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ORIGINAL ARTICLE

Assessment of DNA degradation and the genotyping success of highly degraded samples

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Abstract DNA becomes progressively more fragmented as biological tissue degrades resulting in decreasing ability to gain a complete DNA profile. Successful identification of samples exhibiting very high levels of DNA degradation may be complicated by presenting in minute quantities. The industry standard method for human DNA identification utilising short tandem repeats (STR) may produce partial or no DNA profile with such samples. We report a comparative study of genotyping using STRs, mini-STRs and single nucleotide polymorphisms (SNPs) with template at different levels of degradation in varying amounts. Two methods of assessing quantity and quality of a DNA sample prior to genotyping were investigated. The QIAxcel capillary gel electrophoresis system provided a rapid, cost effective screening method for assessing sample quality. A real-time quantitative PCR (qPCR) assay was able to simultaneously quantify total human DNA, male DNA, DNA degradation and PCR inhibition. The extent of DNA degradation could be assessed with reasonable accuracy to 62.5 pg and genomic targets could be quantified to a lower limit of 15.6 pg. The qPCR assay was able to detect male DNA to a lower limit of 20 pg in a 1:1,000 background of female DNA. By considering the amount of DNA and the degradation ratio of a sample, a general prediction of genotyping success using AmpFlSTR® Profiler Plus®, MiniFiler™ kits and SNP analysis can be made. The results indicate mini-STRs and SNP markers are usually more successful in typing degraded samples and in cases of extreme DNA degradation (≤200 bp) and template amounts below 250 pg, mini-STR and SNP analysis yielded significantly more complete profiles and lower match probabilities than corresponding STR profiles.

Keywords Degraded DNA · DNA Quantification · STR · Mini-STR · SNPs · Quantitative PCR

Introduction

In the case of natural and mass disasters, missing persons and forensic casework, highly degraded biological samples are encountered. Conventional methods of identification such as fingerprint, forensic anthropology and dental matching can be inadequate when remains are highly fragmented and decomposed. DNA typing using industry standard short tandem repeats (STRs) often becomes the principle means of identification [1]. DNA becomes progressively more fragmented as biological tissue degrades and this results in a decreasing ability to gain a complete STR profile [2]. The successful genotyping of samples exhibiting very high levels of DNA degradation can be further complicated by the availability of only minute quantities of material. Such highly degraded samples commonly produce incomplete or no STR profiles and therefore the less informative mitochondrial DNA typing or less reliable low copy number typing have been

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used for these samples [3]. The most favoured approach to increasing the success of DNA typing of highly degraded samples is to decrease the target amplicon size. Based on the principle that the smaller the target for PCR, the more likely the sequence will be intact and detected, much focus has been placed on designing reduced-length amplicon (~70–280 bp) STR multiplexes (mini-STRs) by moving primers closer to the target region[4]. Mini-STRs have proven to be a useful tool for genotyping degraded samples [5–8]. The European DNA Profiling (EDNAP) collaborative study recommended that existing STR loci be reengineered to provide smaller amplicons and new mini-STR loci be added to the European core loci for the purpose of increasing the chances of amplifying highly degraded DNA [9]. However, single nucleotide polymorphisms (SNPs) may provide the greatest genotyping success for degraded samples due to their potentially very short amplicon lengths (45–80 bp) [9–13]. In an effort to increase the amount of genetic information and/or discrimination power of forensic testing, the utility of other types of DNA markers are also currently being investigated by the wider forensic community. These include tightly linked SNPs (haploblocks) [14], tri-allelic SNPs [15] and nucleotide variability within STRs [16].

Multiplex real-time quantitative PCR (qPCR) assays which simultaneously quantify total human genomic DNA, male DNA, the extent of DNA degradation and the presence of PCR inhibitors have been previously described [17, 18]. Further to Hudlow et al, 2008 [17] we report a capillary gel electrophoresis method of assessing DNA degradation in addition to a qPCR assay. We also investigate the comparative genotyping success of highly degraded samples using STR, mini-STR and SNP typing systems. This type of study should prove valuable as a diagnostic tool for choosing which DNA typing systems may be the most informative whilst minimizing sample consumption.

Materials and methods

Sonication

DNA was extracted from whole blood (n=4) using QIAamp® Blood Maxi Kit (Qiagen, Hilden, Germany). Degraded samples were generated by sonication (Branson SLPt Sonifier®, Danbury, CT, USA). The sonicator probe was treated with 10% bleach and UV irradiation before and after each sample. DNA extract (0.5–1 ml) was sonicated on ice with 3/32" Microtip at 30% amplitude for up to 25 min in cycles of 30 s. Three size ranges were generated, <200 bp (extremely degraded), 200–400 bp (highly degraded) and >400 bp (moderately degraded).

Capillary electrophoresis

DNA samples were analysed via capillary electrophoresis using a High Resolution gel cartridge on a QIAxcel system (Qiagen, Hilden, Germany). Aliquots (1–3 μL) of neat DNA extract were combined with DNA dilution buffer (Qiagen) to 10 μL total volume. The QIAxcel system produces a digital gel image and an electropherogram for fragment analysis.

qPCR

Degradation assessment utilised a modified quadruplex qPCR assay previously described [17]. The four targets included two autosomal (TH01 and CSF), one male-specific target (SRY) and a synthetic oligonucleotide internal PCR control (IPC). PCR product was detected using dual labelled hydrolysis probes (TaqManMGB®, Applied Biosystems, Foster City, CA and TaqMan®, Operon, Huntsville, AL). The assay was performed on the Rotor-Gene 6000 (Qiagen) real-time thermocycler in a 20 μL reaction volume using QuantiTect Multiplex PCR Mastermix (Qiagen). Modifications to the previously published assay include five-prime dyes and primer concentrations (Online Resource 1). Primer and probe sequences were unchanged as in Hudlow et al. except for the SRY probe ([Cy3.5]TTGCCCTGCTGATCTGCCTCCC [BHQ2A]) [17]. The two-step qPCR protocol consisted of an initial 15 min 95°C polymerase activation step, followed by 40 cycles of 60 s of denaturation (94°C) and 90 s of combined annealing/extension (60°C). Pre-quantified, high molecular weight human genomic male DNA (Promega, Madison, WI) was used as a qPCR quantification standard and no template controls were included to monitor contamination. The ability of the assay to quantify the amount of male DNA in a background of female DNA was assessed by combining male and female DNA (1:1, 1:10, 1:100 and 1:1,000). Both undegraded high molecular weight (HMW) and degraded mixture samples were tested. HMW mixtures utilised Human Genomic Male DNA (Promega, Madison, WI), and female K562 DNA (Promega, Madison, WI). Moderately degraded male and female DNA were used to generate degraded mixture samples.

Genotyping

The AmpFlSTR® Profiler Plus®, and AmpFlSTR® Mini-Filer™ PCR Amplification kits (Applied Biosystems) were used for STR and mini-STR genotyping respectively. PCR was performed in 25 μL reaction volumes on a GeneAmp 9700 thermocycler (Applied Biosystems) with cycling protocols as per kit manufacturer instructions. Electrophoresis was performed on a 3130 Genetic Analyser (Applied

Biosystems). Samples were prepared for fragment analysis as per AmpFlSTR® Profiler Plus®, and AmpFlSTR® MiniFiler™ PCR Amplification kit recommendations. Data analyses were performed using GeneMappper ID v 3.2.1 software (Applied Biosystems) with a 50 relative fluorescence units (RFU) peak amplitude threshold for all dyes. A partial profile was defined as the loss of one or more alleles or loci. SNP analysis (53 SNPs—Online Resource 2) was performed in 4 separate multiplex reactions (in triplicate) using Sequenom Mass Array with iPLEX GOLD chemistry by the Australian Genome Research Facility (AGRF, St. Lucia, QLD). PCR cycling conditions consisted of denaturation at 94°C for 4mins, followed by 45 cycles of 94°C for 20 s, 56°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 3 min. The shrimp alkaline phosphatase incubation was at 37°C for 40 min and 85°C for 5 min. The iPLEX single base extension reaction consisted of 94°C for 30 s, followed by a 200-short-cycle program of one loop of five cycles (94°C for 5 s and 52°C for 5 s) sitting inside a loop of 40 cycles with 80°C for 5 s and then a final extension at 72°C for 3 min. The 53 SNP panel used was not designed for human identification purposes. It is an assay previously used in our laboratory and was utilised in this study solely to determine the success rate of SNP typing from highly degraded samples. As such, no population data were used. Instead an average heterozygosity value of 0.45 was assumed to estimate the type of matching probability (pM) that could be expected from SNPs chosen for identification purposes (SNPs with high heterozygosity values). The pM of each locus was determined by adding together the square of each possible genotype. With the assumption of locus independence and Hardy–Weinberg equilibrium, the pM for the set of SNPs was calculated as the product of the pM for each individual marker. Statistical analyses were performed using Microsoft Office Excel 2007 and SPSS Statistics 17.0.

Results and discussion

A series of artificially degraded samples ranging from moderate to extreme levels of degradation was achieved with 5, 15 and 25 min sonication, respectively. Degradation was assessed in a two-step process. The QIAxcel system (Qiagen) produces a digital gel image for fragment analysis (Fig. 1a). The electropherogram provides a profile of the fragment composition of each sample (Fig. 1b). Samples with greater than 90% of fragments sized below 200 bp were considered extremely degraded. Those samples with more than 90% of fragments sized between 200 and 400 bp were considered highly degraded and samples with greater than 90% of fragments sized above 400 bp were considered moderately degraded. These size ranges were used to test the genotyping success of the STR (100–400 bp) mini-STR (~100–250 bp) and SNP (~80–120 bp) genotyping methods. The QIAxcel system provided a rapid, cost effective (less than 50c/sample) screening method for assessing sample quality.

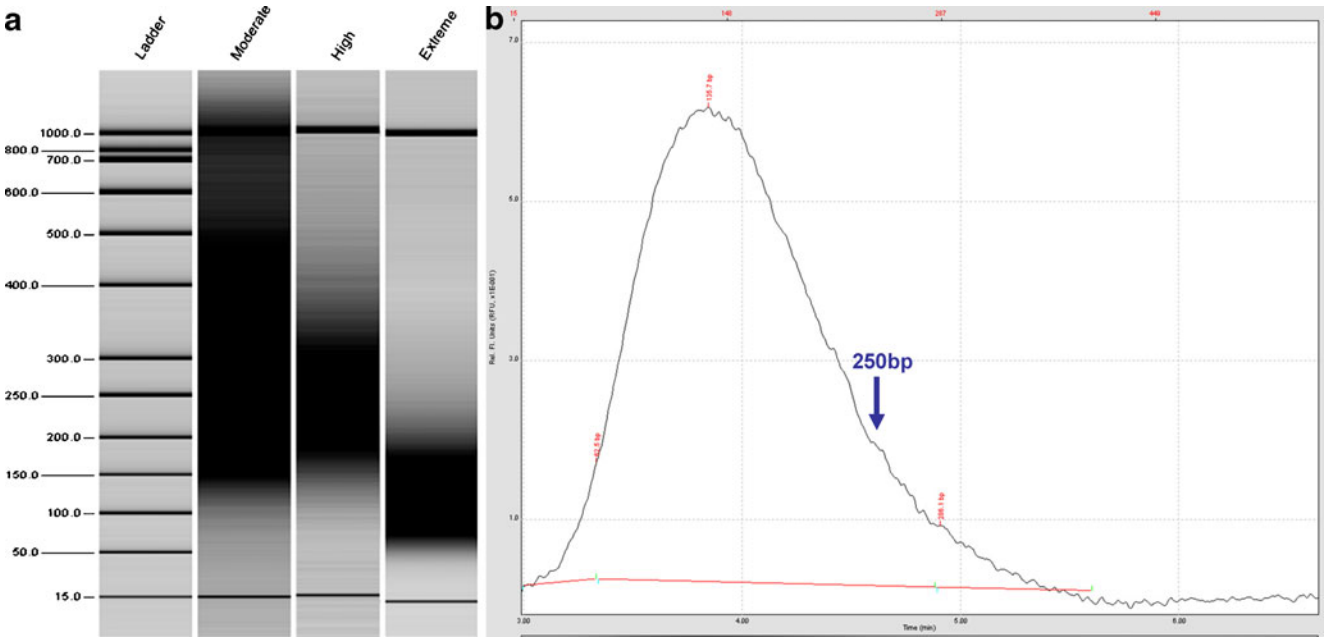

Fig. 1 Digital electropherograms from QIAxcel system of artificially degraded samples. **a** Digital gel image. **b** Fragment analysis showing extreme levels of DNA degradation

qPCR degradation assay

The modified quadruplex qPCR assay was able to simultaneously quantify total human DNA, male DNA and the extent of PCR inhibition and DNA degradation with reasonable accuracy and sensitivity. Calibration curves for each assay, namely; TH01, SRY, CSF, IPC showed good linearity with R^2 values above 0.99. Between assay reproducibility was assessed with three separate assays each in triplicate and data for each sample were used to calculate the mean and standard deviation.

To assess the sensitivity of the assay DNA template amounts ranging from 2 ng to 15.6 pg were used. Precise quantification, as indicated by standard deviations in C_T of less than one cycle was achieved for all four targets to 62.5 pg (data not shown). These results are concordant with the reported lower limit of 44 pg of Hudlow et al. [17]. Quantification of DNA at 15.6 pg was unreliable showing wide variation or failure to detect. This is consistent with stochastic effects expected at such low levels of template (reviewed in [19]). At 15.6 pg of template TH01 was the most common failure (63%) followed by SRY (54%) and CSF (18%). These results are consistent with expectations based on the amplicon length of the targets (TH01; 170–190 bp, SRY; 137 bp, CSF; 67 bp). The failure to detect these amplicons may be due to a combination of stochastic effects and lack of intact template at such low amounts. All targets of undegraded DNA were detected at 15.6 pg but the failure to detect at this template level increased as the sample quality decreased (44% moderately, 66% high and 77% extremely degraded samples).

The ability of the CSF-TH01-SRY-IPC assay to indicate the level of DNA degradation in a sample was evaluated. As expected the degradation ratio (CSF quantity/TH01 quantity) increased as the level of degradation increased (Fig. 2). No statistically relevant correlation between the degradation ratio and sample quality was observed. This is not surprising however given the testing of only three levels of DNA degradation, in addition to undegraded DNA.

To assess the sensitivity and accuracy of the CSF-TH01-SRY-IPC assay to predict the degree of degradation, DNA template amounts from 2 ng–15.6 pg were tested (Online Resource 3). The assay produced reliable degradation ratios to a lower limit of 15.6 pg in high quality samples and to a level of 62.5 pg for all degraded samples. This is likely because while CSF was detected at 15.6 pg TH01 was often not detected at all, making it impossible to generate a ratio.

It should be noted that the combination of extreme DNA degradation and low template amounts (<250 pg) resulted in an apparent overestimation of sample quality (Fig. 2). This may be explained by the fact that extreme degradation effectively decreases the amount of intact template and exacerbates the stochastic effects which result from amplification of low template samples.

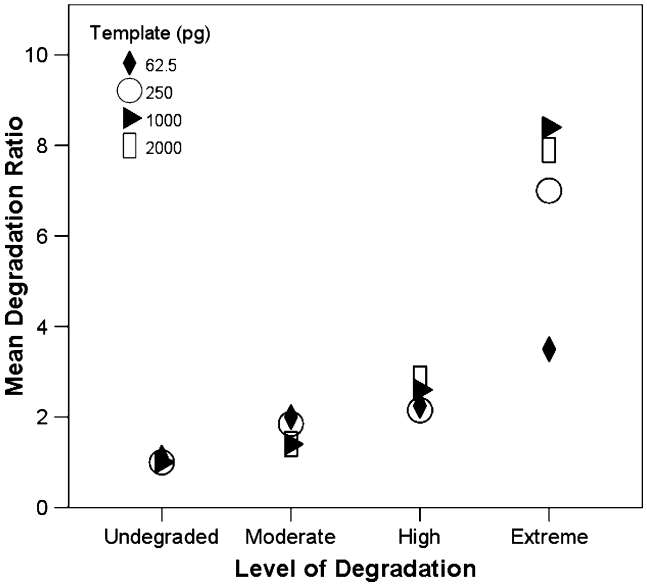

Fig. 2 Sensitivity of the quadruplex qPCR assay in assessing DNA degradation. A ratio of 1 indicates equal amplification of both long and short PCR targets. A ratio >1 indicates greater amplification of the smaller target and therefore possible DNA degradation

Mixtures

The ability of the qPCR assay to quantify a minor male component of a mixture was investigated. Known mixtures were prepared using HMW standards and highly degraded DNA in ratios from 1:1 to 1:1,000 to simulate low copy number female:male mixture samples. Each mixture was then quantified with the TH01-CSF-SRY-IPC assay in triplicate. The male component was kept constant at 20 pg as this was considered the lower limit of detection of the assay. Online Resource 4 indicates that the quadruplex assay was able to detect male DNA to a lower limit of 20 pg in a 1:1,000 mixture. However a large standard deviation was commonly observed which may be expected for amounts below 100 pg due to stochastic effects. This is also consistent with variation observed in previous studies [17]. It should be noted that a consistently higher male:female ratio (quantity of SRY/quantity TH01) was also observed in degraded mixtures compared to the corresponding HMW samples. This may indicate a relative resistance of the Y chromosome (or at least the region where SRY gene is located) to degradation. An alternate explanation is that this difference is accounted for by the smaller amplicon size of SRY (137 bp) vs TH01 (170–190 bp) and/or reduced competition of reagents.

Genotyping

Sensitivity and comparative studies of genotyping success rates across STR, mini-STR and SNP typing were performed using intact and degraded samples. Evaluation of differentially degraded samples identified that overall SNP analysis provided the highest rate of loci retrieval compared to AmpFlSTR® Profiler Plus® and AmpFlSTR® MiniFiler™ amplification kits.

All typing methods performed well with intact single source DNA in amounts as low as 62.5 pg. Significant improvement was observed in the genotyping success of undegraded DNA using SNP analysis or mini-STRs over STR typing at 15.6 pg (72% vs 48% vs 23%, respectively). The AmpFlSTR®MiniFiler™ PCR amplification kit is known to offer increased sensitivity over STR kits (0.2–0.6 ng vs 1–2.5 ng) [20]. Smaller amplicon size, enhanced kit chemistry, increased cycle number and differing cycling conditions contribute to this improvement in performance [21]. These results indicate that mini-STR and, in particular SNP typing would probably provide a better alternative to STR profiling for routine low copy number samples in addition to highly degraded DNA.

Within the recommended input range of commercial STR kits (1–2.5 ng) complete profiles were achieved with moderate and highly degraded DNA. However amplification of extremely degraded samples in this template range resulted in partial profiles (55–65% loci). An almost two-fold improvement in genotyping success with MiniFiler™ and SNP typing over that of Profiler Plus® was observed with these samples (Fig. 3). Complete typing success was seen with MiniFiler™ and SNP typing compared with only 55–65% with Profiler Plus® STR typing. It should be noted that all MiniFiler™ profiles showed evidence of off-scale alleles and pull-up artifacts with DNA amounts greater than 250 pg (data not shown). This may be expected due to the increased cycle number and sensitivity of kit chemistry.

A substantial reduction in genotyping success is observed with all degraded samples below 250 pg. Mini-STRs and SNPs showed considerable increases in loci retrieval across all input amounts <1 ng compared to STRs. These results support previous studies demonstrating the enhanced performance and sensitivity of the AmpFlSTR® MiniFiler™ amplification kit [20, 21] and SNP genotyping [2, 13, 15, 22] for typing degraded samples. This may be attributed to reduced amplicon size, improved kit chemistry, genotyping platforms or a combination of all factors. Although MiniFiler™ yielded more complete profiles from template amounts <250 pg compared with Profiler Plus®, the MiniFiler™ profiles showed an increase in baseline artifacts and allele drop-in.

SNP analysis yielded the highest overall rate of loci retrieval for all samples tested with the one exception being extremely degraded DNA in very low amounts (15.6 pg). This result may be explained by a combination of exacerbated stochastic effects and possible limitations of the SNP platform used. Of all SNP mis-calls, 71% were due to failure of detection and 29% were false homozygotes due to allelic dropout. 80% of all SNP genotyping mis-calls occurred with 15.6 pg DNA. The SNPs analysed ranged in length from 82–120 bp with a mean length of 99 bp. As expected, the success rate of loci retrieval increased as the number of loci in each multiplex reaction decreased. The 26 loci multiplex on average retrieved 89.8% of the SNP loci,

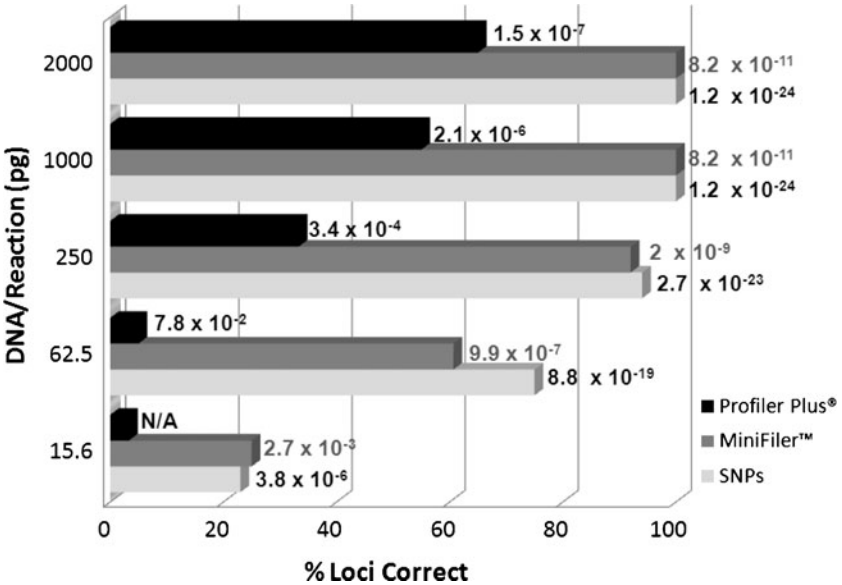

Fig. 3 Comparison of successful loci retrieval and associated matching probability (pM) values generated by AmpFlSTR® Profiler® Plus, MiniFiler™ and SNP analysis of extremely degraded DNA. US Caucasian population

the 15 loci multiplex 91.9%, the 10 loci multiplex 93% and the duplex assay successfully called 95.6% of the loci. The five least successful (70–80% correct) SNPS (RS7541041, RS2634041, RS1805007, RS732774, RS17435026) were not solely in the largest multiplex, but included SNPs in the three larger reactions. There was no correlation between SNP amplicon length and genotyping success. Primer and assay design is therefore an equally important contributor to the success of SNP typing of highly degraded samples as amplicon size.

AmpFlSTR® Profiler Plus® showed a considerable decline in retrievable loci as sample quality and template amount decreased (Fig. 3). As expected the longer amplicons were more susceptible to locus drop-out as the level of degradation increased. The four longest loci included in the Profiler Plus® kit (D18, D7, FGA and D21) showed ≥80% locus drop-out with extremely degraded DNA with template amounts ranging from 2 ng down to 15.6 pg. Due to reduced length, the four longest target loci in the AmpFlSTR® MiniFiler™ kit (FGA, D7, D18 and D21) showed comparatively reduced locus dropout rates of 40%, 27%, 27% and 13% respectively.

An interesting comparison is the success rates of STRs, mini-STRs and SNPs of equivalent size. The success of retrieval of the smallest loci (≤~150 bp) in the Profiler Plus (Amelogenin, D3S1358, D5S818, D8S1179) and MiniFiler kits (Amelogenin, CSF, D13S317, D16S539, D2S1338) are compared to that of the SNP panel (<120 bp) with extremely degraded samples (Fig. 4). At input amounts of ≥1 ng, all typing methods yielded 100% alleles. At lower amounts (250, 62.5, 15.6 pg), the small loci of the Profiler Plus kit showed a much higher allele dropout rate (33.6%, 87.5%, 95.8% respectively) than those of the SNP assay (6%, 25% and 77% respectively) and MiniFiler kit (0%, 20%, 63.3%). This comparison suggests that regardless of amplicon size, the robustness of assay design and chemistry have a significant impact on the profiling success of highly degraded samples. When comparing similiar sized amplicons across the three assays, the small loci of the MiniFiler kit performs the best. However, when considering the success rates of the kits as a whole, SNP typing provided the highest rate of loci retrieval from highly degraded samples presumably because all of the loci are small amplicons.

It has been suggested that lower match probability (pM) values may be obtained with a partial STR profile compared to a full mini-STR profile [8]. This may be the case with low or moderate levels of DNA degradation when >80% loci are retained. However our results show that consistently lower pM values were obtained with MiniFiler™ than those generated from partial Profiler Plus® profiles with≤60% loci (Fig. 3). The MiniFiler™ kit is designed to function as an adjunct to STR typing kits for challenging samples. Therefore maximal matching probabilities may be achieved with a combined partial STR profile and a complete mini-STR profile. Analysis of the 53 SNP panel described in this paper (assumed average heterozygosity of 0.45) generated pM values significantly lower than complete MiniFiler™ profiles (1.2×10^{-24} vs 8.2×10^{-11}). The pM values obtained with ≥70% of this 53 SNP set successfully genotyped are as discriminatory as a full 15-plex STR kit (AmpFlSTR® Identifiler PCR kit, Applied Biosystems; 8.8×10^{-19} vs 5.01×10^{-18} [23]). These results suggest that SNP analysis could provide not only

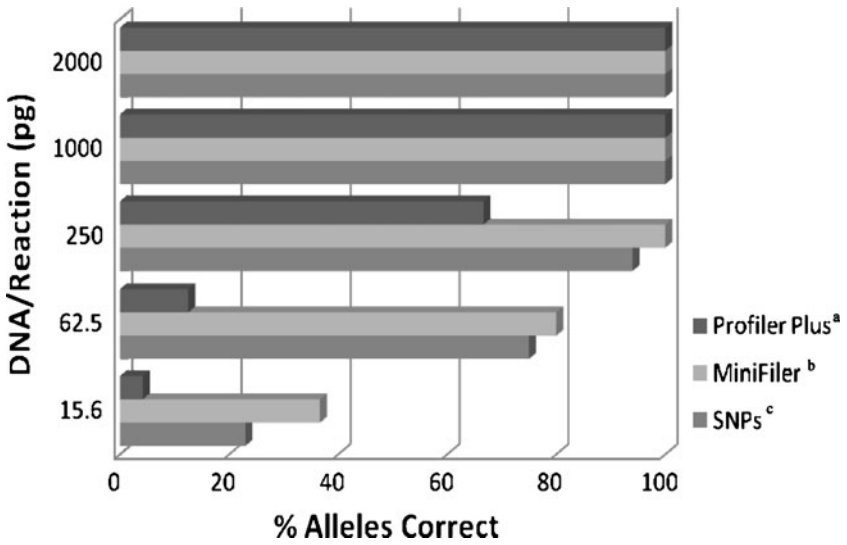

Fig. 4 Comparison of the combined success of the smallest loci (≤150 bp) in each of the Profiler Plus and MiniFiler kits with the full 53SNP panel with extremely degraded samples. *a* Profiler Plus loci (Amelogenin, D3S1358, D5S818, D8S1179). *b* MiniFiler loci (Amelogenin, CSF, D13S317, D16S539, D2S1338). *c* All 53 SNPs included (<120 bp)

greater genotyping success, but also greater powers of discrimination when typing severely degraded and/or DNA in low amounts.

While a statistical correlation between the degradation ratio generated by the qPCR quadruplex and downstream genotyping success was not observed, a general trend is seen which may assist in the prediction of the genotyping success of each method. This lack of statistical correlation is to be expected since only three levels of DNA degradation plus intact DNA were tested. Samples with amounts of DNA >1 ng, and low degradation ratios (<2.5) would be expected to yield complete profiles with all three typing methods. However samples with the same low degradation ratios but <1 ng template would be predicted to generate more complete profiles using MiniFiler™ or SNP analysis. An extremely high degradation ratio (>7) would predict low success with Profiler Plus® amplification regardless of template amount. Such extremely degraded samples would generate more complete profiles with MiniFiler™ amplification or SNP typing. SNP analysis would be expected to generate the most complete genetic profile from all degraded samples in low amounts (≤250 pg). The broad prediction of genotyping success using STR, mini-STR and SNP analysis could assist in the choice of method and thereby maximise the genetic information gleaned from challenging samples.

Conclusion

Levels of DNA degradation were assessed with the QIAxcel capillary electrophoresis system and a quadruplex qPCR assay. The QIAxcel provides a rapid, cost effective screening method and the more sensitive quadruplex qPCR assay was able to simultaneously quantify total human DNA, male DNA as well as the extent of DNA degradation. Minute amounts of male DNA in a background of female DNA were able to be detected and showed some evidence of protection to DNA degradation. By considering the amount of DNA and the degradation ratio of a sample, a general prediction of genotyping success using AmpFlSTR® Profiler Plus®, MiniFiler™ kits and SNP analysis can be made. This assay could assist in determining which genotyping method may be more informative thereby maximising the evidentiary value of each sample. For good quality and moderately degraded samples in amounts within the recommended input amounts for PCR, STR profiling would be the most appropriate choice. However in cases of extreme degradation and/or template amounts below 250 pg, mini-STR or SNP analysis should yield significantly more complete profiles and generate lower match probabilities. Although not currently used for routine human identification by the forensic community, SNP typing has been suggested as an adjunct to traditional STR and mini-STR profiling for analysis of highly degraded samples [10, 13]. The results of this study demonstrate the substantial improvement in loci retrieval of extremely degraded and low level samples via SNP analysis with the potential to generate comparable powers of discrimination to mini-STR profiles. Coupled with the ability to obtain genetic information regarding ancestral origin and phenotypic characteristics (reviewed in [12]), SNPs could become a valuable addition to human identification testing.

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Any Australian ethical permission necessary for this project has been granted and ethical standards have been adhered to.

Conflict of interest The authors declare that they have no conflict of interest.

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