Application of a Forensic DNA Extraction System for Cannabis sativa Seed Identification

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ABSTRACT

Identification of *Cannabis sativa* seeds is crucial for law authorities fighting drug abuse and global trafficking. However, because they lack chemicals that are routinely used to pinpoint *C. sativa* plants, their identification becomes far more challenging. Germinating the seeds requires tremendous resources, is time consuming and is not effective when dealing with nonviable seeds. Botanical identification relies on well-trained experts. In recent years, laboratories have started to use specific DNA markers to achieve this goal. Real-time quantitative polymerase chain reaction (qPCR) was found to be a simple and efficient approach. However, seed DNA extraction is often labor intensive and involves many steps, which may also lead to DNA loss and contaminations. In the present study, we explored whether the PrepFiler™ Express Forensic DNA Extraction Kit, which is used in our DNA casework forensic laboratory, can reduce the work required for this critical step. We show that this kit has several advantages when compared with a dedicated plant extraction kit. In addition, we show that the combination of this extraction method and qPCR can enable high-throughput *C. sativa* seed identification.

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INTRODUCTION

In recent years, self-purchasing *Cannabis sativa* seeds became easy and available because of global trading through online services, social media, and the dark net.[1] As a result, the legal demand for *C. sativa* seed identification has increased worldwide.[2] Although there is a growing trend of legalizing marijuana in certain countries, in many others, possession and trafficking of *C. sativa* seeds is still illegal and poses a challenge for law enforcement authorities. Because *C. sativa* seeds present minute levels of the specific psychoactive substance, delta-9-tetrahydrocannabinol (THC), with maximum values below 0.02% (less than 2 µg/g),[3] it cannot be identified by standard analytical means.

Several approaches are currently used worldwide to characterize *C. sativa* seeds:

1. Morphological seeds are identified by botanic experts.[4]·[5] This approach is relatively quick when performed by a well-trained expert; however, it heavily relies on this unique area of expertise and cannot be evaluated by objective scientific tools.
2. Seeds are germinated followed by analytical tests or cystolithic hair identification of the plant.[6] The main drawback of this approach is the time resources and growing facilities required for germination and plant growth. In addition, some plant seeds may not germinate because of various reasons and hence would not be identified by this method.
3. Specific *C. sativa* DNA markers are identified by molecular methods. This is a relatively new approach with high specificity and may enable large-scale seed analysis and can identify nonviable seeds.[6]· [7]· [8] However, it requires multiple steps, including DNA extraction, which may be expensive and time consuming.

C. sativa seed identification in our national DNA casework forensic laboratory is done morphologically by an outsourced botanical expert. In terms of cost effectiveness, employing such an expert may not be worthwhile. Nowadays, many forensic DNA laboratories are equipped with instruments, technology, knowledge, and expertise to conduct in-house genetic identification of *C. sativa* in large scales. In searching for the most efficient method to identify *C. sativa* seeds, which will allow high-scale identification, the DNA casework forensic laboratory explored the possibility of using the genetic markers approach. In preliminary studies, we found that the extraction of DNA from seeds can be tedious, as it requires time and effort, and the yield may be low. *C. sativa* seeds (and therefore their embryos) contain high concentrations of substances that can negatively interfere with DNA extraction, such as proteins (approximately 25%), carbohydrates (>25%), and fats (>35%),[9] as well as significant amounts of phenolic compounds.[10] These compounds degrade the quality of DNA by precipitating along with it, thus reducing yield.[11] An array of DNA isolation protocols have been optimized and were used in various combinations to isolate quality DNA from plants for analyses.[12]·[13] Liquid nitrogen plays an important role in obtaining high-quality DNA, and hence it has been used extensively for DNA extraction from fresh leaf and other tissues; however, it is expensive and requires liquid nitrogen supply, experience, and expertise for large-scale work. Although methods for DNA extraction from whole *C. sativa* seeds are available,[6] we searched for an easier and robust extraction protocol using instruments and technologies that already exist in our laboratory, the PrepFiler Express Forensic DNA Extraction Kit. This magnetic bead extraction isolation technology has several unique features that make it particularly fit

to this purpose.[\[14\]](#),[\[15\]](#) Most notably, it is an automated, easy to use system designed to efficiently extract DNA from challenging forensic samples, and the purified DNA is free from polymerase chain reaction (PCR) inhibitors.

In this work, we explored methods for DNA seed extraction combined with real-time quantitative PCR (qPCR) of specific genetic markers. qPCR is a well-established method used in genetic research, diagnostic medical applications, and more. This approach is easy to implement, offers an efficient and robust method for *C. sativa* seed identification, and enables large-scale working volume.

MATERIALS AND METHODS

DNA extraction

Botanically characterized *C. sativa* and *Humulus lupulus* seeds were collected. Single seeds were placed for wash in a 1.5-mL microcentrifuge tube with 400 μ L of nuclease-free ddH₂O water and then dried on filter paper. Each seed was placed in a new clean folded filter paper and inserted into a disposable plastic bag. The seeds were slightly crushed by a pestle and placed in a 1.5-mL microcentrifuge tube or in a PrepFiler Column (Applied Biosystems), depending on the extraction kit. DNA was extracted by DNeasy Plant Mini Kit (Qiagen; Valencia, CA) or PrepFiler Express Forensic DNA Extraction Kit, using the AutoMate Express nucleic acid extraction system (ThermoFisher Scientific; Waltham, MA), according to the manufacturer's protocol (20 *C. sativa* seeds were extracted in each assay).

DNA quantitation

Extracted DNA samples were quantified using a Qubit 2.0 fluorometer (Invitrogen; Carlsbad, CA) with Qubit dsDNA BR Assay Kit according to the manufacturer's protocol.

Primers and probes

The green plant- (a general sequence found in all green plants) and *C. sativa*-specific sequences were based on previous studies conducted by Johnson et al.[\[6\]](#)

qPCR assay

qPCR was carried out in a single-plex assay in contrary to the duplex assay carried out by Johnson et al.[\[6\]](#) The reaction working solution contained 10 μ L of 1X TaqMan Environmental Master Mix, 0.6 μ L of 10 μ M green plant-specific/*C. sativa*-specific

forward and reverse, 1 μL of 5 μM green plant-/*C. sativa*-specific probe, and 5.8 μL of double-distilled water (DDW) per sample. Extracted DNA samples (2 μL , 2 ng/ μL) were added to 18 μL of working solution to achieve a final reaction volume of 20 μL per well.

The amplifications were carried out in GeneAmp PCR System 9700 (Applied Biosystems) instrument with initial hold of 50°C for 2 minutes and then 95°C for 10 minutes, cycling at 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 30 seconds for 35 cycles. The analysis was carried out with ABI HID real-time PCR analysis software (version 1.2). All qPCR reagents were purchased from ThermoFisher Scientific.

RESULTS AND DISCUSSION

C. sativa seed DNA extraction

C. sativa seeds are often seized from shipments, dealers, or individuals and transferred to forensic laboratories for characterization. Germination and examination of plant morphology or by analytical means requires time, space, and resources. Genetic DNA analysis offers a quick and robust approach for the discrimination of the seed's genus and source. DNA extraction is a critical step in the forensic process of DNA evidence, and the extraction efficiency should be high and allow high-throughput sample handling. [16] [17] To address this point, we performed a comparison between a plant-dedicated DNA extraction kit (hereinafter "plant kit") and a casework item forensic extraction platform (hereinafter "forensic kit"). The plant kit (DNeasy Plant Mini Kit) involves several manual liquid handling and transfer steps, whereas the forensic kit (PrepFiler Express) is semiautomated and allows for the extraction of multiple samples in parallel. An additional step of seed mechanical mashing was performed prior to extraction, as described in the Materials and Methods section.

To compare DNA extraction yields, 40 botanically identified seeds were randomly assigned into 2 sets and then subjected to extraction by the plant and forensic kits. The DNA amount in the extraction solution was quantified by Qubit Fluorometer. As shown in Fig. 1, the mean DNA concentration obtained using the forensic kit was almost 4 times higher than that of the plant kit ($P < 0.01$ according to Student's *t* test). Because seeds are not always shipped and stored in optimal conditions, their DNA quality may be low because of degradation and other factors. Therefore, obtaining higher DNA quantities is crucial to increase the likelihood of successful genetic characterization and to avoid loss of evidence. Reagent control quantification was

below the detection limit in both kits, a necessary indication to exclude DNA contamination.

FIGURE 1. Quantification results of DNA extracted from *C. sativa* seeds extracted by the extraction kits and measured with the Qubit Fluorometer kit. Results represent mean DNA concentration $\pm$ standard error.

Apart from higher DNA yield, using the forensic kit has several additional traits that make it suitable for working in a high-throughput manner, partially summarized in

[Table 1](#). Forensic laboratories worldwide are coping with an increasing amount of cases and samples, which leads to an increasing backlog accumulation and extends case processing duration.[\[18\]](#) Reducing the amount of work required to obtain results will necessarily aid forensic laboratories to reduce the backlog and provide better services to their clients—investigative units and courts. Although the total estimated time required for the forensic kit is higher, it is actually less time consuming. The forensic kit is composed of 2 main steps, incubation and separation, which are approximately 40 and 30 minutes long, respectively. During these steps, the laboratory worker is free to carry out other assignments. The plant kit, on the contrary, requires the laboratory worker’s constant attention with very short breaks, if any. Taken together, the high DNA yield and reduced labor make the forensic kit a useful and efficient tool for large-scale high-throughput seed DNA extraction.

TABLE 1 Estimated work required for seed DNA extraction using the forensic and plant kits.						
Kit	Chemical principle	Extraction time estimate per 12 seeds (min)			Number of manual steps	Additional work for seed batches
		Instrument time	Manual working time ¹	Total estimated time		
Forensic kit	Magnetic separation	80	10	90	4	Negligible up to 12 seeds
Plant kit	Silica DNA extraction	36	35	71	13	Each additional seed requires work

***C. sativa* genetic profiling**

After obtaining suspected seed DNA, it is necessary to genetically characterize its source. This step is also important in order to demonstrate that the extracted DNA contains the seed’s DNA rather than bacteria or fungi, which may reside on its outer surface. Several approaches, including sequencing and single-nucleotide polymorphism (SNP) analysis, were previously used to achieve this goal.[\[19\]](#) Johnson and colleagues used qPCR to distinguish suspected *C. sativa* from other plants.[\[6\]](#) The method is useful when no subspecies identification is needed and is relatively simple compared with other profiling methods. To examine the suitability of qPCR in our workflow, we adopted a similar protocol that involves amplification of 2 genes, one specific for *C. sativa* (hereafter “Cannabis probe”) and the other a general plant gene

(hereafter “green probe”), as previously described.[\[6\]](#) In contrary to their protocol, we performed 2 separate amplification reactions rather than a duplex and took a fixed volume of DNA extract (2 µL). Taking a fixed volume enables easier workflow automation when using liquid handling robots or manual distribution. To assure the protocol is specific for *C. sativa*, we extracted DNA from a close species, *H. Lupulus* seeds,[\[20\]](#) and amplified them under identical conditions. [Table 2](#) summarizes the possible results of the qPCR reaction and the respective conclusions. A positive result for *C. sativa* is determined when both probes are beyond the reaction threshold.

TABLE 2 Possible qPCR results of suspected seeds		
Cannabis probe	Green probe	Conclusion
+	+	<i>C. sativa</i>
+	–	Inconclusive
–	–	Inconclusive
–	+	Not <i>C. sativa</i>

A “+” sign represents detection of a fluorescent signal observed above the point determined as a threshold by thHID software (“threshold cycle,” also called C_T). A “–” sign represents a condition in which the fluorescent signal did not reach the C_T .

A total of 31 *C. sativa* single-seed DNA extractions were quantified, amplified, and analyzed. As shown in [Table 3](#), all results were positive for *C. sativa*. Amplified *H. Lupulus* seed DNA extractions were negative for the Cannabis probe and positive for the green probe, as expected. No false-positive or false-negative results were recorded. These results reinforce the results of Johnson and colleagues and demonstrate the usefulness and simplicity of this tool for this purpose.

TABLE 3 Summary of qPCR results			
	Seeds extracted and amplified	<i>C. sativa</i> probe	Green probe
<i>C. sativa</i> seeds	31	31/31	31/31

<i>H. Lupulus</i> seeds	16	0/16	16/16
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Conclusions

Using a common forensic DNA extraction kit combined with qPCR, as presented in this study, offers a powerful tool for *C. sativa* seed genetic identification. This approach has several benefits:

1. It reduces time and labor, scarce resources in forensic laboratories.
2. It displays higher DNA yields, which may be critical when handling damaged or nonviable seeds.
3. The forensic kit and qPCR equipment are available in many forensic laboratories worldwide, so no separate kit purchasing is required. This seed identification protocol can be easily incorporated into the regular workflow of the laboratory, especially if it uses automated liquid handling instruments, thus allowing large-scale processing while maintaining high-quality assurance standards. In addition, using existing equipment and methods is expected to significantly decrease the time and effort required for validation and personnel training.
4. The significantly lower number of steps in the forensic kit reduces DNA loss and risk of contaminating samples. In addition, it minimizes the risk of human error and does not require the use of liquid nitrogen.
5. Although this method can be more expensive and time consuming than expert botanical identification, the results obtained can be objectively scientifically examined by another party or in court, if required.

In all, we believe that DNA extraction using the forensic kit combined with qPCR provides an efficient, high-throughput approach for *C. sativa* seed identification. To our knowledge, this approach has not been previously demonstrated. This protocol may also be useful when aiming to identify other parts of *C. sativa* that display low THC levels, such as plant roots. This challenge is currently being addressed in our laboratory, and preliminary results are promising. Applying a modified version of the method presented in this manuscript can perhaps be helpful for discriminating specific cultivars of *C. sativa*, hence reducing breeding costs.[\[21\]](#) Beyond the specific use of this approach to identify *C. sativa* seeds in the criminal context, we hypothesize that using this extraction platform in other life sciences research disciplines has great

potential. In some academic institutes, forensic laboratories (which may be using this platform) and biology research laboratories work side by side, so examining this application can be relatively simple.

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Ilan Feine and Jonathan Roth contributed equally to this work.

Footnotes

1. Estimated time required for liquid or tube transfers and other manual process. [↵](#)

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