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Technical note

Identification and persistence of *Pinus* pollen DNA on cotton fabrics: A forensic application

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ABSTRACT

Advances in plant genomics have had an impact on the field of forensic botany. However, the use of pollen DNA profiling in forensic investigations has yet to be applied. Five volunteers wore a jacket with *Pinus echinata* pollen-containing cotton swatches for a 14-day period. Pollen decay was evaluated at days 0, 3, 6, 9 and 14 by microscopy. Pollen grains were then transferred to slides using a portable forensic vacuum handle. Ten single grains per swatch were isolated for DNA analysis. DNA was extracted using a high throughput extraction method. A nine-locus short tandem repeat (STR) multiplex system, including previously published primers from *Pinus taeda*, was developed. DNA was amplified by PCR using fluorescent dyes and analyzed by capillary electrophoresis. Pollen counts from cotton swatches in a 14-day period exhibited an exponential decay from 100% to 17%. The success rate of PCR amplification was 81.2%. Complete and partial STR profiles were generated from 250 pollen grains analyzed (44% and 37%, respectively). Due to the limited amount of DNA, drop-in events were observed (1.87%). However, the rate of contamination with pollen from other pine individuals originating from environmental sources was 4.4%. In conclusion, this study has shown that pollen can be a stable source of forensic DNA evidence, as a proof-of-principle, and that may persist on cotton clothing for at least 14 days of wear. This method can be applied in forensic cases where pollen grains larger than 10 μm (e.g., from herbs or trees) may be transferred to clothing (worn by suspect or victim) by primary contact.

1. Introduction

Forensic palynology, a sub-discipline of forensic botany, utilizes pollen grains as trace evidence for criminal investigations. First used in the 1950s, pollen evidence is analyzed for forensic purposes prevailingly in countries such as Australia, New Zealand and the UK [1]. Pollen may be present as a fine or coarse powder, and consists of the microgametophytes of seed plants (which produce the male gametes). Grains with diameters between 7 and 200 μm have hard coats made of sporopollenin, which protect the gametes during their movement and migration. An individual tree can produce thousands to millions of grains in a single pollinating season. Wind-pollinated plants produce an abundance of pollen dispersed over long distances, while insect-pollinated plants produce smaller amounts dispersed over shorter distances. Due to its strong cellular wall, pollen deposited can persist for months to years after initial dispersal, even after the original plant is not present anymore [2]. Therefore, the distribution and variation of pollen populations in two different locations only a few miles apart may be considerably different [3]. Moreover, the seasonality of pollen can be studied with the help of pollen calendars [4]. A study of Montali et al. demonstrated that pollen calendars can be used as reference tools for determining the season of death in case of murders or other crimes [5].

The persistence of small trace particulates on clothing is of forensic importance, and is dependent on multiple factors, including size, texture, and the type of fabric [6]. In addition, Houck [6] identified that following particulate transfer, the time and degree of activity of the subject (host) until recovery are crucial factors that are dependent on one another. While many studies describe the persistence and transfer of fibers and other trace evidence on clothing [7–13], few have investigated pollen grains [14–17]. According to Bull et al., pollen persistence in clothing follows an exponential decay curve similar to other trace evidence [15]. In another study, Zavada et al. demonstrated that "clothing acts as a quantitative and qualitative collector, and produces a pollen profile that exhibit regional seasonal trends similar to patterns generated using volumetric collectors" [17].

Traditionally, pollen is identified using microscopy techniques based on morphological characteristics. As described by Morgan et al. [16,18] and Mildenhall [19,20], palynologists use an "assemblage" consisting

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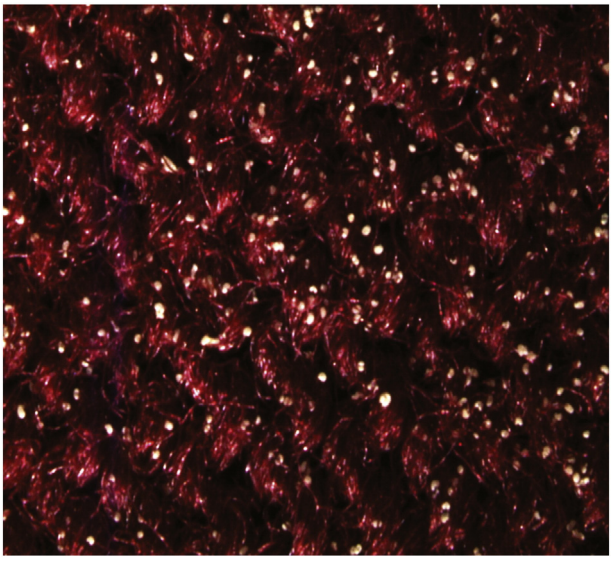

Fig. 1. Microphotograph, magnified at 25×, displaying *P. echinata* pollen grains brushed on a cotton swatch at day 0.

of hundreds of grains to determine the types and percentage of the various pollen species present. However, in forensic cases, hundreds of grains are not always available, and individualization of pollen assemblages based on morphology alone can be very difficult. Male sex cells (containing DNA) are trapped inside a rigid pollen grain wall and protected until the grain is crushed. In the case of *Pinus*, one pollen grain can include many cells; however, the sperm nuclei may develop with or without cells [21]. Currently, the application of molecular biology techniques to pollen grains is being used in other scientific areas such as environmental sciences, ecology and agriculture [22–24]. A DNA fragment found on the *rpoB* region of chloroplast DNA has been used to genotype *Pinus* pollen grains collected off glaciers [22]. The internal transcribed spacer (ITS) has been used to identify Chenopodiaceae pollen grains in surface soil down to the species level [23]. Short tandem repeat (STR) analysis has been used to monitor the dispersion of single pollen grains from one area to another by insects [24].

The aim of this project was to study the persistence and stability of *Pinus* pollen DNA on cotton fabrics during a 14-day time period. For this purpose, we combined the use of a new forensic device for pollen collection, a high-throughput method for DNA extraction and PCR amplification, and a newly developed nine-multiplex STR system for genotyping.

2. Materials and methods

2.1. Sample collection

Reference needle and pollen samples were collected from *Pinus echinata* trees in Huntsville, Texas during March and April, 2014. Needle samples were cut from the pine branch and stored in paper envelopes. Pollen grains were collected directly from pine male cone buds. Pine buds were excised from the base of the needles and transferred to a 50-mL Falcon tube. Every individual bud was shaken onto a white paper to allow release of pollen. Pollen was then transferred to 1.5-mL microcentrifuge tubes. Both pine tissues were stored at −20 °C until processing.

2.2. Persistence study and pollen processing

The persistence of pollen DNA in cotton fabrics was assessed following a previously published report with minor modifications [15]. Pollen from a previously genotyped single tree was used for these experiments. Four clean (tested) cotton swatches (approximately 60 mm × 30 mm) per individual were used in this study. All swatches were sieved with pollen on day 0 (approximately 1 mg), brushed with a small brush and taped to a jacket in the front section. Five volunteers wore a jacket with pollen-containing cotton swatches for a 14-day period. Jackets were worn indoors only to prevent contamination from other pollen sources. The swatches were collected on day 0, 3, 6, 9, and 14 and stored in a petri dish at room temperature prior to DNA extraction. Pollen counts in swatches were estimated from photographs taken at 25× magnification under a Leica stereomicroscope MZ1 (Leica Microsystems Inc., Buffalo Grove, IL, USA). Replicate counts per sample were performed in two areas of 12 mm^2 each, and the obtained values were averaged. After both photographs were taken, the grains were collected using the BioTX TED vacuum handle with the TED powder/fiber tips (BioTX Automation, Inc., Livingston, TX, USA) and quantitatively transferred from the cotton swatches to a slide. The complete transfer of the grains from the swatch to the slide was then confirmed with microscopy (Fig. 1). The tip was removed from the vacuum after collection and a 5-mL syringe containing aniline dye solution (0.7 mM aniline blue, 30 mM sucrose, Sigma-Aldrich, St. Louis, MO, USA) was attached to the base. Approximately 200 μL aniline solution was passed through the filtered tip onto a microscope

Table 1
Primer information on the nine STR loci present in the multiplex system.

Marker	GenBank accession	Dye/primer sequence (5′–3′)	Primer concentration (μM)	Amplicon size (bp)
PtTX4228	AF455080	F-FAM-ATATCATGTTTAGGTTGGTGTG R-AGTTAGGCTTTTTGTCC	0.10	140–168
PtTX3034	AF143974	F-FAM-TCAAAATGCAAAAGACG R-ATTAGGACTGGGGATGAT	0.30	184–220
PtTX3025	AF143970	F-FAM-CACGCTGTATAATAACAATCTA R-TTCTATATTCGCTTTTAGTTTC	0.30	245–298
PtTX4181	AF442374	F-FAM-CTCTCCCCTTTATTTACACATTG R-AAAGATTGGTCGGTTGGTTAT	0.20	362–450
PtTX2037	AF143959	F-VIC-GCCTTTAGATGAATGAACCAA R-TAAGCGGGATATTATAGAGTT	0.10	143–188
PtTX3011	JX486754	F-NED-AATTTGGGTGTATTTTTCTTAGA R-TAGTAAAAGTTGAAGGAGTTGGTG	0.30	150–211
PtTX3052	AF441514	F-NED-CCTCACTAGGAGGCTACGGAAGAG R-AAAGACTCCTTGATGTTGTGAACA	0.10	230–262
PtTX4058	AF324774	F-PET-AAGTGTTGGGAGAAAAATGTAAT R-CTCCTTCTGTCCCTATCCTCT	0.20	123–159
PtTX2123	JX486756	F-PET-GAAGAACCCACAAACACAAG R-GGGCAAGAATTCAATGATAA	0.05	189–201

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