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Testing the recoverability of grass DNA transferred to textiles for forensic purpose

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Abstract: Botanical evidence such as grass stains on textiles is sometimes present in the crime scene and can allow investigators to establish an association between persons linked to the criminal event and the crime scene. In this study, extraction of grass DNA from stains on textiles was undertaken. DNA extraction was performed on four grass species conserved both indoors and outdoors for 7, 14 and 30 days after staining. Once the extracted DNA was quantified, a polymerase chain reaction (PCR) amplifying a fragment of the internal transcribed spacer was performed.

DNA extraction was successful in 97.5% of samples. No significant differences in the amount of extracted DNA were detected among species or stain ages. However, one grass species (*Cynodon dactylon*) showed a significant diminution in the mean DNA concentration between indoor and outdoor samples (439.9±137 ng/µL vs. 318.9±177 ng/µL respectively; p=0.041). PCR was successful in 89.2% of samples.

This study has thus demonstrated the recoverability of grass DNA from stains on cloths and its stability in the first month after staining in both outdoor and indoor environments, as well as its suitability for PCR amplification that could allow correct species identification.

Keywords: grass DNA, forensic DNA analysis, forensic science

INTRODUCTION

Criminal investigation involves the recovery and processing of evidence from different origins and of different characteristics. Among this wide range of supporting data, forensic investigation often involves botanical traces that can potentially be useful for establishing links between individuals involved in a criminal event or at the scene of the crime. Among the huge variety of plants that could be

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used as forensic evidence, grass species are of great importance as they are commonly present in both urban and rural areas and because traces of them can be transferred to several objects such as clothes and shoes.

Thus, efforts have been directed at setting up reliable, reproducible and standardised protocols and techniques of DNA analysis to correctly identify the vegetal species potentially useful as evidence. These techniques have been successfully applied to several plant samples, grass species among them [1]. For this species identification, the study of genetic sequences as internal transcribed spacer (ITS) has been proposed [2]. This study utilises leaf fragments as problem samples without considering the possibility that the plant, and specifically grass traces, may be present as textile stains, this latter case being commonly observed in daily forensic casework.

In the case of grass stains, morphological identification is obviously impossible, so DNA techniques are crucial to achieve species identification. To our knowledge, grass stains have not been evaluated for possible DNA recovery, nor has it been known whether the quantity and quality of recovered DNA (in the event of extraction being possible) will allow investigators to perform a successful polymerase chain reaction (PCR). Thus, the aim of this study is to evaluate these possibilities.

MATERIALS AND METHODS

Grass Species

Stains obtained from four grass species that are very common in the Iberian peninsula and other Mediterranean regions were tested. Three of these species (*Festuca arundinacea, Zoysia japonica and Stenotaphrum secundatum*) are frequently found in garden areas. The fourth, *Cynodon dactylon,* is used as ornamental grass in gardens, but it is also present as a ubiquitous Mediterranean wild grass, commonly found in natural environments and uncultivated fields. Species identification was done by morphological methods (quantitative and qualitative).

Sample Preparation

Grass stains were obtained by rubbing leaf samples against a white piece of cotton fabric. The rubbing was made with moderate pressing until a visible green stain was produced. From each grass species, ten stained pieces of fabric were made; five of them were kept in an indoor environment while the other five were placed outdoors. The outdoor samples received direct sunlight 3 hours a day. No rain fell in the month of the experiment so water was sprayed on the samples twice in order to compensate for this.

At the end of the 7^{th} day, a 1×1 cm stained sample was cut from each of the 10 stained pieces of the fabric and DNA extraction was performed on each sample. The process was repeated at the end of day 14 and day 30. Thus, a total number of 30 samples for each grass species were extracted for DNA.

DNA Extraction

DNA extraction was performed according to Ahmed et al. [3]. All reagents were provided by Sigma-Aldrich, USA. A 1×1 cm piece of grass-stained fabric of was placed in a reaction tube. An extraction buffer (400 μL: composed of 100 mM EDTA, 250 mM NaCl and 100 mM Tris-HCL) were

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added, together with 10 µL of dithiotreithol, 10 µL of proteinase K (10 µg/µL), 8 µL of 98% 2-mercaptoethanol and 10 µL of 20% sodium dodecyl sulphate. After being gently mixed in a vortex for one minute, the mixture was incubated overnight at 65ºC.

After incubation, the textile piece was eliminated from the tube. Phenol-chloroform-isoamyl alcohol (25:24:1, 400 µL) was added to the remaining liquid phase and the two phases gently mixed for 20 seconds. The tube was then centrifuged at 15000 rpm for 5 minutes and the upper phase was collected and poured into a Microcon filter (100-µ pore diameter, Millipore Inc., USA) that had been previously hydrated with 100 µL of doubly distilled water. After centrifugation at 3000 rpm for 25 minutes, the filter was placed in another reaction tube and 200 µL of doubly distilled water were added and the tube centrifuged again at 3000 rpm for 25 minutes in order to wash the filter. Finally, 60 µL of doubly distilled water were added to the filter before placing it in inverted position in a new tube. A final centrifugation at 4000 rpm for 5 minutes allowed the recovery of DNA from the filter.

The DNA concentrate was automatically quantified by spectrophotometry using a Nanophotometer™ (Implen GmBH, Germany). Absorbance at 260 nm was used. The function describing the absorbance-to-concentration relation was a modification of the Lambert-Beer equation: $c_{nuc} = A_{260}$ * factor nuc * dilution factor, where c_{nuc} is nucleic acid concentration (ng/µl); A_{260} is absorbance (AU) of nucleic acids; and factor nuc is substance specific factor for nucleic acids (ng * cm/µl), which equals 50 in the case of double-stranded DNA.

PCR Testing

In order to check for the PCR suitability of the stain-derived grass DNA, an explorative PCR was performed on all samples. A pair of angiosperm universal primers was selected for this purpose [4]. These oligonucleotides amplify an inter-species constant fragment of the ITS, thus generating a constant 589-bp amplicon.

The PCR conditions and reagents were as follows. DNA was amplified in a 96-plate thermocycler (Eppendorf, Germany). The reaction mixture used in the PCR consisted of 10 ng of genomic DNA, 0.2 µM of each primer (Roche Diagnostics GmBH, Germany), 0.6 unit of Go Taq polymerase (Promega Inc., USA), 2.5 mM of $MgCl_2$, 10 mM of Tris-HCl (10x), 50 mM of potassium chloride, and 200 µM of deoxynocleotide triphosphate (Roche Diagnostics GmBH, Germany), in a total volume of 25 µL. After an initial denaturation at 94ºC for 6 minutes, 30 PCR cycles were performed with 45 seconds of denaturation at 94ºC, 45 seconds of annealing at 58°C, and an extension for 45 seconds at 72°C. Finally, a single extensional step at 72°C for 10 minutes was performed.

Amplified products were separated by horizontal electrophoresis (80-100 mA) in a 2% agarose gel (Sigma-Aldrich, USA) and diluted in TAE buffer (2 M Tris-acetate and 100 mM sodium EDTA, National Diagnostics, USA). After a 10-minute exposure to ethidium bromide solution (0.5 µg/mL, Sigma-Aldrich, USA), amplified DNA fragments were visualised with UV light (254 nm).

Statistical Analysis

All statistical analyses were performed by using the SPSS software package (SPSS Inc., Chicago, Illinois). The Kolmogorov-Smirnov test [5] was undertaken in order to assess the normality of the DNA concentration value distribution. ANOVA and t Student tests were performed in order to

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study mean DNA concentration differences between groups. Statistical significance was defined as $p<0.05$.

RESULTS AND DISCUSSION

Table 1 shows mean DNA concentrations obtained from each grass species in both indoor and outdoor environments at different analysis times. In three cases (2.5%), no DNA was obtained. Eight samples (6.7%) gave DNA concentrations under 20 ng/µL.

Table 1. Mean DNA concentration values obtained for each grass species under different conditions

	Indoor condition				**Outdoor condition**			
	Day 7	**Day 14**	**Day 30**	***p***	**Day 7**	**Day 14**	**Day 30**	***p****
Cynodon dactylon Mean±SD(ng/µL)	412.6±78.8	458.2±160.9	448.0±152.1	0.856	324.2±129.0	303.3±178.0	329.0±248.5	0.976
Stenotaphrum secundatum Mean±SD(ng/µL)	363.6±309.9	511.2±246.7	561.8±367.8	0.606	317.8±157.7	455.8±139.9	406.8±338.7	0.181
Zoysia japonica Mean±SD(ng/µL)	540.2±171.5	419.6±280.8	422.6±330.9	0.728	340.4±166.3	367.0±225.9	554.6±330.2	0.368
Festuca arundinacea Mean±SD(ng/µL)	459.8±196.6	562.0±322.8	360.4±344.7	0.574	427.8±107.6	547.2±378.1	390.2±275.2	0.655

Note: *p* and *p** are significance values of the comparison between means obtained at the three different times for indoor and outdoor samples respectively.

After the normal distribution of DNA concentration values had been confirmed, the difference between indoor and outdoor mean concentrations for each species was assessed. DNA concentrations from *C. dactylon* showed significantly lower values in outdoor samples (318.9±177 ng/µL vs. 439.9±137 ng/µL; p=0.041). The other three species did not show significant differences between mean outdoor and indoor values (*S. secundatum*: 386.8±158.2 ng/µL vs. 472.9±295.0 ng/µL, p=0.411; *Z. japonica*: 420.7±251.7 ng/µL vs. 460.5±256.1 ng/µL, p=0.608; *F. arundinacea*: 455.1±285.7 ng/µL vs. 460.7±286.4 ng/µL, p=0.956). When the indoor-outdoor comparison was undertaken taking the sample age separately (7days, 14days and 30days), no differences were found in any of the four species. (Means are displayed in Table 1 with the following p values: *C. dactylon* - 0.227, 0.188 and 0.385; *S. secundatum* - 0.776, 0.674 and 0.417; *Z. japonica* - 0.098, 0.753 and 0.545; *F. arundinacea* - 0.758, 0.949 and 0.884 respectively). Furthermore, when DNA values for the four species were taken together, neither indoor nor outdoor means showed significant difference (458.3±243.3 ng/µL for indoor samples vs. 396.1±223.4 ng/µL for outdoor samples, p=0.159).

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When the possible effect of storage time in both environments was assessed, it was found that none of the grasses showed significant difference in the final DNA concentrations of samples at different ages (data shown in Table 1). Also, there was no statistical difference in the mean amounts of extracted DNA between the species, either for indoor or outdoor samples ($p=0.987$ and $p=0.395$ respectively).

The first PCR assay was successful in 107 samples (89.2%). After further repeating the PCR twice, seven samples still did not provide any PCR product. Three of them had previously shown no DNA in the spectrometric measurement while the four remaining samples, despite containing an appropriate DNA concentration, repeatedly failed in the PCR assay. In all other cases, the PCR product had the expected 589-bp size.

In forensic investigation, DNA extraction from biological stains is of great importance. In the case of samples of human origin, protocols and techniques are well defined and characterised. However, little attention has been paid to DNA extraction from plant-originated stains. In the case of grass species, friction between plant and textile or other materials usually results in the transfer of biological traces to this items and species identification can often provide some evidence owing to the restricted and specific geographic distribution of each kind of grasses. Short tandem repeats (STR) and single nucleotide polymorphisms (SNP) have been proposed as useful techniques for species identification [6]. DNA barcoding [7], however, is the most commonly reported species identification technique in recent researches [8-9]. Grasses have also been successfully tested with this new technique [1]. However, these studies employed leaves or leaf fragments as samples and have not taken into account the possibility that plant material may be present in stain form. This possibility is especially frequent in the case of grass samples that have been transferred by friction with clothes, shoes or other materials. In contrast to intact leaves, stain production may hypothetically alter cell walls and membranes and thus lead to greater DNA exposure to degrading agents in the environment, which may affect its recoverability and integrity.

In the present investigation, we set out from the hypothesis that friction between grass leaves and textiles can be considered analogous to the grinding of leaf fragments with a pestle performed in standard plant DNA extraction protocols. Thus, following this hypothesis, we used a standard extraction buffer with very good results as in only three cases did the DNA extraction register a complete failure. DNA from both indoor and outdoor samples was successfully extracted in nearly all samples and in all species at all three storage periods. We did find a decrease in the mean extracted DNA concentration for outdoor samples in all four species. However, only in the case of *C. dactylon* did the decrease reach statistical significance. The causes of this phenomenon could possibly be attributed, apart from the characteristics of this particular species, to slight variation in the amount of plant material attached to the fabric when the stain was made, the intensity of received sunlight, and the level of humidity applied to the samples.

Another aspect that should be had in mind is the suitability of extracted DNA for amplifying moderately heavy DNA fragments. In old stains or outdoor conditions, the possibility of DNA degradation must not be underestimated. In these cases, amplification of heavy DNA fragments may fail and species identification will have to be achieved by using lighter amplicons. In all tested environments,

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species and stain ages, the PCR of a moderately heavy product was successful in nearly all samples, thus suggesting that no significant degradation occurred in these samples.

CONCLUSIONS

The recoverability of grass DNA from cloth stains has been demoustrated, which shows the stability of this DNA during the first month after the stain was made in both outdoor and indoor environments, and also its suitability for PCR amplification of relatively heavy DNA fragments that could allow correct species identification.

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