of 50 apparent male samples.

Keywords : Forensic science, STR typing, DNA extraction, Skeletal remains

I. Introduction

Extraction of DNA from damaged bone sample is of great importance in the identification of human remains, but is particularly difficult[1-10]. Due to the environmental conditions to which human remains are exposed, many could be limited in quantity or quality. The quality of the bone samples obtained from human remains will vary substantially, from apparently pristine to highly degraded. Protocols for testing STR loci and mitochondiral DNA are well developed and figure prominently identification cases. However, additional demands may be placed on analytical processes particularly because of the extreme condition of some of the bones. Consideration might focus on the extraction procedures, alternate analytical methods for challenging samples.

The success of DNA typing relies on isolation of DNA of sufficient quantity, quality and purity. The sample condition often is out of the control of the scientist. Some samples will yield sufficient high molecular weight DNA without chemical contaminants that might inhibit the analytical process. For others, the environmental insults may be so great that little or no

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DNA may be available for subsequent typing. Extraction methods that minimize loss of the DNA are the most desired. DNA extraction protocols that overcome, remove or dilute enzyme inhibitors, also should be considered. Standard DNA extraction procedures exist for the types of materials that may be encountered and include organic solvents, chaotropics, and ion exchange resins.

After extraction, the DNA is first subjected to amplification by the PCR. If possible, the components of the PCR should be optimized to overcome the vagaries of environmentally contaminated samples. A larger reaction volume dilutes inhibitors that impact PCR, additives, such as BSA, also could be included routinely in the PCR to overcome the effects of some inhibitors that may be present.

In this study, a novel technique based on the phenol-chloroform, QIAquick PCR purification kit is described. The method was applied to bones exhumed from cemetery.

II. Materials and Methods

1) Bone specimens

The femur shafts (4 cm) were collected in early 2005 from the unidentified remains of people who had been buried. Exhumed bones came from several cemeteries in South Korea. Burials had taken place between 1994 and 2004. Every unknown remain have been differently affected by environment because they were found totally different conditions (see appendix) such as scene (river, forest, road, sea, et al.), and various degree of putrefaction from fresh to skeletal remain. When these remains were exhumed after various period of burial (11 month to 10 years), all remains became skeletalized.

The femur shaft (4 cm) was decalcified for 6 days in 0.5 M EDTA and washed 3 times in distilled water. Each bone sample was manually processed using a scalpel to obtain 1~2 mm bone particles.

2) DNA extraction with QIAamp mini kit

DNA extraction was performed with the QIAamp mini kit (Qiagen, Germany) in accordance with the manufacturer's instructions[11]. Buffer ATL 180 ㎕ and proteinase K 20 ㎕ were added to each 0.2~0.4 g of bone particles. The solution was heated in a 56°C water bath for overnight. Buffer AL 200 ㎕ were added to above solution and was heated in a 70°C water bath for 10 min. Ethanol 200 ㎕ were added and then vortexed. The supernatant was transferred to the column, and centrifuged at 8000 rpm for 1min. The flow-through was discarded and buffer AW1 500 ㎕ was added to the column, and centrifuged at 8000 rpm for 1min. The flow-through was discarded and buffer AW2 500 ㎕ was added to the column, and centrifuged at 8000 rpm for 1min. The flow-through was discarded and DNA was eluted from the column by the addition of 60 ㎕ of DW and microcentrifuged for 1min. We added the volume of template DNA to 1 ㎕ of the 60 ㎕ total DNA extract per 25 ㎕ PCR reaction.

3) DNA extraction with phenol/chloroform plus QIAquick PCR purification kit

Approximately 0.2~0.4 g of bone particles was digested with 450 ㎕ of DNA lysis buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.5% SDS, 39 mM dithiothreitol, and 0.4 mg/ml proteinase K). The solutions were incubated overnight at 56°C without agitation. Undigested bone debris was removed by centrifugation. After traditional extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), the DNA was concentrated using Microcon YM-100 (Millipore, U.S.A). Further purification was performed with the QIAquick PCR purification kit (Qiagen, Germany) in accordance with the manufacturer's instructions[12]. DNA was dissolved in a total volume of 30 ㎕ distilled water. We optimized the volume of template DNA to 10 ㎕ of the 30 ㎕ total DNA extract per 25 ㎕ PCR reaction.

4) STR genotyping

DNA was amplified using an AmpFLSTR Identifiler kit (Applied Biosystems, Foster City, CA) in accordance with the user's manual (28 cycles). The AmpFLSTR Identifiler kit allows simultaneous amplification of 15 STR loci (D19S433, D3S1358, D8S1179, D5S818, THO1, vWA, D21S11, D13S317, TPOX, D7S820, D16S539, CSF1PO, D18S51, FGA, and D2S1338) and the amelogenin locus[13]. The PCR products were separated using capillary electrophoresis with the 3100 ABI Prism Genetic Analyzer, and the results were analyzed by the GeneMapper ID software v3.1.

5) Analysis of STR profiles

Genotypes of all 15 STR loci and the amelogenin locus were analyzed based on relative fluorescence units (rfu). The peak heights were classified into three categories as described by Schneider et al[14]. strong signal, >150 rfu; low signal, 150-30 rfu; and very low or no signal, <30 rfu. The amplification of a locus was termed "successful" if the peak height exceeded 30 rfu. (Do not interpret as "correct signals" from the bones sample due to the missing references for the bones.) Contamination with extraneous DNA, detectable PCR artifacts such as heterozygotic peak imbalance, and unexpected results (such as a false female) were not included in the peak height analysis.

III. Results and Discussion

We compared the efficiency of DNA extraction from bone using a QIAamp mini kit with the novel phenol/chloroform plus QIAquick column method. Figure 1 shows the Identifiler STR electropherogram of a DNA sample extracted from a bone (E-92 sample) analyzed 4.11 years of burial period at cemetery using mini kit method. Figure 2 corresponds to the Identifiler STR electropherogram obtained when DNA from the same one was extracted with the phenol/chloroform and purified with the QIAquick column. STR electropherogram obtained when using template DNA extracted with new method was substantially better than electropherograms extracted with the alternative method.

We did experiment with QIAamp mini kit to compare the new method with an old method (Mini kit without optimization PCR volume of template DNA) in respect of extraction or typing efficiency. However, we did not obtain any satisfied results with QIAamp mini kit (Figure 3), so we could not compare the analysis of the same bone sample side by side with an old and the improved method. The results of this experiment demonstrate that DNA from the exhumed bones was degraded or contaminated with PCR inhibitors or low quantity.

The results of multiplex STR genotyping obtained from the recorded peak heights with new method are

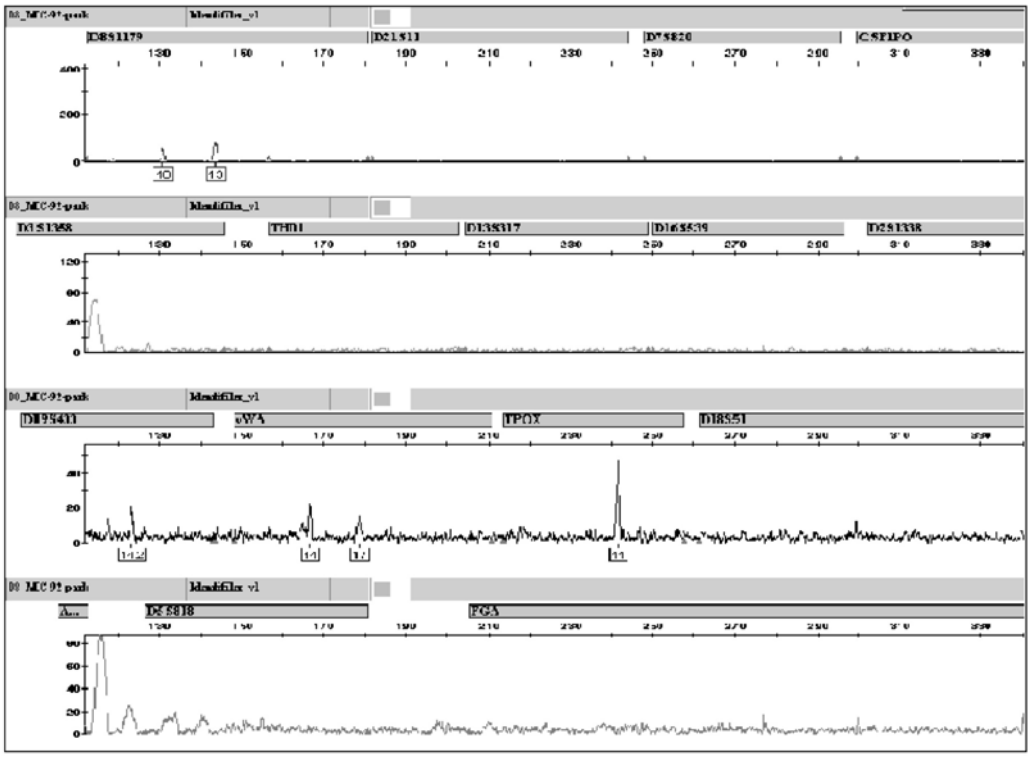

Fig. 1 STR electropherogram of a DNA sample from bone (E-92 sample) analyzed 4.11 years of burial period. DNA was extracted with QIAamp mini kit.

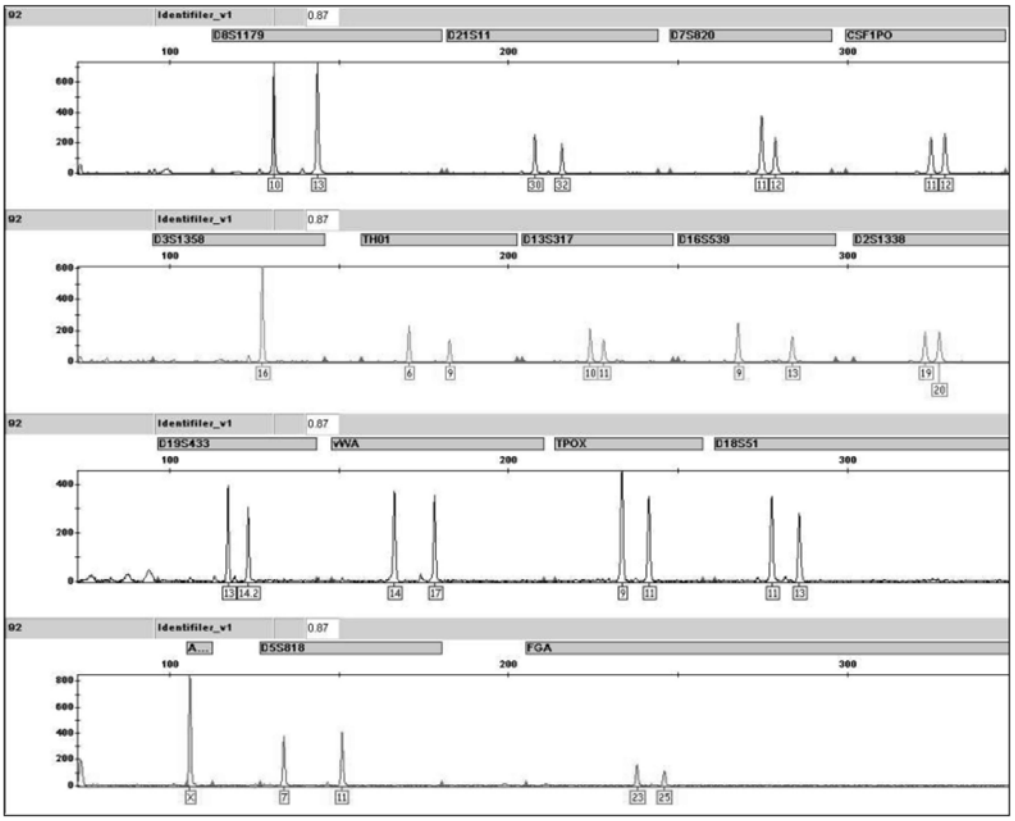

Fig. 2 STR electropherogram of a DNA sample from the same bone as in Fig.1. DNA was extracted with the phenol/chloroform plus QIAquick PCR purification kit.

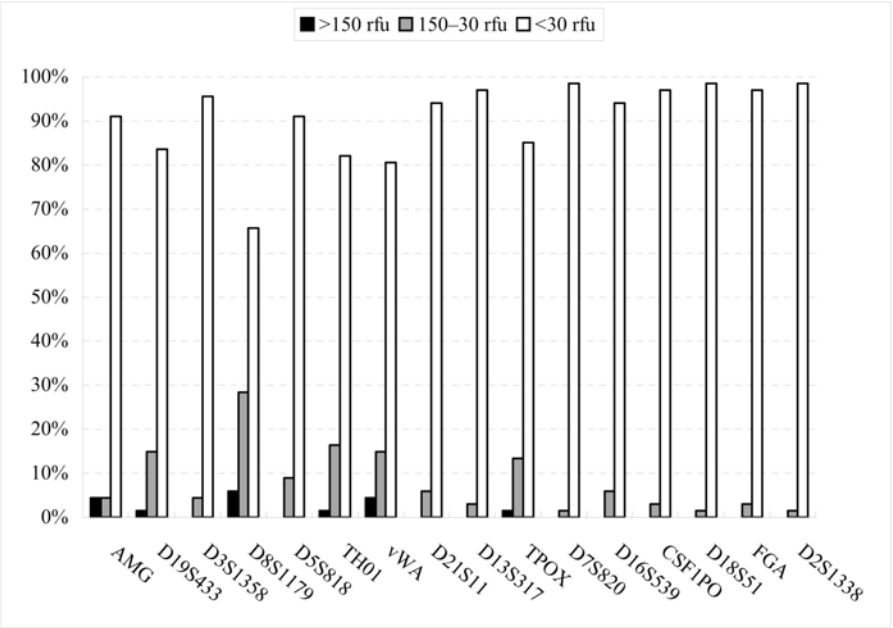

Fig. 3 Results of STR peak height analysis of DNA samples from exhumed bones extracted with QIAamp mini kit. For each locus, data are summarized for the following three peak height categories: >150 rfu (black columns), 150-30 rfu (grey columns), and <30 rfu (white columns). The results for all STR loci are arranged from left to right in the order of increasing fragment sizes for the three categories. An aliquot of 1 $\mu\ell$ DNA and 28 cycles were used for the PCR amplification.

depicted in Figure 4. The mean proportion of peak heights was as follows: strong signal 65.2% (699/1072 loci), low signal 22.1% (237/1072), and very low signal 12.7% (136/1072). Therefore the success rate of STR typing is 87.3%. As expected, the overall pattern of successfully amplified loci showed less variation in terms of STR size ranges. Almost 90% successful amplification was achieved up to the D8S1179 locus (123-169 bp), and 80% of the successful amplification was occurred up to the FGA locus (214-355 bp). So, we face allele drop-out problem at larger STR amplicon less frequently, that is a important point for the analysis of relevant forensic samples.

Additional purification of extracted DNA using silica-based column was performed by several authers. The QIAquick PCR purification kit with cetyltrimethylammonium bromide (CTAB) buffer, which was originally developed for plant DNA extraction was demonstrated to improving the results of STR typing of forensic bone samples obtained 2-9 years after death[10]. Other commercial kit, CleanMix PCR purification kit was used in the ancient DNA extraction. QIAquick spin column uses a kind of silica gel-based affinity chromatography that retains DNA fragments (100 bp to 10 kb), while excluding nucleotides, proteins, and salts; this was originally designed for the purpose of PCR product purification[12]. Further purification of the degraded DNA extract using this kit has potential to remove the PCR inhibitors from the bone sample as suggested in previous paper[10, 19].

Apart from the success rate of STR typing of the degraded skeletal remains achieved in the present study, it was noted that amelogenin gender determination was wrong in 3 cases among 50 male bones (Appendix). Figure 5 shows that the Y-chromosome allele at the amelogenin locus, i.e., a 112 bp amplicon, was not amplified in apparent male remains, but the electropherogram displayed a complete STR profile. In these 3 cases, the apparent male remains falsely appeared as a female profile. These results are good agreement with those of previous studies, where sexing errors were exclusively false females and this phenomenon predominated in the case of degraded forensic bone samples[7, 18]. Therefore, in future studies, sex determination from degraded bone samples should be accompanied with AMGX/AMGY real-time PCR.

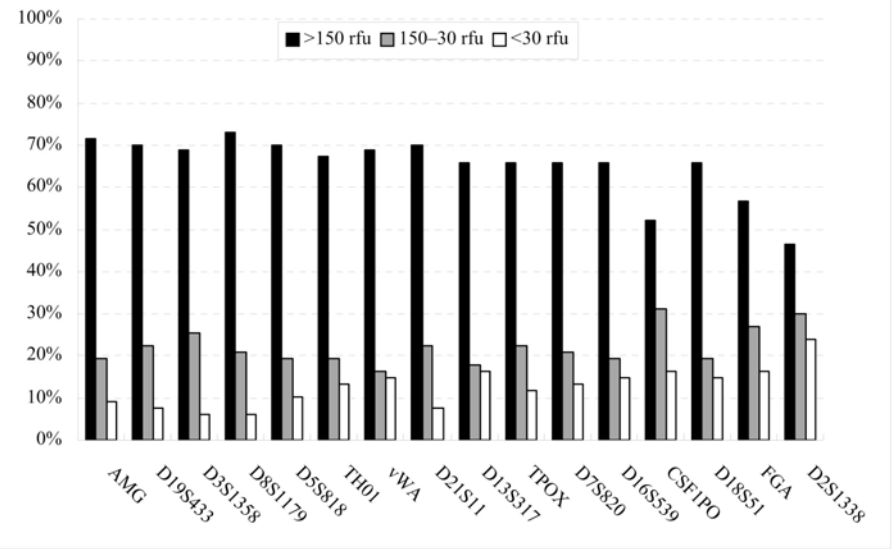

Fig. 4 Results of STR peak height analysis of DNA samples extracted with the phenol/chloroform plus QIAquick PCR purification kit. For each locus, data are summarized for the following three peak height categories: >150 rfu (black columns), 150-30 rfu (grey columns), and <30 rfu (white columns). The results for all STR loci are arranged from left to right in the order of increasing fragment sizes for the three categories. An aliquot of 10 $\mu\ell$ DNA and 28 cycles were used for the PCR amplification.

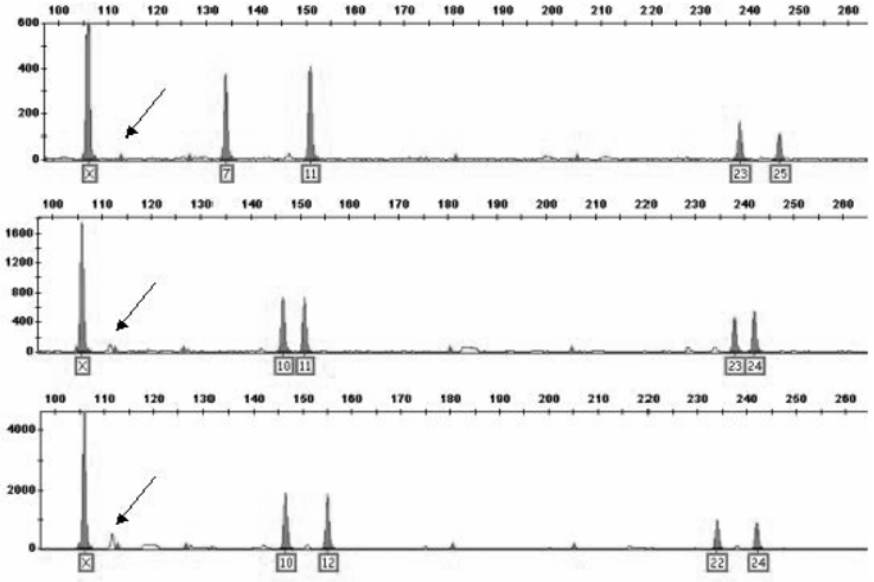

Fig. 5 Partial electropherograms of three false female cases obtained from apparent male skeletal remains. The arrows indicate the allele drop-out of the Y- chromosome at the amelogenin locus. The 112-bp amplicon was not amplified. Top panel: STR result of specimen E-92, Middle panel: STR result of specimen E-126, and Bottom panel: STR result of specimen E-127.

APPENDIX - Information and results of STR profile of exhumed bones extracted with phenol/chloroform plus QIAquick PCR purification kit.

Specimen	Period of burial (y mo)	Age	Sex	Scene	Results															
					Amel	D19S433	D3S1358	D8S1179	D5S818	THO1	vWA	D21S11	D13S317	TPOX	D7S820	D16S539	CSF1PO	D18S51	FGA	D2S1338
1	4.9	40-50	M	Toilet	L	L	L	L	L	L	L	L	L	L	L	V	V	V	L	V
2	5.1	30-40	M	River	L	L	L	S	L	L	V	L	V	V	V	V	V	L	V	V
3	4.1	50-60	M	Railroad	S	S	S	S	S	S	S	S	S	S	S	S	L	S	L	L
4	3.7	20-30	M	Highway	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
5	3.6	30-40	M	-	S	S	L	L	L	L	L	L	V	L	V	L	V	L	L	V
6	4.10	20	M	River	L	L	L	S	L	L	L	L	V	V	L	L	V	L	V	V
7	4.11	20	M	River	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8	4.11	20	M	River	L	L	L	S	S	L	L	S	V	L	S	L	L	S	L	L
9	3.11	30	M	River	S	S	S	S	S	L	L	S	S	S	S	S	S	S	S	L
10	4.11	18-25	F	River	S	S	S	S	S	S	S	S	S	S	S	S	L	S	L	S
11	4.7	65-80	M	River	S	S	S	S	S	S	S	S	L	S	S	L	L	S	L	L
12	1.10	-	M	Road	V	L	V	V	V	V	V	V	L	L	V	L	V	V	V	V
13	2.3	40-50	M	Highway	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
14	4.11	60-70	F	Forest	S	S	S	S	S	S	S	S	S	S	S	S	L	S	S	L
15	1.3	-	M	Road	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	L
16	3.6	50	M	Road	S	S	S	S	S	S	S	S	S	S	L	S	L	S	L	L
17	6.1	-	M	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	L
18	4.11	40-50	M	Forest	S	S	S	S	S	S	S	S	S	S	S	S	S	S	L	S
19	7.5	10-20	M	-	V	V	L	L	V	V	V	V	V	V	V	L	L	V	V	V
20	7.11	10-20	M	River	S	S	S	S	S	S	S	S	S	S	V	S	L	V	S	S
21	8.2	10-20	M	River	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

22	3.9	60-70	M	Forest	S	V	S	S	S	S	S	S	V	S	S	S	S	S	S	S
23	7.2	10-20	M	Railroad	L	S	L	L	L	L	L	L	S	L	S	L	S	S	L	L
24	4.1	30-40	M	Forest	V	S	L	L	L	V	V	L	S	L	L	L	L	V	V	V
25	3.10	-	M	Ditch	S	S	S	S	S	S	S	S	S	S	L	S	V	V	L	S
26	2.11	20-30	M	Forest	L	L	L	L	L	L	L	L	L	L	L	V	L	L	V	V
27	2.4	10-20	M	Forest	S	L	S	S	S	S	S	S	L	S	L	S	L	L	S	L
28	2.1	50-60	M	-	S	S	S	S	S	S	S	S	S	S	L	S	S	S	S	S
29	3.5	60-70	M	Forest	S	L	L	L	L	V	V	S	V	L	S	S	S	S	L	L
30	1.2	40-50	M	River	S	S	S	S	S	S	S	S	S	S	S	S	L	L	S	S
31	1.5	50-60	M	River	V	S	V	V	V	V	V	V	S	V	S	V	S	S	V	V
32	3.5	50-60	M	Forest	S	L	S	S	S	S	S	S	L	S	V	S	V	V	S	S
33	3.4	-	M	Sea	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	L
34	3.4	-	F	Sea	L	V	L	L	V	V	L	L	V	L	S	L	S	S	L	L
35	5.11	10-20	F	River	L	S	L	L	L	L	L	L	S	L	L	L	L	L	L	V
36	0.11	-	-	River	S	S	S	S	S	S	S	S	S	S	L	S	L	L	S	S
37	1.3	60-70	F	Forest	S	L	S	S	S	S	S	S	V	S	S	S	S	S	S	S
38	2.5	40-50	M	Forest	S	L	S	S	S	S	S	S	L	S	S	S	S	S	S	S
39	3.6	50-60	M	Forest	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	L
40	6.2	-	M	Home	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
41	5.10	-	F	Forest	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
42	5.11	-	M	Road	V	S	L	L	L	L	V	L	S	V	V	V	S	S	L	V
43	2.2	-	-	Forest	S	S	S	S	S	S	S	S	S	S	S	S	L	V	S	S
44	3.1	20-30	M	Forest	L	S	L	L	L	L	L	L	S	L	L	L	S	S	L	L
45	3.1	50-60	M	Forest	S	L	S	S	S	S	L	S	L	L	S	S	L	L	S	S
46	2.9	60	F	Forest	S	S	S	S	S	S	S	S	S	S	S	S	L	S	S	S
47	1.10	-	-	Forest	S	L	S	S	S	S	S	S	L	S	S	S	S	S	S	L
48	3.9	40	M	Forest	S	S	S	S	S	S	S	S	S	S	S	S	L	S	S	S
49	3.5	50	F	Road	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
50	5.2	-	-	Hotel	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
51	4.1	60-70	M	Street	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
52	3.9	60-70	M	River	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
53	3.8	-	M	River	L	L	L	L	L	V	L	L	L	L	L	V	L	L	L	V
54	10.1	40-50	M	Forest	L	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
55	9.5	30-40	F	Sea	L	L	L	L	V	L	V	V	V	V	V	V	V	V	V	V
56	7.4	20-30	F	Sea	S	S	S	S	S	S	S	S	L	L	S	L	S	L	L	L
57	6.6	40-50	M	Forest	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
58	4.5	40	M	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
59	10.5	-	-	Fire	S	S	S	S	S	S	S	S	S	S	S	S	L	S	S	L
60	5.3	-	-	Hospital	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
61	3.	-	-	Highway	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
62	6.2	-	M	Road	S	S	S	S	S	S	S	S	S	S	L	L	L	L	L	L
63	3.9	-	M	Hospital	S	S	S	S	S	S	S	S	S	S	S	S	L	S	S	L
64	2.6	-	M	Forest	L	V	L	L	L	L	L	L	L	L	L	L	V	L	V	V
65	1.8	-	M	Forest	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
66	-	-	M	Sea	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
67	-	-	M	River	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

S: strong signal (>150 rfu), L: low signal (150-30 rfu), V: very low or no signal (< 30 rfu).

Detectable contamination with extraneous DNA was occurred at E-115 sample. Detectable PCR artifacts, i.e. heterozygotic peak imbalance were occurred frequently in locus with low signal or very low or no signal but rare in locus with strong signal. Unexpected results (such as false female) were occurred at E-92, E-126, E-127 sample.

This study demonstrates a phenol/chloroform plus QIAquick PCR purification kit capable of recovering nuclear DNA from a variety of exhumed femur samples from 11 month to 10 years old.

IV. References

1) Bender K, Schneider P.M, Rittner C. Application of mtDNA sequence analysis in forensic casework for the identification of human remains. *Forensic Sci. Int.* **113**,103-107 (2000).
2) Butler J.M. Forensic DNA typing. 2nd ed., Elsevier Academic Press, Burlington. (2005).
3) Budowle B, Bieber F.R. Eisenberg A.J. Forensic aspects of mass disasters: Strategic considerations for DNA-based human identification. *Leg. Med.* **7**,230-243 (2005).
4) Butler J.M. Shen Y. McCord B.R. The development of reduced size STR amplicons as tools for analysis of degraded DNA. *J. Forensic Sci.* **48**,1054-1064 (2003).
5) Chung D.T. Drabek J. Opel K.L. Butler J.M. McCord B.R. A study on the effects of degradation and template concentration on the amplification efficiency of the STR miniplex primer sets. *J. Forensic Sci.* **49**,733-740 (2004).
6) Holland M.M, Cave C.A. Halland C.A. Bille T.W. Development of a quality, high throughput DNA analysis procedure for skeletal samples to assist with the identification of victims from the World Trade Center attacks. *Croat. Med. J.* **44**,264-272(2003).
7) Cattaneo C. Craig O.E. James N.T. Sokol R.J. Comparison of three DNA extraction methods on bone and blood stains up to 43 years old and amplification of three different gene sequences. *J. Forensic Sci.* **42**,1126-1135 (1997).
8) Hoff-Olsen P. Mevag B. Staalstrom E. Hovde B. Egeland T. Olaisen B. Extaction of DNA from decomposed human tissue. An evaluation of five extraction methods for short tandem repeat typing. *Forensic Sci. Int.*;**105**,171-183 (1999).
9) Evison M.P. Smillie D.M. Chamberlain A.T.Extraction of single-copy nuclear DNA from forensic specimens with a variety of postmortem histories. *J. Forensic Sci.* **42**,1032-1038 (1997).
10) Ye J. Ji A. Parra E.J. Zheng X. Jiang C. Zhao X. Hu L. Tu Z. A simple and efficient method for extracting DNA from old and burned bone. *J. Forensic Sci.* **49**,754-759 (2004).
11) QIAamp DNA mini kit handbook, QIAGEN.
12) QIAqucik spin handbook, QIAGEN.
13) AmpFLSTR Identifiler PCR amplification kit, User's Manual, PE Biosystems (2001).
14) Schneider P.M. Bender K. Mayr W.R. Parson W. Hoste B. et al. STR analysis of artificially degraded DNA-results of a collaborative European exercise. *Forensic Sci. Int.* **139**,123-134 (2004).
15) Hagelberg E. Mitochondrial DNA from ancient bones, in: Herrmann B. Hummel S. Ancient DNA. Springer-Verlag press, New York, pp.195-204. (1994)
16) Graw M. Weisser H.J. Lutz S. DNA typing of human remains found in damp environments. *Forensic Sci. Int.***13**, 91-95 (2000).
17) Crainic K. Paraire F. Leterreux M. Durigon M. Mazancourt P. Skeletal remains presumed submerged in water for three years identified using PCR-STR analysis. *J. Forensic Sci.* **47**, 1025-1027 (2002).
18) k. HanaokaY. Maruyama S. Nonaka I. Kajiwara M. Takagi T. Sato Y. DNA analysis of neonatal human remains wrapped and kept in a vinyl bag for 15 years. *Leg. Med.* **5**,183-186 (2005).
19) Keyser-Tracqui C. Ludes B. Methods for the study of ancient DNA, in: A. Carracedo. Forensic DNA typing protocols. Humana Press, Totowa, New Jersey pp. 253-264 (2005).