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**HUMAN SKELETAL REMAINS:  
DEVELOPMENT OF DNA EXTRACTION AND  
TYPING METHODS**

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## **ABBREVIATIONS**

BSP: Bone Sialo Protein

CBQVS: Component-Based Quality Values System

CCD: Charge-Coupled Device

CODIS: Combined DNA Index System

DNA: Deoxyribonucleic Acid

EDTA: Ethylene Diamine Tetra-acetic Acid

ENFSI: European Network Forensic Science International

EOF: Electro-Osmotic Flow

ESS: European Standard Set

ISFG: International Society for Forensic Genetics

LCN: Low Copy Number

mtDNA: Mitochondrial DNA

PCR: Polymerase Chain Reaction

RGD: Arginine-Glycine-Aspartic site

RMP: Random Match Probability

SNP: Single Nuclotide Polymorphism

STR: Short Tandem Repeat

## **ABSTRACT**

In forensic cases human remains are often the only biological material available for identification of missing persons or unknown remains found in different circumstances such as mass disasters, wars or socio-political events and to solve paternity issues. DNA extracted from bones is often present in low copy number (LCN) and in various states of degradation due to chemical and physical damages produced by intrinsic and extrinsic bone characteristics. Efficient DNA extraction procedures, as well as accurate DNA amplification, are critical steps involved in the process of successful DNA analysis of skeletal samples. Unfortunately, at present there is not an infallible method to recover DNA from very degraded samples due to variations in DNA yield from larger bone fragments that may be attributed to heterogeneity within a bones.

In this study different types of human bones ranging in age from few months to 90 years post mortem, found in various states of preservation and conserved in different places, were analyzed. We developed a modified silica based spin columns protocol, consisting in an initial separation of DNA from proteins and waste material, by using phenol-chloroform to better purify samples. Moreover, as the recovery of information from these degraded samples is enhanced by the use of smaller PCR products (Mini Short Tandem Repeats) rather than conventional STRs, eight STR markers included in available commercial multiplex PCR kits, were redesigned by moving forward and reverse primers in close proximity to the STR repeat region. Two PCR quadruplexes were assembled to obtain PCR products less than 130 bp in size.

Our modified protocol was successfully employed to extract DNA from long bones of different ages and preservation state. Importantly the use of phenol chloroform consistently increased the amount of DNA that could be extracted from long bones, because it allowed to clean samples preventing that waste material interferes with columns or magnetic beads. Environmental conditions under which remains were exposed, had stronger influence on the state of DNA quality than the age of skeletal remains.

Moreover the use of miniSTRs has proposed here could be used in addition to commercial kits, to increase as much as possible the number of markers analyzed. Using

amplification commercial kits and the two new mini-STR quadruplex systems we always obtained genetic profiles of at least 12 STR from DNA typing of femur samples.

The improvement of DNA extraction methods and the inclusion of robust and powerful miniSTR loci in addition to the commercial available kits, are effective solutions for forensic practices of degraded DNA samples because ensure that difficult casework samples with low amounts of degraded DNA can be fully typed.

# 1. INTRODUCTION

## 1.1 Identification of human skeletal remains in forensic genetic investigations

The introduction of new DNA analysis techniques for human identification, is a recent development in forensic investigations. The application of an identification method based on DNA analysis is essential when recognition cannot be based only on the examination of personal effects or other physical characteristics. In the last twenty years substantial efforts have continuously been made to identify human remains found in different circumstances such as mass disasters, wars, socio-political events, to identify people responsible for violent crimes and to solve paternity issues.

The first detection of deoxyribonucleic acid (DNA) polymorphism in forensic casework investigation was performed by Jeffreys et al. in 1985 (1) and since its first use it has become a powerful tool in identification tests. The development and validation of new technology for detection of DNA polymorphisms have been very rapid. Over the last twenty years DNA profiling has become the main method for forensic human, animal and plant identification, in particular by introducing the study of microsatellite regions - Short Tandem Repeat (STR) loci - in routine paternity testing, as well as human identification (2).

Genetic investigations are usually performed with enzymatic amplification and analysis of STRs regions, using Polymerase Chain Reaction (PCR) technique and DNA sequencers, respectively. DNA intron regions are usually examined in forensic genetic practice because do not code for any known characteristics. The polymorphism of an STR mainly results from differences in the number of the repeated sequences, that leads to variations in the total length of the STR regions from person to person (3). Data obtaining by DNA typing are highly reliable and can be used as a powerful tool for producing valuable results. Multiplex PCR amplification of all or a subset of these STR markers is possible with a variety of commercial STR kit using spectrally resolvable fluorescent dyes (4). Moreover, the availability of commercial STR kits has greatly simplified the use of STRs, in recent years and led to development of DNA population databases (5). The use of STR markers for DNA profiling has a number of advantages over previously used methods, including highly polymorphic nature, ease of genotyping and ability to obtain results from degraded DNA samples or extremely small amounts of DNA. For the comparison of DNA profiles between different European

countries, the use of DNA kit containing the European Standard Set (ESS) or the Interpol Standard Set of Loci, is necessary and recommended by European Network Forensic Science International (ENFSI) (6).

The use of human skeletal remains as sources for detection of DNA polymorphism is a relatively recent advance in forensic identification. Intrinsic features of bones, such as physical and chemical robustness to environmental degradation and biological attack, make DNA analysis the only way to determine the identity of a person when traditional methods such as facial recognition, dactyloscopy or odontology, cannot be established (7).

Forensic identification case studies of human remains are usually referred both to identification of skeletal remains found in different circumstances and exhumation of remains from public or private cemeteries for investigation of paternity civil processes, where the task is usually to determine whether the people being investigated are related as parent-child (8). A common problem with these kinds of analysis is the preservation of DNA. The majority of DNA in bone is located in the osteocytes and Hochmeister et al. (1991) estimated that microgram quantities of DNA could potentially be extracted from a gram of bone (9). Osteocytes are embedded in a calcified matrix that is a barrier for access to DNA during the extraction process. Therefore, it is necessary to remove the matrix to improve the yield of DNA. Moreover the skeletal fragments recovered from mass fatality incidents often undergo series of decomposition changes that degrade both nuclear and mitochondrial DNA. For this reason the quality of DNA in old bones is highly variable and often substantially limited. This reflect the fact that remains are buried or disposed in many different environmental context, with differential exposure to potentially harsh extrinsic factors such as temperature, UV radiation, humidity and exposure to animals, insects and microbes. Different disposal conditions are marked by burial in different soil types, complete or partial immersion of remains in water, or in contact with fire. Microbial degradation is evidenced in old bones by both morphology and co-extraction of variable amount of microbial DNA and DNA inhibitors which are variable among samples (10). Qualitative differences in DNA content of different samples can be determined by the degree of reproducibility of allele determinations such as the presence or absence of artifacts (i.e. stutter bands or allelic dropouts and



drop in) (11). Finally, since airborne contamination can be caused by bone powder, highly stringent contamination control measures must be included when handling bone powder. The possibility of laboratory contamination increases particularly when processing a number of bone samples simultaneously, such as in mass fatality incidents.

The ideal method for purification and concentration of DNA should at the same time maximize DNA recovery and minimize the co-purification of PCR inhibitors. Low yields in the quantitative comparison may either indicate low amounts of template DNA or high amounts of inhibitions. In addition to decomposition by bacteria and other microorganisms, the simultaneous exposure to environmental agents results in DNA degradation in postmortem tissues. The state of preservation varies from putrefying cadavers, but still complete, to bones with little or no soft tissues (12). In cadavers, DNA degrades very quickly, even in early postmortem periods. The degradation of soft tissues is particularly evident after short intervals of time, a consequence of the rapid bacterial increase that is natural in decomposing corpses, especially in those that are exposed to hot temperatures.

In conclusion it is possible to suppose that the same characteristics of bone correlated with its general long term survival, i.e. its resistance to morphological degradation at the macroscopic and microscopic level, are those that contribute to protect DNA from degradation and therefore make difficult the extraction process.

## **1.2 Structure of bone**

Bone is a complex, highly organized and specialized connective tissue. It is characterized physically by the hardness, rigidity and strength, and microscopically by the presence of relatively few cells and much intercellular substance formed of collagen fibers and stiffening substances. Bone tissue is primarily composed of proteins and minerals and the association of organic and inorganic substances, which form the bone matrix, gives bone its hardness and resistance. (13)

### **1.2.1 Classification of bones**

There are five types of bone in the human body: long, short, flat, irregular and sesamoid.

**Long bones** are characterized by a long shaft (diaphysis) that is the result of the primary ossification center of bone. The expanded, flared ends of the shaft are called

metaphyses, while the ends of long bone are called epiphyses because they develop from secondary ossification center of the bone (the articular surfaces of the epiphysis are parts of joints).

The outer part of a long bone is made of compact bone which makes up 80% of the human skeleton. Microscopically, compact bone is riddled with canals and passageways that serve as conduits for nerves, blood vessels, and lymphatic vessels. The structural unit of compact bone is called osteon or Haversian system that is an elongated cylinder oriented along the long axis of the bone. Osteons appear as tiny weight-bearing pillars composed of a group of hollow tubes (lamella) of bone matrix, one placed inside the next. Running through the core of each osteon there is a canal (central or Haversian canal) which contains small blood vessels and nerve fibers. Running at right angles to the long axis are canals (perforating or Volkmann's canals) which connect the vascular and nerve supplies of the periosteum to those in the central canals and medullary cavity.

The interior part of a long bone is composed of spongy structure which consists of plates (trabeculae) and bars of bone adjacent to small, irregular cavities that contain red bone marrow. The canaliculi connect to the adjacent cavities, instead of a central Haversian canal, to receive their blood supply. It may appear that the trabeculae are arranged in a haphazard manner, but they are organized to provide maximum strength similar to braces that are used to support a building. The trabeculae of spongy bone follow the lines of stress and can realign if the direction of stress changes.

**Short bones** are roughly cube-shaped, and have only a thin layer of compact bone surrounding a spongy interior. The bones of wrist and ankle are short bones, as are the sesamoid bones.

**Flat bones** are thin and generally curved, with two parallel layers of compact bones sandwiching a layer of spongy bone. Most of the bones of the skull are flat bones, as is the sternum.

**Irregular bones** do not fit into the above categories. They consist of thin layers of compact bone surrounding a spongy interior. As implied by the name, their shapes are irregular and complicated. The bones of the spine and hips are irregular bones.

**Sesamoid bones** are bones embedded in tendons. They are tight, inflexible fibrous joints between bones that are united by bands of dense fibrous tissue in the form of

membranes or ligaments. Examples of sesamoid bones are the patella and the pisiform (14).

### 1.2.2 Organic composition of bone matrix

**Collagen** constitutes about 90% of the organic content of bone matrix and it is included in the amorphous substance made of proteoglycans and glycoproteins of which osteonectin binds to collagen and hydroxyapatite to form type I collagen fibers. Type I collagen is synthesized as tropocollagen and then exported, forming fibrils. Individual collagen molecules contain three polypeptides of about 1000 amino acids per chain with a high glycine and hydroxyproline content. The chains are aligned and linked into fibrils that subsequently rearranged into layers in which crystals of mineral are deposited. Collagen molecules intertwine to form flexible, slightly elastic fibers to give strength and flexibility to the bone.

The organic matrix of bone contains, in addition to type I collagen, includes other proteins such as osteonectin, a glycoprotein of molecular weight 32000 Dalton which constitutes about 3% of the organic matrix, osteocalcin and bone sialoprotein (BSP) (14,15).

**Osteonectin** binds collagen, hydroxyapatite, and growth factors; it regulates cell proliferation and can stimulate angiogenesis and the production of matrix metalloproteinases.

**Osteocalcin** is secreted by osteoblasts and thought to play a role in mineralization and calcium ion homeostasis. Its specific interaction with hydroxyapatite, osteocalcin has been thought to affect the growth or maturation of calcium phosphate mineral phases. Moreover it has been also stipulated that osteocalcin may also function as a negative regulator of bone formation, although its exact role is unknown.

**BSP** contains both mineral and arginine-glycine-aspartic (RGD) integrin-binding sites. The protein has been localized in vivo at the mineralization front, concurrent with and just prior to mineralization. Based on its affinity for type I collagen and hydroxyapatite, some believe BSP to be a de novo nucleator. Other possible functions of BSP relate to its ability to mediate cell attachment and it has been suggested that the protein is involved in the metastasis of tumor cells in bone (16).

### **1.2.3 Inorganic composition of bone matrix**

Approximately 70% of the mineral portion of the bone is composed of hydroxyapatite, which includes calcium phosphate, calcium carbonate, calcium fluoride, calcium hydroxide and citrate. Initially the main mineral constituent of new bone is calcium phosphate that laid down in amorphous form and subsequently, in mature bone, it is organized into crystals of hydroxyapatite that impregnate the collagen matrix. Calcium carbonate is the mineral usually found in the hard tissues of invertebrates, but calcium phosphate and apatite do occur in other groups than vertebrates. The composition of the mineral matrix of bone varies with age and in relation to various dietary factors. Mineral crystals are distributed as particles along the adjacent sheaves of collagen fibrils but also along individual fibrils and in the thickness of the fibrils themselves with which they combine chemically.

Structural arrangement of bone tissue is such that the mineral portion provides structural support to the protein portion in the bone and, by doing so, physically excludes exogenous/extracellular agents/enzymes that are potentially harmful to the protein portion of the bone (17). DNA has a very strong affinity for hydroxyapatite and its degradation is linked to the loss of crystallinity in the hydroxyapatite and also related to the loss of collagen (18).

### **1.2.4 Cellular composition of bone**

There are several types of cells constituting bone. Three primary cells involved in forming and maintaining bone tissue, are described below.

**Osteoblasts** descend from osteoprogenitor cells and are mononucleate bone-forming cells responsible for synthesizing and depositing bone material. They make a large quantities of a material known as osteoid (prebone tissue), an uncalcified organic matrix rich in collagen. Calcification of bone take place as crystal of hydroxyapatite that is deposited into the osteoid matrix. Once surrounded by bony matrix, the osteoblast are called osteocytes, cells that reside in lacunae and are responsible for maintaining bone tissue. Osteoblasts also manufacture hormones, such as prostaglandins, to act on the bone itself. Bone lining cells are essentially inactive osteoblasts. They cover all of the available bone surface and function as a barrier for certain ions.

**Osteocytes** originate from osteoblasts that migrate into and become trapped and surrounded by bone matrix that they themselves produce. The spaces they occupy are known as lacunae. Osteocytes have many processes that reach out to meet osteoblasts and other osteocytes probably for the purposes of communication. Their functions include to varying degrees: formation of bone, matrix maintenance and calcium homeostasis. They have also been shown to act as mechano-sensory receptors — regulating the bone's response to stress and mechanical load. They are mature bone cells.

**Osteoclasts** are those cells responsible for bone resorption (remodelling of bone to reduce its volume). Osteoclasts are large, multinucleated cells located on bone surfaces in what are called Howship's lacunae or resorption pits. These lacunae, or resorption pits, are left behind after the breakdown of bone surface. All skeletal elements change dramatically during ontogeny and continue to be capable of change in childhood. Bone formation takes place throughout the life and the remodelling of bone occurs at the cellular level as osteoclasts remove bone tissue and osteoblasts build bone tissue. The opposing processes of bone formation and resorption allow bones to maintain or change their shape and size during growth (14).

### **1.3 Molecular damage in old bone: DNA degradation processes**

As described before the major constituent of bone are protein and collagen and the relationship between these constituents involves complex features and chemical bonds. Alterations of bone proteins cause the complete structural and chemical breakdown, responsible for the post mortem changes in bone.

Enzymatic repair processes maintained the integrity of DNA molecules within living cells and after the death of an organism cell compartments that normally sequester catabolic enzymes, breakdown (19). In post mortem period the DNA is rapidly degraded by enzymes such as lysosomal nucleases together with endogenous nucleases that are the first agents to start the process of DNA fragmentation (20). Moreover due to the exposure to exogenous agents, like microorganisms, humidity and many organic compounds, the amount of informative DNA available is drastically reduced. Spontaneous degradation by hydrolysis and oxidation will further modify DNA structure at a much slower speed and escape enzymatic and microbial degradation under

rare circumstances, such as when a tissue becomes rapidly desiccated after death or DNA is adsorbed to a mineral matrix. In these cases chemical processes start affecting the DNA slower but still relentless. Many of DNA degradation processes are similar or identical to those that affect DNA in living cell but after death they are not counterbalanced by cellular repair processes. Damages accumulate progressively until DNA loses its integrity and decomposes, with an irreversible loss of nucleotide sequence information (Table 1) (21). With modern molecular biology techniques such as PCR reaction, it is possible to recover partial or complete information from DNA not yet completely degraded.

DNA degradation into small average size, generally between 100 and 200 bp, is the most common type of DNA damage of old skeletal remains (22). The reduction in size is due to both enzymatic processes that occur shortly after death and nonenzymatic **hydrolytic cleavage** of phosphodiester bonds in the phosphate-sugar backbone (21) that generate single-stranded nicks. The glycosidic bonds between nitrous bases and the sugar backbone are the most susceptible bond hydrolytic cleavage that result in depurination and depyrimidination of bases, producing an apurinic and apyrimidinic site (19). Once a nucleotide is released, the abasic site can undergo a chemical rearrangement that can lead to strand scission if it is not immediately repaired.

In addition to these modifications, DNA bases are also susceptible to **hydrolytic deamination** of cytosine (uracil), 5-methyl-cytosine (thymine) and adenine (hypoxanthine), that are of particular relevance for DNA amplification since they cause incorrect insertion of bases (A instead of G, and C instead of T) when new DNA strands are synthesized by DNA polymerase (21).

Endogenous damage of DNA are also induced by **oxidative reactions** such as superoxide radical ( $O_2^{\cdot-}$ ), hydrogen peroxide ( $H_2O_2$ ) that are produced by ionizing radiation or aerobic metabolism of microorganisms that colonize post mortem tissue. Ionizing radiations can also produce hydroxyl radicals ( $\cdot OH$ ) in cells and tissues by interacting with cellular water. Pyrimidines (in particular thymine) are more sensitive to oxidative damage than purines. Oxidative damage mostly includes modifications of sugar residues, conversion of cytosine and thymine to hydantions, removal of bases and cross linkages (23). It has been shown that skeletal remains from different range of environments and ages contain oxidized base residues. Specifically, no DNA sequences

could be amplified via PCR from samples with higher amounts of two oxidized pyrimidines (5-hydroxy-5-methylhydantoin and 5-hydroxyhydantoin), which block the *Taq* DNA polymerase (24).

Another type of damage are ***crosslinks***, that occur when various exogenous or endogenous agents react with two different positions in DNA. This can either occur in the same strand (intrastrand crosslink), in the opposite strands of the DNA (interstrand crosslink) and also between DNA and protein. The cross linking reaction between the two DNA strands is temperature dependent and is slower in low temperatures (25). DNA strands would also react through their bases with reducing sugars in a nonenzymatic glycation reaction followed by the generation of abasic sites (23). An effect of crosslinking reaction is represented by Maillard products that have been identified in ancient fecal remains (coprolites) by head space gas chromatography–mass spectroscopy. Maillard products are formed by condensation reactions between sugars and primary amino-groups in proteins and nucleic acids. Treatment with a reagent, N-phenacylthiazolium bromide, which breaks Maillard products, allows DNA sequences to be amplified from some ancient remains that otherwise are not amenable to amplification (21).

All of these DNA modifications are problematic because they could cause incorrect incorporation of bases during the PCR, or produce the total loss of expected DNA fragment during PCR amplification.

**TABLE 1-** Overview over different types of damage in aged DNA (21,23).

<b>TYPE OF DAMAGE</b>	<b>CAUSES</b>	<b>EFFECT ON DNA</b>	<b>EFFECTS ON PCR</b>	<b>TROUBLE SHOOTING</b>
Strand breaks	Nuclease activity; degradation by microorganisms	Reduction of overall template copy numbers; size reduction	PCR failure; increased stutter peaks; inconsistent PCR outcomes	Resampling, multiple independent PCRs; size reduction; Mini-STRs; mtDNA; SNPs; increase PCR cycles up 34; Extra dose of Taq polymerase; DNA repair
Oxidative lesions	Bacterial metabolism; radiation	Base or sugar fragmentation; nucleotide modification	PCR failure	
DNA crosslinkage	Hydrolytic base loss; Reduced sugar residues;	DNA to DNA or DNA to other biomolecules linkage	PCR failure; Millard products	
Hydrolytic lesions	Hydrolytic loss of amino group	Change of coding potential	PCR miscoding lesions	



#### 1.4 PCR amplification of degraded DNA

Bone samples may contain highly fragmented DNA molecules caused by different forms of damage, summarized before. The modern forensic analysis of human remains is based on size or sequence analysis of PCR products. PCR based protocols although highly effective may encounter complications through the low copy numbers of the template or modifications imposed on the template during the degradation process. Compared with contemporary DNA preparations from fresh samples of saliva or blood, old bones DNA is generally of shorter length. The length of DNA sequences that can be amplified by PCR is in fact limited not only by strand breaks but also by lesions that present blocks to the elongation of DNA strands by the Taq polymerase. A diploid human cell contains ~ 6.6 pg of genomic DNA and template DNA concentration < 100 pg genomic DNA (about 15-17 diploid copies of nuclear DNA) is considered as Low Copy Number (LCN) (26). When processing a small number of starting templates during the PCR, stochastic effects may occur. The result is that the following events may appear: allelic dropout, allelic imbalance, increased stutter or non-template addition.

The preferential amplification condition could happen in heterozygote individuals when one of the two alleles fail to amplify properly. This effect is known as *allelic dropout* and the result is the incorrect genotyping of the individual as homozygote (23).

When the ratio of the expression levels of 2 alleles at a given heterozygous locus is not 1 to 1, the final effect is an *allelic imbalance*.

Other frequent artifacts that occur are *stutter products* that are originated from the slippage of the Taq polymerase enzyme during DNA replication. Thus, for tetranucleotide repeat units, stutter products are 4 base pairs shorter or higher than the main allele band and less than 10% of the main peak (27).

*Non-template addition* is another type of frequent artifact and refers to the addition of an extra nucleotide to the 3' end of PCR product. Because the non-template addition is often adenine (A), sometimes it is referred to as adenilation. This plus A modification is primer specific and results in a product that is one base pair longer than the target sequence (28).

Optimal template amounts are well defined and typically range from 200 pg to 2 ng of input DNA (1 ng is considered the optimum amount for most commercial kits) with 28-

30 PCR amplification cycles (29). One nanogram of DNA is approximately equal to 660 copies of genomic DNA and when the starting DNA amount is less than 60 copies an increased incidence of PCR failures is observed (30). DNA amplification displays a similar efficiency for loci such as those used in forensic applications, characterized by a length less than 500 bp and a GC content of 45-56%. Commercial multiplex kits used in forensic DNA typing amplify fragments in the size range of 100-450 bp and multiplexing the PCR reaction reduces the amount of sample material necessary for analysis and minimizes the experimental time and costs.

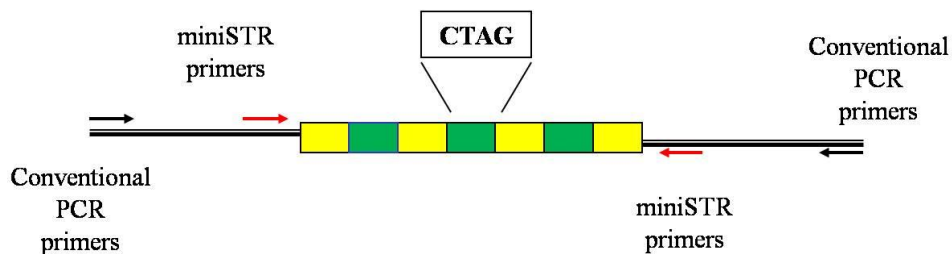
In low copy number samples repeated extractions and amplifications are recommended to authenticate the results and other analytical approaches recommended include reducing PCR volume, increasing PCR cycle number and nested PCR. Whilst low copy number typing is appropriate for identification of missing persons and human remains and far developing investigative leads, future developments are required to overcome its limitations in other applications. Occurrence of artifacts discussed here could be reduced if quantification of samples indicate that template DNA contains fragments several hundred base pairs to initiate the PCR. Such quantification of template molecules can be achieved by using competitive PCR and more recently, by using real-time quantitative PCR (23).

### **1.5 Application of miniSTRs on degraded samples**

The current commercially available multiplex STR kits used in forensic DNA typing, can generate amplicons in the size range of 100 bp to 450 bp. Within the forensic community, a core set of STR markers have been developed for the use in forensic casework, and large databases such as the Combined DNA Index System (CODIS). In situations where samples are badly degraded that STR analysis is not possible, the analysis of the mitochondrial DNA (mtDNA) hypervariable regions is typically used (31). However, mtDNA testing is a time-consuming process and, due to the haploid, non-Mendelian nature of mtDNA inheritance, data are not as powerful for identification purposes as a full 13-locus STR match of CODIS. Furthermore recovery of information from degraded DNA samples is theoretically possible also with single nucleotide polymorphism (SNP) typing, since a smaller target region is needed. The most significant disadvantage exists with SNP markers is due to their low power of

discrimination compared to STR markers. Numbers on the order of 40–60 SNPs have been suggested to reach equivalent power of discrimination or random match probabilities of 13-15 STR loci as are commonly in use today (32).

Although PCR target size reduction increases amplification efficiency, it may also increase the chances of amplifying contaminating DNA, especially in highly degraded DNA specimens. An additional approach to try to recover information from degraded DNA samples is to reduce the size of the PCR products by moving primers in as close as possible to the STR repeat region (fig.1). These reduced sized targets are referred to as miniSTRs (33).



**Fig.1.** MiniSTR primers are closer to STR repeat region than conventional STR primers, producing reduced size PCR amplicons that enables higher recovery of information from degraded DNA samples.

Because the main problem associated with DNA degradation is the fragmentation of the DNA template, primers which produce smaller PCR products have higher possibility of obtaining a profile from shorter DNA fragments. The advantage of miniSTRs is that the fragments are likely to survive degradation, hence it is more likely that a complete DNA profile will be observed using a standard number of cycles. Due to their wide use, CODIS STR markers would thus be ideal for candidates in designing new primers that could create smaller PCR products. The newly designed primers are combined into various multiplexed sets, referred to as “miniplexes,” usually with a single locus in each dye colour. A major advantage of these miniSTRs, is that database compatibility could be maintained with convicted offender samples processed using commercial STR multiplexes. However, a few of the CODIS loci cannot be made into smaller amplicons due to the fact that some of the CODIS loci have large allele ranges (e.g. FGA) (34).

In conclusion miniSTR assays should offer a new potential tool for recovering useful information from samples that generated partial profiles with present STR multiplexes.

## **1.6 Aim of this study**

In this study different types of human bones ranging in age from few months to 90 years post mortem, found in various states of preservation and conserved in different places, were analyzed. Extraction and successful PCR amplification of DNA from human remains in historical and forensic cases has great importance, but is particularly difficult because the methods employed at present are not always satisfactory. Several of them are in fact complicated and time consuming and none methods has reached on acceptance level such that they are routinely used on a widespread basis. Bone extraction protocols currently employed in forensic laboratories tend to be limited because they often fail to give reproducible results, e.g. in cases where bones are exposed to environmental conditions for a long time (35).

Different kind of bone, femur, humerus, tibia, jaw, rib, belonging to different cadavers were analyzed. We established a semi-standardized protocol for DNA extraction from bones, verifying which kind of bone yields the best quality of DNA and evaluating different characteristics such as preservation, place of conservation and age of skeletal remains. A comparison in terms of quality of electrophoretic products, was performed from human skeletal remains considering five different DNA extraction methods and starting from a low amount of bone powder (50 -100 mg). In addition to the traditional phenol–chloroform organic method for DNA extraction, four commercial kits were evaluated: QIAamp® DNA Mini kit (Qiagen, Hilden, Germany), QIAamp® DNA Investigator kit (Qiagen, Hilden, Germany), DNA IQ™ System (Promega, Milan, Italy) and PrepFiler™ Forensic DNA Extraction Kit (Applied Biosystems, Foster City, CA). Experiments were also performed with a modified protocols developed by Forensic Genetic Laboratory of University of Verona, consisting in an initial separation of DNA from proteins and waste material, by using phenol-chloroform to better purify samples. The new step was introduced after incubation in lysis buffer of different kit solutions. The phenol-chloroform step allowed to clean samples avoiding that the waste material would interfere with columns or magnetic beads.

Moreover as recovery of information from degraded samples is enhanced by the use of smaller PCR products called miniSTR, primer pairs of eight STR markers, included in available commercially multiplex PCR kits, were redesigned by moving forward and reverse primers in close proximity to the STR repeat region. They were

assembled in two PCR-multiplexes to obtain PCR products less than 130 bp in size. The choice of this size was determined by the observation that in degraded samples amelogenin is usually the only marker amplified because it is the lowest in size (106-112 bp). In addition to the new miniSTRs multiplexes all samples were amplified also with two kits widespread in forensic use such as AmpFlSTR<sup>®</sup> Identifiler<sup>™</sup> PCR Amplification Kit (Applied Biosystems), AmpFlSTR<sup>®</sup> MiniFiler<sup>™</sup> PCR Amplification Kit (Applied Biosystems) and PowerPlex<sup>®</sup> ESI 17 System (Promega).

The improvement of DNA extraction methods and increasing number of miniSTRs in addition to the commercial available kits, may be effective solutions for forensic practices of degraded DNA samples. The application of the DNA extraction protocol based on the use phenol-chloroform for bones exposed to critical environmental conditions for long periods and for low amounts of bone gave good results. Moreover the use of miniSTRs has proposed here could be used in addition to commercial kits, to increase as much as possible the number of markers analyzed.

## 2. MATERIALS AND METHODS

### 2.1 Samples

The present study was conducted on skeletal remains of 14 persons that were recovered from very different geographical and environmental areas (wooded area, corn field, exhumed from zinc coffin, buried under soil and burned body). Due to variability of conservation of each sample and post mortem intervals that ranged from few months to 90 years (eg. bones of unknown soldiers of World War I), skeletal remains were found in extremely different state of preservation. Moreover, the type of skeletal elements could be different from case to case (Table 2).

To ensure quality standards and prevent contamination in Forensic Genetics Laboratory, were followed recommendations by DNA commission of the ISFG (36).

All samples were initially examined in the autopsy room, where about 2 mm of the external and internal surfaces of all bones were grounded away with sandpaper to eliminate potential contaminants. Bone fragments were then transferred to Laboratory of Forensic Genetics and pulverized.

First of all, bones were cut into small pieces and placed into 7 ml Shaking flask, made of stainless steel (Sartorius), and frozen in liquid nitrogen. Then Shaking flask together with fragments of bone and a grinding ball, provided with the flask, was placed into the homogenizer Mikro-Dismembrator S (Sartorius) that was set at maximum frequency of  $3.000 \text{ min}^{-1}$ . Due to the high efficiency of shaking with grinding balls, samples were rapidly disintegrated and 100-150 mg of powder transferred to a sterile 2 ml tubes. Pulverized bones were then decalcified in 1.5 ml of 0,5 M Ethylene Diamine Tetra-acetic Acid (EDTA), incubated overnight at  $56^{\circ}\text{C}$  in a water bath and after centrifugation, the supernatant was discarded and the remaining decalcified pellet was extracted with different kits.

**Table 2.** Case profiles

Case	Specimen type	Maturity	Post mortem period	Location where found	Reference information
1	Femur	Adult	I World War	Buried in pauper grave	Completely skeletonized
2	Femur, humerus, tibia	Adult	I World War	Buried in pauper grave	Completely skeletonized
3	Cranium Compact bone,	Fetus	26 years	Buried in an ossuary	Completely skeletonized
4	Femur, cranium, humerus, ribs, vertebrae	Adult	22 years	Wooded area on mountain	Completely skeletonized
5	All skeleton	Adult	20 years	Exhumed from zinc coffin	Mummified
6	All skeleton	Adult	20 years	Exhumed from zinc coffin	Partially skeletonized
7	Spongy bone, humerus, femur	Adult	19 years	Buried under soil	Completely skeletonized
8	Compact bone, femur	Adult	12 years	Buried in pauper grave in the mountains of Colombia	Completely skeletonized
9	Cranium, femur	Adult	4 months	Burned, homicide in a car	Burned, Partially skeletonized
10	Fragment of femur	Adult	2 months	Burned, Suicide at home	Burned, Completely skeletonized
11	Fragment of femur	Adult	2 months	Burned, Suicide in a car	Burned, Completely skeletonized
12	Femur, tibia	Adult	1 months	Burned Plane crash	Burned, Partially skeletonized
13	Cranium, femur, tibia	Adult	1 months	Burned, homicide in a car	Burned, Partially skeletonized
14	Cranium, femur	Adult	2 months	Discarded in a corn field during summer	Partially skeletonized

## **2.2 DNA extraction**

All bone powder samples were extracted by the use of conventional phenol/chloroform procedure and four different commercial kits such as QIAamp<sup>®</sup> DNA Mini kit (Qiagen), QIAamp<sup>®</sup> DNA Investigator kit (Qiagen), DNA IQ<sup>™</sup> System (Promega) and PrepFiler<sup>™</sup> Forensic DNA Extraction Kit (Applied Biosystems), following standard protocols. Subsequently from the same samples, DNA was extracted using the same kits with minor modifications. Both standard and modified protocols are described below.

### **2.2.1 Conventional phenol/chloroform DNA extraction procedure**

1. 1 ml of TNE (10 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 2% sodium dodecyl sulfate), 20 µl of proteinase K and 10 µl of DTT were added to the samples and incubated overnight at 60°C.
2. 1 ml of phenol-chloroform-isoamylalcohol (25:24:1) was added and the tube was centrifuged at 14000 rpm for 5 minutes to separate the phases.
3. About 90% of the upper, aqueous layer was removed and placed in a clean tube, carefully avoiding proteins at the aqueous phenol interface.
4. Step 2 was repeated twice.
5. The upper layer was transferred and mixed with 1 ml chloroform isoamylalcohol (24:1) before the third centrifugation at 14000 rpm for 5 minutes
6. The supernatant was again transferred to another tube and DNA made insoluble by the addition of twice-three times the solution's volume of cold absolute ethanol (Carlo Erba, Milan, Italy).
7. This solution was then centrifuged at maximum speed for 30 min.
8. DNA was rinsed by centrifugation in 70% isopropanol at maximum speed.
9. Subsequently sample was dried up before it was made soluble in 50 µl of distilled water (37).

### **2.2.2 QIAamp<sup>®</sup> DNA Mini kit**

1. 700 µl of Buffer ATL , 20 µl of proteinase K and 10 µl of dithiotreitol (DTT) 1 M (Sigma Aldric, Milan, Italy) were added to the samples.



2. Samples were incubate overnight at 56°C in a water bath (Vetrotecnica, Verona, Italia).
3. 500 µl of Buffer AL were added and mixed by pulse-vortexing for 10 s.
4. The tube was place in a water bath (Instruments s.r.l.), and incubated at 70°C with for 15 min.
6. Samples were centrifuged at full speed 14,000 rpm for 2 min (Eppendorf) and carefully the supernatant was transferred to a new 1.5 ml microcentrifuge tube.
7. 500 µl of ethanol (96%) were added and mixed by pulse-vortexing for 20 s.
8. Carefully the entire lysate was transferred to the QIAamp MinElute column (in a 2 ml collection tube).
9. Sample was centrifuged at 8000 rpm for 1 min. If the lysate was not completely passed through the membrane after centrifugation, it was centrifuged again at a higher speed until the QIAamp MinElute column was empty.
10. 500 µl of Buffer AW1 were added to the QIAamp MinElute column and centrifuged at 8000 rpm for 1 min.
11. 500 µl of Buffer AW2 were added to the QIAamp MinElute column and centrifuged at 14,000 rpm for 1 min.
12. QIAamp MinElute column were placed in a clean 2 ml eppendorf and incubated at room temperature for 1 min.
13. 70 µl of Buffer AE were applied to the center of the membrane and incubate at room temperature (15–25°C) for 1 min.
14. Samples were centrifuged at 8,000 rpm for 1 min (38).

### **2.2.3 QIAamp<sup>®</sup> DNA Investigator kit**

1. 360 µl of Buffer ATL, 20 µl of proteinase K and 10 µl of dithiotreitol (DTT) 1 M were added to the samples.
2. Samples were incubate overnight at 56°C in a water bath.
3. 300 µl of Buffer AL were added and mixed by pulse-vortexing for 10 s.
4. The tube was place in a water bath (Instruments s.r.l.), and incubated at 70°C with for 10 min.
6. Samples were centrifuged at full speed 14,000 rpm for 2 min (Eppendorf) and carefully the supernatant was transferred to a new 1.5 ml microcentrifuge tube.

7. 150 µl of ethanol (96%) (Carlo Erba) were added and mixed by pulse-vortexing for 15 s.
8. Carefully the entire lysate was transferred to the QIAamp MinElute column (in a 2 ml collection tube).
9. Sample was centrifuged at 14000 rpm for 1 min. If the lysate was not completely passed through the membrane after centrifugation, it was centrifuged again until the QIAamp MinElute column was empty.
10. 600 µl of Buffer AW1 were added to the QIAamp MinElute column and centrifuged at 8000 rpm for 1 min.
11. 700 µl of Buffer AW2 to the QIAamp MinElute column and centrifuged at 8,000 rpm for 1 min.
12. 700 µl of ethanol (96%) were added to the QIAamp MinElute column and centrifuged at 8,000 rpm for 1 min.
13. Samples were centrifuged again but at full speed 14,000 rpm for 3 min to dry the membrane completely. This step was necessary since ethanol carryover into the eluate could interfere with some downstream applications
14. QIAamp MinElute column were placed in a clean 2 ml eppendorf and incubated at 56°C for 3 min.
15. 40 µl of Buffer ATE were applied to the center of the membrane and incubate at room temperature (15–25°C) for 1 min.
16. Samples were centrifuged at 14,000 rpm for 1 min (39).

#### **2.2.4 DNA IQ™ System**

1. 100 µl of Bone Incubation Buffer (10mM Tris pH 8.0, 100mM NaCl , 50mM EDTA, 0.5% SDS), 20 µl of Proteinase K and 10 µl of DTT (Sigma Aldrich) were added and incubated overnight at 56 °C.
2. Samples were placed in a 1.5 ml eppendorf, added 200 µl of prepared Lysis Buffer and incubated at 70°C for 1 hour.
3. Samples together with incubation and lysis buffers were transferred to a DNA IQ™ Spin Basket seated in a 1.5ml Microtube.
4. Samples were centrifuged at room temperature for 2 minutes at maximum speed and then the spin basket was removed.

5. The stock resin bottle was vortexed until resin is thoroughly mixed. 15µl of DNA IQ™ Resin were added to the samples.
6. samples/Lysis Buffer/resin mixture were vortexed for 3 seconds at high speed and incubate at room temperature for 5 minutes. Vortex tube for 2 seconds at high speed.
7. The tube was placed in the magnetic stand and separation occurred instantly.
8. All of the solution was carefully removed and discarded without disturbing the resin pellet on the side of the tube.
9. 100µl of prepared Lysis Buffer were added, the tube was removed from the magnetic stand and vortex for 2 seconds at high speed.
10. The tube was returned to the magnetic stand and all Lysis Buffer discarded.
11. 100µl of prepared 1X Wash Buffer were add, the tube was removed from the magnetic stand and vortex for 2 seconds at high speed.
12. The tube was returned to the magnetic stand and all Wash Buffer discarded.
13. Steps 11 and 12 were repeated two more times for a total of three washes.
14. With the tube in the magnetic stand and the lid open, the resin was air-dry for 5 minutes.
15. 25µl of Elution Buffer were added and the tube vortexed for 2 seconds.
16. The tube was incubated at 65°C for 5 minutes.
17. The tube was vortexed for 2 and immediately placed on the magnetic stand.
18. The DNA-containing solution was carefully transferred in a clean microtube (40).

### **2.2.5 PrepFiler™ Forensic DNA Extraction Kit**

1. 300 µL of PrepFiler™ Lysis Buffer, 20µl of Proteinase K and 10 µl of DTT were added to the samples and incubated overnight at 60 °C.
2. Samples together with incubation buffer and lysis buffers were transferred to a PrepFiler™ Filter Column into a new 1.5 ml PrepFiler™ Spin Tube.
3. Samples were centrifuged at room temperature for 2 minutes at maximum speed and then the Filter Column was removed.
4. The stock magnetic particles bottle was vortexed until resin is thoroughly mixed. 15µl of PrepFiler™ Magnetic Particles were added to the samples.
5. Samples were vortexed for 10 seconds, then centrifuged briefly.

6. 180  $\mu$ L isopropanol were added to the sample lysate tube and vortexed for 10 seconds, then centrifuged briefly.
7. After binding the DNA to the magnetic particles, the tube was returned to the magnetic stand and all visible liquid phase was discarded.
8. 300 $\mu$ l of prepared PrepFiler™ Wash Buffer were add, the tube was removed from the magnetic stand and vortex for 2 seconds at high speed.
9. All of the solution was carefully removed and discarded without disturbing the resin pellet on the side of the tube.
10. Steps 8 and 9 were repeated two more times for a total of three washes.
11. With the tube in the magnetic stand and the lid open, the resin was air-dry for 5 minutes.
12. 30  $\mu$ l of PrepFiler™ Elution Buffer were added and the tube vortexed for 2 seconds.
13. The tube was incubated at 65°C for 5 minutes.
14. The tube was vortexed for 2 and immediately placed on the magnetic stand.
15. The DNA-containing solution was carefully transferred in a clean 2 ml eppendorf (41).

### **2.2.6 Introduction to minor modifications to standard protocols**

After incubation in lysis buffer an equal volume of phenol-chloroform-isoamylalcohol was added in each sample. Samples were vortexed and then centrifuged at 14000 rpm for 3 minutes to separate upper aqueous phase containing the nucleic acids and a bright pink lower phase containing the proteins and the lipids dissolved in phenol-chloroform. This step was repeated one or two times depending on the contamination of bones with soil. The aqueous phase were then transferred in a new tube and the same quantity of chloroform-isoamylalcohol was added. After centrifugation at 14000 rpm for 3 minutes the aqueous phase was transferred in a new tube and 500  $\mu$ l of ethanol (96%) were added and vortexes for 20 seconds. Samples were then transferred in the columns, or in an tube depending on the type of kit and processed following the standard protocol.

## 2.3 DNA amplification

DNA amplification and STRs typing of autosomal DNA were performed using various commercial kits: AmpFISTR Identifiler™ PCR Amplification Kit (Applied Biosystems), PowerPlex® ESI 17 System (Promega), AmpFISTR MiniFiler™ PCR Amplification Kit (Applied Biosystems) and two new miniSTR quadruplexes developed and validated by Forensic Genetic Laboratory of Verona.

PCR negative and positive controls (K562 and 9947A, Promega) were included in each amplification reaction to verify the purity of amplification reagents.

### 2.3.1 AmpFISTR® Identifiler™ PCR Amplification Kit

The AmpFISTR® Identifiler™ PCR Amplification Kit (Applied Biosystems) is a STR multiplex assay that amplifies 15 tetranucleotide repeat loci and the Amelogenin gender determining marker in a single PCR amplification (Table 3).

PCR amplifications were performed in reaction volumes of 25 µl using 10.5 µl of AmpFISTR® PCR Reaction Mix, 0.5 µl of AmpliTaq Gold DNA Polymerase (5U/ µl) (Applied Biosystems), 5.5 µl of AmpFISTR® Identifiler Primer Set. 15 µl of master mix were dispensed in each PCR tube and 10µl of DNA sample were added. Thermal cycling was performed with the GeneAmp® 9700 (Applied Biosystems) using the following conditions:

- *Initial incubation step:* 95°C for 11 min;
- *28-34 cycles:* denaturation 94°C for 1 min, annealing 59°C for 1 min, extension 72°C for 1 min;
- *Final extension:* 60°C for 60 min;
- *Final hold:* 4°C forever.

**Table 3-** Loci amplified with AmpFISTR® Identifiler™ PCR Amplification Kit, the range of PCR products expressed in base pair and the corresponding dyes used (42).

Locus	Range of PCR product sizes (bp)	Dye label
D8S1179	123-169	6-FAM™
D21S11	185-240	
D7S820	255-291	
CSF1PO	305-341	
D3S1358	112-140	VIC®
TH01	163-202	
D13S317	217-245	
D16S539	252-292	
D2S1338	307-359	
D19S433	102-135	NED™
vWA	155-207	
TPOX	222-250	
D18S51	262-346	
Amelogenin	106/112	PET®
D5S818	134-172	
FGA	215-355	

### 2.3.2 AmpFISTR® MiniFiler™ PCR Amplification Kit

The AmpFISTR MiniFiler™ PCR Amplification Kit (Applied Biosystems) contains eight STR loci shared with the AmpFISTR Identifiler™ PCR Amplification Kit, but uses shorter amplicons, which makes them more likely to be successful on fragmented DNA (Table 4).

PCR amplifications were performed in reaction volumes of 25 µl using 10.0 µL of AmpFISTR® MiniFiler™ Master Mix, containing 1X GeneAmp® PCR Gold buffer (Applied Biosystems), 5.0 µl AmpFISTR® MiniFiler™ Primer Set and 10 µl of DNA sample.

Thermal cycling was performed in the GeneAmp® 9700 (Applied Biosystems) using the following conditions :

- *Initial incubation step:* 95°C for 11 min;
- *28-34 cycles:* denaturation 94°C for 20 sec, annealing 59°C for 2 min, extension 72°C for 1 min;

- *Final extension*: 60°C for 45 min;
- *Final hold*: 4°C forever.

**Table 4-** Loci amplified with AmpFlSTR® MiniFiler™ PCR Amplification Kit, amplicon length reduction of STR markers compared to the Identifiler® and expressed in nucleotides and finally the corresponding dyes color (43).

Locus	MiniFiler Kit Amplicon Reduction (nt)	Dye label
D7S820	-129	6-FAM™
D13S317	-99	
Amelogenin	0	VIC®
D21S11	-33	
D2S1338	-183	
D18S51	-168	NED™
D16S539	-157	
FGA	-87	PET®
CSF1PO	-201	

### 2.3.3 Two new miniSTR quadruplexes

Eight conventional STR markers (D8S1179, D3S1358, TPOX, TH01, D5S818, CSF1P0, D13S317, D16S539) included in multiplex PCR kits commercially available, were redesigned and converted into Mini-STRs, in order to reduce or eliminate the polymorphism's flanking regions. The eight STRs were amplified in two quadruplex: D8S1179, D3S1358, TPOX, D16S539 and CSF1P0, TH01, D13S317, D5S818 (Table 5). Two quadruplexes were designed with one miniSTR locus in each dye color (Fam, Vic, Ned Pet) to avoid overlap and interference with other loci. Three of eight mini-STRs (CSF1P0, D8S1179 and D13S317) described in this work, were previously validated and tested (44).

DNA amplification of the two quadruplexes was performed in a reaction volume of 12.5 µl using Qiagen® Multiplex PCR kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations in terms of primers and DNA concentrations (45). Thermal cycling was performed with a GeneAmp® 9700 (Applied Biosystems) using following conditions:

- *Initial incubation step*: 95°C for 15 min;

- 28-30 cycles: denaturation 94°C for 30 sec, annealing 59°C for 1.30 min, extension 72°C for 1 min;
- Final extension: 60°C for 30 min;
- Final hold: 4°C forever.

Allelic ladders for mini-STRs were created using 1:1000 dilution of allelic ladder from Identifiler® kit. 2 µl of these diluted ladders were amplified in the two quadruplex sets using the thermal cycling parameters outlined for the PCR above, except amplified for 15 cycles instead of the standard 28 cycles (31). The cell line samples K562 and 9947A (Promega) were used as control DNA for calibrating allelic ladders.

Two macros for the new quadruplexes were specifically written for the use of two new mini multiplexes in order to make the allele calls from the allelic ladders.

All PCR amplifications, together with positive and negative control samples, were performed on Gene Amp® PCR System 9700 (Applied Biosystem). Each miniplex consists of four different loci, with each locus labeled using different colored dyes (44).

**Table 5-** Primer sequences and PCR product sizes, reduction amplicon length compared to STR markers of Identifiler® kit, of the two Mini-STR quadruplex systems.

Locus	mini-STR primers (5'–3')	Mini-STR size (bp)	Reduction size
D8S1179	F [6-FAM] GTATTTTCATGTGTACATTCG R GATTATTTTCACTGTGGGG	68–112	59
D3S1358	F [VIC] CAGAGCAAGACCCTGTCT R GAAATCAACAGAGGCTTGC	73–117	24
TPOX	F [NED] AGGCACTTAGGGAACCCT R GTCAGCGTTTATTTGCC	64–92	153
D16S539	F [PET] CAGACAGACAGACAGGTG R GTATCTATCATCCATCTCTG	86–114	178

Locus	mini-STR primers (5'–3')	Mini-STR size (bp)	Reduction size
CSF1P0	F [6-FAM] CAGTAACTGCCTTCATAGATAG R GACCTGTTCTAAGTACTTCC	82–118	223
TH01	F [VIC] ATTCCCATTTGGCCTGTTC R GTCACAGGGAACACAGACTC	69–115	95
D13S317	F [NED] GCCTATCTGTATTTACAAATAC R CCCAAAAAGACAGAAAGAA	92–120	113
D5S818	F [PET] CCTCTTTGGTATCCTTATGT R CTTTATCTGTATCCTTATTTATACC	84–120	50



Microsatellites and their adjacent regions have mutation rates higher than other genomic regions (46) and when the primers are redesigned problems may occur in despite of well known polymorphisms have been taken in account. For this region only comparison study can verify the presence of previously undetected polymorphisms. If the polymorphism is close to 3' end of the primer the allele may not be amplified and stochastic event may occur such as null allele or allele dropout (47). On the other hand, if the polymorphism is close to 5' end of the primer only small reduction in amplification efficiency will be observed.

We performed a concordance study on 100 buccal swab provided by healthy donors and previously typed with Identifiler<sup>®</sup> and Minifiler<sup>™</sup>. The comparison of typing results between the two new quadruplexes and conventional STRs showed no genotype differences with good balance between alleles, no double peaks due to +A/-A and stutter products higher less than 15% of the main peak, confirming that changes in the dimensions of the STRs do not influence the profile.

#### **2.3.4 PowerPlex<sup>®</sup> ESI 17 System**

The PowerPlex<sup>®</sup> ESI 17 System allows co-amplification and four-color fluorescent detection of seventeen loci (sixteen STR loci and Amelogenin) described in Table 7. The markers typed comprise the 15 STRs of Identifiler<sup>®</sup> kit plus five recently introduced European Standard Set (ESS) STRs: D1S1656, D2S441, D10S1248, D12S391 and D22S1045. Moreover this kit includes also the marker SE33.

PCR amplifications were performed in reaction volumes of 25 µl using 5.0 µl of PowerPlex<sup>®</sup> ESI 5X Master Mix, 2.5 µl PowerPlex<sup>®</sup> ESI 10X Primer Pair Mix, 10 µL of DNA sample and water up to final volume.

Thermal cycling was performed with the GeneAmp<sup>®</sup> 9700 (Applied Biosystems) using the following conditions :

- *Initial incubation step:* 96°C for 2 min;
- *28-34 cycles:* denaturation 94°C for 30 sec, annealing 59°C for 2 min, extension 72°C for 90 sec;
- *Final extension:* 60°C for 45 min;
- *Final hold:* 4°C forever.

**Table 7-**The table shows the loci amplified with PowerPlex® ESI 17 System, the range of PCR products expresses in base pair and the corresponding dyes used (48).

Locus	Range of PCR product sizes (bp)	Dye label
Amelogenin	87/93	Fluorescein
D3S1358	103-147	
D19S433	163-215	
D2S1338	223-295	
D22S1045	306-345	
D16S539	84-132	
D18S51	134-214	
D1S1656	226-273	
D10S1248	286-330	
D2S441	347-383	
TH01	72-115	TMR-ET
vWA	124-180	
D21S11	203-259	
D12S391	291-343	
D8S1179	76-124	
FGA	143-289	
SE33	309-459	

## 2.4 Samples preparation

Samples were prepared for electrophoresis on the ABI Prism 3100 Avant and 3130 Genetic Analyzer (Applied Biosystems) using 10 µl of Hi-Di formamide (Applied Biosystems), 0.5 µL of GS500 LIZ Size Standard (Applied Biosystems) and 1 µl of PCR products of Applied Biosystems and homemade miniSTRs kits, or 10 µl of Hi-Di formamide (Applied Biosystems), 0.5 µl of CC5 Internal Lane Standard 500 Size Standard (Promega) and 1 µl of PCR products when PowerPlex® 17 ESI was used.

The reaction plate was heated in a thermal cycler for 3 minutes at 95 °C and then was cooled at 4 C° to ensure that the denaturation process had occurred.

Prior to running any multiplex samples on the ABI 3130, a five dye matrix was established under the “G5 filter” (Applied Biosystems or Promega). PCR products were run using the default module HIDFragmentAnalysis36\_ POP4\_1 which performs an electrokinetic injection onto the 4-capillary array for 10 s at 3000 volts. The STR alleles

were then separated at 15 000 volts for approximately 30 min with a run temperature of 60°C using the 3130 POP™-4 sieving polymer, 1X Genetic Analyzer Buffer with EDTA, and a 36-cm array (Applied Biosystems).

## **2.5 Capillary electrophoresis on ABI Prism 3100 Avant and 3130 Genetic Analyzer**

All samples were separated on an ABI Prism 3100 Avant and/or 3130 Genetic Analyzer, a fluorescence-based, multi-capillary electrophoresis instrument that allow to analyze 4 samples simultaneously. In both instruments all steps are automated, including polymer loading, sample injection, washing and filling of polymer syringes, separation and detection and data analysis.

The completely automated system consists in the following steps:

1. Flowable polymer is loaded into the capillaries prior to each run.
2. Samples are injected and run according to specified conditions
3. Data are collected and analyzed by GeneScan/Genotyper v 3.7 or GeneMapper ID-X v 1.1 software and the files are then available for direct transfer to a database for further analysis and reporting.
4. Up to 96 samples can be scheduled to run without interruption.

Amplification products were separated in four capillaries of 36 cm in length and 50 µm of internal diameter. The efficient heat dissipation of the narrow capillaries, along with a detection cell heater, provides enhanced thermal control, which results in more consistent runs and faster run times.

In these capillaries, the ionic double layer at the surface of the capillary wall is immobilized to suppress electro-osmotic flow (EOF), created by residual charges on the silica surface, that induce a flow of the bulk solution toward the negative electrode. The EOF creates problems for reproducible DNA separations because the velocity of the DNA molecules can change from run to run. Capillaries contain charged silanol groups, that are dynamically coated to prevent EOF in DNA separations. The ion boundary at the surface of the capillaries used in the ABI PRISM Genetic Analyzers instruments are immobilized by specially formulated polymers, which are renewed each time the capillaries are filled with polymer. The polymer used was a poly-dimethylacrylamide POP-4 (POP-4™ Polymer for 3100 and 3130/3130xl Genetic Analyzers, Applied Biosystems) commercially available, which is successfully used in DNA genotyping because it provides a sieving matrix for the separation of single-stranded DNA and, at

the same time, suppress the EOF. POP-4™ consists of 4% linear dimethylacrylamide, 8 M urea, 5% 2-pyrrolidinone. For STR analysis, the run temperature was set at 60°C to further help keep the DNA strand denatured.

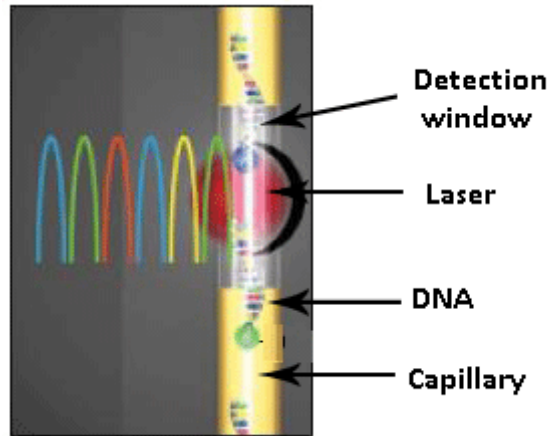
The polymer was dissolved in the electrophoresis capillary systems with a Buffer 10X with EDTA (Applied Biosystems) diluted 1X to stabilize and solubilize the DNA, to provide charge carriers for the electrophoretic current and to enhance the injection. Following electrophoresis, the automated polymer delivery system automatically flushes and replenishes the capillaries with fresh polymer. After the capillary array has been filled, the next set of samples is automatically injected directly from either 96-well sample plates.

Samples were automatically loaded onto the separation medium from sample plates. The electrokinetic injection of the samples consists to applied a voltage for a defined time, to move charged molecules from the sample into the capillary. As DNA is negatively charged, a positive voltage is applied to draw the DNA molecules into the capillary. Electrokinetic injections produce narrow injection zones, but highly sensitive to the sample matrix. The result is the simultaneous loading of samples into the parallel 4 capillary array in less than 30 seconds reducing the sample usage.

PCR products were separated by size and dye color using electrophoresis followed by laser-induced fluorescence with multiwavelength detection. The covalent bind of different dye onto the 5'(nonreactive) end of each primer allowed to detect contemporary a multiplex of different STR markers including loci that have alleles with overlapping size ranges.

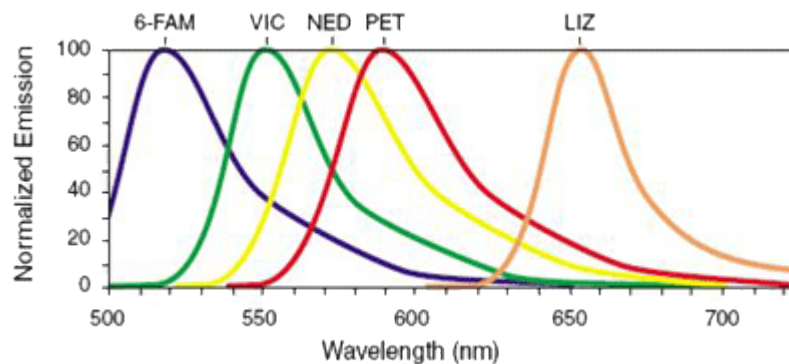
All forward STRs and miniSTR primers were labeled with a dye color and an internal standard, containing DNA fragments of known size was labeled with a different dye color which enabled the use of available matrix standards and reliable color separations on the ABI PRISM instruments. The dyes are all excited by a single argon-ion laser, with primary excitation lines at 488 and 514.5 nm, yet fluoresce in different regions of the spectra. Each of these fluorescent dyes emits its maximum fluorescence at a different wavelength. During data collection on the ABI PRISM® instruments, the fluorescent signals are separated by a diffraction grating according to their wavelengths and projected onto a charge-coupled device (CCD) camera that allow to determine which dye is present, based on the emission of each fragment as it passes the detector

window. CCD camera simultaneously detects all wavelengths from 525 to 680 nm (fig. 2).



**Fig. 2.** The laser excites the colored fluorophores, when DNA fragments pass by the detection window. ([http://www.nfstc.org/pdi/Subject05/pdi\\_s05\\_m02\\_01\\_e.htm](http://www.nfstc.org/pdi/Subject05/pdi_s05_m02_01_e.htm))

The multicomponent analysis separates the five different fluorescent dye colors into distinct spectral components. Although each of these dyes emits its maximum fluorescence at a different wavelength, there is some overlap in the emission spectra between the dyes (fig. 3).

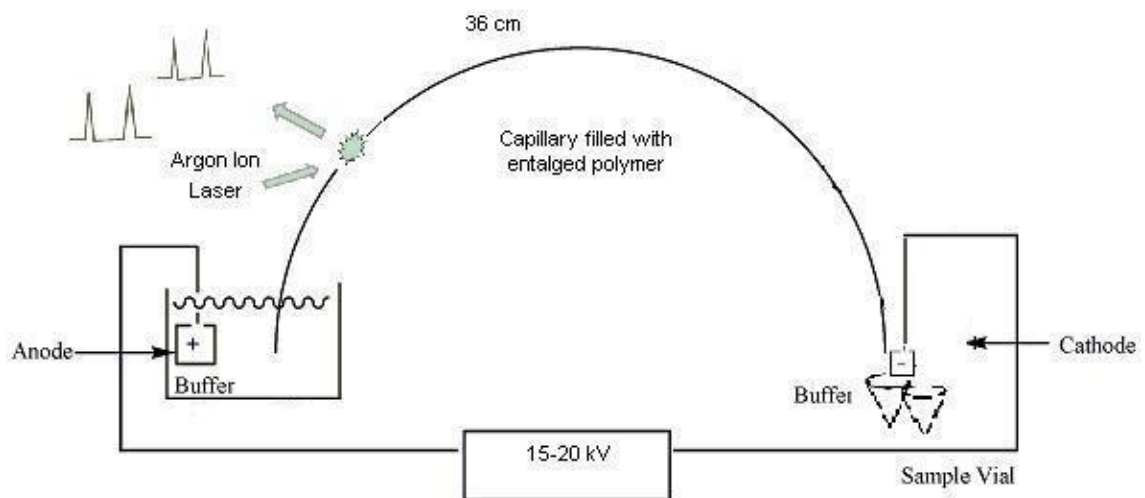


**Fig. 3.** Example of emission spectra of 6-FAM<sup>™</sup>, VIC<sup>®</sup>, NED<sup>™</sup>, PET<sup>®</sup> and LIZ<sup>®</sup> dye used with Amp-FISTR Identifiler<sup>®</sup> PCR Amplification Kit and Amp-FISTR<sup>®</sup>MiniFiler<sup>™</sup> PCR Amplification Kit (Applied Biosystems).

The goal of multicomponent analysis is to effectively correct for spectral overlap. Alleles for overlapping loci are distinguished by labeling locus-specific primers with different colored dyes

The position of the primers defines the overall PCR product size as does the number of repeats present in the STR region. The internal standard was coelectrophoresed with each sample to calibrate sizes from run to run. The collected data in the form of multicolored electropherograms were analyzed by software that automatically determines STR allele sizes based on a standard curve produced from the internal size standard. STR genotyping is performed by comparing the allele sizes in each sample to the sizes of alleles present in an allelic ladder, which contains common alleles that have been previously sequenced.

When the fluorescent light has been collected and dispersed across the CCD, the data are transferred to the instrument computer where they are transformed by chemometric algorithmic processing into 5-dye electropherograms. This method of collecting and imaging light has several advantages such as reducing the experimental noise in the dye electropherogram through the use of spectral over-sampling, preventing the time-interpolation problem by simultaneously collecting all the colors and providing the versatility required to adapt new chemistries and dye sets as they become available without requiring changes in the optical hardware (4,49,50). The capillary electrophoresis system is schematically summarised in figure 4.



**Fig. 4.** Capillary electrophoresis system.

### **2.5.1 GeneScan and Genotyper programs**

The GeneScan and Genotyper programs were used with ABI PRISM<sup>®</sup> 3100 Avant<sup>™</sup> Genetic Analyzer to convert the raw data into the appropriately colored peak and to generate STR genotyping information.

**GeneScan software** performs three primary functions. It calls peaks based on threshold values specified by the user; it separates the peaks into the appropriate dye color based on a matrix file and it sizes the STR allele peaks based on an internal size standard labeled with a different colored dye that is run in every sample. It is important to be consistent in the use of an internal size standard because all STR allele peaks are measured relative to this internal size standard. The default sizing algorithm, and one most commonly used, with the GeneScan program is the local Southern method. The local Southern method measures the size of an unknown peak relative to its position from two peaks in the internal standard that are larger than the unknown peak and two that are smaller than the unknown peak. GeneScan software contains six different screens that may be used as part of data analysis and evaluation: processed data (color-separated), size standard curve, electrophoresis history, sample information, raw data (no color separation) and an analysis log file.

**Genotyper software program** takes GeneScan data and converts the sized peaks into genotype calls. Genotyping is performed by comparison of allele sizes in an allelic ladder to the sample alleles. The manufacturer of a particular STR kit normally provides Genotyper macros in order to make the allele calls from the allelic ladders. These macros can be designed to filter out stutter peaks that may interfere with sample interpretation (4).

### **2.5.2 GeneMapper ID-X v.1.1**

GeneMapper ID-X v.1.1 is an automated genotyping software running on the Windows XP<sup>®</sup> platform and used with ABI PRISM 3130 Genetic Analyzer. GeneMapper ID-X v.1.1 DataCollection v.3.0 combines functions of GeneScan<sup>®</sup> and Genotyper<sup>®</sup> Softwares, and it includes additional features and enhancements.

GeneMapper<sup>®</sup> ID-X v.1.1 database stores the following data:

1. predefined and custom designed size standard definitions;
2. panel, marker (loci), and allele bin definitions;
3. analysis methods;
4. table profiles (for generating tabular reports);
5. saved projects with sizing and genotyping results;
6. plot settings;

Process Component-Based Quality Values System (CBQVS) automatically assigns values to the quality of data in respect to sizing and allele calling. Poor quality samples are those below the user defined thresholds.

GeneMapper<sup>®</sup> ID-X software compares genotype concordance between overlapping loci among different kits for the same sample, or concordance of genotype calls from duplicate amplifications or duplicate injections of the same sample. Positive and negative controls give the expected allele calls.

The software displays different types of labels. In addition to traditional allele labels, artifact labels have been introduced which are applied automatically to data spikes (identified through a rule set). Artifact labels are visible on the electropherogram (identified by a pink box) to help the review process but are not transferred to the genotypes table (51).



### **3. RESULTS and DISCUSSION**

The most important variables upon which DNA identification depends are: extent of time after death, environmental conditions, preservation of skeletal remains and available type of bone.

When working with DNA from old bones, the main problem stems from the low amount of starting molecules, the presence of PCR inhibitors and DNA degradation. All these events often make the extraction process very difficult and the success of DNA typing mostly depends on selection of appropriate DNA isolation protocols (52). Efficient DNA extraction procedures, as well as accurate DNA amplification, are critical steps involved in the process of successful DNA analysis of skeletal samples. The extraction techniques that have had most impact on general degraded DNA work have focused on purifying extracted DNA with silica binding and decalcifying bone with EDTA (53).

In this study three DNA extraction methods have been tested and compared to evaluate the ability to recover DNA from skeletal samples, that also depend on the capacity of the extraction method to remove PCR inhibitors.

In order to develop a reliable method to extract DNA and to verify the reproducibility of results obtained, we analyzed femur, humerus, tibia, jaw, rib, cranium belonging to different cadavers. The authenticity of profiles obtained from bone samples were confirmed by comparison of DNA profiles obtained from saliva sample of presumptive living relatives, when disposables, as in a routine paternity analysis.

#### **3.1 Consideration on human skeletal remains.**

The lack of precise information on the best samples for DNA testing from degraded bone, is due to the difficulty in performing controlled experiments, with regard to the long post mortem periods, the effect of relevant environmental variables, inter-individual variations, such as sex or age, and the need of a large sample size.

In this study skeletal samples were obtained from 14 severely decomposed cadavers from 1 month to 90 years old with a large variety of post mortem histories.

In our cases post mortem periods, environmental conditions, preservation of skeletal remains and availability of bone specimens, were widely variable.

Bones buried in the soil appeared in brown colour, internal structures were completely decomposed and all agents of surrounding soil matrix such as humic acids, fungus or bacteria living in the soil, have caused the total bone crumbly. Differently, skeletal remains found during summer in a corn field and exposed for 1 month to environmental conditions, still presented shreds of soft tissues and intact bone structures. Bodies enclosed in zinc coffins were completely saponified, with a white and crumbly aspect caused by organic degradation produced by the enclosed space. Finally burned bones were extremely fragile and exhibited warping, shrinkage, fracturing and color change.

The most significant images of caseworks discussed in this study are shown below (figures 5, 6a, 6b, 7a, 7b, 8, 9a and 9b), where are evident the wide different conditions of bone preservation in different cases.

Differences in quantity and quality of DNA could be attributed to the respective prevailing environmental factors or to the respective storage conditions. Temperature, humidity, and pH value are environmental factors that play an important role on DNA preservation. In the case of finds embedded in soil, it could be relevant to know the amount of post mortal organic substances in the soil (classified as humic and fulvic acids) and the general degree of microbial infestation in the respective soil.

The relative small sample size evaluated in this study showed that success rates of DNA extraction seemed have no association with post mortem intervals, while environmental conditions, preservation and type of bones would be important factors influencing success.



**Fig. 5.** Skeletal remains of soldiers dating to the World War I, found in a mass grave.



**Fig.6a) and b).** Cranium a) and femur b) found in a corn field 2 months after death.



**Fig. 7 a) and b)** Comparison between two femurs: a) saponified bone, aged 20 years old and preserved in a zinc coffin; b) bone 19 years old buried in the soil.



**Fig 8.** Human skeleton found in a mass grave in Columbia, 12 years after death.



**Fig.9 a) and b).**Skeletal remains of a 9 months fetus buried 26 years ago.

### **3.2 Evaluation of DNA extraction methods and amplification protocols**

Careful considerations must be given not only to the environmental inhibitors or other metal ion-rich environments that often accompany bone samples found in soil, but also to the inhibitors that are naturally inherent in bones, such as calcium ions and collagen derivatives.

The surfaces of skeletal remains were cleaned by soaking, to reduce the possibility of bone sample source contamination. This procedure improves the quality of DNA because it reduces the possibility to have PCR inhibitors and other components that may interfere with accurate isolation and amplification of nucleic acids.

100-150 mg of decalcified and undecalcified bone powder samples were prepared before the digestion to evaluate which would be better for DNA typing. Decalcification treatment removes calcium ions from bone specimens and should improve bone powder dissolution during the digestion to enhance DNA recovery. However, in our cases, decalcification resulted in lower detectability during DNA typing. This was probably due to the inhibitory effect of decalcification on amplification, which can be enhanced by excess amounts of impurities in the dissolved bone. These results suggest that, if possible, should be better processing a set of undecalcified bone powder samples simultaneously with a set of decalcified powder samples.

Because of the extraction methods affects the quality and quantity of DNA recovered, different procedures have been tested to maximise the yield of DNA and eliminate PCR inhibitors.

DNA extractions were performed using three different DNA extraction methods that include: silica based spin columns (QIAamp<sup>®</sup> DNA mini kit, QIAamp<sup>®</sup> DNA Investigator kit by Qiagen), magnetic bead-based treatment (DNA IQ<sup>™</sup> system kit by Promega, PrepFiler<sup>™</sup> Forensic DNA Extraction Kit by Applied Biosystems) and conventional organic extraction (phenol-chloroform).

An additional step with organic solvents was apply to the first two DNA extraction methods to improve DNA purification.

The QIAamp<sup>®</sup> columns provided in QIAamp<sup>®</sup> DNA Mini kit and QIAamp<sup>®</sup> DNA Investigator kit use a silica membrane to bind the DNA template. Binding to silica is widely used when clean DNA is required or samples contain large amounts of chemical substances of a poorly understood chemical nature but which are known to inhibit PCR.

Investigator kit was used because it should ensure better purification of genomic DNA from small sample volumes or sizes as reported in the kit handbook (39). Buffers, pH and salt conditions are optimized to ensure proper binding of DNA to silica membrane, while other proteins and contaminants, which can inhibit PCR, are not retained. Purified DNA is then eluted with an elution buffer under basic conditions and low salt concentration. DNA samples adsorb to the silica membrane in the presence of high salt concentrations, low pH (< 7.5) and do not require organic components. Silica filters are convenient if are used with a filtration system, but require extensive washing to remove the guanidine-based lysis buffer.

The other two DNA extraction methods such as PrepFiler™ and DNA IQ™ system are based on magnetic bead particles. The two kits use silica coated magnetic beads and a magnetic support to separate DNA template from other cellular debris and contaminants. These particles are made up of crosslinked polymer with magnetic material and should offer a large surface area with high and efficient DNA binding capacity that should result in a maximum for DNA isolation. Problems concerning this DNA extraction method are due to persistence of contaminant inhibitors in final DNA extract when magnetic-beads are not properly washed and the capacity to capture only a constant amount of DNA. In situation where samples recovered have been exposed to a variety of environmental insults, this latter effect can decrease the yield of human DNA as the ratio of bacterial DNA to human DNA increases.

The last purification method applied in this study was a traditional extraction with phenol-chloroform. This method is based on the difference of solubility of nucleic acids and proteins in two different organic phases. Addition of phenol allows to remove proteins and lipids leaving nucleic acids in aqueous layer. The use of chloroform improves protein removal by further denaturing and removing residual phenol from the aqueous layer. Isoamyl alcohol is added to help reduce foaming of chloroform. The pH of PCIA is important because higher pH and salt concentration help strip DNA molecules from histone proteins (54).

Despite of its efficiency to ensure the complete removal of proteins and other PCR inhibitors, the method has several disadvantages such as the use of hazardous organic chemicals (phenol) and multiple centrifugation steps that may result in significant loss of DNA amount.

We observed that none of these methods, following standard DNA extraction procedure, provided reproducible results in all skeletal remains analyzed, probably because variables between different bones were extremely emphasize. DNA extraction methods could not solve all problems due to intrinsic characteristics (i.e. collagen type I) described above and probably to their inability to remove inhibitors.

As mentioned before an additional step with organic solvents was therefore apply to the silica based and magnetic beads DNA extraction methods to improve DNA purification.

The introduction of an intermediate step performed with organic solvents could improve DNA purification. Both silica and magnetic beads standard methods were modified, introducing one purification step only with phenol-chloroform-isoamyl alcohol after lysis buffer incubation. This step does not determine the loss of material and the possibility of contamination as traditional PCIA procedure. Furthermore, it seems to ensure an efficient separation of the waste material from nucleic acids and allows to maximize the recovery of DNA, probably due to the less interference of proteins and contaminants with columns or magnetic beads. The presence of PCR inhibitors such as Maillard products is often, but not always, indicated by a discoloured DNA extract, usually tinted yellowish to reddish-brown (55). Moreover, it is known that bone contains long fibrils of type I collagen that under specific environmental conditions such as low pH and relative high temperature, swell and collapse to form gelatine (56). These conditions may occur also during DNA extraction, because some DNA extraction kits involving the use of low pH lysis buffer ( $\text{pH} \leq 5$ ) and cell lysis at 60 °C.

In our experience, Maillard products and degraded collagen may cause DNA trapped and the reduction of DNA extraction efficiency due to the saturation of columns and magnetic beads that could prevent with phenol-chloroform-isoamyl alcohol treatment.

For each sample analyzed, we evaluated the presence of partial profiles, unbalanced peaks, allelic drop out and drop in, the presence of artifacts caused by the increased number of PCR cycles and the reproducibility of results.

Initially, to evaluate the best DNA extraction protocol in term of number of STRs obtained and reproducibility of results, DNA from all type of samples (short, flat

and long bones) were amplified only by AmpFlSTR<sup>®</sup> Identifiler<sup>®</sup> PCR Amplification Kit.

All three standard DNA extraction procedures failed STRs amplification except for amelogenin gene (106-112 bp in size), probably due to low amount and highly fragmentation of DNA template. These results were confirmed also increasing PCR number cycles from standard 28 to 34.

It is well known that the success of amplification is determined by the average size of DNA template and by the absence of PCR inhibitors.

Moreover we observed that short and flat bones processed with modified silica and magnetic bead protocols, failed to give positive results, in fact none marker was amplified or in rare cases only amelogenin marker. The attention was therefore focused on long bones and in particular on femur.

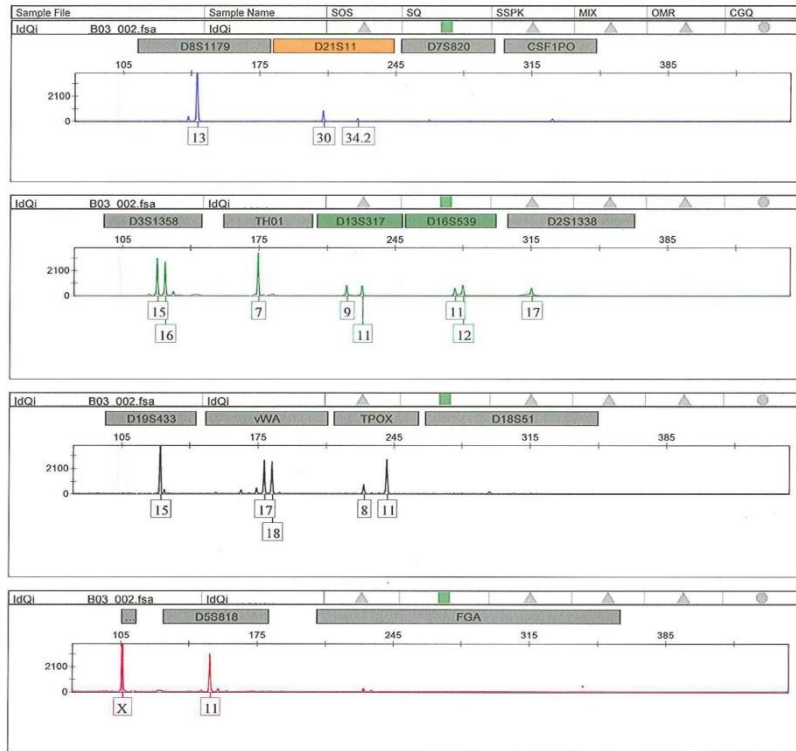
Better results were obtained when the modified protocols were used for DNA extraction from long bones. In this situation partial genetic profiles consisting of the lowest STRs in size with peaks at very low Relative Fluorescence Unit (RFU) below the detection threshold (<100 RFU in homozygote genotype), were obtained.

Nevertheless, both modified magnetic beads and QIAamp<sup>®</sup> DNA Investigator kit DNA extraction protocols produced insufficient results. Magnetic beads procedure failed probably due to the inability of lysing both undecalcified and decalcified bone powder specimens, that were completely undissolved after overnight incubation. Differently, using silica method, bone powder was almost completely digested in the extraction buffer, even if lower DNA amount was recovered from QIAamp<sup>®</sup> DNA Investigator kit than QIAamp DNA mini kit. This result was probably due to the small diameter of column filter membrane, which can be easily occluded by debris, soil and all other contaminants that cause insufficient DNA recovery.

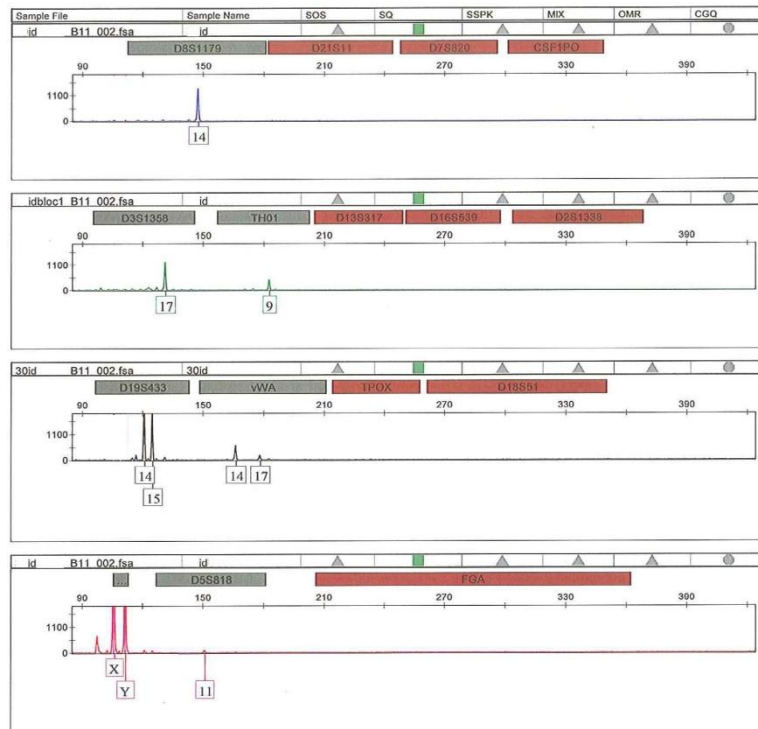
Only QIAamp DNA mini kit modified protocol gave better results in term of reproducibility of electropherograms than all other DNA extraction methods. In this study the best results were obtained combining the positive aspects of phenol chloroform step with the high efficiency of QIAamp<sup>®</sup> DNA Mini kit in recovering DNA. Using this kit the number of STR systems obtained from all long bone samples, significantly increased. The new silica based protocol produced a variable number of 5-11 markers plus amelogenin in all femurs analyzed.



We found that the quality of DNA seems not to be influenced on the age of skeletal remains. Femur 22 years old, found in a wooded area (fig.10), in fact, produced a profile of 11 STRs, while a femur 12 years old but buried and exposed to drastic environmental conditions produced only 6 STRs (fig. 11).



**Fig. 10.** Partial profile of 11STR allele peaks plus amelogenin, obtained from Identifiler kit on a 22 years old femur found in a wooded area



**Fig. 11.** Partial profile of 6 STR allele peaks obtained from Identifiler kit on 12 years old femur.

It was difficult to predict DNA yield or profiling success from appearance and age of bones. As mentioned before, the quality of DNA changes between different type of bone: femur in comparison with humerus, tibia, short and flat bones was always the best sample for obtaining the high number of STR systems and reproducible results.

After evaluation of all DNA extraction methods based on results obtained in the first step of amplification with AmpFISTR® Identifiler® PCR Amplification Kit, the attention was focused on other amplification protocols that allowed to increase the number of STR markers to obtain a complete genetic profile.

A better approach to improve the success rate for degraded DNA encountered with current STR systems, would be to redesign primer to produce smaller PCR products (miniSTR). Because the main problem associated with DNA degradation is the fragmentation of DNA template, primer producing smaller PCR amplicons could increase the probability of obtaining a profile from shorter DNA fragments.

Moreover, European Network of Forensic Science Institutes (ENFSI) has recommended to use the European Standard Set (ESS) of Loci as minimum number of markers to enable international comparison of DNA profiles. ESS contains only 7 STRs, that should be extended by 5 additional loci after EU-Prum-Decision (57).

A similar recommendation was already proposed by DNA Commission of the International Society for Forensic Genetics (ISFG) which established that a minimum set of 12 markers (plus Amelogenin) should be attempted on degraded samples (36).

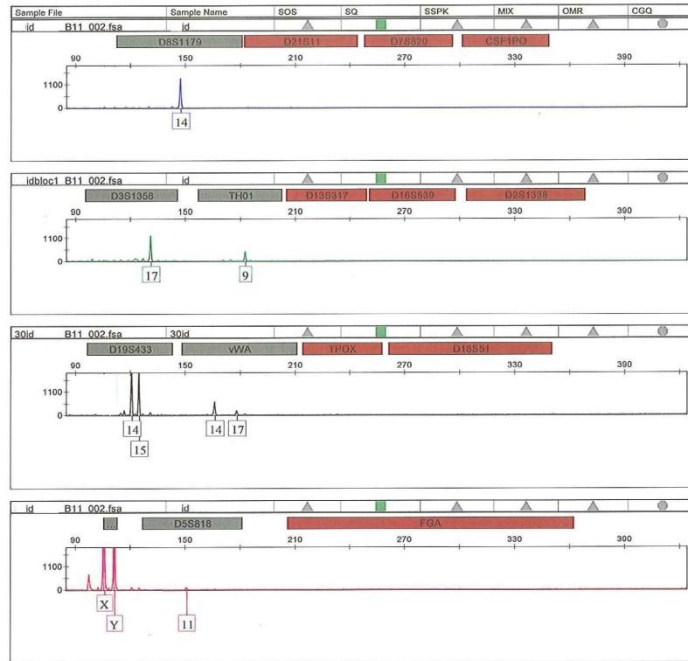
In order to observe these recommendations, we decided to use MiniFiler™ commercial kit that consists of 8 miniSTR (D13S317, D7S820, D2S1338, D21S11, D16S539, D18S51, CSF1P0 and FGA) plus amelogenin, and two new homemade miniplexes, each of which includes 4 miniSTR (CSF1P0, TH01, D13S317, D5S818 and D8S1179, D3S1358, TPOX, D16S539). Minifiler and two quadruplex allowed amplification of 13 miniSTR overall. We knew that three miniSTR (D13S317, D16S539 and CSF1P0) of the two homemade multiplexes were already present in Minifiler kit, but we decided to redesigned them also to have a positive control on profiles obtained from each sample.

To test the effect of PCR cycle numbers on the sensitivity of MiniFiler kit and two miniSTR quadruplexes, DNA was amplified at 28, 30, 32 and 34 cycles. At 28 cycles amplification products were observed, but the signal intensity was quite low (RFU<100 in homozygote genotype), while good signal intensity (RFU>700) and good peak imbalance ratio were obtained at 30-32 cycles. Although increasing the cycle number can increase signal intensity, lower cycles can achieve better peak imbalance. In fact, at 34 PCR cycles many artefacts were observed and the quality of profiles was not good. The benefit of increased sensitivity derived from increasing the number of cycles of amplification has to be balanced against a reduction of profile quality (58).

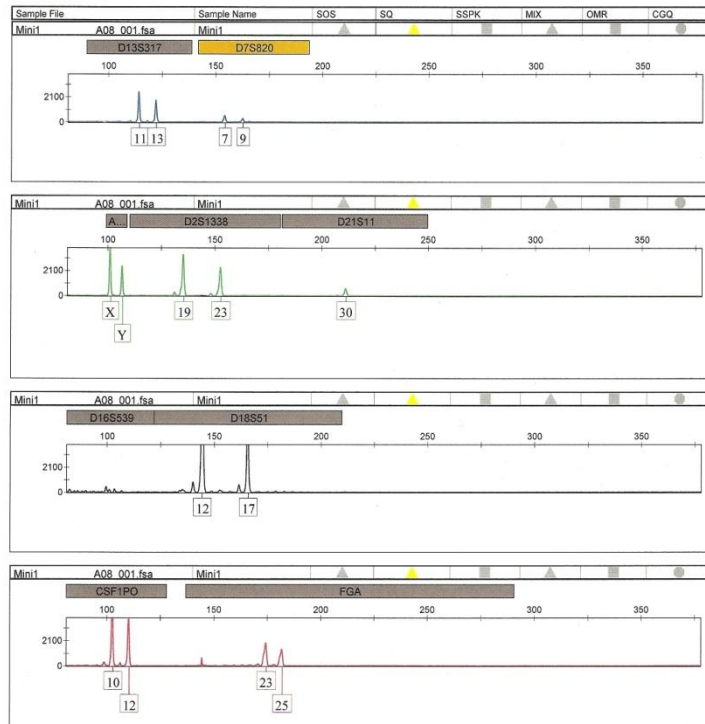
Using MiniFiler™ and the two new mini-STR quadruplex systems we obtained genetic profiles of at least 12 STR from DNA typing of femur samples in all those cases in which Identifiler® provided a partial profile consisting of the lowest STR in size.

Figures below refers to a genetic profile obtained from femur sample aged 12 years (case 8), found in a mass grave. At 32 PCR cycles Identifiler® kit provided only 6 STRs (D8S1179, D3S1358, TH01, D19S433 and vWA) plus amelogenin marker (fig. 12a), while Minifiler kit allowed to obtain 7 of 8 STRs and amelogenin (D13S317, D7S820, D2S1338, D21S11, D18S51, CSF1P0 and FGA) (fig. 12b). Due to the high DNA degradation locus D16S539 failed to amplify. The two new homemade quadruplexes amplified all 8 loci and also D16S539 because it was shorter than Minifiler (12c and 12d). This demonstrated that the two new multiplexes could be successfully employed in degraded samples and could provide additional information on skeletal remain samples.

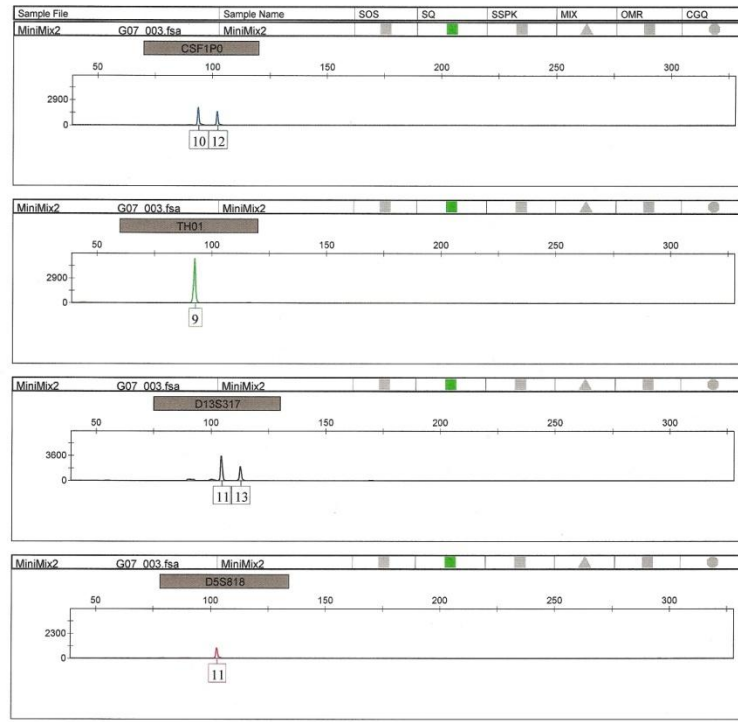
12a)



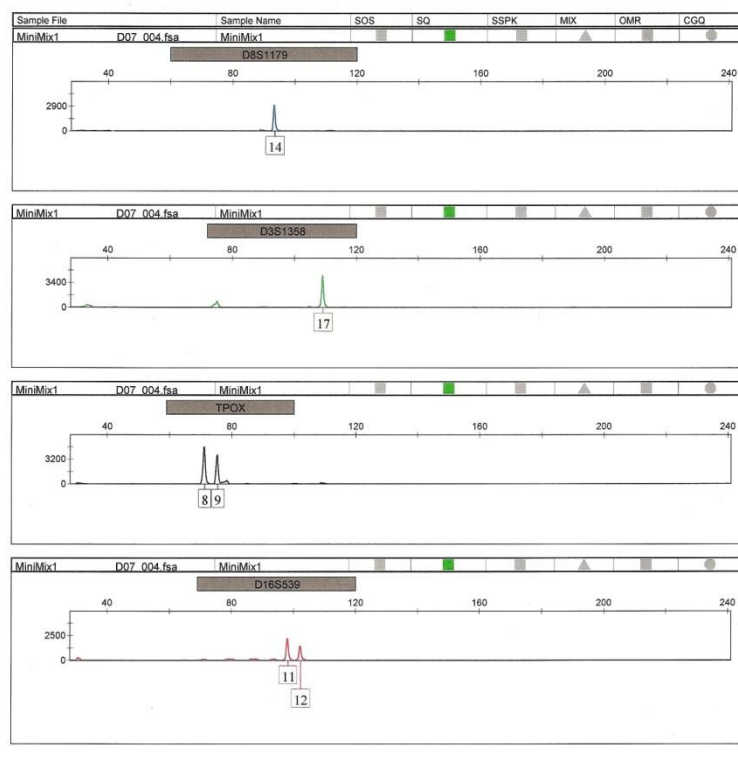
12b)



12c)



12d)



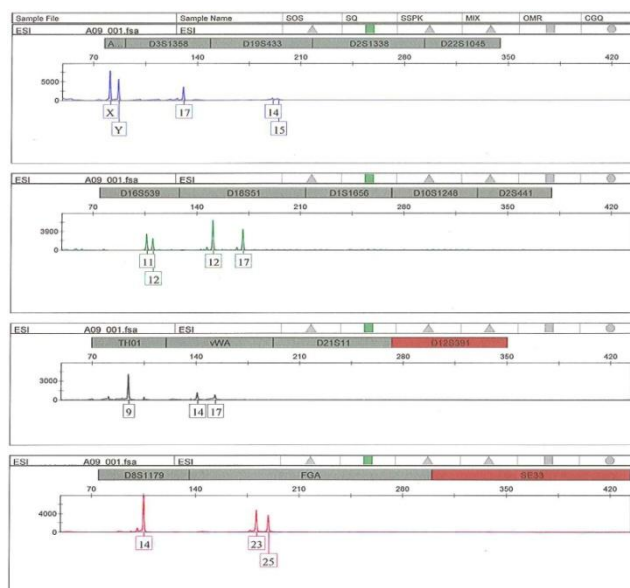
**Fig. 12.** Results obtained from Identifiler® kit (a), Minifiler kit (b) and two new quadruplexes (c and d) on a 12 years old femur extracted with new protocol consisting of phenol chloroform and QIAamp DNA mini kit.

Last year Promega Corporation developed a new STR multiplex kit (PowerPlex® ESI 17 System) containing the five new loci selected by ENFSI and EDNAP (D10S1248, D22S1045, D2S441, D12S391 and D1S1656) as well as 11 other loci commonly used throughout Europe, plus SE33 marker. PowerPlex® ESI 17 Systems focus on miniaturization of current ESS loci, which can be very useful on degraded DNA. For this reason we decided to extend STR analysis also using PowerPlex ESI 17 Systems on all our DNA samples.

In all cases the new commercial kit amplified a greater number of STRs than Identifiler kit, due to the reduced size of markers and probably to an higher PCR amplification efficiency.

Results obtained from PowerPlex ESI 17 Systems on femur 12 years old, showed the amplification of two more STRs (D16S539 and FGA) compared to Identifiler (fig. 13).

In conclusion, considering all commercial and homemade kits, a genetic profile of 15 STRs plus amelogenin (X/Y) was obtained on 12 years old femur, which corresponds to the Identifiler complete profile. Using Italian population database the probability of founding a man with the same genotype (Random Match Probability - RMP) was  $5,26678 \cdot 10^{-20}$ . Identification was also confirmed comparing the genetic profile with that of parents.

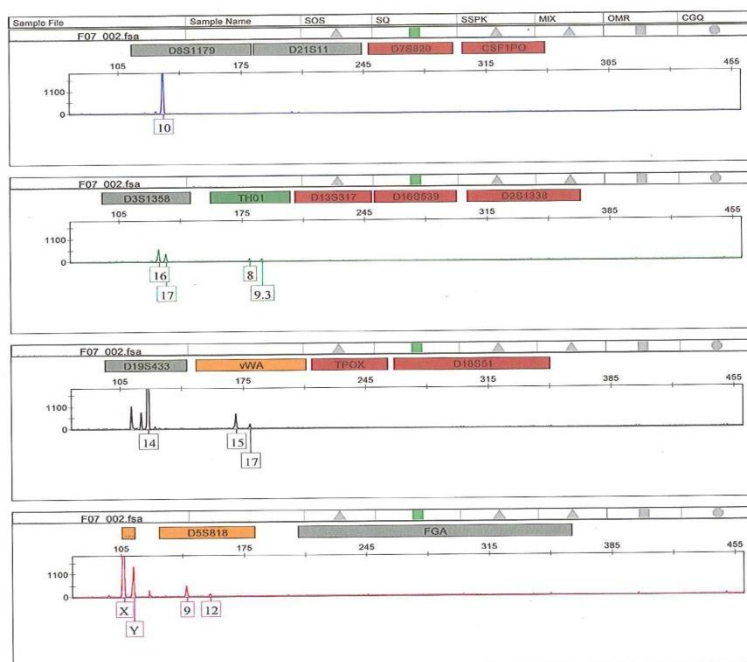


**Fig. 13.** PowerPlex ESI® 17 kit partial profile generated from a femur 12 years old extracted with the new protocol

Similar results were obtained in another case of a saponified body exhumed from zinc coffin after 20 years after death (case 5).

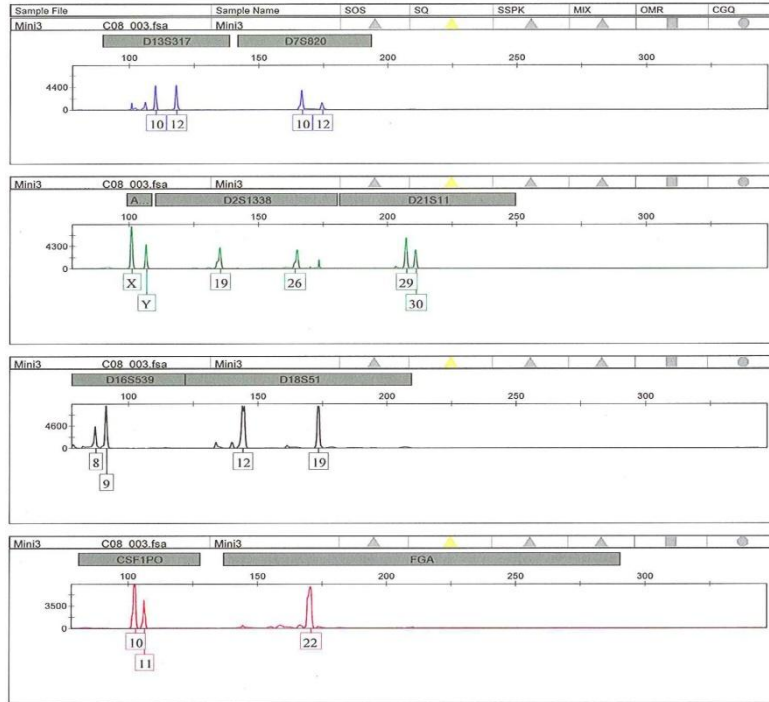
Identifiler kit, generated a partial profile of 6 STRs (fig.14a) plus amelogenin. Increasing the analysis with Minifiler kit and the two new homemade quadruplexes we obtained 15 loci (14b, c and d).

14a)

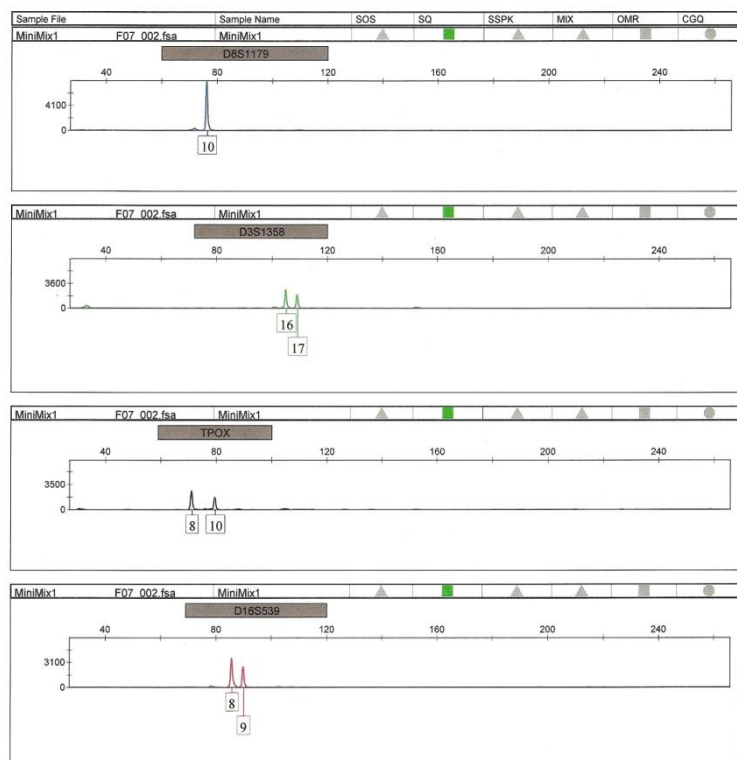




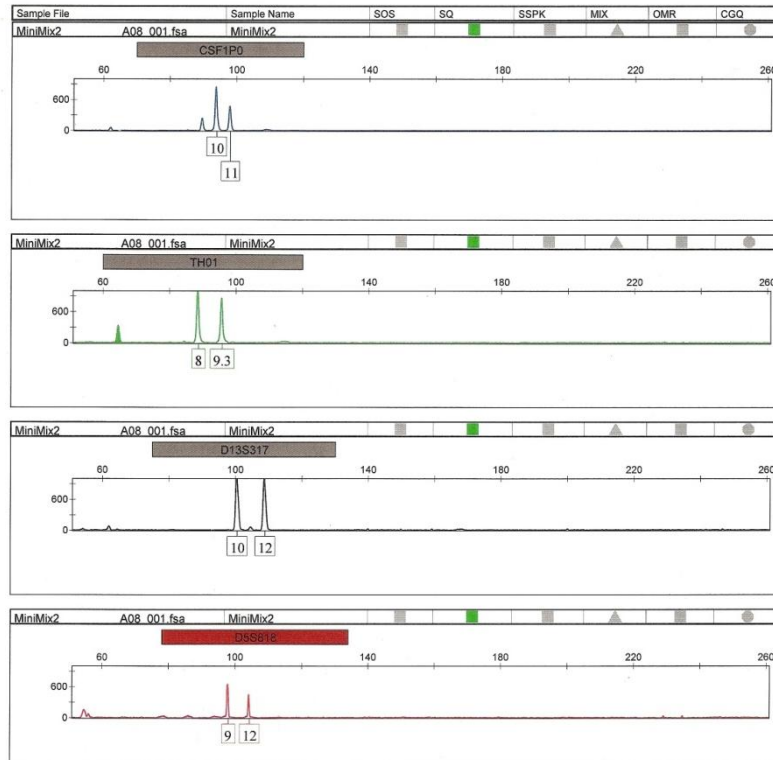
14b)



14c)

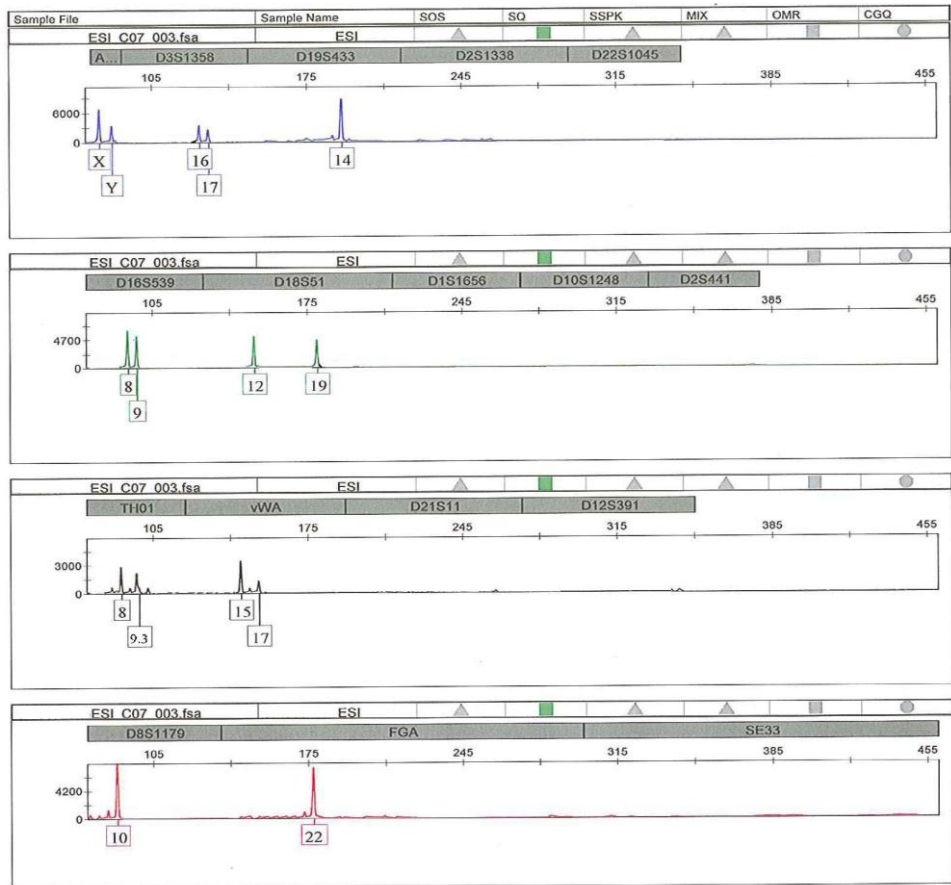


14d)



**Fig. 14.** Results obtained from Identifiler® kit (a), Minifiler kit (b) and two new quadruplexes (c and d) on a 20 years old saponified femur, extracted with new protocol consisting of phenol chloroform and QIAamp DNA mini kit.

Also in this case results obtained from PowerPlex ESI 17 Systems showed the amplification of three more STRs (D16S539, D18S51 and FGA) compared to Identifiler kit (fig.15). Using Italian population database the probability of founding a man with the same genotype (Random Match Probability -RMP) was  $8,11 \times 10^{-23}$ .



**Fig.15.** PowerPlex ESI® 17 kit partial profile generated from a 20 years old femur extracted with the new protocol.

Complete autosomal genetic profiles (21 STR loci and amelogenin) were only obtained from 2 cases, partial profiles with one or two STR loci missing were obtained from 2 bones (primarily longer loci were missing), and in the remaining 10 cases 12 to 17 loci were amplified (Table 8).

**Table 8.** Efficiency of autosomal DNA typing (AmpFI STR Identifiler™ PCR Amplification Kit (Applied Biosystems) and PowerPlex ESI 17 System (Promega), expressed as the number of successfully typed autosomal short tandem repeats (STRs) Minifiler kit (Applied Biosystems) and two homemade quadruplexes.

Case	Specimen type	Maturity	Post mortem period	Location where found	Number of autosomal STRs (Identifiler, ESI, Minifiler, two mini quadruplexes)
1	Femur	Adult	I World War	Buried	12/21
2	Femur	Adult	I World War	Buried	13/21
3	Femur	Fetus	26 years	Buried	12/21
4	Femur	Adult	22 years	Wooded area	15/21
5	Femur	Adult	20 years	Zinc coffin	15/21
6	Femur	Adult	20 years	Zinc coffin	17/21
7	Femur	Adult	19 years	Buried	19/21
8	Femur	Adult	12 years	Buried	15/21
9	Femur	Adult	4 months	Burned	14/21
10	Fragment of femur	Adult	2 months	Burned	16/21
11	Fragment of femur	Adult	2 months	Burned	15/21
12	Femur	Adult	1 months	Burned	20/21
13	Femur	Adult	1 months	Burned	21/21
14	Femur	Adult	2 months	Corn field	21/21

Considering all STR markers used (Identifiler<sup>®</sup>, MiniFiler<sup>™</sup>, PowerPlex ESI<sup>®</sup> kit and two new miniplexes) in most cases we obtained a profile of 13 STRs, the same number of markers contained in Combined DNA Index System (CODIS), which is the system adopted by the FBI.

The good quality of results were confirmed both repeating all PCR amplifications at least three times (58) and comparing genetic profile with parents and relative living persons, when they were available.

#### 4. CONCLUSIONS

Purification of DNA from a variety of samples is still a rate-limiting step in obtaining useful genotypes. Several procedures are currently used to purify and extract DNA from degraded samples such as skeletal remains (59-62). Variations in DNA yield from larger bone fragments are inevitable and may be attributed to heterogeneity within a bone. For this reason, unfortunately, at present there is not an infallible method to recover DNA from very degraded samples.

Many paper regarding DNA extraction protocols from ancient and modern bones are present in literature, but they are frequently in contrast between them. Some are based on bone extraction protocols that involve the incubation of powdered material in a lysis buffer, followed by the collection of the supernatant. Other protocols obtain DNA by full demineralization of bone, resulting in full physical dissolution of the bone powder and recovery of all DNA released by the complete demineralization procedure (63).

Recently a new experimental DNA extraction kit was developed to extract DNA from degraded skeletal remains without the need of powdering the samples (64). These results are in contrast with another study where it is demonstrate that the fine powder produced higher yields compared with samples ground to pieces one to a few millimeters in diameter (65). The fact that so many different extraction techniques are in use, indicates that no single procedure has emerged yet as having clear advantages that would lead to it becoming standardized.

Our new DNA extraction method based on combining phenol-chloroform-isoamyl alcohol and QIAamp<sup>®</sup> DNA Mini kit can provide an alternative method to standard protocols and traditional method of phenol-chloroform, but always with the limitations related to the technique that failed on such bone samples (short and flat). This method, in fact, produced genetic profiles consisting of at least 12 STRs on long bones especially femur, but only amelogenin gene on the other bones. The extraction procedure used in new protocol, yielded higher amounts of DNA of starting material from femurs than the conventional phenol/chloroform method, magnetic beads and silica based QIAmp Investigator methods and allowed the simple extraction of DNA from small quantity of bone powder.

The difference in success rates between long bones of upper body and lower body, in particularly femur, were already shown in previous studies (10) and, importantly,

environmental conditions under which remains were exposed, had stronger influence on the state of DNA quality than the age of skeletal remains.

Moreover it was difficult to predict DNA yield or profiling success from the appearance or characteristics of bones. In some cases, in fact, very aged femurs produced unexpectedly partial profiles while other more recent bones failed to give partial results. In concordance to a previous study (66), samples that gave lower amounts of DNA or partial profiles showed no characteristics with regard to morphology that would permit speculation as to why they may have failed, or why the correlation between yields of different methods varied among samples.

However, given a representative sampling of bones, it is clear that the higher level of DNA recovery with the silica method in some cases is essential to recover a full DNA profile. In choosing a DNA extraction method it is important that the method would allow to remove as many inhibitors as possible and at the same time maximize the yield of DNA template.

Amplification with reduced size fragments included in Minifiler™ kit, and the two new homemade quadruplexes, have proved to be a very useful resource when traditional marker fail to give a genetic profile of at least 12 STRs as recommended by ENFSI and ISFG Commission.

Unfortunately not all conventional STRs included in Identifiler® amplification kit can be reduced less than 130 bp, but only those that are characterized by a limited number of repetitive units. The two new miniSTRs multiplexes were also able to give more complete profiles for bone samples that had been exposed to different environmental conditions. DNA template in low copy number (LCN) can be successfully amplified with high sensitivity and good peak imbalance. The improvement in amplification of smaller sized fragments was evidently compared with that of traditional kits such as Identifiler or Powerplex ESI 17 systems.

In this study a limited sample size were analyzed, and moreover the size was variable among different type of skeletal elements. Large sample size is extremely important to observe the differences and understand the difficulties in performing experimental studies of DNA degradation in different bone and properly controlling the effect of environmental variables. Unfortunately the effect of environmental variables

was not controlled for at all, since the samples originated from a very wide range of contexts.

Finally the high success rate for nuclear STR typing reported here further confirmed that STRs and in particular mini-STRs could be considered a good method of choice in casework with missing persons, exhumation or identification involving degraded skeletal remains. As a consequence, an extended of European Standard Set (ESS) of DNA Database Loci were defined for the following reasons: 1) to improve the discrimination power; 2) improve the sensitivity of testing so that smaller amounts of DNA are detected, and 3) improve robustness or the quality of the result (67).

Only the inclusion of robust and powerful mini STR loci will ensure that difficult casework samples with low amounts of degraded DNA can be fully typed to avoid unacceptable rates of adventitious matches due to increasing numbers of partial profiles in the databases.



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