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A quantitative assessment of a reliable screening technique for the STR analysis of telogen hair roots

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ABSTRACT

Human telogen hairs are commonly recovered as trace evidence but currently have limited use for forensic DNA analysis. Recent studies have revealed that telogen roots may be shed with adhering material that may contain cells, thus providing a potential source of nuclear DNA. A simple histological stain can be used to screen telogen roots for the presence of nuclei, thus increasing the chance of selecting roots that may yield nuclear DNA. Using this method to visualise nuclei, we surveyed 998 hairs from 136 individuals, quantified the number of nuclei, extracted DNA and evaluated corresponding DNA yield and STR profiling success. Of the hairs screened, 35% of telogen roots contained nuclei and in total 6% of all roots screened had more than 100 nuclei. The number of nuclei associated with telogen roots was independent of the presence or absence of visibly adhering material, highlighting the importance of using histological staining rather than simple microscopic examination. DNA yield and STR profiling were significantly and positively correlated with nuclei number. The methods presented here can be incorporated into routine trace and DNA analysis providing an efficient and cost effective method to screen telogen hairs, and predict STR profiling success prior to destructive DNA analysis. The results of this study indicate telogen hairs may provide a reliable source of nuclear DNA for use in routine casework.

1. Introduction

Telogen hairs, otherwise known as resting stage hairs, represent the final stage of the growth cycle of hair. Contrary to hairs in the active growth stage (i.e. anagen hairs) telogen hairs dislodge from the head with relative ease, with up to 150 telogen hairs shed daily [1,2]. Despite telogen hairs accounting for up to 95% of all hairs collected as forensic evidence they are not frequently utilised for identification during forensic investigations. Unlike hairs in the active growth stage (i.e. anagen), which can be routinely used as a source of nuclear DNA, telogen hairs are fully keratinised, containing little cellular material and as such, the use of standard methods for analysis of DNA from telogen hairs is difficult [3]. Analysis is currently limited to comparison of qualitative features using microscopy and in some cases mitochondrial DNA (mtDNA) analysis [4]. If possible, the ability to use even a small fraction of telogen hairs recovered as evidence for nuclear DNA analysis would enhance the value of telogen hairs as forensic evidence and enable this common form of trace evidence to be used for individual identification.

Comparative microscopy is currently the primary technique for evaluating multiple hair samples for similarities between known and questioned samples. Comparative microscopy is an important screening method for establishing the origin of hair (human or animal), the root type (essential for determining if further analyses will be performed), the nature or shape of the hair tip and numerous other features including pigment pattern, colour and damage conditions that directly relate to hair as evidence [5]. However, the results are considered qualitative as objective classification and assignment of a probability value to such features is challenging [6–10]. Furthermore, the comparison of the microscopic features of hairs may be insufficient to exclude an individual as the donor of a hair. In contrast, the use of a panel of short tandem repeat (STR) markers for forensic DNA analysis is a highly discriminatory method for individual identification. DNA methods are generally repeatable, robust and reliable, and can provide information about ancestry, relatedness and discriminate between closely related individuals [11,12]. Consequently, these approaches can provide complementary information – microscopic hair comparison

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can be used to compare hairs for consistent microscopic features, and subsequent DNA analysis can provide a quantitative likelihood of an individual's identity.

DNA is usually recovered from hairs by complete digestion of the root or hair shaft [13]. Telogen hairs can be used as a source of mtDNA [4,14,15], however the standard practice of comparing the two hypervariable regions (HV1 and HV2) with reference or database sequences offers limited discriminatory power when compared to STR profiling of nuclear DNA [11,16–19]. Furthermore, typing inconsistencies and heteroplasmy of mtDNA extracted from hair have been identified as potential issues for DNA identification purposes [20–23]. Nuclear DNA analysis has been successfully performed using large samples of hair shaft and telogen hairs (up to several grams) [24]. However, in forensic casework often only single hairs are available providing a limited amount of nuclear DNA, thereby making analysis more challenging. Attempts have been made to evaluate single telogen hairs for nuclear DNA analysis, with a focus on STR analysis for identification purposes [25–30]. This work has yielded inconsistent results with limited success using standard techniques. The effects of environmental weathering and hair treatment may also have an impact on the DNA quality and quantity further confounding issues of fragmentation and DNA concentration [27]. Additionally, melanin, the natural pigment of hair, is a potent inhibitor of PCR [31,32].

Rather than target the degraded DNA in the hair shaft using the standard approach of digestion, material adhering to the root may serve as a source of nuclear DNA. A key advantage of targeting this material is that the hair is not destroyed through the DNA extraction process, and therefore can be retained for further analysis. Recent studies examining the morphology of telogen hairs have revealed the presence of material adhering to the roots in some cases [33–35]. A classification scheme for telogen root types has been established based on the presence and type of material adhering to the root: Type 1, telogen roots with no visible adhering material; Type 2, material adhering to the hair shaft; and Type 3, tissue below the club, otherwise known as a germinal tag [34,35]. In order to confirm this adhering material contained cellular material, Bourguignon et al. [34] used the DNA binding dye 4′,6-diamidino-2-phenylindole (DAPI) and stained a large cohort of telogen roots of various types after which nuclei were visualised in adhering material. However, this stain is costly and requires a fluorescent microscope that is not generally standard equipment in forensic laboratories. Brooks et al. [35] substituted haematoxylin in place of DAPI and found no significant difference for screening hairs and detecting nuclei. The use of haematoxylin is an ideal alternative because it is more economical and can be visualised using a standard light microscope, a piece of equipment that is commonly found in forensic laboratories. Both studies demonstrated that the presence of visibly adhering material did not correlate with DNA yield and that the short amount of time and minimal cost of the staining procedure increased the chance of DNA recovery and the successful STR profiling of telogen roots.

While these previous studies demonstrated the validity and usefulness of using histological staining to screen telogen roots, information about the expected frequencies of nuclei in telogen hairs and the consequent correlation of nuclei to DNA yield and STR profiling is still not clear. Furthermore, little is known about the minimum number of nuclei required to generate an informative STR result. In the present study, we provide additional support for the use of a simple staining procedure for the selection of single telogen hairs for STR analysis. We correlate the number of visible nuclei from single telogen roots with DNA yields and consequent STR profiling success. An evaluation of concentration of DNA extracts has also been undertaken, and results indicate that concentration of extracts improves STR results.

2. Methods

All pre-PCR work including staining was undertaken in a dedicated pre-PCR laboratory, housed in a separate building to post-PCR work, and protocols for the handling of low template and degraded DNA samples were followed.

2.1. Nuclei staining

Head hairs and two reference buccal swabs were collected from each of 136 volunteer donors in accordance with ethics approval from the University of Adelaide Human Ethics Committee (Project # HM-138-2009) and the University of Canberra Committee for Ethics in Human Research (Project # 08-65). Hairs were collected by brushing rather than plucking in order to maximise the recovery of telogen hairs. Participants brushed hair onto white paper using a fine tooth comb for collection. All collected hairs were examined to confirm the hairs were in telogen stage. Depending on the number of hairs determined to be in telogen stage, up to 10 hairs were used per individual (mean of seven hairs per individual). At no stage were hairs cleaned or decontaminated with sodium hypochlorite (bleach), detergent or UV light. To determine the presence and frequency of adhering material and nuclei on the root and hair shaft, 2 cm of the proximal end of telogen hairs was removed for staining following the method described by Brooks et al. [35]. Single or multiple hairs from one individual were placed into a 1.5 mL screw cap tube and 1 mL molecular biology grade Absolute Ethanol (Sigma–Aldrich, Australia) was added to the samples and incubated at room temperature for 30 min. The ethanol was added to histologically fix the samples to prevent loss of tissue or degradation. The ethanol was then removed and 1 mL of Harris Haematoxylin (Crown Scientific, Australia) was added for 3 min. Haematoxylin was removed and the hairs rinsed with 1 mL molecular biology grade water (Invitrogen, USA), and mounted on glass slides in glycerol. Haematoxylin binds to the chromatin within the DNA/histone complex and stains nuclei a dark violet [36].

Hair roots were examined under 100×, 200× and 400× magnification using a compound light microscope with fluotar dry objectives (Leica Microsystems, Germany). Hairs were examined across several focal planes in order to confirm whether nuclei were present. Once visible, fine focus adjustments were used to confirm the presence of nuclei and perform counts. In the case of nuclei that were well spaced or in small numbers, refocusing was used to improve the view of nuclei. When large number of nuclei were present, in order to prevent counting nuclei twice, a suitable plane of focus was chosen with the majority of nuclei visible, followed by only minimal refocusing.

Visible nuclei were counted and hairs were classified according to previously published criteria as Type 1, Type 2 or Type 3. Nuclei numbers were binned into categories for graphic and tabular representation (0 nuclei, 1–20 nuclei, 21–40 nuclei, 41–60 nuclei, 61–80 nuclei, 81–100 nuclei and >101 nuclei). These categories were chosen in order to provide an improved resolution of frequency distribution, as the previous studies by both Brooks et al. and Bourguignon et al. only binned nuclei into a small number of categories [34,35]. The upper maximum of 100 nuclei was used with counting stopped at >101 nuclei as enough DNA is generally recovered from large numbers of nuclei to generate consistently reportable full or partial profiles [34,35]. In total, 998 telogen hairs were stained and examined.

2.2. DNA extraction, quantification, QIAQuick® concentration and STR profiling

A subset of hairs (n = 110), containing a range of nuclei from 0 to 100 nuclei was used for DNA extraction. DNA from single hairs was extracted using the Chargeswitch® Forensic kit (Invitrogen, USA) according to manufacturer's instructions, with the following exceptions: (a) to prevent digestion of the hair shaft, DTT was not added; (b) 500 μL lysis buffer, 10 μL proteinase K (20 mg/mL), 20 μL of magnetic beads, 100 μL purification buffer and 250 μL wash buffer were used instead of the recommended amounts; and (c) in order to increase efficiency of elution of DNA from the beads, DNA was eluted in a final volume of 50 μL at 55 °C with agitation. Extraction negative controls were included with every set of extractions.

Reference buccal swabs were extracted using a Chelex method modified from Walsh et al. [37]. Swabs were cut from their handles and then incubated at room temperature with 1 mL of TE buffer (10 mM Tris, pH 7.5, 1 mM EDTA) on a rotary wheel on high speed for 30 min, followed by centrifugation at 8000 × g. All but 20 μL of supernatant was discarded and 180 μL of 5% Chelex and 5 μL proteinase K (20 mg/mL) was then added. Samples were vortexed then incubated at 56 °C for 1 h, followed by 8 min at 100 °C. After brief centrifugation at 8000 × g, the supernatant was transferred to a new tube. An extraction negative control was included for each set of extractions. Both reference and root extracts were stored at 4 °C.

Quantification of DNA from hair root and reference extracts was performed using quantitative PCR (qPCR) with SYBR® green chemistry. A previously published 67 bp nuclear target was used to quantify the DNA [38]. The qPCR mix consisted of 5 μL 2× Brilliant II SYBR® green master mix (Agilent Technologies, USA), 0.15 μM forward primer (GGGCAGTGTTCCAACCTGAG), 0.15 μM reverse primer (GAAAACTGAGACACAGGGTGGTTA), 400 ng/μL rabbit serum albumin, 3.3 μL water and 1 μL DNA extract to make a total of 10 μL. All samples were run in triplicate, and negative (PCR blank) and positive controls (dilutions of male genomic control DNA, Applied Biosystems, USA) were included on all runs. Extraction negative controls were also quantified. Cycling was performed using a Corbett 6000 Rotorgene real-time PCR thermocycler and consisted of an initial 5 min denaturation step at 95 °C, followed by 45 cycles consisting of 95 °C for 10 s, 59 °C for 20 s and 72 °C for 15 s. Nuclear DNA concentration was determined using the comparative CT method; unknown samples were compared to a standard curve with a range of 0.033–8.848 ng/μL. The standard curve was created using male genomic control DNA (Applied Biosystems, USA).

2.3. Concentration of extracts prior to DNA analysis

To examine the effect of concentrating DNA extracts, DNA from 25 samples with a range of nuclei numbers was extracted and quantified as per the methods described above. The samples were then concentrated using QIAquick® columns (QIAGEN, Germany) as per manufacturer's instructions, from 50 μL to 25 μL. Post-concentration, samples were again quantified and then treated identically to other samples.

2.4. STR profiling

STR profiling was performed using AmpFℓSTR ProfilerPlus™ (Applied Biosystems, USA) hereby termed a "standard" reaction. All reactions were performed in 25 μL reaction volumes containing 9.6 μL of ProfilerPlus™ reaction mix, 5 μL of ProfilerPlus™ primer mix, 0.4 μL AmpliTaq Gold™ and 10 μL of DNA. Cycling was performed on a 9700 GeneAmp thermal cycler and consisted of an initial denaturation at 95 °C for 10 min followed by 28 cycles of 94 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min, followed by a final extension at 60 °C for 45 min.

Two microlitres of PCR product was analysed on a 3130xl Genetic Analyser in a 17 μL final volume that also included 15 μL HiDi™ Formamide and 0.3 μL ROX-500 Size Standard (Applied Biosystems, USA). Results were analysed using Genemapper ID software (v3.2.1). A peak amplitude threshold of 50 relative fluorescence units (RFU) was used. Profiles were considered a full profile if all alleles seen in the reference profile were present, and an informative partial profile for identification purposes was defined as having a minimum of six alleles matching the reference profile.

2.5. Inhibition test

Ten extracted DNA samples with a range of nuclei numbers were spiked with either 50 or 100 pg of control DNA (9947A, Applied Biosystems, USA) to determine if the haematoxylin stain produced any inhibitory effects, and quantified using qPCR as per other samples. The control DNA concentrations were chosen to emulate the low-level amounts of DNA seen in the samples used in this study.

2.6. Analysis

Data was compiled in Excel (Microsoft, USA). Statistical tests were performed using RCMDR v1.6-x [39], a statistical interface for R version for Mac OS X GUI 1.40 [40]. The threshold for statistical significance was set at 99% confidence or a p value of 0.01. A Shapiro–Wilk test of normality was performed which revealed a non-normal distribution of nuclei numbers ($p = < 0.01$). Due to this non-normal distribution, non-parametric significance testing was used to determine significance and correlations of nuclei to DNA yield and STR profiles. Significance testing was performed using the Kruskal–Wallis or Friedman Rank Sum test depending on the data being tested. Correlations were tested using Spearman's rank correlation coefficient and are reported as Spearman's rho.

3. Results

3.1. Nuclei frequency

Using the haematoxylin stain and viewed under a light microscope, nuclei were easily visualised and were found both on roots, and within adhering material (Fig. 1). In all but two cases,

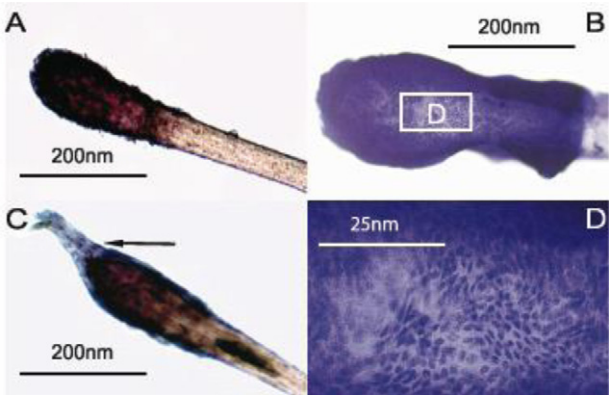

Fig. 1. Telogen hair root types and nuclei after haematoxylin staining. (A) Type 1 root, no nuclei; (B) Type 2 root, >100 nuclei; (C) Type 3 root, <10 nuclei, nuclei in germinal tag indicated by arrow; and (D) typical appearance of stained nuclei, enlarged from inset of (B).

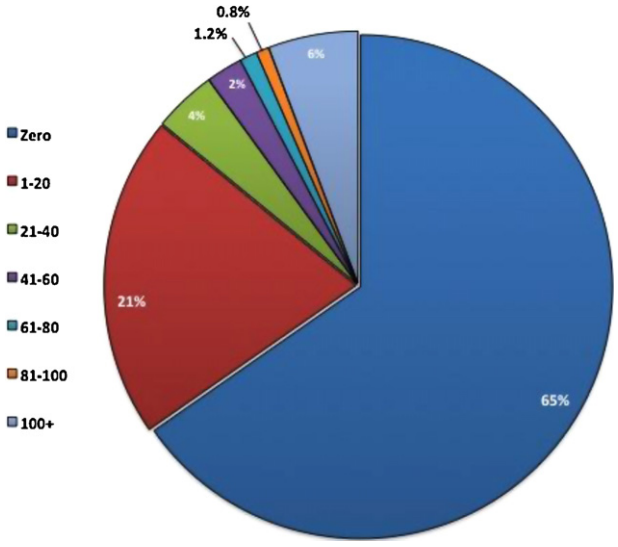

Fig. 2. Frequency of seven classes of nuclei counts (0, 1-20, 21-40, 41-60, 61-80, 81-100, >100) on 998 telogen hair roots after haematoxylin staining.

nuclei were found in material adhering to the first few millimetres of the proximal end of the hair. When present, nuclei were mostly in low numbers; of 998 hair roots examined, 65% had no associated nuclei, 21% yielded 20 or less nuclei, 8% contained 20-100 nuclei and 6% had more than 100 nuclei (Fig. 2). The presence of nuclei on telogen roots was independent of the presence of tissue that was visible prior to staining (Fig. 3). The majority (93%) of the 136 individuals screened had at least one hair with no nuclei and only 24% of individuals had at least one hair with greater than 100 nuclei (Suppl. Table 1). Intra- and inter-individual variation in nuclei frequency per hair root was substantial – 21 individuals had no nuclei on all hairs, while only one individual had greater than 100 nuclei for every hair sampled. Individuals with more than 100 nuclei on one hair generally had other hairs with greater than 100 nuclei but hairs with large numbers of nuclei were also seen from individuals who had other hairs with few or no nuclei. As only a small representative sample (mean of seven hairs) was taken from each individual, statistically testing the intra-individual association of nuclei numbers between hairs was not performed, as the result generated would not be an accurate representation of the true intra-individual difference.

Telogen roots were classified as in previous studies, according to the presence or absence of adhering material: Type 1 (no adhering material); Type 2 (material attached to shaft); and Type 3 (tag attached below the club root) (Fig. 1). The most predominant root type seen was Type 1 comprising 59% of samples, with Types 2 and 3 occurring at lower frequencies (18% and 23%). Type 1 accounted for the majority of roots across all nuclei categories except for 1-20 and 21-40 nuclei, for which Type 3 roots were the most frequent (Fig. 3). Type 2 roots produced on average, the highest numbers of nuclei (mean = 17 nuclei), followed by Type 3 roots (mean = 11 nuclei) and Type 1 roots (mean = 10 nuclei). Significance testing with Kruskal–Wallis Test revealed a significant difference in nuclei number between all the root types (i.e. Types 1, 2 and 3 roots, $p = < 0.01$). A significant difference was also found between Type 1 and Type 2 roots, and Type 1 and Type 3 roots. The difference between Type 2 and Type 3 roots was not significant ($p = > 0.01$).

Although the difference in nuclei number between all the root types was significant, and all categories of nuclei number were found across all root types, the majority of all root types either yielded no or small numbers of nuclei (Suppl. Table 2). Of the hairs screened and classified, 7% of type 1 roots had more than 100 nuclei and 76% had zero nuclei. Similarly in type 2 roots, 6% had more than 100 nuclei and 62% had zero nuclei. Type 3 roots were the most likely to contain nuclei with only 38% having no nuclei. However, the majority of Type 3 roots yielded low numbers (1-40 nuclei, 55% of type 3 roots) and this root type had the lowest amount of roots with >100 nuclei (3% of type 3 roots).

3.2. DNA yield and inhibition test

Nuclear DNA recovery from hairs with visible stained nuclei varied from undetectable to 0.17 ng/μL (Table 1). Samples that contained no nuclei yielded no detectable DNA. Many of the hairs with low numbers of nuclei (i.e. 1-40 nuclei) also yielded no detectable DNA, or very low DNA concentrations (mean of 0.007 ng/μL). The Spearman's correlation test revealed a strong and highly significant correlation (rho = 0.732, $p = < 0.01$) between nuclei number (0-99 nuclei) and DNA yield (Fig. 4A).

The inhibition test demonstrated no inhibition was present, with the average DNA quantified being at least the amount of control DNA added to the qPCR reaction (Suppl. Table 4). Furthermore, qPCR amplification plots did not exhibit any features which are indicative of inhibition, such as depressed amplification curves [32].

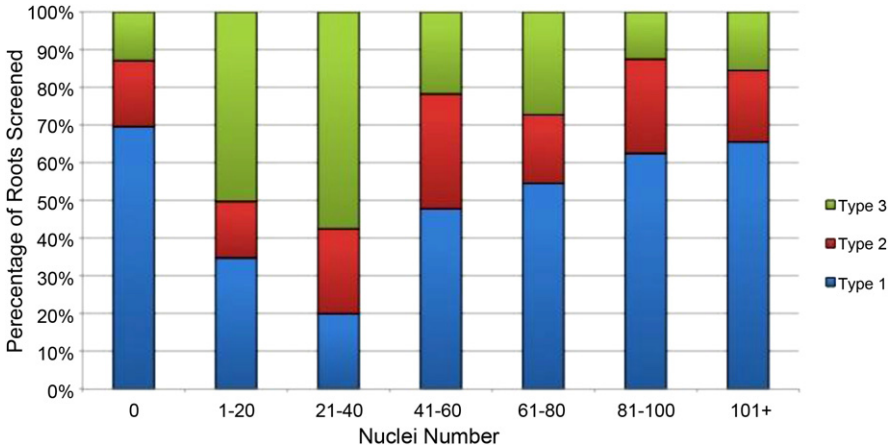

Fig. 3. Percentage of telogen Type 1 roots (no adhering material), Type 2 roots (adhering material on the shaft) and Type 3 roots (adhering tag at base of root) associated with seven classes of nuclei counts (0, 1-20, 21-40, 41-60, 61-80, 81-100, >100) after haematoxylin staining (see also Suppl. Table 2).

Table 1
Nuclei counts, DNA yield and STR profiling from telogen hair roots after haematoxylin staining.

Nuclei range	Number of hairs stained	Mean nuclei ± SE	Number of hairs extracted	Mean DNA yield ± SE (ng/μL)	Percent of samples producing alleles	Mean allele number ± SE (>50 RFU)	Mean heterozygous peak height ± SE (RFU)	Mean homozygous peak height ± SE (RFU)
0	651	0[a]	16	Undetected[b]	0	0	0	0
1-20	207	9 ± 6	48	0.006 ± 0.013	4	0	0	0
21-40	40	30 ± 6	21	0.014 ± 0.019	33	2 ± 4	88 ± 23	118 ± 66
41-60	23	50 ± 5	15	0.061 ± 0.069	33	2 ± 4	76 ± 19	133 ± 76
61-80	11	71 ± 7	6	0.048 ± 0.061	33	5 ± 7	118 ± 97	534 ± 281
81-100	8	87 ± 7	4	0.046 ± 0.037	75	11 ± 3	249 ± 133	453 ± 555

SE, standard error.
[a] All hairs examined in this range had zero nuclei, therefore calculation of SE is not applicable.
[b] Undetected result. No target DNA was detected using the qPCR assay, either due to DNA being outside range of sensitivity of the qPCR or no target DNA being present.

3.3. STR profiling

The STR profiles generated were generally incomplete with low peak heights, allele dropout and in some cases complete STR profiling failures (Table 1). In total, 34% of the DNA extracts produced an STR result. Of the samples that did yield a result all alleles matched the reference profile of the donor, and no allelic drop in was observed. The minimum number of nuclei that produced an STR result was 16, with the sample producing two alleles (>50 RFU). An informative partial profile (minimum six alleles) was generated from 22 nuclei, indicating it is possible to generate a partial profile from a small number of starting nuclei.

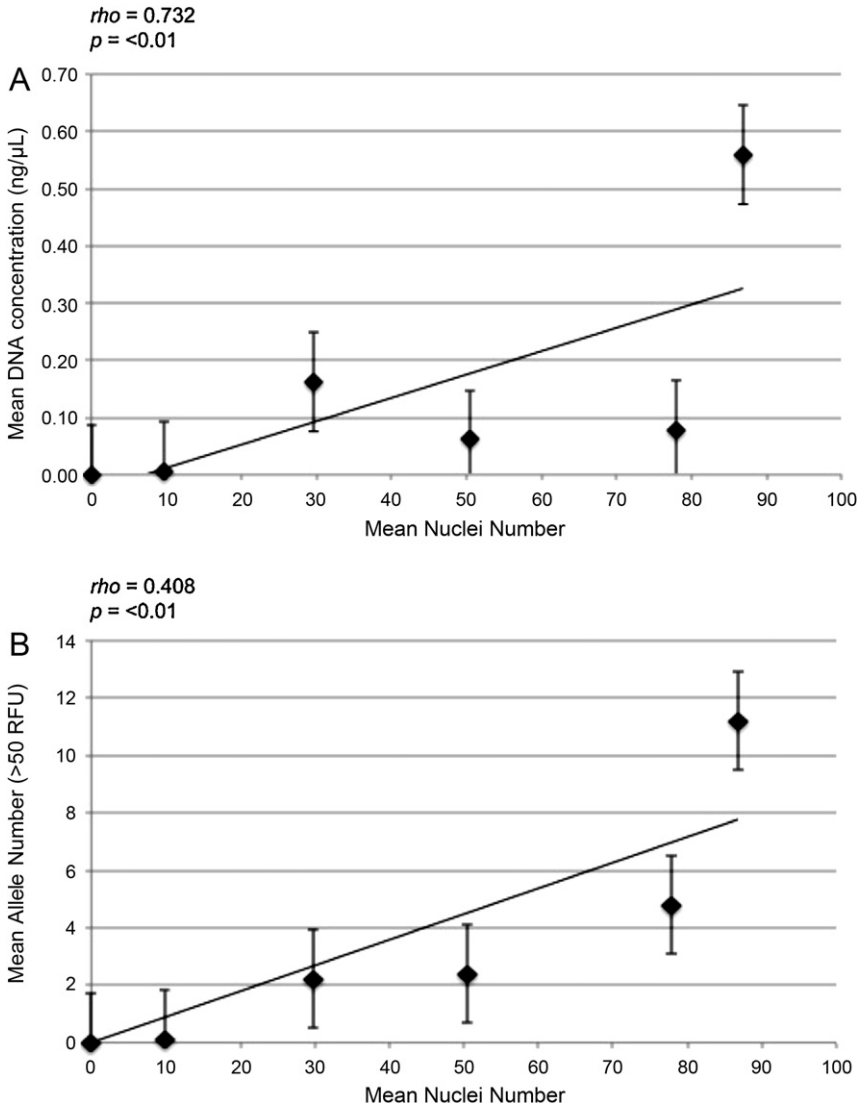

Fig. 4. Correlation between nuclei number, DNA yield and STR profiling success (mean ProfilerPlus™ allele number, n_{max} = 20) for 110 telogen hair roots after haematoxylin staining. (A) mean nuclei counts and DNA yield per hair; and (B) mean nuclei counts and mean ProfilerPlus™ allele number typed.

Table 2
Nuclei counts, DNA yield and STR profiling from telogen hair roots after haematoxylin staining and subjected to post-extraction DNA concentration.

Nuclei range	Number of samples	Mean nuclei ± SE	Initial DNA concentration ± SE (ng/μL)	Post-Qiagen DNA concentration ± SE (ng/μL)	Mean difference ± SE (ng/μL)	Mean allele number ± SE (>50 RFU)	Mean heterozygous peak height ± SE (RFU)	Mean homozygous peak height ± SE (RFU)
0	2	0[a]	Undetected[b]	0	0	0	0	0
1-20	10	12 ± 1	0.003 ± 0.01	0.054 ± 0.039	0.052 ± 0.012	1 ± 1	88 ± 22	NA
21-40	3	31 ± 4	0.011 ± 0.02	0.232 ± 0.137	0.164 ± 0.011	7 ± 5	232 ± 23	462 ± 99
41-60	5	58 ± 2	0.019 ± 0.09	0.1 ± 0.037	0.065 ± 0.113	4 ± 2	134 ± 24	160 ± 37
61-80	4	72 ± 1	0.023 ± 0.04	0.085 ± 0.096	0.056 ± 0.014	4 ± 4	246 ± 32	486 ± 159
81-100[c]	1	82	0.060	0.135	0.076	12	341	324

SE, Standard error.
NA, no data were available for mean homozygous peak heights. This is a result of low success rates of STR profiling due to low the numbers of nuclei and consequent DNA yield.
[a] All hairs examined in this range had zero nuclei, therefore calculation of SE is not applicable.
[b] Undetected result. No target DNA was detected using the qPCR assay, either due to DNA being outside the range of sensitivity of the qPCR or no target DNA being present.
[c] Only a small number of roots contained nuclei in this range, therefore only one sample was able to be used for the evaluation of concentrating extracts. Numbers reported here are the values for the one sample used. No standard error or mean could be generated for DNA concentration, difference or allele number.

However, this result was only for a single sample indicating that this is not a consistent result. On average, samples with a similar number of nuclei only resulted in two alleles (Fig. 4B, Table 1). In order to produce partial profiles consistently (i.e. mean of six alleles) upwards of 60 nuclei were required (Table 1).

The Spearman's correlation test between nuclei number and allele number was highly significant (Fig. 4B), however the correlation was much lower than between nuclei number and DNA yield (rho = 0.408, $p = < 0.01$). Due to the low template amounts of DNA (mean DNA yield = 0.02 ng/μL), there was greater potential for increased stochastic behaviour and profiling results tended to exhibit some inconsistency and in some cases, differences in profiling results occurred between samples where nuclei numbers and DNA yields were similar. This variance is reflected in the resulting lower correlation between nuclei number and alleles generated, compared with the stronger correlation of nuclei number to DNA yield (Fig. 4A and B).

No full STR profiles were produced (Suppl. Table 5) unless samples with more than 100 nuclei were used (data not shown). Overall, using standard profiling techniques, 28% of hairs lie within the required nuclei range (>20 nuclei) to possibly produce at least an informative partial STR profile, however it is generally expected lower nuclei numbers will result in less than six alleles and these profiles may have limited discriminatory power.

3.4. Concentration of extracts prior to DNA analysis

QIAquick® concentration of extracted telogen hair root DNA produced a significant increase in DNA per microlitre of extract ($p = < 0.01$, mean increase = 0.068 ng/μL, Table 2). However, for some individual samples the increase in yield was much larger than this (Suppl. Table 6).

In comparison to non-concentrated samples, the QIAquick® concentrated samples yielded more alleles from less nuclei, with increased peak heights (Table 2). As also seen with non-concentrated samples, STR profiling from concentrated extracts resulted in partial profiles and also complete failures. A minimum of 12 nuclei was required to produce an STR result from a concentrated extract, and resulted in a partial profile with six alleles being called (peaks > 50 RFU) thus meeting the requirement for an informative partial profile. Correlation analysis produced similar results to the non-concentrated samples, with a significant correlation between nuclei number and DNA concentration (rho = 0.448, $p = < 0.01$) although this correlation was weaker than the profiling results of non-concentrated extracts. The strength of correlation between nuclei number and allele number was similar to that seen with non-concentrated extracts, despite only a small sample size for the concentration experiment (rho = 0.449, $p = < 0.01$). Due to low sample numbers and consequent low success of profiling, statistical analysis of the significance between allele numbers from non-concentrated compared to concentrated samples was not performed, as the result would not be statistically valid. Based on these results, hairs with as few as 12 nuclei may yield an informative STR result. This means up to 40% of all roots that have nuclei may produce enough DNA for an STR result if the DNA extract is concentrated prior to STR amplification.

4. Discussion

Telogen hairs are a common source of trace forensic evidence but are considered an unreliable source of DNA for STR profiling due to low DNA yield and DNA degradation. This study supports the use of a simple, rapid and cost-effective method to screen telogen hairs for the presence of cell nuclei, which can serve as a viable source of nuclear DNA for identification. The combination of staining/microscopic examination and DNA analysis on the same hairs has not only identified the frequency of nuclei on telogen roots across a large group of individuals, but also a threshold number of 16 nuclei that is likely to produce an informative STR profile under standard conditions. Thirty five percent of telogen hairs yielded associated nuclei and the presence of nuclei was in many cases independent of the presence or absence of visible tissue adhering to the root. Results demonstrate that the use of haematoxylin stain is highly advisable in order to confirm that sufficient numbers of nuclei are present prior to destruction of the hair and costly STR analysis.

Hairs were mounted directly onto the slide without sectioning, and therefore it is possible that nuclei may be hidden from focus or view by being on the other side of the hair. The method described here included screening hairs through a variety of focal planes to determine where nuclei were, and using the "best view" as the basis for counting. This is a necessary step, as simply focusing to the top cuticle layer of the hair may mean that nuclei adhering to hair are not brought into focus, and may be missed. The possibility that some nuclei were missed due to being out of view in part accounts for the standard error of nuclei categories (Table 1) and may explain the imperfect correlation of nuclei to DNA yields. Despite these possible sources of error, observed nuclei number is a strong indicator of DNA yield and nuclear DNA typing success.

All DNA recovered belonged to the donor of the hair with all STR profiles generated from nuclei matching the DNA analysed from reference buccal swabs. This is important for two reasons; firstly as an indication that the additional steps of staining did not introduce

any contaminating DNA and secondly, that despite the nuclei being from the outside of the hair, the nuclei were from the donor of the hair, and therefore must be shed from the follicle already adhering to the hair. The DNA recovered is not the result of contamination from the surrounding environment despite the lack of a decontamination procedure prior to staining or extraction. Decontamination of the telogen root may in fact be detrimental to DNA yield, as decontamination procedures could destroy any DNA within nuclei adhering to the roots (for a discussion of this issue see [27]). In most cases, nuclei were found adhering to the root, or within a few millimetres of the root end of the hair. This indicates that shorter telogen hair fragments could be used provided the root is intact, and this method should be successful when applied to shorter pieces of hair.

The hairs utilised in this study were sorted, stained and DNA extracted within a few months of collection, and results are not expected to be affected by cellular degradation. DNA typing success rates for equivalent nuclei-count forensic casework telogen hair samples may be lower or more variable due to longer times between scalp detachment and adverse environmental conditions prior to collection and DNA analysis. Further investigation is required in order to determine factors that affect the persistence and recovery of material adhering to telogen roots over a longer period of time.

The strong correlation of nuclei to DNA yield indicates that nuclei numbers can be used to estimate DNA yield. However, a weaker correlation was seen between nuclei number and allele number. The vast majority of nuclei screened were in low numbers, and therefore yielded very low DNA amounts that were generally well below the manufacturer's recommended amount for Profiler Plus™ [41]. The DNA yields seen can therefore be considered low template amount DNA (LT DNA). Most of the DNA extracts utilised for STR profiling yielded profiling results consistent with using LT DNA and a standard profiling method i.e. dropout, inconsistent profiling results and complete profiling failures [42–44]. These failures and inconsistencies negatively impacted the correlation between nuclei number and alleles produced. The stochastic effects seen, in particular the inconsistent results between samples with similar LT DNA amounts make prediction of STR outcome from starting nuclei numbers far more difficult than using nuclei to predict DNA yield. While this study has shown that it is possible that a result can be generated from as few as 16 nuclei, at least 60 nuclei were needed to overcome stochastic effects and reliably produce an informative partial profile using different samples. Methods that are optimised for LT DNA may increase the number of informative profiles and the consistency of results [45–48]. Work is currently underway to assess whether common LT DNA strategies can improve allele numbers, increase allele peak heights and limit stochastic variability between similar samples. Overall, the results presented in this study demonstrate that it is possible to recover nuclear DNA from telogen roots, and that with standard methods informative STR results can be obtained.

Aside from strategies to improve profiling results through application of LT DNA methods, concentration of the DNA extract prior to PCR shows promise. Although a small number of samples were evaluated in this study, concentrating the extract significantly increased DNA, profiling sensitivity and allele peak heights, but overall it did not increase the number of alleles or consistency between samples with similar nuclei numbers. In some cases, DNA yields increased by more than expected (i.e. more than double) when concentrated. While inhibition was not detected, it is possible that concentration of the extracts purified the DNA further, producing more efficient amplification during the qPCR. However, the most likely explanation is stochastic sampling effects for the qPCR from the unconcentrated DNA extract producing underestimates of the actual DNA concentration. In addition, the lower end of the standard curve used for quantification will result in less reliable qPCR results. Upon concentration of the DNA, the qPCR result generated is more reliable, with less stochastic behaviour due to having a larger concentration of template molecules per microlitre of extract. Despite an increase in the DNA due to concentration of extracts, the majority of DNA yields were still in the LT DNA range and still below the recommended amounts for profiling. STR results were also affected by stochastic behaviour and the limited sensitivity of the standard profiling method, and exhibited the same features as seen in STR profiles from non-concentrated samples. Despite the lower correlation, this initial evaluation demonstrates that concentration of the DNA extract warrants further investigation and may prove to be an alternative to the low copy number (LCN; 34-cycle) profiling method. The LCN method has been shown to cause artefacts and generate results that are challenging to interpret [49–52] and our alternative may improve the consistency of STR profiles generated from LT DNA without complicating interpretation.

STR typing success of telogen hair roots may be also further improved through the application of new STR technology with improved sensitivity and tolerance to inhibition [53,54]. As these kits are relatively new to the forensic community, work is still underway to validate and demonstrate these kits on casework. The numbers of nuclei adhering to telogen roots and STR alleles should still be correlated and therefore STR profiling outcomes should still be predictable with this new technology. Finally, it is possible that partial STR results generated due to LT DNA could be complemented by mtDNA analysis. It is expected that along with nuclear DNA, mtDNA would also be extracted from adhering cellular material so the same extract that was used for STR typing could be used for mtDNA analysis. Alternatively, as the hair shaft is not destroyed during the extraction process, mtDNA could be extracted and analysed from the hair shaft. However, STR profiling is far more discriminatory and should be explored as a first option.

5. Conclusion

The results of this study add further support to previous work evaluating histological stains for analysis of material adhering to telogen roots and has established an improved resolution of the frequency at which nuclei are found and the corresponding DNA yield and STR profiling outcome. A strong correlation between number of nuclei and both DNA yield and number of STR alleles has been shown, demonstrating that nuclei numbers can be used to gauge profiling success. Based on the frequencies obtained in this study, up to 35% of hairs contain nuclei, and with standard techniques, 28% of the roots containing nuclei could potentially produce at least a partial STR profile. Preliminary results demonstrate that improvements to STR profiling outcomes may be possible through concentration of the extracts using a silica column method. Further improvement may be possible with the use of LT DNA profiling strategies and these are currently being evaluated. The simple and cost effective method described here allows single telogen hairs to be efficiently screened prior to STR analysis, thus reducing profiling failures from hairs with low or no nuclei. The method also has the added benefit of being non-destructive to the hair shaft, enabling retention for further analysis if required. Finally, the method demonstrated in this study provides a means to utilise certain single telogen hairs for STR DNA analysis, which when combined with comparative microscopy promises to increase the ability to identify individuals from a very common form of trace evidence.

Conflict of interest

The authors declare no conflict of interest.

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Authors contributions

JE performed haematoxylin staining, DNA extractions, DNA quantification, STR profiling, statistical analysis and drafted the manuscript. EB conceived experiment and initial experimental design. CM performed volunteer hair collection. EB and CM screened hairs. EB, DM, JR, AC and JA assisted with experimental design, analysis and manuscript preparation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fsigen.2012.10.001.

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