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Analysis of artificially degraded DNA using STRs and SNPs—results of a collaborative European (EDNAP) exercise

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Abstract

Recently, there has been much debate about what kinds of genetic markers should be implemented as new core loci that constitute national DNA databases. The choices lie between conventional STRs, ranging in size from 100 to 450 bp; mini-STRs, with amplicon sizes less than 200 bp; and single nucleotide polymorphisms (SNPs). There is general agreement by the European DNA Profiling Group (EDNAP) and the European Network of Forensic Science Institutes (ENFSI) that the reason to implement new markers is to increase the chance of amplifying highly degraded DNA rather than to increase the discriminating power of the current techniques.

A collaborative study between nine European and US laboratories was organised under the auspices of EDNAP. Each laboratory was supplied with a SNP multiplex kit (Foren-SNPs) provided by the Forensic Science Service®, two mini-STR kits provided by the National Institute of Standards and Technology (NIST) and a set of degraded DNA stains (blood and saliva). Laboratories tested all three multiplex kits, along with their own existing DNA profiling technique, on the same sets of degraded samples. Results were collated and analysed and, in general, mini-STR systems were shown to be the most effective.

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Accordingly, the EDNAP and ENFSI working groups have recommended that existing STR loci are reengineered to provide smaller amplicons, and the adoption of three new European core loci has been agreed.

Keywords: Degraded DNA; Short tandem repeats; Single nucleotide polymorphisms; Mini-STRs

1. Introduction

Existing short tandem repeat (STR) systems used in European national DNA databases (NDNADBs) include seven core STR loci recommended by the European Network of Forensic Science Institutes (ENFSI) and agreed by Interpol [1]. The core loci are included in commercially available multiplexes. However, all current markers have relatively large amplicon sizes (between 150 and 450 bp) [2]. It has been demonstrated that smaller amplicons are much more likely to be amplified in samples containing degraded DNA [3–11]. There are two kinds of markers that can bring the size of the amplicon substantially below 150 bp: 'mini-STRs' that have short flanking regions to the tandem repeat sequence and single nucleotide polymorphisms (SNPs). See Butler [4] and Budowle [12] for an extensive review of existing technologies. A small number of validated SNP assays are used in casework and these include mini-sequencing assays for mitochondrial DNA (mtDNA) [13–16], Y chromosome [17], a red hair marker assay [18] and autosomal multiplexes [19]. There has been some debate about which is the best approach [20]. Some existing high molecular weight markers have already been converted into low molecular weight (<130 bp) 'mini-STR' multiplexes simply by moving the primer binding sites closer to the STR repeat region [3,7,21]. The advantage of this approach is that it is possible to maintain consistency with existing core loci that are used in NDNADBs. To achieve the ultimate lower limit of small amplicons (ca. 40 bp), SNPs are preferable, but the downside is that a panel of 45–50 loci would be needed to achieve match probabilities comparable with existing STR multiplexes [22,23]. Furthermore, the larger the multiplex, the more difficult it is to reliably and to reproducibly construct [12]; loss of amplification efficiency may ensue, effectively defeating the object of the exercise. To circumvent this problem, several SNP multiplexes of a dozen loci each can be used in concurrent multi-tube reactions, however, the sample size needs to be sufficient to allow this option [24,25]. Large amounts of DNA from, e.g., bones, can be analysed in this way, but the study of many small forensic stains is precluded as the amount of DNA extract available is limited. In addition, the binary nature of SNPs means that their statistical characteristics are not amenable to the interpretation of complex samples such as mixtures. A robust, highly quantitative SNP assay would be required to allow determination of mixtures using an interpretation strategy based on heterozygous balance and homozygous thresholds [22].

Accordingly, a collaborative EDNAP study was carried out to compare some different DNA profiling techniques for their usefulness in genotyping artificially degraded samples. The study was primarily designed to assess the effectiveness of new techniques (especially SNPs and mini-STRs).

2. Materials and methods

2.1. Degraded DNA samples

All laboratories were provided with sets of artificially degraded blood and saliva samples. Aliquots of 5 μl blood or 10 μl saliva were pipetted onto 4 mm^2 cotton squares and degraded at 37 °C in a 100% humid environment over a period of 12 and 16 weeks, for saliva and blood, respectively. After set periods of 0, 2, 8, 12 [saliva] and 16 [blood] weeks, degradation was suspended by storing the samples at −20 °C until the time course was complete. Laboratories extracted 3–4 stains at each time-interval, combining the extracts together. This protocol was used to average out variation that may be inherent between different stains.

2.2. Extraction and quantification

Standard protocols of laboratories carrying out the analyses were used (Table 1). Methods included: QIAamp or QIAshredder supplied by Qiagen™ [26,27] and phenol–chloroform [28]. Quantification was carried out using Picogreen [29], Quantifiler™ Human DNA Quantification kit (according to manufacturer's protocol) or Slot-blot methodology [30]. One laboratory performed quantification using a real-time quantitative PCR assay with a fluorogenic Taqman probe, targeting the human Alu repetitive sequence, with PCR primers adopted from Nicklas and Buel [31].

2.3. SNP and STR kits and protocols

The following STR kits were used in the study, according to manufacturer's protocol: AMP*Fl*STR® SGM Plus™ (SGM+) (Applied Biosystems) [7 labs][32]; AMP*Fl*STR® Identifiler (Applied Biosystems) [1 lab] [33]; Powerplex®16 system (Promega) [1 lab] [34]; plus mini-SGM and miniNC01 (National Institute of Standards and Technology (NIST), US) [9 labs] [3,6,21]. The 21 loci 'Foren-SNP™' multiplex kit (The Forensic Science Service®, UK) was used as described by Dixon et al. [9 labs] [19].

Table 1
Extraction and quantification methods and results, provided by participants. Grey boxes indicate that no information was received. UND = undetermined data value

Lab ID	Extraction protocol	Quantification values (ng/uL)																
		Ref 1 blood					Ref 1 saliva				Ref 2 blood				Ref 2 saliva			
		Quant method	0 weeks	2 weeks	8 weeks	16 weeks	0 weeks	2 weeks	8 weeks	12 weeks	0 weeks	2 weeks	8 weeks	16 weeks	0 weeks	2 weeks	8 weeks	12 weeks
1	Qiagen (manual)	Picogreen	1.91	0.22	0.22	0.03	1.03	0.07	0.01	0.01	2.13	0.23	0.23	0.02	0.36	0.03	0.03	0.01
2	Qiagen (robot)	Quantifiler	0.63	0.01	0.00	0.00	0.82	0.00	0.00	0.00	0.72	0.01	0.01	0.00	0.36	0.00	0.00	0.00
3	Phenol–chloroform	Quantifiler	2.31	0.22	0.06	0.02	6.59	0.00	0.00	0.00	5.16	0.92	0.25	0.46	8.95	0.06	0.01	0.03
4	Phenol–chloroform	Quantifiler	2.18	1.29	0.00	0.00	UND	0.00	0.00	0.00	3.65	2.35	0.96	0.00	8.31	0.00	0.00	0.00
5	Phenol–chloroform	qPCR	9.67	0.93	0.53	0.12	19.00	0.10	0.03	0.03	10.29	1.64	1.97	1.58	15.84	0.06	0.15	0.06
6	Chelex	None																
7																		
8	Phenol–chloroform	Slot-blot	11.79	0.57	1.00	1.68	33.46	0.12	0.03	0.05	10.15	2.33	4.13	0.80	14.88	0.36	0.23	0.05
9	Qiagen (manual)	None																

In addition, two laboratories carried out low copy number AMP*Fl*STR® SGM Plus™ profiling (34 PCR amplification cycles) using the method described by Gill et al. [35]

All PCR products were electrophoresed on AB 3100 capillary electrophoresis (CE) sequencers (Applied Biosystems) with either POP-4 or POP-6 polymer. Results were analysed using Genescan™ and Genotyper™ analysis software (Applied Biosystems).

2.4. Data analysis

Each laboratory was given an identifier number and genotyping results for each DNA profiling system for each laboratory were collated on Microsoft® Excel spreadsheets.

Genotypes were analysed as percentages—e.g. for SGM+ a full genotype comprised 22 alleles, thus a profile with 11 alleles was 50% of a full profile. Converting into percentages allowed direct comparisons between different multiplex systems.

Data were analysed with Minitab™ Release 14 using ANOVA, box–whisker plots, and the median polish method [36]. Box–whisker plots are a convenient method to display the main features of a set of data and facilitate the comparison of multiple sets. A box–whisker plot comprises a box, whiskers and outliers. A line is drawn across the box to represent the median; the bottom of the box is the first quartile (Q_1) and the top is the third quartile (Q_3)—hence half of the data are represented in the inter-quartile (IQ) range Q_3–Q_1; 25% of the data values are less than or equal to the value of Q_1; and 75% are less than or equal to the value of Q_3. The whiskers are lines extending from the top and bottom of the box. The lower whisker extends to the lowest value within the lower limit, whilst the upper whisker extends to the highest value within the upper limit. The limits are defined by: $Q_1 - 1.5(Q_3 - Q_1)$ (lower limit) and $Q_3 + 1.5(Q_3 - Q_1)$ (upper limit). The outliers are unusually high or low data values that lie outside of the lower and upper limits, these are represented by asterisks.

Identifiler® and Powerplex®-16 were omitted from the final results analysis, except for the inter-laboratory comparison, because only one laboratory used each multiplex. Low copy number (LCN) SGM+ results were also disregarded from intra-laboratory analyses, because only two laboratories submitted data.

3. Results

3.1. Extraction methods

Details of extraction techniques and corresponding quantification values were submitted by six of the laboratories (Table 1). These ranged from 0 ng/μL for heavily degraded samples to 33 ng/μL for a reference sample stain (Fig. 1).

The inter-quartile (IQ) range for degraded saliva samples (≥2 weeks incubation) varied between 0.03 and 0.17 ng/μL, compared to 0.5–2.3 ng/μL for blood samples indicating that DNA in the saliva stains degraded much more rapidly than in blood.

In comparison, undegraded control (time zero) reference samples showed considerable variation in the amount of DNA recovered between laboratories. More DNA was recovered with phenol–chloroform compared to Qiagen™ but the variation was much greater in the former (IQ range = 27 and 1.4 ng/μL, respectively) (Fig. 1). The method of quantification may have affected the DNA quantification values gained. Both laboratories using phenol–chloroform extraction followed by Quantifiler™

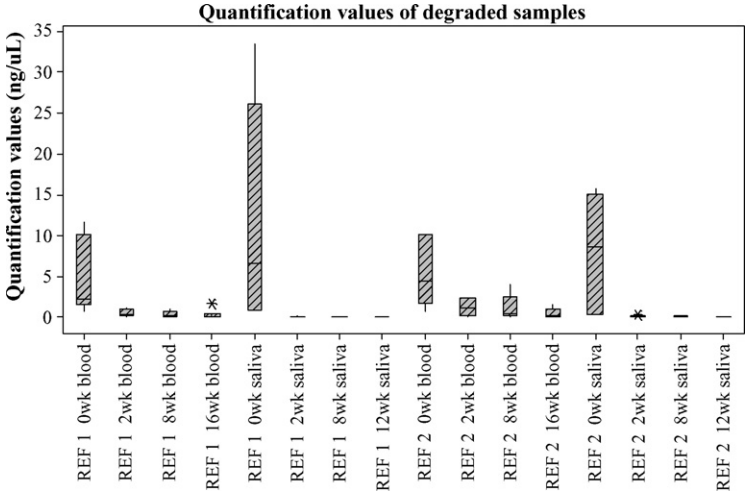

Fig. 1. Box–whisker plot showing the range of quantification values received for each reference individual for each sample type. Calculations are based on data from the six laboratories that submitted data.

Table 2
ANOVA results for percentage profile data for each laboratory for each sample type using each multiplex kit. Significant P-values are denoted in bold

Analysis of variance (ANOVA) tests	Degrees of freedom (DF)	Sum of squares (SS)	*F* ratio	Probability (*P*)
Multiplex	3	11705	2.49	0.061
Lab ID	7	96328	8.78	<0.001
Ref ID	1	11829	7.55	0.006
Sample type	1	177422	118.4	<0.001
Degradation time	3	280931	59.78	<0.001
Multiplex * lab ID	21	20122	0.61	0.909
Multiplex * ref ID	3	553	0.12	0.950
Multiplex * sample type	3	2293	0.51	0.676
Multiplex * degradation time	9	2949	0.21	0.993
Lab ID * ref ID	7	5841	0.53	0.809
Lab ID * sample type	7	15431	1.47	0.176
Ref ID * sample type	1	6126	4.09	0.044
Lab ID * degradation time	21	47521	1.44	0.098
Ref ID * degradation time	3	2867	0.61	0.609
Multiplex * lab ID * ref ID	21	3545	0.11	1.000
Multiplex * lab ID * degradation time	63	20937	0.21	1.000
Multiplex * ref ID * degradation time	9	2765	0.2	0.994
Lab ID * ref ID * degradation time	21	30254	0.92	0.566
Multiplex * lab ID * ref ID * degradation time	63	13408	0.14	1.000
Multiplex * lab ID * sample type	21	8114	0.26	1.000
Multiplex * ref ID * sample type	3	262	0.06	0.981
Lab ID * ref ID * sample type	7	14042	1.34	0.231
Multiplex * lab ID * ref ID * sample type	21	3571	0.11	1.000

quantification (labs 3 and 4) gave similar values, whereas quantification with qPCR (lab 5) and slot-blot (lab 8) produced much greater values (Table 1). However, all phenol–chloroform values (for control samples) were greater than those gained with Qiagen™, regardless of the quantification method.

3.2. Analysis of variance (ANOVA) calculations

ANOVA analysis on percentage profile data (Table 2) showed major significant differences as follows: (a) between different laboratories ($p < 0.001$); (b) between the two donating individuals (ref ID) ($p = 0.006$); (c) between the

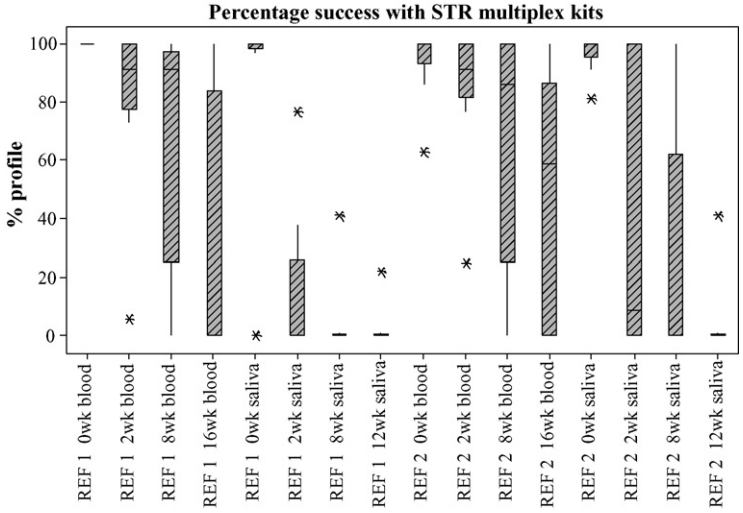

Fig. 2. Box–whisker plot showing the variation in percentage profiles per sample between the participating laboratories, using standard STR multiplex DNA profiling kits.

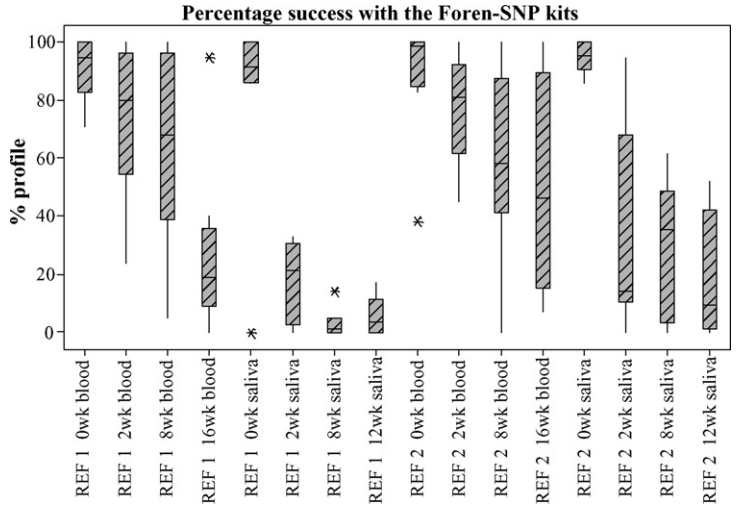

Fig. 3. Box–whisker plot showing the variation in percentage profiles per sample between the participating laboratories, using the Foren-SNP multiplex DNA profiling kit.

sample types—blood and saliva ($p < 0.001$); and (d) between different degradation times ($p < 0.001$). There was a smaller (borderline) significant two-way interaction between ref.ID and sample type ($p = 0.044$), i.e., there may be a significant difference between blood and saliva samples that are dependent on the reference individual. Otherwise, higher order interactions were not obvious in the data-set. Differences between the performance of the four multiplexes were almost significant using ANOVA ($p = 0.061$). Although there were differences between laboratories, these differences were consistent when averaged across different multiplexes, i.e., if a lab performed well with one multiplex then it would also perform well with another, and vice versa.

3.3. Intra-laboratory variation

Laboratories obtained full DNA profiles from control reference samples (time zero). As samples degraded, there was an increase in the amount of variation between the different laboratories in terms of percentage profile observed (Figs. 2–5). After several weeks, virtually all the DNA had degraded and no profile was obtained.

The most consistent multiplex across all laboratories was the mini-STR NC01 kit (Fig. 5). This multiplex consisted of three STR loci, D10S1248, D14S1434 and D22S1045, which are not available in commercial STR kits. The small number of loci present in the multiplex, compared to the 21

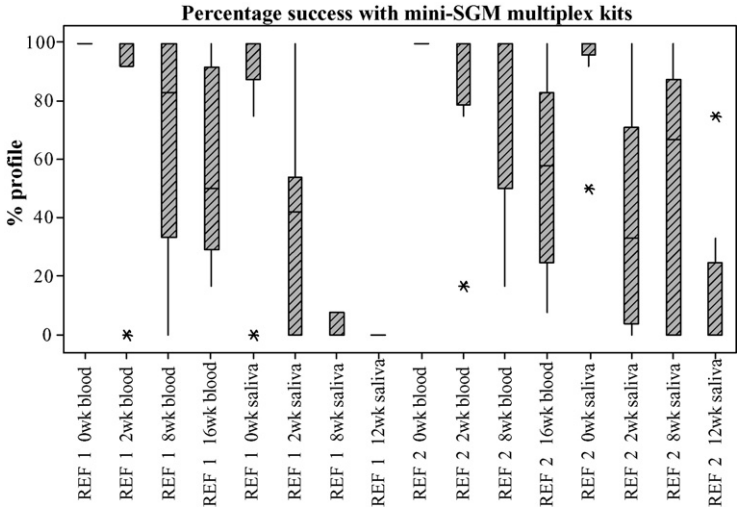

Fig. 4. Box–whisker plot showing the variation in percentage profiles per sample between the participating laboratories, using the mini-SGM DNA profiling kit.

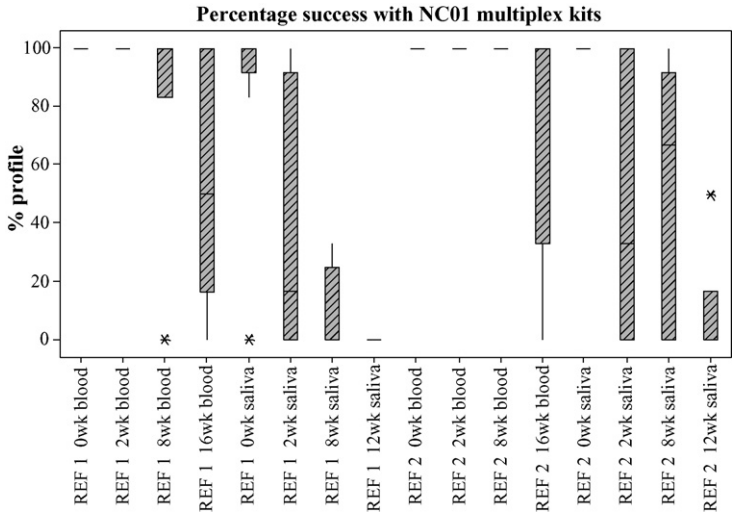

Fig. 5. Box–whisker plot showing the variation in percentage profiles per sample between the participating laboratories, using the NC01 mini-STR DNA profiling kit.

loci found in the Foren-SNPs™ kit, eleven loci in SGM+ and seven loci in the mini-SGM multiplex, may have increased the robustness of the system.

In order to standardise the data and allow the different laboratories to be compared without bias, median polish analysis [36] was used. Median polish is similar to analysis of variance tests except that medians are used instead of means, thus adding robustness against the effect of outliers. The degradation time course of each sample averaged across all laboratories was compared and consequently, the performance of each multiplex—noting that the ANOVA showed no higher order interactions to complicate analysis. All four degradation profiles were quite different from each other (Fig. 6). As indicated by the ANOVA, saliva degraded faster than blood. It can be further generalised that the mini-STR systems performed better than SGM+. The SNP multiplex was inconsistent, but appeared to work better with the saliva compared to blood, possibly due to the presence of inhibitory factors in the blood samples. Interestingly, LCN SGM+ (34 amplification

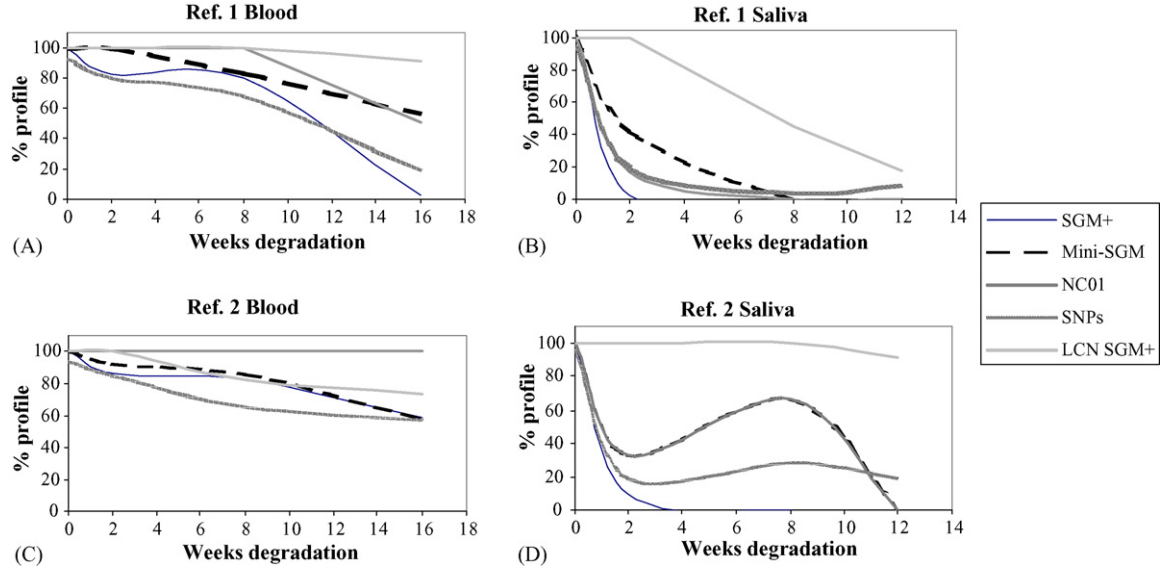

Fig. 6. Percentage profiles obtained across all labs for all samples and sample types. (A) Reference 1 blood. (B) Reference 1 saliva. (C) Reference 2 blood. (D) Reference 2 saliva. Values were calculated using median polish analysis to standardise the data obtained from all laboratories.

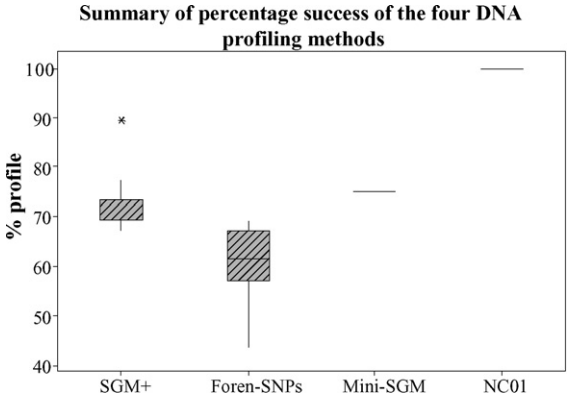

Fig. 7. Median polish results showing the variation in median percentage profiles across laboratories.

cycles) worked significantly better in three out of four samples (Fig. 6), compared to any other profiling method. Likewise, the single laboratory that reported LCN results with Powerplex® 16 achieved results that compared favourably to the overall results for the mini-STR multiplexes in this study.

3.4. Inter-laboratory variation

Median polish analysis (Fig. 7) was again used to provide a relative comparison of individual laboratory performance (averaged across all samples) (Table 3).

The analysis confirmed that the NC01 mini-STR multiplex kit performed the best, giving a median value of detected genotypes of 100% followed by mini-SGM (75%). The Foren-SNP™ multiplex gave the lowest median value of detected genotypes (61.6%). This was attributed to the complexity of the SNP multiplex. The multimix comprised 65 separate primers of which 63 were >35 bp in length. Transporting the multimix to different countries may have permitted freeze-thawing of the solution, causing shearing of the large primers. Consequently, SNPs also showed the greatest variation between the different labs (Fig. 7).

To evaluate further, we ranked the SNPs in order of success, and selected the best ten for separate statistical analysis, irrespective of amplicon size. This modified system gave equivalent results to the miniSTR systems (data not shown). The Foren-SNPs™ loci ranged in size from 56 to 146 bp and the maximum amplicon size for mini-STRs was 170 bp. Thus, we concluded that good markers for degraded DNA were dependent upon the small size of the amplicon, and not on the choice of SNP or a mini-STR (unless the former was used to achieve the smallest amplicon size possible).

Table 3
Median polish analysis results for each laboratory in the study, including median values gained for each multiplex across all labs

Lab ID	SGM+ (%)	Foren-SNPs (%)	Mini-SGM (%)	NC01 (%)
1	69.5	69.3	75.0	100
2	69.5	58.2	75.0	100
3	69.5	*	75.0	100
4	69.5	43.5	75.0	100
5	89.5	60.0	89.6	100
6	69.5	64.4	75.0	100
7	67.2	56.5	35.4	91.5
8	77.4	68.3	75.0	100
9	69.5	63.1	75.0	100
Median across labs	69.5	61.6	75.0	100

3.5. Total allele dropout across degradation periods

Allele dropout was measured for each sample at each stage of degradation, averaged across laboratories plotted against molecular weight (bp). Data from the two mini-STR systems (mini-SGM and NC01) were combined under a general heading of 'mini-STRs', with a maximum amplicon size of 170 bp. Linear regressions were plotted for reference 1 blood sample (Fig. 8a–c), confirming a general trend that lower molecular weight loci were more likely to stay intact. Allele dropout increased with increasing times of degradation for all three DNA profiling techniques. Foren-SNPs™ was the only multiplex to show allele dropout in control samples (time zero). Mini-STRs showed decreased allele dropout with the more degraded samples compared to SGM+.

4. Discussion

A previous EDNAP study using DNA degraded by sonication and DNAse I [37], and other studies using degraded body fluid stains [3,5,6,8–11] and telogen hair roots [7], have demonstrated the efficacy of low molecular weight amplicons to analyse degraded DNA. The experiment described in this paper followed a different design to those previously described, as it simulated a time-course series of degraded stains in their 'natural state'. This was achieved by incubating material spotted with saliva and blood in 100% humidity at 37 °C. Under these conditions, degradation was greatly accelerated compared to the dried-state process and total degradation was achieved within a short time period of 12–16 weeks. By taking samples at regular intervals, a complete time-course was produced and a point reached which corresponded to the time where little or no amplifiable DNA remained. We showed that saliva degraded faster than blood, but this is not surprising as this body fluid contains enzymes such as lysozymes, amylases, peroxidases and histatins, as well as numerous bacteria, which contribute micrococcal nuclease. Micrococcal nuclease is a non-specific endonuclease, that cuts adjacent to any base, with the rate of cleavage reported to be 30 times greater

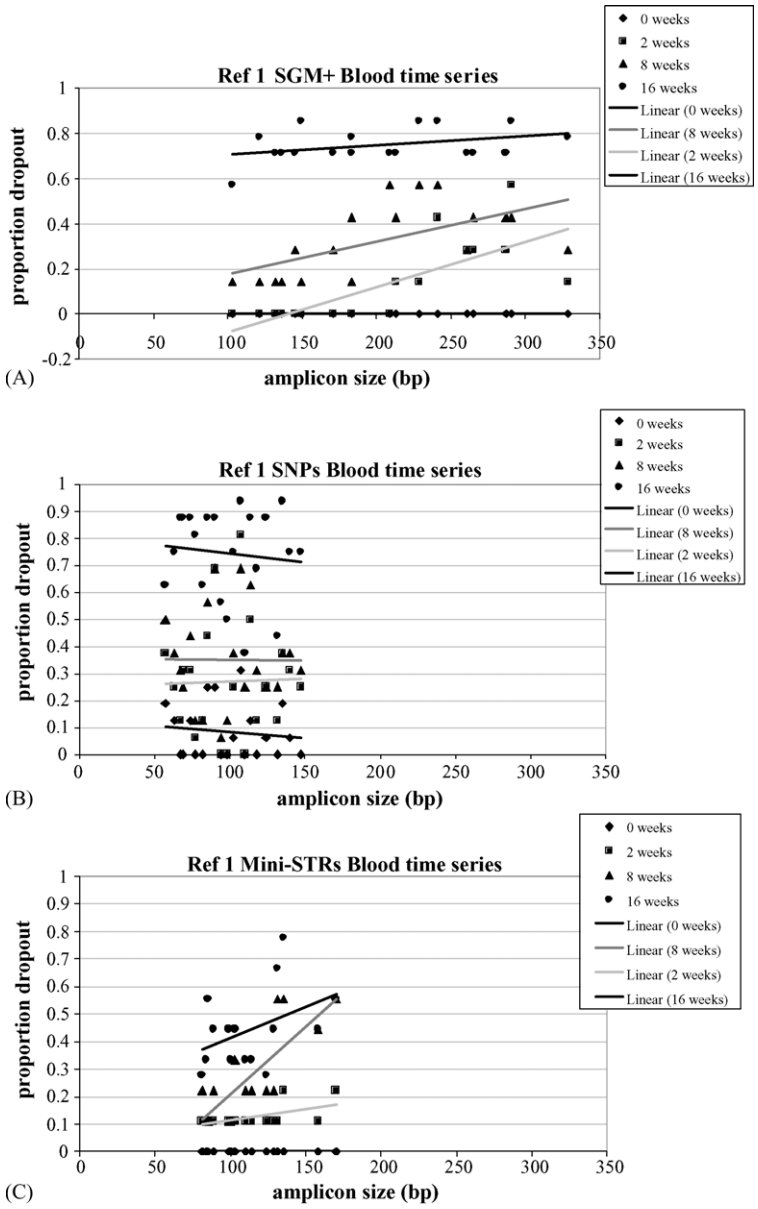

Fig. 8. Degradation plots for reference 1 blood investigated with SGM+, Foren-SNPs and mini-STR multiplexes. Graphs indicate the proportion of allele dropout compared to amplicon size. (A) SGM+ profiles. (B) SNP multiplex profiles. (C) mini-STR profiles. Mini-SGM & NC01 were combined for the mini-STR analysis.

at the 5′ side of A or T rather than G or C (fortunately most STR sequences tend to be GC-rich). Mammalian cells contain two additional DNAses that cleave non-specifically; DNAse I, which slightly favours purine–pyrimidine sequences [38] and DNAse II, an enzyme found in lysozomes associated with cell apoptosis [39].

Median polish analysis was carried out in order to standardise the data, allowing data sets from all laboratories to be compared regardless of variability in laboratory techniques, operator differences and sampling limitations [36]. Transformed data was analysed to investigate degradation rates, allele dropout and performance of the four assays used in this study. The artificially degraded samples gave similar results across all laboratories, showing the method produced samples with consistent levels of degradation across all sets.

The mini-STR assays tested gave the best results overall, when compared with standard SGM+ profiling and the Foren-SNPs^{TM} kit. Low copy number (LCN) DNA profiling proved to be the most successful method of amplification,

although this technique was only carried out by three laboratories; one using Powerplex® 16 and two using SGM+. LCN profiling only differs from standard DNA profiling by the number of cycles used for PCR amplification [40]. By increasing the number from 28 cycles to 34 cycles, the chance of amplifying the few molecules present in the DNA extract is improved. However, when LCN conditions are used, the allelic balance concurrently deteriorates and the chance of allele dropout is increased compromising interpretation. The advantage of mini-STRs is that more 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. The mini-STR assays tested in this study used 32 cycles in PCR amplification, hence the method was intermediate between LCN and standard testing, which may have increased the percentage profiles obtained [3,6,41]. It is possible that the mini-STR assays performed better as a consequence of an increased cycle number compared to standard profiling methods, as opposed to the smaller amplicon sizes targeted, however it is likely that a combination of both factors contributed to the increased success rates of these assays. Fig. 8 demonstrates the mini-STR loci give a negative regression in relation to amplicon size after 8 weeks of degradation, whereas the higher molecular weight SGM+ loci begin to show allele dropout after 2 weeks The mini-STR assays were also the most robust in this study as the number of loci targeted was lower than the other DNA profiling methods tested. NC01, giving the highest percentage profiles overall, only contained three STR loci and therefore would generally have been easier to optimise than the Foren-SNP™ multiplex containing 21 loci.

The Foren-SNP™ kit performed poorest out of the four assays tested in this study. This particular kit was used as it was the only fully validated forensic SNP multiplex available [19]. Other SNP multiplexes have been developed, but lack the quantitative and qualitative properties for forensic use [12,24,42]. SNP assays based on primer extension biochemistry, such as GenomeLab™ SNPStream® (Beckman Coulter) and SNaPshot™ multiplex system (Applied Biosystems™), are capable of genotyping thousands of SNPs in a single analysis but require an increased volume of either initial DNA template or PCR product, both of which are limited in crime scene samples. They also have the disadvantage of being multi-stage procedures, with sample tubes needing to be opened at various stages within the process. The Foren-SNPs™ kit allowed amplification of all 21 loci in a single tube reaction which were then analysed on an electrophoresis instrument. The potential certainly exists to further optimise SNP multiplex systems, as loci do benefit from being single base sites, therefore much smaller amplicons can be targeted [22,23,43]. The ability to obtain a result using SNPs would be beneficial, especially if the sample failed to give a profile using standard STR DNA profiling. However, the biallelic nature of SNPs makes it difficult to interpret mixtures and a well balanced assay would be required to make this feasible [22]. Consequently, for crime stain work where mixtures are often encountered, STRs are preferable. STRs also benefit from being consistent with, and therefore comparable to, current national DNA databases. For identification of discrete samples, such as bones, teeth and highly degraded tissues commonly encountered in mass-disasters, there is no reason why SNPs may not be used [20,44]. It is therefore proposed that further research is focussed on reducing the size of STR amplicons, so that degraded samples can be amplified with an increased chance of success using both conventional and LCN conditions.

5. Conclusions

The EDNAP and ENFSI groups have recommended that new multiplexes can be made more efficient to detect degraded DNA by re-engineering the STR amplicons so that primers lie closer to the repeat region. To achieve the best sensitivity, amplicons should be lower than 150 bp. Not all existing core loci (e.g. HUMFIBRA/FGA) can be engineered to be this small. Since the number of core loci in Europe is currently insufficient for an effective pan-European database the EDNAP and ENFSI groups have recommended that the three NC01 loci, D10S1248, D14S1434 and D22S1045 be adopted as European standards [45,46].

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