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Twelve-year Experience in Identification of Skeletal Remains from Mass Graves

Šimun Anđelinović, Davorka Sutlović, Ivana Erceg Ivkošić, Vedrana Škaro, Ante Ivkošić, Frane Paić, Boja Režić, Marija Definis-Gojanović, Dragan Primorac[1]

Laboratory for Clinical and Forensic Genetics, Split University Hospital, Split, Croatia; and [1]Ministry of Science, Education, and Sports of the Republic of Croatia

Aim To present twelve-year (1993-2005) experience in identification of human remains found in mass graves in Croatia and Bosnia and Herzegovina (BH), as well as remains that presumably belonged to Croatian citizens given by Serbia and Montenegro. The unique experience of identification of more than a thousand of skeletal samples is valuable for better organization of post-mortem identifications.

Methods Standard forensic methods and methods based on DNA analysis were used for identification of human remains from mass graves. DNA was isolated using standard phenol/chloroform/isoamyl alcohol extraction. In some cases, decalcification and repurification were used prior to the extraction to overcome inhibition of amplification process. Different DNA systems were used for DNA quantitation and amplification (AluQuant, short tandem repeats (STR) commercial systems, Y chromosome STRs, and mitochondrial DNA [mtDNA]). Typing of PCR products was performed on AmpliType®PM and AmpliType® DQA1 DNA probe strips, ABI PRISM® 310 Genetic Analyzer and immobilized sequence-specific oligonucleotide (SSO) probes.

Results Up-to-date analysis of 1,155 skeletal samples resulted in 703 positively identified bodies: 577 using standard forensic methods, 109 by DNA typing, and 17 by combination of these two methods. The majority of identifications from 1993 to 1999 was, as usual, achieved by standard forensic methods. Later on, these methods were not sufficient and DNA analysis was requested. It was performed in 42% of all cases in 12 years. The crucial step in DNA analysis is extraction of genomic DNA. Standard phenol/chloroform/isoamyl alcohol extraction, complemented with other methods and modifications, proved as the most successful method for this step. In certain cases, the quality and/or quantity of nDNA was not satisfying and the analysis of the mtDNA was performed.

Conclusion Our experience demonstrated that the advent of forensic DNA analysis methods greatly increased our ability to positively identify previously unknown skeletal remains by a comparative genetic analysis with presumptive relatives.

Significant efforts are currently underway to identify the remains of missing individuals after the 1991-1995 Serbian aggression on Croatia (1) and Bosnia and Herzegovina (BH) (2), discovered in 135 mass graves situated throughout Croatia and southern BH. Recently, we have been challenged with the samples received from Serbia and Montenegro that presumably belonged to Croatian citizens, war victims murdered and buried in towns of Novi Sad and Sremska Mitrovica.

According to the most recent statement from the Government of the Republic of Croatia, by the end of 1992 there were more than 11,000 missing persons in Croatia as a result of the war (3). Consistent with the records, 11,834 persons were killed. Up to date, 3,502 bodies were exhumed

and 2,944 were identified. However, another 558 bodies still wait for identification. Also, there are 1,160 persons still considered missing.

A variety of methods are used to identify human remains, depending on the circumstances and the state of remains. The most commonly used methods include: identification of the remains by a living person who knew the deceased by direct facial recognition or recognition of special features, individual scars or marks (e.g., tattoos); matching of fingerprints (provided pre-mortem inked prints are available) or dentition (provided representative pre-mortem dental records are available). In many situations, these methods cannot be used either because of extensive putrefaction or destruction of the remains, or because appropriate medical or dental records are not available. In war circumstances, with high number of the dead, mostly buried in common graves and often without ante-mortem data, the identification is much more difficult (4).

Due to the lack of ante-mortem data and body decomposition, common methods for human identification was not sufficient in approximately 42% of all cases and DNA identification was requested (5).

The ability to analyze trace amounts of human DNA from old teeth and bone samples offered the opportunity to identify unknown skeletal remains by a comparative genetic analysis with presumptive relatives (6). However, degradation and contamination of DNA extracted from bone and teeth samples could make that process difficult. DNA isolation from bones and teeth samples was performed using standard phenol/chloroform/isoamyl alcohol procedure, as well as using some advanced methods. Some samples that failed to give results after second phenol/chloroform/isoamyl alcohol extraction were subjected to decalcification method with ethylenediaminetetraacetic acid (EDTA) or to the NaOH repurification method (7). A new DNA extraction and DNA quantitation procedures such as: Promega's DNA IQ™ System (Promega Corporation, Madison, WI, USA) (8) and AluQuant™ Human DNA Quantitation System (9) were successfully tested.

From the early beginning of the identification process, the following DNA amplification systems were used: AmpliFLP™D1S80, AmpliType®PM + DQA1 PCR Amplification and Typing Kit (10), AmpFLSTR Profiler™ PCR Amplification Kit, AmpFLSTR Profiler Plus™ PCR Amplification Kit, GenePrint® PowerPlex™ 16 System, AmpFLSTR Identifiler™ PCR Amplification Kit, Y-Plex™6, and Immobilized sequence-specific oligonucleotide (SSO) probe analysis for the mitochondrial DNA (mtDNA) control region (11,12).

We believe that through experience in the identification of skeletal remains from mass graves, we gained valuable data which may provide a solid starting point for any country or medico-legal team that finds itself under similar set of unfortunate circumstances, trying to identify huge number of casualties in a short period of time. At the same time, it clearly shows the importance of new identification methods and advocates their rapid application in case work.

Material and Methods

The post mortal remains of 674 persons were excavated from different common graves at more than thirty locations in Croatia and BH two or more years after their death and were transported for forensic identification to the Department of Pathology and Forensic Medicine, Split University Hospital, Split, Croatia. The basis for identification was the list of persons who had been killed or missing from the beginning of the 1991-1995 war. At the same time, ante-mortem records were available in about 50% of cases. They included the data collected from relatives, as well as prewar medical and dental records. Forensic expertise was performed on all collections of the remains, including a detailed examination of the clothes, footwear, and other belongings found with the remains. Careful photographic documentation was made of all the characteristic details. External examination and autopsies were performed in cases where post mortal changes did not result in a complete decomposition of the body.

In cases of skeletal remains we estimated sex, stature, and age by using the usual methods and formulas (13,14). Anthropological characteristics of the bones, pathological changes and signs of trauma were described in detail. Further, the comparisons of ante-mortem dental records with post-mortem dental status, as well as X-ray comparisons were done. Video superimposition (10) was done in cases where skulls of the victims and their photographs were available.

After all these procedures, we organized exhibitions of evidence to family members and close friends, presenting them all relevant data and the victims' belongings.

DNA Sources

Bone samples, mostly long bones and teeth, were obtained from the remains excavated from mass graves situated throughout Croatia and southern BH or returned from Serbia and Montenegro. From 5 to 20 g of bone samples were collected for DNA analysis, and approximately 2 g of powdered bone was used for DNA extraction.

DNA Extraction

All bone surfaces were cleaned from the remnant soft tissue and soil traces, and additionally brushed in warm water with mild detergent. The bones were rinsed with distilled water several times and left to air-dry. Bone fragments were washed in commercial bleach, then in deionized water three times and twice in the 70% ethanol, and left to air-dry for 24 hours. Frozen samples were pulverized in a bone grinder and 3 mL of extraction buffer (10 μmol/L Tris, pH 8.0; 100 μmol/L NaCl; 50 μmol/L EDTA, pH 8.0; and 0.5% sodium dodecyl sulphate [SDS]) and 100 μL of 20 mg/mL proteinase K was added to the 2 g of pulverized bone sample. This solution was incubated at 56°C for 48 hours, and DNA was isolated by organic phenol/chloroform/isoamyl alcohol extraction (15).

DNA from bloodstain reference samples of living relatives was isolated by standard Chelex 100 protocols (16).

DNA Isolation System Procedure

DNA IQ Isolation System (Promega, Madison, WI, USA) was used for DNA isolation, with some modifications. In 2 g of pulverized bone, 3 mL of Lysis Buffer or Extraction Buffer and 200 μL of proteinase K solution (20 mg/mL) were added and incubated 15 minutes at 56°C. The samples were centrifuged 4 minutes at 4,000 rpm. Two volumes of prepared Lysis Buffer and 15 μL of Resin were added to clear supernatant and incubated at room temperature for 10 minutes. The tubes were placed on the magnetic stand and, after separation of solution and resins, all solution was discarded.

Lysis Buffer (100 μL) was added to resin. The tubes were placed on the magnetic stand and, after separation of the resins and buffer, all lysis buffer was discarded.

The same was done with 100 μL of Wash Buffer. The procedure with Wash Buffer was repeated two more times and the resin was left to air-dry on a magnetic stand for 5-15 minutes.

Elution Buffer (30 μL) was added to the resin and incubated at 65°C for 5 minutes. The tubes were immediately placed on the magnetic stand and, after the separation of resins and buffer DNA, solution was transferred to a container of choice.

EDTA Decalcification Procedure

To 2 g of bone powder, 16 mL of 0.5M EDTA, pH 7.5 was added and left on a shaker at room temperature for 24 hours. The solution was centrifuged for 15 minutes at 2,000 rpm, supernatant was discarded, and another 16 mL of EDTA was added. The procedure was repeated for the next 3-5 days. The pellet was rinsed in 16 mL of distilled water and centrifuged 15 minutes at 2,000 rpm. The supernatant was discarded and procedure was repeated two more times. The usual incubation and DNA extraction procedures followed.

NaOH Repurification Procedure

DNA from the substrates that failed to amplify was subjected to NaOH treatment. Approximately 30-50 μL of DNA was placed into a Centricon-100 microconcentrator (Amicon Corp., Danvers, MA, USA), along with 200 μL of 0.4 mol/L NaOH. The volume was reduced to 5 μL by centrifugation at 2,000 rpm and the filtrate was discarded. The chamber was refilled with 400 μL of 0.4 mol/L NaOH and centrifuged once more, as described (7). The sample was neutralized by washing once with 400 μL of 10 mmol/L Tris (pH 7.5) and recovered in 15 μL of 10 mmol/L Tris, pH 7.5.

DNA Quantification

Agarose gel electrophoresis and ethidium bromide staining were used for the evaluation of total DNA. The quantity of human DNA was determined by spectrophotometry, slot-blot hybridization with the primate-specific D17Z1 alfa-satellite probe by use of Quanti Blot™Human DNA Quantitation Kit (Roche Molecular Systems, Inc., Branchburg, NJ, USA) or by Promega AluQuant™ Human DNA Quantitation System (Promega).

Electrophoresis was performed on 1% agarose gel with 1×TBE (Tris-Borate-EDTA buffer) (0.089 mol/L boric acid, 0.002 mol/L EDTA, pH 8.3), for genomic DNA or on a 3% NuSieve GTG, 1% SeaKem GTG gel (Roche Molecular Systems, Inc) in 1×Tris Acetate-EDTA buffer (TAE) (0.04 mol/L boric acid, 0.001 mol/L EDTA, pH 8.3) for mtDNA. The tank buffers contained ethidium bromide (EtBr) (Sigma-Aldrich Corp., St. Louis, MO, USA), allowing for the visualization of the DNA fragments on a transilluminator after electrophoresis. Lambda Hind III fragments were used as molecular weight markers (MWM) to determine the average fragment size of nuclear DNA and 444 bp HVI and 415 bp HVII fragments of mtDNA.

Dilutions were prepared and their absorbencies determined at 260 and 280 nm on a spectrophotometer (Ultrospec 2000, Pharmacia, Biotech Ltd, Cambridge, UK). The quantity of DNA was estimated by using the absorbance at 260 nm in the equation A260 ×50×dilution factor=μg/μL. The A_{260} /A_{280} ratios were used to evaluate the quality of extracted DNA. Aliquots of extracted DNA were transferred to a new tube and the sample was adjusted to 20 μL.

Promega AluQuant™ Human DNA Quantitation System. Into each microcentrifuge tube, 5 μL of the Denaturation Solution (NaOH) (Promega) and 5 μL of DNA sample were added and incubated at room temperature for 10 minutes (9). Two tubes were used for the same sample: one for the sample to be treated with the Master Mix (for each reaction: 5 μL of AluQant Neutralization Solution 5 μL of AluQuant probe Mix, and 10 μL of Aluquant Enzyme Solution; and the second for the Master Mix Control (for each reaction: 5 μL of AluQant Neutralization Solution, 5 μL of Nuclease-Free Water, and 10 μL of Aluquant Enzyme Solution.

The Master Mix (20 μL; with the probe) was added to the appropriately labeled tubes and 20 μL of the Master Mix Control (without the probe) was added to the control labeled tubes. Tubes were incubated at 55°C for 60 minutes.

The reaction (25 μL) was then transferred into TD-20/20 luminometer tube (Turner Designs, Sunyvale, CA, USA), filled with 50 μL of reconstituted L/L Reagent (Promega) and the tubes were placed in the luminometer. Luciferase reaction gives a light output that is read by luminometer, giving a numeric readout that allows the human DNA in a sample to be easily calculated.

DNA Amplification and Typing

Amplifications were performed on the Perkin-Elmer GeneAmp PCR System 9600 (Perkin Elmer, Branchburg, NJ, USA) using the AmpliType PM + DQA1 PCR Amplification and Typing Kit, AmpliFLPD1S80 (Perkin Elmer), the AmpFLSTR Profiler PCR Amplification Kit, the AmpFLSTR Profiler Plus PCR Amplification Kit, the AmpFLSTR Identifiler PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA), (17-19) GenePrint-PowerPlex 16 System (Promega) (20) or the Y-Plex6 (ReliaGene Technologies, Inc., New Orleans, LA, USA), according to the manufacturer's protocols available with the kits (21).

Typing of the PCR products was performed on AmpliType PM (Perkin Elmer) and AmpliType DQA1 DNA probe strips (Perkin Elmer) and ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The recommended parameters for GeneScan and Genotyper (Applied Biosystems) analysis were followed. Automatic assignment of genotypes was performed with GeneScan and Genotyper softwares (Applied Biosystems).

Immobilized SSO probe analysis (Roche Diagnostics, Alameda, CA, USA) was used for mtDNA.

Immobilized SSO Probe Analysis of mtDNA

Forward primer and a reverse primer amplified two hypervariable regions of mtDNA. PCR product yield was compared to a MWM 1 and 2 (MWM 1 is a 1:2 dilution of MWM 2) and 5 μL was used for a further analysis performed in four steps:

1) PCR product was prepared for hybridization by mixing 1:1 with Amplicor [1] DN denaturation solution (1.6% NaOH) (Roche Diagnostics) and incubated with 3 mL of wash buffer (100 mL sodium phosphate buffer (SSSP) concentrate and 25 mL sodium dodecyl sulphate (SDS) concentrate, Roche Diagnostics, in 875 mL deionized water) in each tray well containing a linear array at 55°C for 15 minutes.

2) Binding of streptavidin-horseradish peroxidase (SA-HRP) enzyme conjugate (Roche

Diagnostics) (8 μL per strip) to hybridized PCR products at 55°C for 5 minutes.

3) Washes after hybridization and binding were performed with 3 mL of wash buffer (Roche Diagnostics) per linear array.

4) Color development was performed with a 3 mL volume of color development solution (Roche Diagnostics): 3 mL of citrate buffer, 0.4 μL of 30% hydrogen peroxide, and 0.16 mL of Chromogen-TMB Solution per linear array.

Analysis of Typing Results

DNA profiles from the bone and teeth were analyzed and compared to the DNA profiles from living relatives. DNA genotypes from living relatives were obtained from the analysis of the DNA isolated from blood and bloodstains.

The database was kept in the Microsoft Access 2000. Microsoft Excell 2000 was used for statistical calculation.

Calculations for statistical probability were performed according to the following protocols (22-25):

1) Identification of skeletal remains using children if the missing persons were parents: in parentage testing, the frequency of potential parents was determined by the Random Men Not Excluded [RMNE = + 2p(1-p)] frequency or the population of potential allele donors (23).

2) If the missing person was the father of the children, we calculated the system index (SI) and paternity index (PI), which indicated how many remains were likely to be the child's father (23). Testing of the mother and child indicated how many of non-father males, but not the remains, could be excluded.

3) Identification of remains using parents: in the process of identifying remains it was necessary to determine the frequency of one obligate allele in the population of potential fathers or mothers (RMNE or Random Female not excluded, RFNE) in each of the two parents. Calculation the RMNE/RFNE for each allele and then multiplying the respective RMNE for the first allele times the RFNE for the second allele gave the likelihood of getting two parents who had those alleles, ie, the Random Parents Not Excluded (RPNE). The formula for calculating RPNE is $[p^2+2p\ (1\text{-}p)]\times[q^2+2q(1\text{-}q)]$ (23).

Results

We analyzed 1,155 bone samples for identification purposes during the period from 1993 to 2005 (Table 1). Positive identification was achieved for 703 persons: 577 by standard forensic methods, 109 by DNA typing, and 17 by a combination of these two methods.

From 1993 until the end of 1999, identification by standard forensic methods was performed for 674 human bodies exhumed from mass graves, 577 (85%) of whom were positively identified.

From 1994 until today, 481 samples have been subjected to DNA analysis, 385 have been successfully amplified, and 109 bodies have been identified (28.3%). After 1999, prior to the DNA analysis, all samples deriving from skeletal remains had been subjected to standard forensic

Table 1. Summary of twelve-year identification process of skeletal remains from mass graves found after the war throughout Croatia and southern Bosnia and Herzegovina or given by Serbia and Montenegro

	Samples analyzed		No. (%) of	DNA	Percent of positive identifications		
Year*	standard forensic methods	DNA typing	successful DNA amplifications	amplification system	standard forensic methods	DNA typing	combined methods
1993	118	/	/	/	77.1	/	/
1994	120	11	1 (9.1)	AmpliType PM+DQA1	90.8	/	9.1
1995	44	12	1 (8.3)		86.4	/	8.3
1996	177	15	2 (13.3)		73.5	/	13.3
1998	117	15	3 (20.0)		94.9	/	20.0
1999	98	16	10 (62.5)	AmpFLSTR Profiler™	100.0	/	62.5
2000	/	47	41 (87.2)		/	17.1	/
2001	/	152	131 (86.2)	AmpFLSTR Profiler™, AmpFLSTR Profiler Plus™, GenePrint®PowerPlex™ 16, Y-Plex™6	/	16.8	/
2002	/	99	86 (77.8)	AmpFLSTR Identifiler™, AmpFLSTR Profiler™, AmpFLSTR Profiler Plus™, GenePrint®PowerPlex™ 16 Immobilized SSO probe analysis	/	36.0	/
2003	/	89	86 (96.6)	AmpFLSTR Identifiler™ Immobilized SSO probe analysis, Y-Plex™6	/	34.9	/
2004	/	25	24 (96.0)	AmpFLSTR Identifiler™, Y-Plex™6	/	79.1	/
Total	674	481	385 (80.0)		85.6	28.3	5.6

*During 1997, only blood samples and bloodstains were analyzed for the purposes of creating the Croatian population database.

methods before they were delivered to our laboratory.

Several Croatian population databases, with a total of more than 3,000 profiles, have been created for forensic analyses to estimate the frequency of a multiple locus DNA profile (26-28). Our laboratory contributed with approximately 500 profiles.

A genotype from each skeletal remain was compared to all the genotypes in the database in order to positively identify a missing person (23,29).

In addition to standard phenol/chloroform/isoamyl alcohol procedure used for DNA extraction, Promega DNA IQ System, modified in our laboratory, was successfully tested in 22% of the cases.

During 2003 and 2004, 132 bone samples were subjected to decalcification method with EDTA, as an additional procedure prior to extraction of DNA. Its effectiveness was 84.9%.

NaOH repurification, another additional procedure (7), also showed better results in 10 cases; however, in some cases not all 16 loci were amplified. This method was used for DNA samples that failed to amplify initially or after decalcification procedure.

Gel electrophoresis was performed to evaluate the quality of extracted DNA. The average fragment size of nuclear DNA was 477 bp. The size of mtDNA fragment was 444 bp for HVI region and 415 bp for HVII region.

Spectrophotometric analysis showed that the total amount of the DNA extracted by phenol/chloroform/isoamyl alcohol ranged from 20 to more than 100 ng, with the average of 64 ng. For DNA extracted by modified DNA IQ System, the total amount ranged from 20 to almost 200 ng, with the average of 100 ng.

AluQuant Human DNA Quantitation System was tested on 46 DNA samples: 26 extracted using phenol/chloroform/isoamyl alcohol procedure and 20 extracted using modified Promega DNA IQ System.

During the last 12 years, different DNA amplification systems were used. In the beginning of our work, we used AmpliTypePM+DQA1 PCR Amplification and Typing Kit and AmpliFLP D1S80. Although this approach was successful with 24% of samples, positive identification was accomplished only when standard forensic method and DNA analysis were combined.

Better results were accomplished by using multiplex STR systems. The success of AmpFLSTR Profiler PCR Amplification Kit, which amplifies ten loci, was 87%. For the last three years, we have been using AmpFLSTR Identifiler PCR Amplification Kit, which amplifies sixteen loci, including all Combined DNA Index System (CODIS) core loci in a single reaction. So far it has been used for 213 samples and the success of amplification and obtaining full genotypes was about 92%.

We also started the process of applying mtDNA typing for the identification of human remains. Immobilized sequence-specific oligonucleotide (SSO) probe analysis of mtDNA was applied to 21 samples and it was successful in all cases. Seventeen identifications were confirmed exclusively with this method.

Y-Plex™6 Kit, performed for Y-chromosome STR analysis, was helpful in solving 12 cases. In 5 cases, the matching of Y-chromosome STRs from human remains and those from 30 blood samples did not give any positive results.

About 53% of all samples were femurs, 21% were teeth samples, and the rest were skull, long bones (humerus, ulna, radius, or tibia), ribs, mandibula, calcaneus, pelvis, sacrum, and fibula (Table 2). Full genotypes were obtained in 92% cases of all femurs and in 90% cases of all teeth samples. For other bone samples, successfulness varied between 67 and 100%. No genotypes were obtained from rib bones, mandibula, or calcaneus.

Discussion

The identity of 577 (85.6 %) persons was confirmed in the analysis by standard forensic methods. Due to the insufficient ante-mortem and/or post-mortem material, as well as extensive post-mortem changes, the others (14.4%) remained unidentified. Some identification techniques, such as dactiloscopy, could not be performed. The identification was further impaired by the fact that the bodies had been buried and excavated carelessly, which incurred additional trauma to the bodies. Details of the clothes and footwear, as well as other personal belongings were of great importance. This conforms to the reports from some authors, who claim that the distinction of clothing and jewelry helps in the identification

Table 2. Success of DNA amplification for different bone types in last four-year time period

	Number of DNA isolations in year						Percent of successfulness of DNA amplifications in year					
Bone type	2000	2001	2002	2003	2004	total	2000	2001	2002	2003	2004	total
Femur	15	69	46	76	13	219	80	91	87	97	100	92
Teeth	6	63	6	11	0	86	67	89	100	100	/	90
Skull	2	5	5	0	1	13	100	60	60	/	100	69
Humerus	1	6	6	0	3	16	100	67	100	/	67	81
Ulna	1	3	1	1	0	6	0	100	100	0	/	67
Radius	1	1	1	0	0	3	100	100	100	/	/	100
Mandibula	0	1	0	0	0	1	/	0	/	/	/	0
Rib	0	2	0	0	0	2	/	0	/	/	/	0
Calcaneus	0	0	1	0	0	1	/	/	0	/	/	0
Pelvis	0	0	4	0	0	4	/	/	75	/	/	75
Tibia	0	0	9	1	8	18	/	/	89	100	100	94
Sacrum	0	0	1	0	0	1	/	/	100	/	/	100
Fibula	0	0	2	0	0	2	/	/	100	/	/	100
Unknown	21	2	17	0	0	40	100	50	88	/	/	93
Total samples	47	152	99	89	25	412	87	86	87	97	96	89

procedure in almost half of the cases (30). The reasons for the relatively small number of identified persons by teeth examination were missing skulls and the lack of ante-mortem data or insufficient dental records. We found that the method of exhibition of material findings was extremely helpful to family members and friends, because they did not have to undergo the difficult experience of entering the autopsy room to identify actual body remains (31).

Genetic typing by analysis of PCR-amplifiable STR loci is the most promising approach for forensic DNA profiling and has become a method of choice for identification of human remains (32,33). The comparatively short length of the STR loci (up to 250 bp) makes them especially suitable for typing highly degraded DNA.

In the isolation of DNA from skeletal remains, we encountered several problems, such as DNA degradation and contamination.

DNA IQ System for DNA extraction employs a novel technology with magnetic particles to prepare clean samples for STR analysis easily and efficiently. The unique DNA IQ Resin is designed to eliminate PCR inhibitors and other contamination. Samples for IQ testing were randomly chosen among ones that were successfully analyzed before. There was no case when the results were obtained from DNA isolated by DNA IQ System and not obtained from DNA isolated by phenol/chloroform/isoamyl alcohol procedure.

EDTA decalcification method is useful because calcium ions could interfere with DNA amplification and it seems suitable to remove them during isolation process. Several groups of authors described the usage of decalcification process prior DNA isolation from ancient bones (34-36). As the results were ambiguous, we decided to make our own test with some modifications of Hochmeister et al (35) method, such as shorter time of incubation with EDTA. According to our results, EDTA decalcification method is helpful for gaining better results in DNA amplification process.

NaOH treatment described by Bourke et al (7) was used to overcome potential inhibitors of Taq Polymerase when DNA failed to amplify. In some cases, 5-8 loci which originally failed to be amplified, could be amplified after this additional procedure. However, NaOH protocol is not advised when the quantity of DNA is limited, since the treatment results in significant loss of DNA. This treatment should be additionally tested.

STR reactions are optimized for a narrow range of DNA concentration (from 0.25 to 2 ng of human DNA). Too small or too large amounts of human DNA in an STR reaction will give poor or erroneous results and the analysis may fail.

Successful typing of DNA isolated from bone samples mostly depends on selection of appropriate DNA isolation procedure. Increasing the percentage of successful DNA amplification from 9% (in 1993) to 96% (in 2004) is mostly based on the optimization of DNA isolation protocols (Table 1). The average success of DNA amplification in twelve years is more than 80%. According to International Commission on Missing Persons (ICMP), successfulness of DNA amplification can be more than 95%, if modified Qiagen DNA extraction protocol is used (Huffine E, personal communication). ICMP optimized this procedure and

it provides great results for massive DNA identification and it is almost irreplaceable when a daily high throughput of results is required. However, this is a more expensive method, so that cheaper but almost equally successful organic and IQ DNA isolation procedure seems to be more suitable for our laboratory.

To precisely determine the amount of DNA, we tested AluQuant Human DNA Quantitation System. The main features of this system are human-specific probes, sensitive and broad quantitation range (0.1-20 ng), efficiency, and simplicity. It is a precise, novel system specially designed for the use in forensic DNA analysis and human identity testing, prior to performing PCR and STR analysis. It allows the measurement of human DNA through the use of probes to highly repeated sequences present in the chromosomal DNA.

The AluQuant System has a solution-based hybridization format, unaffected by degraded DNA. As opposed to other quantification systems, it uses a luciferase reaction to give a light output that is read by a luminometer, giving a numeric readout that allows the human DNA in a sample to be easily calculated. AluQuant method measures DNA concentration up to 10 times lower than spectrophotometer. It works well with DNA samples extracted by both procedures, maybe slightly better with the samples isolated by modified Promega DNA IQ System. The amplification products showed stronger amplification with the smaller size STR products. Most of the samples are amplified at a concentration of approximately 5 ng.

AmpliTypePM+DQA1 PCR Amplification and Typing Kit frequently encountered either amplification difficulties or nonspecific hybridization that caused ubiquitous data.

AmpliFLP D1S80 was the additional system used together with AmpliType PM+DQA1 PCR Amplification and Typing Kit. Since this system requires greater amount of DNA, analysis with silver staining of the acrylamide gel does not offer discrimination power. Also, this system analyzes only a single locus.

AmpFLSTR Profiler Plus PCR Amplification Kit and GenePrintPowerPlex 16 System, which amplifies sixteen loci were used in the cases where all alleles were not amplified or some of them were questionable. These two identification systems were helpful for confirming results in unsolved cases amplified with AmpliType PM+DQA1 PCR Amplification and Typing Kit. Though AmpFLSTR Profiler PCR Amplification Kit showed good results, it was not completely sufficient: in some cases 10 loci were not enough to confirm the identity, mostly in the cases where relatives were not very close (29).

The identification of skeletal remains by STR analysis is usually sufficient in the large percent of analyzed cases. However, in cases where two-copy nuclear markers fail, mtDNA analysis often succeeds (37-39).

MtDNA forensic testing should be utilized primarily in the situations where nuclear DNA typing is not an option, or in the event that nuclear typing has been unsuccessfully attempted. As mtDNA is maternally inherited, it is mandatory to be used in cases where parents or siblings cannot be reached, and relatives by maternal side are accessible. Prior to the further application of routine mtDNA testing method, haplotype frequency determination within the Croatian human population should be performed.

STR systems located in the non-recombining region of the Y chromosome are widely used in forensic science for the identification of male individuals (40). We used Y-chromosome STR systems in cases of absence of female relatives and in presence of male relatives. The existence of population data, which include allele frequencies for examined Y-STR loci, as well as frequency of detected haplotypes, is essential for statistical calculation and presentation of final results in concluding identification report. Croatian population data for this type of molecular markers are already published and available online (41). In future, these preliminary data perhaps could be extended with new Y-STR profiles from all parts of Croatia.

Although the quality of DNA extracted from teeth is usually higher than that of DNA from bones (42-44), in our experience was often the opposite. The quality of DNA differed between bone types. Femur and teeth samples were the best material for gaining reproducible results from the extracted DNA. We succeeded to obtain good results from the DNA isolated from a delicate fibula. Quality of bone and the conditions under which the remains were exposed had more influence on the state of DNA. The bones with thick compact part usually have better preserved DNA. Also,

when we compared the bones that were buried to the ones retrieved from a water-well, the latter were less decomposed, with adherent parts of tissue on them. Bone samples are exposed to different microbial organisms, and as it was previously demonstrated, high amounts of microbial DNA (in μg quantities) could interfere with the specific hybridization of human sequences, rendering false negative results on the human DNA quantitation of bone and teeth DNA samples (15).

Our experience demonstrated that the advent of forensic DNA analysis methods greatly increased our ability to positively identify previously unknown skeletal remains by a comparative genetic analysis with presumptive relatives.

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Correspondence to:
Vedrana Škaro
Ul. Grada Chicaga 21
10000 Zagreb, Croatia
vedrana.skaro@st.htnet.hr