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TECHNICAL NOTE

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A Simple DNA Extraction Method for Marijuana Samples Used in Amplified Fragment Length Polymorphism (AFLP) Analysis*

ABSTRACT: As a first step in developing a molecular method for the individualization of marijuana samples, we evaluated a plant DNA extraction kit. The QIAGEN plant DNeasy method uses a spin column format for recovery of DNA and is effective for obtaining high molecular weight DNA from leaf, flower (bud), and seed samples of marijuana. The average DNA yield was 125–500 ng per 100 milligrams of fresh plant tissue. The recovered DNA was of polymerase chain reaction (PCR) quality as measured by the ability to generate reproducible amplified fragment length polymorphism (AFLP) profiles. AFLP is a technique used to create a DNA profile for plant varieties and is being applied to marijuana samples by the authors to link growers and distributors of clonal material. The QIAGEN plant DNeasy method was simple, efficient, and reproducible for processing small quantities of marijuana into DNA.

KEYWORDS: forensic science, DNA typing, *Cannabis*, marijuana, plant DNA, amplified fragment length polymorphism

As the initial step in developing and implementing a plant DNA typing method for individualizing marijuana samples, we assessed a plant DNA extraction kit manufactured by QIAGEN. Traditional plant DNA extractions utilize a time-consuming cesium chloride gradient purification or a hexadecyltrimethylammonium bromide (CTAB) extraction (1) that can yield variable results depending on the experience of the scientist and the quality of the plant material. One of the difficulties in plant DNA extractions is a poor yield. This means a large amount (grams) of starting material is required for sufficient DNA recovery. In some drug seizure situations, only a small amount of dried plant material is recovered which makes it difficult for any further testing to be performed. Other complications in plant DNA extractions include the presence of inhibitory compounds such as tannins, phenolics, and polysaccharides in leaves (1). In addition, the large amounts of starch and protein found naturally in seeds may require extra DNA purification steps. These extra purification steps can further reduce yields and recovery of DNA.

It is desirable but difficult to link individual marijuana growers and distributors to specific illicit field and greenhouse operations. Molecular genetics may offer a solution to this problem. In certain regions of Canada and the United States, marijuana is propagated by taking cuttings from a high-THC content "mother" plant and directly rooting them in the soil (personal communication, Dr. Gary Shutler, RCMP). This clonal form of propagation results in large numbers of plants having identical DNA. DNA typing of marijuana in this situation would allow one to link common grow operations and assess distribution patterns by tracking clonal material. Other growers start their marijuana plants from seed. Each seed has it's own unique genetic composition. DNA typing of marijuana grown from seed would allow one to link a leaf found in an individual's vehicle back to a plant from a vacant field near the suspect's home, for example. For cases where small numbers of seed grown marijuana plants can be practically screened, DNA typing would be appropriate. None of the methods currently used to identify marijuana [cystolith hair morphology (2), Duquenois-Levine test (3,4), chromatography (5) or ITS sequences (6,7)] can link the sample to a specific plant or group of clonal plants. The authors wish to emphasize they are developing a technique for individualizing marijuana samples, not for replacing current methods used to identify a sample as marijuana.

In developing such a technique, we have found that the QIAGEN DNeasy plant kit is effective for the extraction of high quality DNA from small quantities of fresh and dried marijuana. The DNeasy plant kit is a rapid, simple procedure for the isolation of high molecular weight total DNA. Total DNA includes genomic, mitochondrial, and chloroplast DNA. This spin column method yields PCR-quality DNA in less than 1 h without the use of chemical solvents or ethanol precipitation.

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To assess the quality of the recovered DNA, amplified fragment length polymorphism analysis (AFLP) was performed (8). Here, the authors define quality as the lack of PCR inhibitory substances (phenolics, tannins, resins) and absence of notable changes in AFLP profiles that might result from random shearing of the DNA.

AFLP is a PCR technique used to simultaneously visualize multiple amplified restriction fragments and is used by plant researchers to create DNA profiles for plant varieties and genetic linkage maps. AFLP fragments (Plant Mapping Kit; Applied Biosystems, Inc., Foster City, CA) generate a fluorescent band pattern using the same dyes and laser technology as short tandem repeat (STR) tests for human identity testing. The fluorescent band patterns are converted to peak profiles using GeneScan software (Applied Biosystems; Inc.). DNA profiles can be compared by superimposing profiles to determine if they match. The AFLP technique is useful for organisms where short tandem repeat (STR) loci have not been identified and has been successfully used on a variety of organisms including: barley (9), potato (10), tomato (11), soybean (12) and bacteria (13–15).

Materials and Methods

Plant Materials

Seizure Samples—Separate marijuana seizure samples were used in this study and designated as #1016, #12335, #12771, #8556 and #AA. These samples were obtained through the Connecticut State Toxicology Laboratory. Each of these samples consisted of leaf, flower (bud) and/or seed material. The Connecticut State Toxicology Laboratory, using gas chromatography/mass spectrometry (GC/MS) to confirm the presence of delta-9-tetrahydrocannabinol (THC), previously identified these samples as marijuana.

Clonal Samples—The mother and clonal marijuana plant samples were provided by the Royal Canadian Mounted Police (RCMP) Drug Section. Samples were collected from four distinct mother plants and two generations of clonally propagated plants.

DNA Extraction

DNA was extracted from 100 mg of starting material using either the CTAB extraction method or the Plant DNeasy kit (QIAGEN; Valencia, CA). For the CTAB extraction, the published protocol was used with the following exceptions: 1) the sample size and volume were scaled down for use with 100 mg of plant material to allow a direct comparison with the DNeasy kit and 2) the isopropanol precipitation step was allowed to proceed overnight at room temperature. For the DNeasy kit, the manufacturer's recommendations were followed. Both methods require mechanical disruption of the plant cell wall by grinding in liquid nitrogen. A ceramic mortar and pestle was used for the seizure sample DNA extractions while a disposable plastic micropestle was used for extraction of the clonal samples provided by the RCMP. Between grinding steps, the ceramic mortar and pestle was cleaned using 10% bleach and rinsed copiously with sterile distilled water to prevent cross-contamination between marijuana samples. DNA yields were estimated by comparison with genomic DNA standards (K562; Life Technologies, Gaithersburg, MD) after electrophoresis on 1% agarose gels containing ethidium bromide for visualization. DNA recovered from each method was resuspended in 100 μL of the appropriately recommended buffer and 10 μL of each sample (1/10 volume) was loaded on the agarose gel.

DNA Amplification and Analysis of AFLP Peak Profiles

AFLP was used to determine if the extracted DNA was of PCR quality and if reproducible profiles could be obtained from independent extractions of clonal material. The amplified fragment length polymorphism (AFLP) method was performed as recommended by the manufacturer (Plant Mapping Kit; Applied Biosystems, Inc.). For all marijuana samples, the following selective PCR primer sets were used: EcoR1-AAG-Joe and Mse1-CAT; EcoR1-ACT-FAM and Mse1-CAA. These primer sets were selected based on the peak complexity (information value) of the AFLP profiles. Amplification products were separated and visualized by laser excitation of the fluorescent dyes on an ABI model 377 DNA sequencer. ABI collection software was used to record the data for analysis. AFLP patterns from each sample were analyzed using GeneScan software and compared by overlaying peak profiles. To facilitate the analysis, profiles were assigned a color using the GeneScan software. The assigned color does not necessarily correspond to the dye color of the PCR primer set.

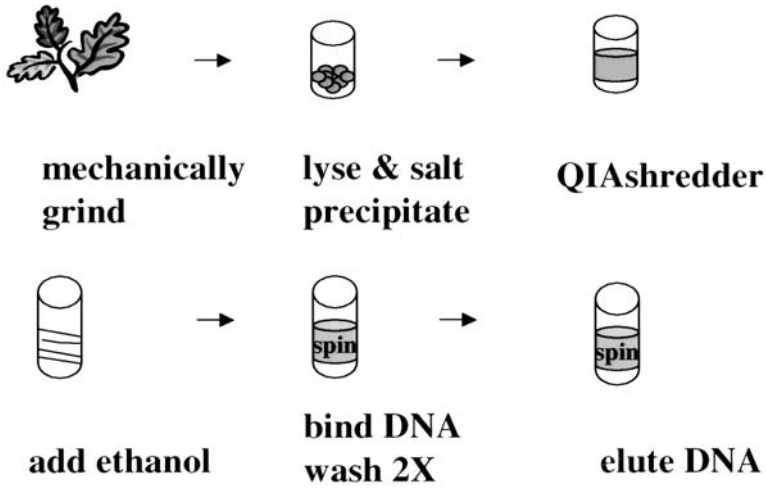

FIG. 1—*Schematic of the QIAGEN plant DNeasy DNA extraction method. The basic steps include tissue disruption, cell lysis and precipitation of cellular debris and salts through a QIAshredder spin column. Ethanol is added to clear the lysate prior to passing the lysate through a second spin column to bind the DNA to a membrane. The DNA remains bound while proteins and polysaccharides are removed by washing. The DNA is finally eluted off the membrane in a small volume of low-salt buffer or water.*

Results and Discussion

The primary benefits of the QIAGEN DNeasy plant kit when compared to the CTAB procedure are the speed of DNA recovery and the high quality DNA yield (low viscosity, lack of tannins). Using the QIAGEN kit, marijuana samples were ground to a fine powder, cells lysed, and proteins and polysaccharides precipitated. Cell debris and precipitates were removed through a filtration unit (QIAshredder) included in the kit. DNA was bound to a silica-gel membrane, washed twice and eluted in sterile, distilled water (Fig. 1). The resulting DNA was typically of high molecular weight but for some dried samples and seeds, low molecular weight fragments were also observed. Fresh tissue and seeds were a particularly good source of intact high molecular weight DNA. Of the forty samples processed using the DNeasy kit; only four samples failed to yield any visible DNA. The lack of DNA recovery from these four samples was most likely due to the starting material (poor seed development). The average yield from both fresh (100 mg) and dried (20 mg) marijuana was 125–500 ng of total DNA per sample (Fig. 2). The average DNA yield from individual seeds was also 125–500 ng of total DNA (Fig. 3).

The CTAB procedure yielded adequate DNA but was more labor intensive than the QIAGEN kit. CTAB extraction involves mechanical disruption of the plant cells by grinding in liquid nitrogen followed by lysis in heated extraction buffer (1). The lysate is extracted with chloroform-isoamyl alcohol and centrifuged. The aqueous phase is removed and isopropanol is added to precipitate the DNA. The precipitated DNA is washed several times before final resuspension. With fresh (non-dried) marijuana leaf samples, a high molecular weight fluorescent material remained in the wells of the agarose gel during electrophoresis (possibly DNA complexed with carbohydrates) (personal communication, Dr. Gary Shutler, RCMP).

The authors noted that the mechanical cell disruption step using liquid nitrogen can significantly affect the final DNA yield. It is important to grind the tissue to a fine, white powder in liquid nitrogen without allowing it to thaw. For the DNeasy kit, there is an optional centrifugation step recommended by the manufacturer after lysing the plant tissue but prior to using the QIAshredder spin column. Performing the centrifugation step significantly enhanced the quality of the marijuana DNA since it prevented shearing due to sam-

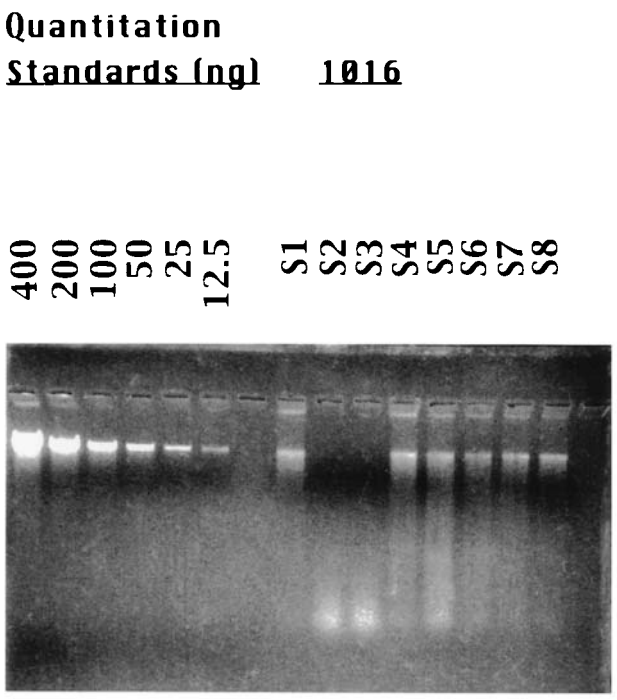

FIG. 3—*The DNA yields (10% of total DNA) from extractions (seizure #1016) of Cannabis seeds using the DNeasy extraction method are visualized on a 1% agarose gel stained with ethidium bromide. S1 through S8 are the sample designations for individual seeds. The average yield was 125–500 ng per seed.*

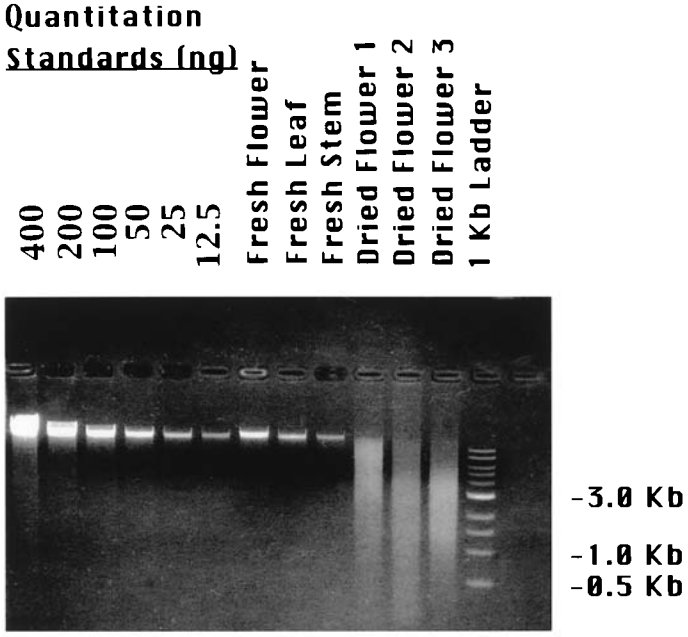

FIG. 2—*Typical DNA yields from fresh and dried Cannabis samples. Ten percent of the extracted DNA per sample was loaded per lane and visualized on a 1% agarose gel stained with ethidium bromide after DNeasy extraction. The average yield was 125–500 ng per sample.*

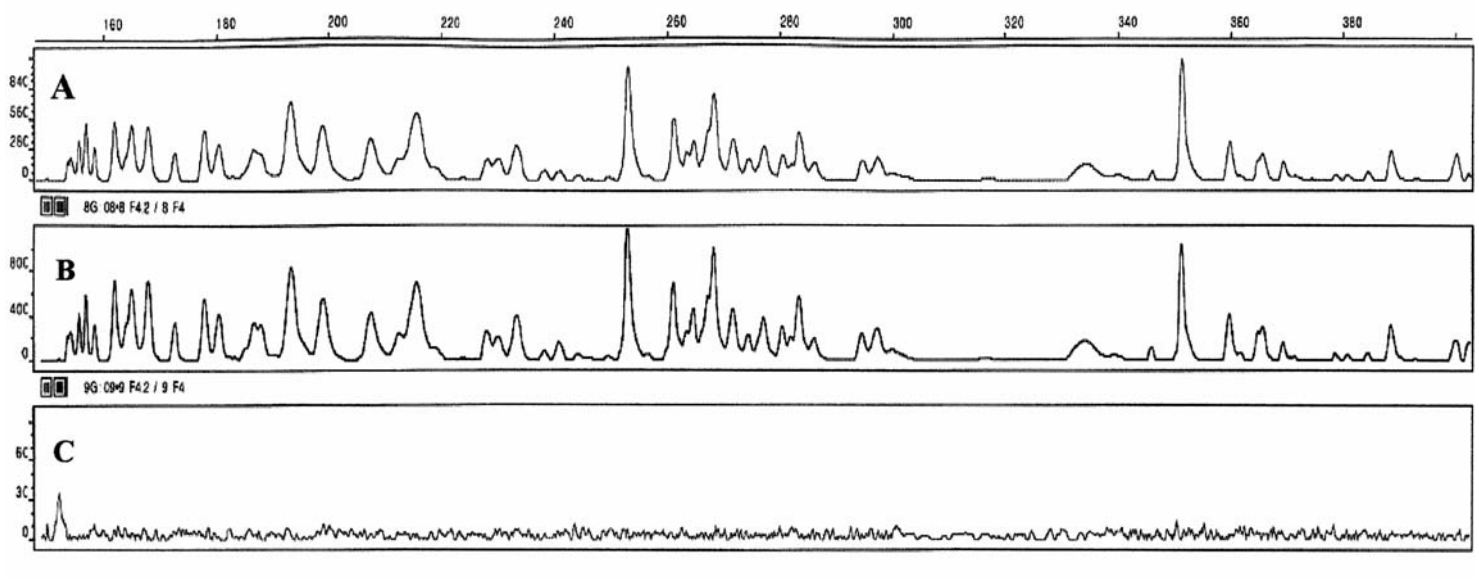

FIG. 4—*Clonal marijuana plants have identical AFLP profiles. The C. sativa variety "Redwood" clones (panel A, generation 2; panel B, generation 3) are compared after DNeasy kit extraction and AFLP analysis. AFLP analysis was performed on 10 ng of DNA from each clone using the selective PCR primers EcoR1-AAG-Joe and Mse1-CAT. Slight variations in the fluorescence intensity (y-axis, relative fluorescence units) of peaks between clones has been observed but sizing (x-axis, bases) of the peaks results in nearly exact profiles. The negative control (panel C, no DNA) exhibits no peaks.*

ple viscosity as it was pulled through the spin column (data not shown).

AFLP analysis was performed on all DNeasy extracted samples that yielded visible DNA on the agarose gel. For these samples, AFLP profiles were successfully generated. For many of these samples, a single PCR primer pair could generate an AFLP profile that distinguished between individuals. Importantly, AFLP profiles generated from clones were identical (Fig. 4) indicating a lack of DNA shearing that might prevent profile reproducibility. Duplicate extractions from the same marijuana sample also yielded reproducible AFLP profiles. The reproducibility of the AFLP profiles from separate extractions and from clonal plants indicated that the steps performed in the plant DNeasy kit do not affect the quality of the DNA for AFLP typing.

The authors are specifically interested in individualizing marijuana samples; however, this DNA extraction method may be useful for other laboratories that wish to use DNA-based identification techniques. A variety of methods are currently employed to identify *Cannabis sativa* L (marijuana). Marijuana can be identified by classical botanical characterization, especially using the type of cystolith hairs present on the leaves. However, presence of cystolith hairs is not a conclusive identification since more than eighty plant species have cystolith hair morphology similar to *Cannabis* (2). A chemical screening test, the Duquenois-Levine color test, is frequently used in combination with cystolith hair observation as a method to identify *Cannabis* (1,3,4). Marijuana can also be identified by chemical methods that test for the presence of tetrahydrocannabinol and other cannabinoids using chromatography or instrumental analysis (GC/MS) (5). Unfortunately, not all *Cannabis* samples exhibit detectable levels of cannabinoids by chromatography due to cannabinoid instability in certain solvents (16,17). An alternate but rarely used strategy relies on molecular genetics to identify *Cannabis*. *Cannabis* species identification has been achieved by cloning and sequencing the nuclear ribosomal DNA internal transcribed spacer regions (6,7). This strategy may be useful for species identification of *Cannabis* seeds and roots that lack cystolith hairs or detectable THC. Previous reports on marijuana species identification by molecular methods have used the CTAB procedure for DNA extractions (6,7). The QIAGEN method described here is significantly faster and easier for obtaining marijuana DNA than the CTAB procedure.

In summary, we have used the plant DNeasy kit from QIAGEN to efficiently process marijuana leaf and seed samples and obtained PCR-quality DNA. Our goal is to develop an easy, reproducible method for individualizing marijuana samples for law enforcement purposes. Identification of a reliable, convenient method for marijuana DNA extraction is the first step toward achieving that goal. The DNeasy kit represents a distinct improvement over the CTAB extraction method. It requires a small amount of sample, has a high recovery rate, and eliminates the need for solvents or time-consuming ethanol precipitation steps. In addition, the DNeasy kit removes many of the PCR inhibitory compounds found in plant samples during the extraction steps thus preventing additional purification steps without loss of quality or yield.

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