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Case report: Identification of skeletal remains using short-amplicon marker analysis of severely degraded DNA extracted from a decomposed and charred femur

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Abstract

Applying two extraction protocols to isolate DNA from a charred femur recovered after a major forest fire, a range of established and recently developed forensic marker sets that included mini-STRs and SNPs were used to type the sample and confirm identity by comparison to a claimed daughter of the deceased. Identification of the remains suggested that the individual had been dead for 10 years and the DNA was therefore likely to be severely degraded from the combined effects of decomposition and exposure to very high temperatures. We used new marker sets specifically developed to analyze degraded DNA comprising both reduced-length amplicon STR sets and autosomal SNP multiplexes, giving an opportunity to assess the ability of each approach to successfully type highly degraded material from a challenging case. The results also suggest a modified ancient DNA extraction procedure offers improved typing success from degraded skeletal material.

Keywords: Degraded DNA analysis; Forensic identification; STR; Mini-STR; SNP

1. Introduction

A frequently encountered challenge in forensic casework is the analysis of highly degraded DNA that requires extraction from difficult material such as bones and teeth from long deceased individuals. In such cases standard STR markers often fail, primarily because of their amplicon lengths, with a direct relationship between the length of amplified fragment and the frequency at which the locus fails to amplify completely or shows signal imbalance between short and long tandem repeats [1,2]. Two alternative approaches are possible for analyzing severely degraded material: mitochondrial DNA typing (mtDNA) and autosomal short-amplicon markers. Until recently, mtDNA offered the only realistic alternative to standard STRs for highly degraded DNA, however it has certain acknowledged drawbacks: (i) sequence variation is much less informative than STRs; (ii) matrilineal inheritance rules out some relationship comparisons such as father and daughter and; (iii) proper interpretation of the significance of mtDNA variation requires large-scale haplotype databases. Alternatively reducing the length of marker amplicons can be achieved by re-designing the primers of existing STRs, commonly termed mini-STRs [3,4] or by analyzing single nucleotide polymorphisms (SNPs) [5,6]. However, while bringing the primers of STRs closer to the repeat region reduces overall amplicon size, loci with the largest range of alleles such as FGA and D21S11 will still suffer from big differences in allele size and therefore disparity in PCR performance between the extremes in repeat number. Although with SNPs designing short-amplicon primers around one base is clearly easier, multiplexes of 45–50 loci are required before a sufficient number of binary polymorphisms can match the discriminatory power of STRs [5].

Since successful typing of severely degraded DNA should be the primary characteristic of new marker sets designed for the purpose regardless of their shortcomings, it is important to

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properly assess how each of the two short-amplicon approaches perform in real cases compared to conventional typing strategies. This report outlines the analysis of DNA extracted from skeletal remains that, once identified, were shown to have undergone a 10-year period of decomposition followed by exposure to the extreme temperatures associated with forest fires.

1.1. Case report

During the summer of 2006 a large number of forest fires afflicted Galicia, NW Spain. Forest fires in this region are characterized by very high temperatures caused by the resinous nature of the wood in mixed Pine and Eucalyptus plantations. After one major fire was extinguished investigators discovered a set of charred skeletal remains uncovered by the burning of underlying foliage. Analysis of the skeleton was initiated to identify if they were from a fire victim or of a man reported as missing from the area 10 years back. The daughter of the missing man was available for comparison, illustrating here the unsuitability of mtDNA and Y-chromosome loci as uni-parental lineage markers for the identification of fathers and their female descendants. A complete femur was the only bone showing some intact areas with reduced charring and was submitted for analysis, despite other signs of exposure to intense heat, as shown in Fig. 1. When discovered this femur was seen to be half buried in the soil, suggesting a degree of protection for part of the bone, while several other bones had been fragmented, with pathology indicating acts of violence.

2. Materials and methods

DNA was isolated with two different extraction methods and using the product of each in parallel analyses, typed with two standard STR kits: AmplSTR® Identifiler™ (Applied Biosystems: AB) and Powerplex 16™ (Promega), then compared to two SNP assays developed by the SNP*for*ID consortium (http://www.snpforid.org), three mini-STRs multiplexes and, solely in this case as a performance benchmark, HV1 mtDNA sequencing.

Fig. 1. Photograph of the femur as received at the laboratory prior to cleaning and DNA extraction.

2.1. Sample preparation

Prior to DNA extraction the femur was cleaned thoroughly using a scalpel and mild-grade sandpaper, limiting attention to a small, 12 cm length of bone in an area visibly less carbonized by the fire. Once the external layer of bone containing possible contaminants had been fully removed the bone was cut into small portions. The internal face of the bone was further cleaned and portions were pulverized in a liquid N_2 mill yielding a total of 8 ml of fine bone dust.

2.2. DNA extraction and quantitation

Two extraction methods were performed in parallel: (A) a normal phenol–chloroform extraction process adapted for bone material, plus: (B) an ancient DNA extraction protocol [7] we have enhanced for forensic use to improve the quality of extracted DNA. (A) Phenol–chloroform samples were digested as two 2.5 ml aliquots of bone dust plus 2.3 ml of a lysis mix comprising 2 ml of 0.5 M EDTA pH 8 as buffer, 80 μl of DTT 1 M, 140 μl of 10% SDS and 50 μl of protease-K 20 ng/ml each. Overnight lysis at 56 °C was followed by standard phenol–chloroform extraction with DNA purified and concentrated with Centricon centrifugal filter devices (Millipore) following the manufacturers protocol. Each dust aliquot was recovered in TE buffer and combined as a single extract. (B) 1.5 ml of bone dust was decalcified with 10 ml of an EDTA solution (pH 8.8) and left at 37 °C overnight. Digestion involved sample centrifugation for 10 min at 15,000 rpm, discarding of the pellet followed by addition of 1 ml 5% SDS, 500 μl 1 M Tris–HCl, 500 μl 0.5 M NaCl, 500 μl 0.1 M $CaCl_2$, 2 × 50 μl protease-K (20 mg/ml), 75 μl of DTT and 7 ml of H_2O. The mixture was incubated for 24 h at 65 °C, then 24 h at 75 °C. The final extraction step was a phenol–chloroform method as detailed in the original protocol [7]. Extraction procedures (A) and (B) were made on individual occasions separated by 24 h with negative extraction controls made in parallel. All extracts were quantified with the real-time PCR Quantifiler™ human DNA quantification kit (AB) using an AB7300 real-time PCR thermal cycler following manufacturers guidelines. Quantifiler™ includes an internal PCR control (IPC) detecting PCR inhibition. Our approach to minimizing inhibition from severely degraded DNA in standard STR typing is to run five dilutions as tandem PCRs, i.e. neat, 1/2, 1/4, 1/8, and 1/16. All other marker sets used only neat extracts.

2.3. Standard STR and mtDNA typing

Identifiler™ and Powerplex 16™ STR typing followed manufacturers guidelines except use of 12.5 μl reaction volumes. PCR comprised 28 cycles and 32 cycles respectively and we note that although increasing cycle number beyond the above numbers can influence PCR yield and quality, these cycles represent the STR amplification conditions we have optimized from numerous challenging DNA cases analyzed. In addition, the optimization of bone and tooth extract preparations in such cases,

Table 1
Genotyping success using SNP multiplexes (shown as total loci genotyped)

SNP multiplex	Total loci	Classical extraction	Ancient DNA extraction
52 plex PCR + Auto1 extension (23 plex)	23	21	21
52 plex PCR + Auto2 extension (29 plex)	29	26	28
Dedicated 23 plex PCR + SBE	23	20	23
Dedicated 29 plex PCR + SBE	29	26	29
34 plex AIMs	34	31	34

detailed elsewhere [8], gives strong indications that inhibition control by running a dilution series together with assessment of the IPC readings is the most effective way to improve yield and quality with standard STR typing. Capillary electrophoresis was performed throughout using an AB3130 with a 50 cm capillary array and POP-7™ polymer, injecting: 1 μl of PCR product, 15 μl of HI-DI™ formamide and 0.35 μl of LIZ GS500™ size standard. Mitochondrial DNA sequencing analysis followed standard protocols outlined previously [9] with primers: 15997L-16236H, 16159L-16401H and 16380L-017H.

2.4. SNP typing

Two validated SNP multiplexes were genotyped using SNaPshot™ primer extension (AB), comprising a 52 plex forensic identification set [10] and a 34 plex ancestry indicative set [11]. The 52 plex assay uses two parallel primer extension reactions detecting 23 and 29 SNPs (termed Auto1 and Auto2, respectively), the 34 plex assay detects all SNPs in a single extension reaction. Both SNP sets were amplified with a single PCR multiplex although the 52 plex can provide improved sensitivity for degraded DNA using reduced-scale 23 plex and 29 plex PCR. We performed both 52 plex and dedicated 23 plex/29 plex amplifications to assess this effect. All SNP analyses used the following modifications to the published protocol [10]: PCR volume was reduced to 13.5 μl, PCR annealing time was 50 s, extension time 40 s. Primer extension was at a reaction volume of 6 μl comprising: 2 μl SNaPshot™ Ready Reaction mix, 1.5 μl extension primer mix and 2 μl purified PCR product. Electrophoresis was as for STRs (AB3130, POP-7™, 50 cm capillary) injecting: 2 μl extension product, 9.5 μl of HI-DI™ formamide and 0.3 μl of AB LIZ™ 120 internal size standard. The 34 plex SNP set used the same reaction conditions and modifications as the 52 plex. Detailed SNP typing protocols are available as a download from: http://www.snpforid.org.

2.5. Mini-STR typing

Three short-amplicon mini-STR sets were used; Mini-NC01 and Mini-SGM, both developed by the National Institute of Standards and Technology (NIST) [12,13 respectively] and the commercial AmplSTR® MiniFiler™ kit (AB). All typing followed the recommended guidelines of NIST (http://www.cstl.nist.gov/biotech/strbase/miniSTR/updated_NC01_protocol.pdf) and AB. Electrophoresis conditions were the same as standard STR analysis.

3. Results

3.1. Bone extraction systems and reproducibility of genotype profiles

Summary genotyping results obtained with each DNA extraction system for SNPs and with ancient DNA extraction for STRs are outlined in Tables 1 and 2, respectively. The most complete electropherograms obtained from each of the short-amplicon genotyping approaches are shown in Fig. 2, which in all cases resulted from the ancient DNA extraction system for STR sets: MiniFiler™, Mini-NC01 and Mini-SGM, and for SNP sets: 23 plex, 29 plex (i.e. the reduced-scale PCRs of the 52 plex) and 34 plex. In particular the SNP results, summarized as total loci typed in Table 1, highlighted the improvement in quality and completeness of profiles obtained from the ancient DNA protocol and suggested enhanced sensitivity with this modified extraction method.

Because short-amplicon systems Mini-SGM and notably MiniFiler™ gave indications that more complete profiles might be obtainable, each of these marker sets was analyzed with

Table 2
Genotyping success using short-amplicon STRs

STR multiplex	Locus	Ladder allele range	Size range	Femur	Daughter
MiniFiler™	CSF1PO	6–15	88–124	10	10
	D16S539	5/8–15	91–119	–	9,13
	Amelogenin	*X–Y*	104/109	*X(Y)*	*XX*
	D13S317	8–15	106–134	11	8,11
	D2S1338	15–28	123–175	16,21	21
	D18S51	7–27	128–208	16	12,16
	FGA	17–33.2/53.2	151–213/293	OL	23,28
	D7S820	6–15	153–189	–	8,12
	D21S11	24–38	190–250	(24,29)	27,29
Mini-NC01	D22S1045	8/10–19	74–109	16	16
	D10S1248	9–19	83–123	14,16	14,16
	D14S1434	9–16	85–106	14	14
Mini-SGM	TH01	3–14	58–102	6,9.3	7,9.3
	D16S539	5–17	79–126	(OL,11)	9,13
	D2S1338	15–30	94–154	–	21
	D18S51	5–29	101–197	–	12,16
	Amelogenin	*X–Y*	124/130	*XY*	*XX*
	FGA	17–33.2/53.2	141–207/287	–	23,28

Individual genotypes are shown for ancient DNA extraction only, listed from shortest to longest first allele. Results in brackets represent peaks in reference positions but with signal strength between 30–50 RFU, these genotypes were not used in paternity calculations. OL: off-ladder peaks outside of reference position.

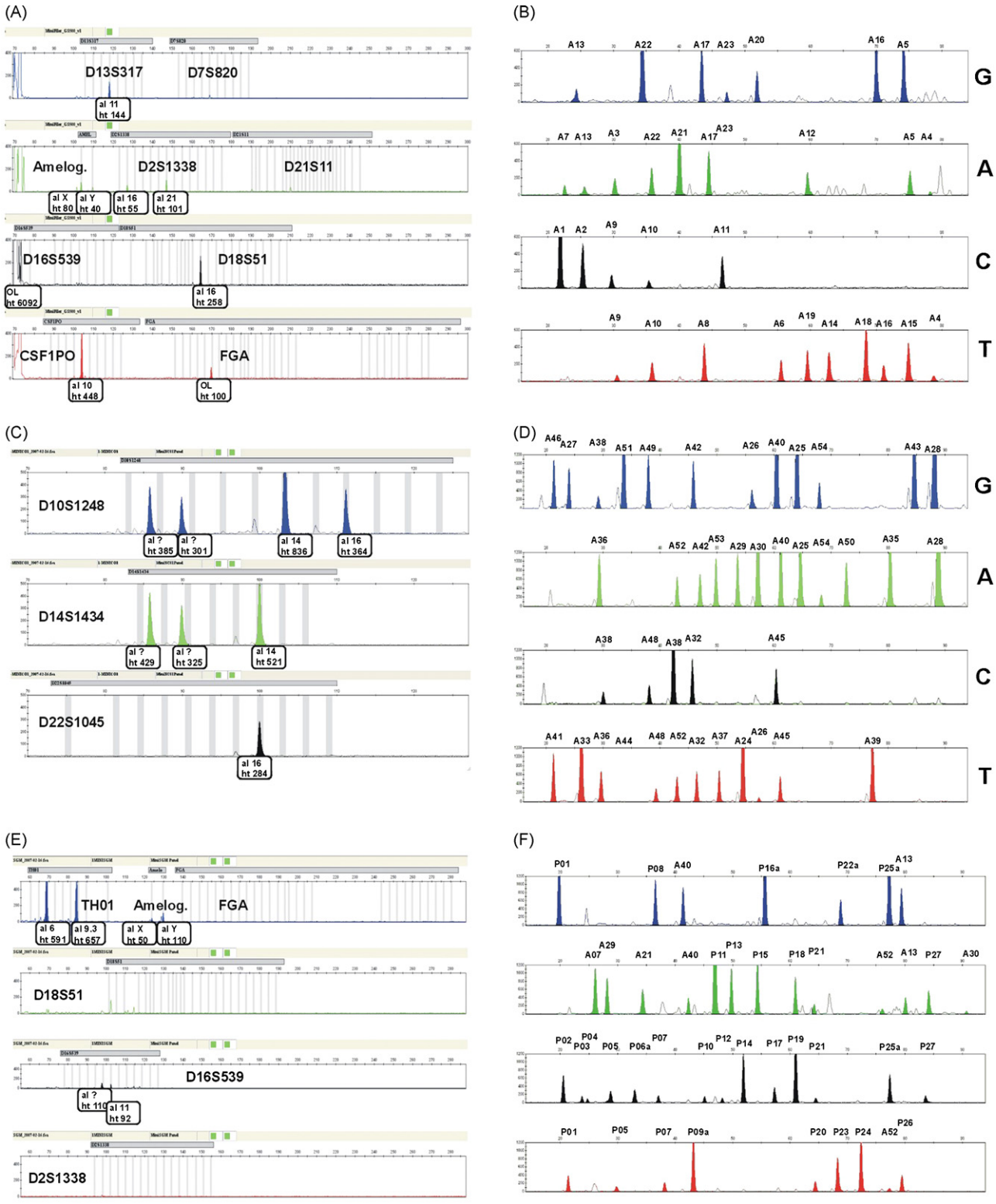

Fig. 2. Genotype results obtained from the femur using the ancient DNA extraction protocol. (A) AmplSTR® MiniFiler™ STR set, (B) SNP*for*ID Auto1 23 plex SNP set using a dedicated 23 plex PCR, (C) NIST Mini-NC01 STR set, (D) SNP*for*ID Auto2 29 plex SNP set (dedicated 29 plex PCR), (E) NIST Mini-SGM STR set, (F) SNP*for*ID 34 plex ancestry indicative marker SNP set. Solid-colour peaks denote identified genotypes except the off-ladder (OL) peaks at FGA (plot A) and D16S539 (E). Solid peaks of STR sets in plots A, C, E are above a pre-determined minimum signal intensity of 50 RFU, solid peaks of SNP sets in plots B, D, F are those within ±0.5 bp of pre-determined size positions. All 52 plex identification set SNPs in (A) and (D) labeled as in the SNP*for*ID browser (http://www.bioinformatics.cesga.es/snpforid/). 34 plex ancestry set loci labeled as follows: P01-rs2304925, P02-rs5997008, P03-rs1321333, P04-rs2814778, A07-rs917118, A29-rs1024116, P05-rs7897550, P06a-rs10843344, A21-rs722098, P07-rs239031, P08-rs12913832, A40-rs2040411, P09a-rs1978806, P10-rs773658, P11-rs10141763, P12-rs182549, P13-rs1573020, P14-rs896788, P15-rs2065160, P16a-rs2572307, P17-rs2303798, P18-rs2065982, P19-rs3785181, P20-rs881929, P21-rs1498444, P22a-rs1426654, P23-rs2026721, P24-rs4540055, A52-rs1335873, P25a-rs16891982, P26-rs730570, A13-rs1886510, P27-rs5030240, A30-rs727811.

duplicate amplifications for the ancient DNA extraction, three analyses per marker set in total. Although Fig. 2 shows the best results, product peaks were consistent amongst analyses in all cases but weaker on other occasions. All SNP sets were analyzed with duplicated amplifications.

3.2. Performance of conventional STRs, short-amplicon STRs, mtDNA and SNPs

Each of the conventional STR sets failed to amplify detectable alleles, including the shortest amplified products of amelogenin, across multiple analyses, comprising duplicate amplifications of each dilution and from both extraction systems. In this identification case the normal recourse of extracting from different bones within the same skeleton or from different segments of the long bones was precluded by the condition of the submitted material and use of most of the best material for two parallel extractions.

Short-amplicon STRs gave detectable alleles in all three marker sets used, although NIST Mini-SGM only gave reliable genotypes for the shortest amplicons of TH01. MiniFiler™ showed partial profiles for both extracts and the signal strength generally correlated with amplicon length with the exception of D16S539: the STR that performed poorly in all cases despite a relatively short amplicon size. The typing results of the NIST sets suggest that while Mini-SGM performs poorly, the Mini-NC01 STRs amplify well with degraded DNA, although extra non-specific peaks are evident in Fig. 2C at two loci. Similarly off-ladder (OL) peaks above 50 RFU are evident in D16S539 in Mini-SGM and FGA in MiniFiler™. Although the D16S539 OL peaks are not particularly strong compared to the TH01 peaks in the same set, the FGA peak appears to be more akin to a well-defined product peak just outside a standard allele bin. Analysis of the FGA peak shown in Fig. 2A and a second replicate indicated a size estimate ~0.2 bp slower than the allele bin left ''edge'' and ~0.65 bp slower than the bin mid-point, while the co-electrophoresed CSF1PO peak ran to the midpoint of its bin. This indicates that the FGA peak was more likely to be artifactual than a slow running 22 allele or a rare intermediate 21.3 allele running fast, both of which would exclude the daughter. The OL peaks in D16S539 and FGA were observed in both ancient DNA extraction replicates but not in the phenol–chloroform extraction.

HVI mtDNA sequencing success is not included in the table but sequencing gave good quality sequence profiles in all analyses with no detectable reduction in peak quality given the challenging condition of the DNA. This is in line with widely published casework results where mtDNA has previously been the most successful means to identify badly burnt human remains.

It is evident that in the analyses of this case SNP genotyping gives the most successful performance. Firstly, the subdivision of the 52 plex into a dedicated 23 plex and 29 plex gave complete profiles compared to using a single combined PCR prior to primer extension but improvements in both success and signal strength were marginal. Secondly, previous observations of the relative performance of Auto1 and Auto2 in a range of degraded DNA analyses indicated that, regardless of the PCR multiplex used, Auto2 is consistently more robust and sensitive as well as providing better quality peak patterns in nearly all cases [8,10]. This is consistent with the results obtained in this study and is illustrated by adjacent electropherograms B and D in Fig. 2. Lastly, the ancestry-indicative 34 plex gave near identical success rates to the subdivided Auto1 and Auto2 PCR indicating that a slightly larger-scale PCR and primer extension reaction does not unduly affect performance with highly degraded DNA. Although some non-specific peaks were seen in each of the primer extension electropherograms, in only a few instances did these reach comparable heights to the SNP alleles and all were well separated from the expected sizes of the alleles. Although the most complete SNP profiles are shown in Fig. 2, ~5% of loci failed in the duplicate amplification runs (slightly less with 34 plex) and in keeping with SNP analysis we have performed in other challenging cases, no consistently good or bad performers were evident amongst the 52 and 34 loci.

3.3. Informativeness of marker sets

Comparison of the genotype profiles from the femur with those of the claimed relative gave a paternity index (PI) of 4625 (probability = 99.978%) for STRs, a PI of 42,645 (probability = 99.998%) for the identification SNP set and a combined PI of 197,350,337 (probability = 99.999999%). The genotyping data from the femur and their assessments of paternity listed above were therefore reported as positive identification of the skeleton as the remains of the man missing from the area 10 years back. The PI and probability values obtained indicate that when a full set of SNP genotypes is obtained the collective power is better than a partial STR profile despite the characteristic that SNPs, as binary polymorphisms, have much lower informativeness per locus. In fact the increased power of SNP sets to differentiate individuals compared to MiniFiler™ STRs is evident from a comparison of complete profiles for each marker set. The eight STRs of MiniFiler™ give an average random match probability of 6.5×10^{-11} for African Americans and 8.2×10^{-11} for Europeans (source: AB product information) compared to 1.0×10^{-18} and 3.7×10^{-21} as equivalent values for the 52 SNPs (source: SNP*for*ID allele frequency browser: http://www.bioinformatics.cesga.es/snpforid/search.php).

The 34 plex ancestry informative set was not used in the paternity analysis since these SNPs were chosen to give little or no variability amongst individuals from the same population-group. However, information about likely population of origin may be useful in the analysis of multiple deceased individuals particularly when applied to the identification of mass disaster victims. For this reason it is important to use the opportunity to assess the 34 plex SNP set with challenging DNA cases and to test the informativeness for ancestry if partial data is obtained [12]. Using a Bayesian classification system imbedded within an interpretative web-portal (http://www.mathgene.usc.es/snipper/) to derive a probability of European, African or East Asian ancestry, expressed as −log likelihoods, (i.e. a smaller value is more suggestive of ancestry), indicated the 34 plex

Table 3
Classification probabilities for SNP profile from femur using the SNP*for*ID 34-SNP ancestry indicative marker set

Training set for calculation	−log likelihood of SNP profile	Likelihoods (as exponentials)	Likelihood ratio	Probability expressed as a verbal predicate
European	41.1095	1.401E−18	European not African:1.644E+11	164 Billion times more likely European than African
African	66.9353	8.518E−30		
European	41.1095	1.401E−18	European not Asian:4.456E+10	44 Billion times more likely European than Asian
Asian	65.6296	3.144E−29		
Asian	65.6296	3.144E−29	Asian not African:3.69	
African	66.9353	8.518E−30		

Ancestry assignment is based on a three population-group comparison and 120 sample training set from each group for the classification algorithm. Likelihood of SNP profile denotes the probability of the individual ancestry matching that of the training set (the lowest −log likelihood value equates to the highest probability).

profile from the femur was 164 billion times more likely to be European in origin than African and 44 billion times more likely European than East Asian (using a three-way differentiation and the three training sets of the classification portal). The statistical values obtained from the Bayesian analysis are outlined in Table 3.

To gauge the reliability of the 52 plex SNP results in the absence of reference genotypes from the deceased, the observed number of heterozygotes was compared to an expected number estimated from a population sample of NW Spain listed in the SNP*for*ID frequency browser. The Auto1 + 2 profiles showed 21 of 52 heterozygous SNPs compared to an expected 24 heterozygotes based on a 46% heterozygosity estimate for NW Spain. This was interpreted as indicating an absence of allele dropout in the SNP profiles obtained from the femur. Although there were insufficient loci in the small-amplicon STR sets to allow the same check to be realistically made with the STR profiles these evidently showed lower heterozygosity than allele frequencies would suggest. An alternative approach to assessing allele dropout is to estimate the expected exclusion rate from the markers used and in the case of the SNP set this is 99.98% in Europeans [9]. Therefore, the lack of exclusions in our analyses suggests an absence of allele dropout.

4. Discussion

Although this is a single challenging DNA case a noticeable difference in typing performance between short and standard amplicon length markers is evident in the DNA analyses described. It is likely from the circumstances of the case that the DNA extracted was severely compromised from the combined processes of decomposition and heat-induced degradation. Therefore, our observations of this case, showing unusually severe degradation, together with other challenging analyses [8] can provide a better way to gauge the success of new marker sets and extraction procedures than trying to reproduce degradatory effects in the laboratory. Considerable effort was made in the original SNP sets primer design process [11,12] to create multiplexes producing amplicons below a 120 bp size limit (average and range for the 52 plex: 88 bp, 59–115 bp and for the 34 plex: 86 bp, 61–117 bp) and the comparable near-complete profiles and peak quality shown in both sets suggests that this is a key factor in reducing locus dropout when typing highly degraded DNA. The reduced-length STR marker sets all provided genotypes from degraded DNA when none could be obtained with standard length amplicons. Although it is inappropriate to draw conclusions from the performance of individual STRs in one case, generally those amplifying above a size ranging from 150 to 180 bp appear to fail more readily in these analyses. A recent study led to the recommendation that short-amplicon STR products should aim to be smaller than 150 bp to ensure the best sensitivity [2]. In our analysis the amelogenin peaks of MiniFiler™ showed a degree of size related imbalance in contrast to the other heterozygous locus of D2S1338 suggesting that stochastic effects between alleles cannot be ruled out as complicating factors in the interpretation of peaks from larger STRs or those showing broader allele size ranges (e.g. FGA). Furthermore FGA in MiniFiler™ and D16S539 in Mini-SGM showed unassigned peaks with good signal strength.

One problem that can limit the proper comparison of marker set performance in real casework is the inability to control for allele dropout when assessing identification cases based solely on bodily remains. Without a reference profile the paternity index for a surviving relative provides a statistical likelihood that both individuals are related as claimed and in this case the failure to detect any exclusion in the 52 SNPs, coupled with a heterozygosity that is a reasonable match to the population as a whole gives persuasive evidence that the SNP profiles obtained from the femur represent the true genotypes of the deceased. Although the possibility of SNP allele dropout cannot be completely discounted, we have observed across a range of challenging DNA analyses [8] that the primer extension reactions of the SNaPshot assays used do not reveal noticeable differences in PCR efficiency, only some imbalance in peak heights related to variation in dye signal strength or extension efficiency between alleles of the same SNP. Furthermore although the background baseline signal can be higher with DNA extracts from degraded sources, these extra peaks always occupy positions well separated from the size bins used to identify the SNP alleles. Therefore, SNP typing tends to show more consistent differences in peak height between loci in the same electropherogram largely independent of the DNA quality and it is difficult to isolate particular SNPs as weak performers with degraded DNA.

The low individual informativeness of SNPs has been a factor hindering their widespread adoption amongst the forensic

community as first-choice markers for degraded DNA analysis. However, this characteristic may become largely irrelevant if full SNP profiles can be reliably obtained from highly degraded material using large-scale multiplexes and simple, easily adopted genotyping systems. This case suggests that short-amplicon approaches offer improved success when typing highly degraded DNA, while SNPs, as part of a range of such marker sets available to the forensic practitioner, could be valuable additions to the more established STRs.

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