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An Overview of DNA Methods for the Identification and Individualization of Marijuana

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The purpose of this review is to summarize the status of DNA-based methods for the identification and individualization of marijuana. In forensics, both identification of a substance as marijuana and the subsequent individualization of a sample may be desired for casework. Marijuana identification methods in the United States primarily include biochemical tests and, less frequently, DNA-based tests. Under special circumstances, DNA-based tests can be useful. For example, if the quantity of seized marijuana is extremely small and/or biochemical tests do not detect any Δ9-tetrahydrocannabinol (THC), DNA identification of plant material as *Cannabis* is still possible. This circumstance can arise when seeds, trace residue, tiny leaf fragments, or fine roots need to be analyzed. Methods for the individualization of marijuana include amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), and short tandem repeat (STR) techniques that link an evidentiary sample to a source. Marijuana growers propagate their plants either by seed or by cloning. Seed-generated marijuana plants are expected to have unique DNA profiles analogous to a human population. Cloned marijuana plants, however, exhibit identical DNA profiles that allow for tracking of plant material derived from a common genetic lineage. The authors have validated the AFLP method for marijuana samples and are constructing a comparative database of marijuana seizure samples to estimate the expected frequency of a DNA profile match between unrelated plants. Continued development of DNA-based methods for plants can be useful for marijuana and other types of plant evidence in forensics.

Key words: *cannabis; polymorphism (genetics); polymorphism, restriction fragment length; random amplified polymorphic DNA technique; tandem repeat sequences*

Cannabis sativa (marijuana) has existed since ancient times and is widely used as a fiber source, a food source, a medicine, and a euphoriant (1-4). Marijuana has been used to treat a variety of ailments, including glaucoma, pain, nausea, asthma, depression, neuralgia, and insomnia (5). Like many crops (e.g., wheat, corn), marijuana was originally a naturally occurring weed species. It was bred and cultivated into a significant cash crop for a multi-billion dollar illicit industry. The hallucinogenic properties of marijuana are derived from Δ9-tetrahydrocannabinol (THC) (6) and potent (high-THC level) marijuana cultivars are sought by the discerning marijuana users (7,8). Before the mid-1970's, the majority of U.S. marijuana was imported from Mexico. When the U.S. and Mexico co-operated in marijuana eradication programs, a domestic growing industry began in the United States. Most of the highly prized United States cultivars originated from a few breeding stocks from the West Coast (5). Now, seeds for marijuana growers are accessible worldwide via the Internet; seed catalogues are posted with extensive descriptions and price lists (e.g., *www.cannabisseeds.cc*). The extent of genetic variation in marijuana populations is unknown due to the illicit nature of this substance and because growers propagate plants secretively. A survey of marijuana seizure samples using DNA profiling methods could be used to assess levels of genetic diversity within this crop.

There are two main steps in most forensic classification schemes that can be applied to marijuana seizure samples. The first step involves identification of a sample. For marijuana, both biochemical (9,10) and DNA tests (11,12) are available to identify a substance as *Cannabis*. The second step is individualization (source attribution). For marijuana, several DNA-based methods are under development and will be described in later sections. Biochemical methods to establish geographic origin of a plant have met with variable success (13-17). However, contaminants (18) and packaging (17) have shown a correlation with marijuana source. Biochemical profiling has also successfully differentiated between resinous and textile *Cannabis* (19), drug subgroups (marijuana, sinsemilla, Thai sticks, ditchweed) (7) and plant gender (20).

Cannabis can be seed-propagated or perpetuated through cloning (1,21,22). Seed-propagated plants are expected to have their own unique genotypes analogous to humans selected from a random population. However, plants that have been propagated through cloning should have identical genotypes like identical twins (21,22). Tracking cloned marijuana based on DNA should be relatively simple; seizure samples with identical profiles should have a common genetic source. The ability to link marijuana growers and users to a common distributor by DNA would be a useful investigative tool for narcotics enforcement. In addition, some forensic cases may be able to link a suspect and victim by matching marijuana samples. The Connecticut State Forensic Science Laboratory, along with several other research groups, is in the process of developing DNA-based methods for the individualization of plant (especially marijuana) samples that are seized from crime scenes. Different DNA-based techniques have different applications, benefits and limitations but all can be utilized to supplement existing forensic methods.

Marijuana Drug Facts

United States teenagers use marijuana more than any other drug according to the U. S. Government Substance Abuse and Mental Health Services Administration (23,24). For example 20% of teenagers aged 12 to 17 years have used marijuana at least once. In comparison, only 3% of teenagers have used Ecstasy and approximately 2% reported using cocaine (23,24). Marijuana prices vary depending on the quantity and quality of what is sold and where the consumer is geographically located, however; it is estimated that marijuana is a multi-billion dollar industry in the United States. One primary source of marijuana is from Mexico where the Border Patrol and U. S. Customs Service seize tons of marijuana worth millions of dollars every year (24). In addition to imported marijuana, the U.S. has a very profitable domestic marijuana growing industry (1,5,24,25).

According to the 2001 National Forensic Laboratory Information System (NFLIS) report, 36% of the analyzed drug items at the national level were identified as *Cannabis* compared to 33% as cocaine, 11% as methamphetamine and 8% as heroin, respectively (26). Considerable variation exists in drug types reported across different regions of the United States; it should be noted that these differences could result from different law enforcement strategies or laboratory analysis policies. In general, *Cannabis* is identified in 25% or more of the drug seizures for the United States regardless of geographical region. In 2001, *Cannabis* estimates for the Midwest, the Northeast, the South, and the West were 47%, 36%, 36%, and 23%, respectively (26).

In 1977, regional narcotics enforcement squads were replaced by a Statewide Narcotics Task Force in Connecticut (27). The Task Force is authorized to enforce the state laws concerning the manufacture, distribution, sale, and possession of narcotics and controlled substances. In addition to enforcement, the Connecticut Statewide Narcotics Task Force collects and provides information regarding drug seizures for Connecticut on an annual basis (25,27). Both indoor and outdoor marijuana grow operations have been identified in Connecticut (25,27). The outdoor grow season in Connecticut begins in April and continues until harvest time in mid-September. The indoor grow season is year-round. The Statewide Narcotics Task Force, in conjunction with the Drug Enforcement Administration (DEA), sponsors and coordinates the Domestic Cannabis Eradication/Suppression Program in Connecticut (25,27). For the first nine months of 2002, statistics for the Statewide Narcotics Task Force domestic *Cannabis* eradication program indicated while greater numbers of outdoor plots were identified compared to indoor grow operations, the number of plants seized were comparable between indoor and outdoor cultivation plots (Table 1) (27). Indoor grow operations may be increasing in number and scale or are more easily detected based on a comparison of data from the years 1999-2002 (Table 1) (27). According to Connecticut statistics for 2002, marijuana distribution and consumption has steadily increased and the demand for high quality hydroponically grown marijuana has also increased despite the greater cost to the consumer (25,27). Marijuana cultivated in Connecticut represents a small fraction of the amount consumed by it's state residents. The majority of consumed marijuana is imported in from California, Texas and Mexico (25,27).

Table 1. Statistics of the Statewide Narcotics Task Force Cannabis Eradication Program for the 1999-2002 period

	Year			
Cultivation	1999	2000	2001	2002
Outdoor				
No. of plots	62	34	32	62
No. of plants	4,606	1,208	1,191	1,772
Indoor				
No. of plots	5	11	2	17
No. of plants	36	333	129	1,117

Although, Connecticut is a relatively small marijuana producing state, marijuana usage still continues to be a substantial drug problem. Based on statistics from the Connecticut Department of Public Safety Controlled Substances and Toxicology Laboratory, the percentage of reported marijuana for the past three years (2000-2002) has remained stable (approximately 27%) (28). Reported marijuana drug items are only exceeded by cocaine which averages 35% of the total drug items reported (28). The majority of *Cannabis* items reported by the Laboratory for 2000-2002 are from four of nine Connecticut counties (Waterbury, New Haven, Hartford, and Fairfield) (28). However, marijuana drug items have been identified and seized from all areas of Connecticut (28). The majority of analyzed drug items reported by the Laboratory are comprised of a single identifiable drug substance; less than 1.5% of drug items were reported as drug mixtures (28).

Marijuana Identification

Identifying a plant sample as *Cannabis sativa* is the first step in determining if an illegal substance has

been seized. Methods for the identification of marijuana include: botanical identification through inspection of the intact plant morphology and growth habit (1,2), microscopical examination of leaves for the presence of cystolith hairs (29-31), chemical screening tests such as the Duquenois-Levine test (32-34), THC identification through biochemical methods (10,19,33,35,36), and the use of molecular sequencing to identify DNA sequence homology to reference marijuana samples (11,12).

Biochemical Tests

Biochemical testing is the most common method for identifying plant material as marijuana. Chemical tests include those developed by Duquenois and other modifications of the original Duquenois test (32-34). Other chemical tests are the Rutgers Identification for Marijuana (RIM) technique and use of gas liquid chromatography (GLC) and high-pressure liquid chromatography (HPLC) to identify cannabinoid compounds (10,15-17,19,35,36). Occasionally, some marijuana samples can't be identified through chemical means because little or no THC is present. Such situations include seizures of seeds not associated with marijuana plant leaves and cases where the plants have been harvested but the roots have been left at the crime scene. In these situations, DNA testing can provide a means for marijuana identification that would otherwise not be possible.

DNA Tests

Although three forms of DNA are present in plant cells (mitochondrial, chloroplast and nuclear), nuclear DNA sequences are most commonly used for plant species identification. DNA-based tests for the identification of marijuana include the molecular analysis of the ITS1, ITS2 and *trn*L intron (11,12, 63,64). A comparison of the ITS1 and ITS2 polymerase chain reaction (PCR) product sizes in five samples of marijuana and in one sample of a close relative (*Humulus lupulus*) revealed a size difference between marijuana and *Humulus* for the ITS2 region (11,12). Other tests using PCR amplification and subsequent restriction enzyme digestion of the trnL region of the chloroplast has shown that marijuana DNA profiles can be generated and compared between samples and may be useful for forensic purposes (11,12).

Marijuana Individualization

After a forensic sample has been identified and classified, it becomes important to individualize the sample. Individualization of a sample in a forensic context means to establish a linkage between the evidentiary sample and the source (Fig. 1). There are several ways that plant samples can be tested by using DNA-based methodologies in forensics: randomly amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), and short tandem repeats (STRs) (Table 2).

Randomly Amplified Polymorphic DNAs

Randomly amplified polymorphic DNA markers are generated in a single standard PCR reaction where the PCR primers consist of random sequences (typically oligomers of 10-15 bases in length). Wherever a PCR primer has sequence homology with the DNA template, it will bind and a PCR product will be formed. The PCR products are of variable size and are separated on a 1% agarose gel and stained with ethidium bromide for detection of the band pattern. No a priori knowledge of an organism's sequence is required to perform randomly amplified polymorphic DNA analysis; however, the PCR amplification conditions must be held constant to generate consistent band patterns. Randomly amplified polymorphic DNA marker analysis requires a single source sample for simple interpretation of band patterns. The method has been used and accepted in court for both criminal and civil cases (37,38). One well-documented plant DNA case involved the use of DNA profiles from *Palo verde* seed pods to link a suspect's vehicle back to a homicide crime scene (37). In the *Palo verde* case, the DNA results were allowed in court but the statistical significance was not used because the representative population database consisted of too few samples (40 plants). While randomly amplified polymorphic DNA marker analysis is inexpensive and simple to perform, the method has suffered from reproducibility problems between laboratories (39). The reproducibility problems may be attributed to differences in thermal cycler ramp speeds that can affect

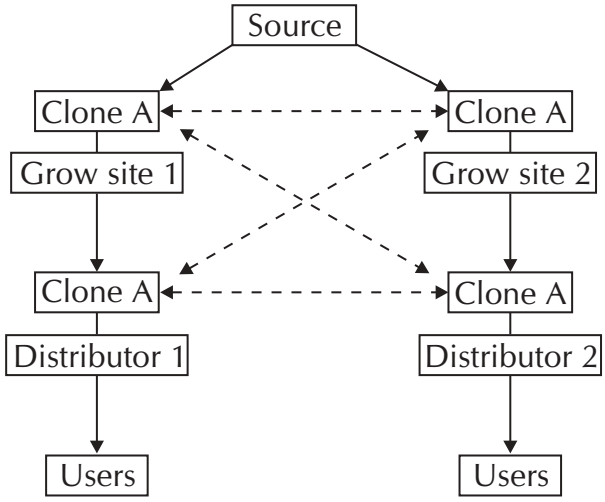

Figure 1. Potential forensic linkages based on individualized marijuana sample information. Growers sharing cloned plants can be associated based on identical amplified fragment length polymorphism (AFLP) profiles. Growers may be linked to major marijuana distributors and marijuana user seizure material may be traced back to a common distributor of clonal marijuana.

Table 2. A comparison of three DNA methods for the individualization of plant samples*

Method	Discrimination power	Relative cost	Input DNA
RAPDs	moderate†	low	1-10 ng
AFLPs	high	moderate-high	1-10 ng
STRs	high	moderate-high	1-10 ng

*Abbreviations: RAPD – random amplified polymorphic DNA; AFLP – amplified fragment length polymorphism; STR – short tandem repeat.
†Ability to distinguish between unrelated individuals.

PCR primer binding to target DNA sequences. In addition, faint bands on agarose gels can be scored differently due to differences in visual assessment between analysts during the detection step (39).

Amplified Fragment Length Polymorphisms

Amplified fragment length polymorphism markers have been used to distinguish between individuals of many species including plants (40-49), insects (50,51), birds (52), fish (53) and bacteria (54-56). These markers are particularly useful for separating closely related individuals from inbred genetic lines (57) and on any single source sample. Amplified fragment length polymorphism analysis requires the PCR amplification of restriction fragments to which adaptor oligomer sequences have been attached. The PCR primers recognize the adaptor oligomers and bind to amplify different sized DNA fragments to generate a band pattern. The DNA fragments are detected with a DNA sequencer. The sequencer has a laser that will excite the fluorescent dye that was incorporated into the DNA fragments during the PCR amplification step. Labeled DNA fragments are captured by a CCD camera as they pass by the laser and the band patterns are recorded by a computer. Computer analysis software is used to aid in interpretation and scoring of the complex band patterns generated by amplified fragment length polymorphisms. Since the extent of genetic diversity is unknown in current marijuana seizure populations, the development and validation of a marker system, such as amplified fragment length polymorphisms, that has a high power of discrimination for closely related individuals is necessary. While the procedure is more complicated than randomly amplified polymorphic DNA or STR analyses, the process utilizes the same equipment and computer analysis software as current STR human identification methods. This means the cost to implement amplified fragment length polymorphisms is minimal for most forensic laboratories with the exception of the amplified fragment length polymorphisms database generation for comparative purposes. Validation of the amplified fragment length polymorphisms method for marijuana samples is complete (21,22) and our analyses of cloned marijuana (courtesy of Dr. Gary Shutler, Royal Canadian Mounted Police) has shown that clonal amplified fragment length polymorphisms profiles are highly reproducible (Fig. 2). In contrast, amplified fragment length polymorphisms profiles from unrelated marijuana plants are easily distinguishable from each other using this method (Fig. 3).

Short Tandem Repeats

Short tandem repeat (STR) sequences refer to repetitive elements found within nuclear DNA that are variable between individuals. The variability in the number of repeated sequences makes these elements useful for distinguishing between individuals of a population. Typically, STR analysis requires a PCR reaction using PCR primers of specific sequence that will bind and recognize a previously characterized site within the nuclear DNA. Short tandem repeat markers are the most common DNA-based method for human identity testing and these sequences are found in many organisms including plants (58,59).

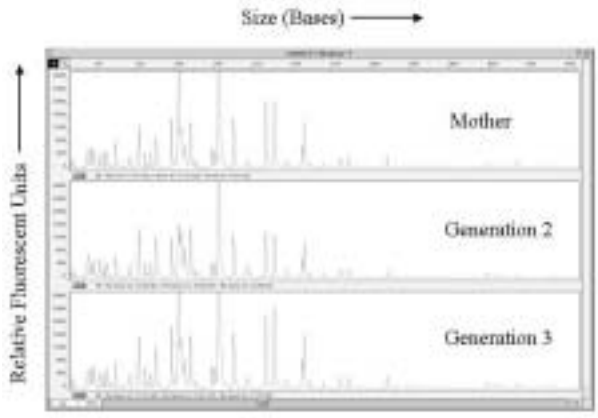

Figure 2. Amplified fragment length polymorphism (AFLP) analysis of known clonal generations exhibit identical DNA profiles. Known clonal marijuana generations were propagated by the Royal Canadian Mounted Police (RCMP, Winnipeg) and were generously provided through collaboration with Dr. Gary Shutler.

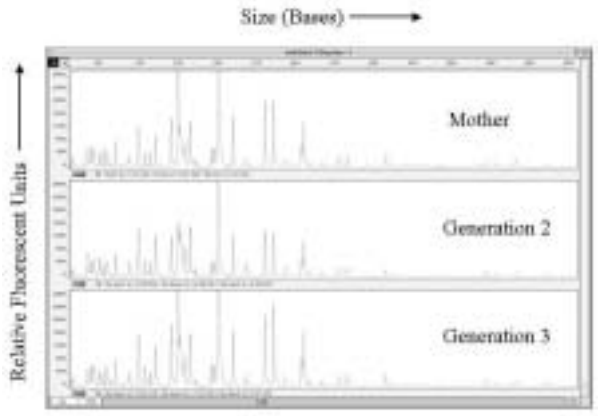

Figure 3. Marijuana samples from unrelated cases have distinct amplified fragment length polymorphism (AFLP) profile differences. Samples #1-3 were generously provided from adjudicated cases by Dr. Eric Buel (Vermont Crime Laboratory).

STRs can be used with mixtures, ie, DNA samples from more than one source.

A few polymorphic loci have been recently identified in *Cannabis sativa* (58-61). One study identifies eleven loci that were screened through a blind test of 40 samples to confirm the reproducibility and accuracy of scoring of these candidate loci (61). This same study showed 100% concordance with our amplified fragment length polymorphisms test results. Another study describes the isolation of a single hexanucleotide repeat sequence in marijuana that was highly polymorphic when screened in a population of 108 marijuana evidentiary samples (59). A third study describes the isolation of ten STRs that were screened against a world-wide population of 255 individuals representing 33 countries (60). Five additional STR markers have been described for *Cannabis* and used to screen 93 marijuana individuals that represent drug and fiber accessions (58).

Although STR markers can be identified in plants, there is significant development and validation time required in establishing this form of testing. For example, genetic mapping to illustrate non-linkage between STR loci is needed for statistical reasons. These candidate marijuana STR markers have only recently been identified, which is the reason why the following experiments for STR loci have not yet been performed:

a) physical mapping to chromosomes;

b) tests for locus independence;

c) typing a core set of population samples for a direct comparison of candidate loci ability to discriminate between individuals;

d) estimation of the extent of inbreeding in various populations; and

e) multiplexing of loci for increased power of discrimination, sample through-put, conservation of evidence, and user convenience in a single PCR amplification reaction.

It is anticipated that these types of developmental and additional validation experiments will be performed prior to adoption for forensic casework. STR testing is recognized and accepted as a valid form of DNA testing in United States courts and is extremely useful for mixed samples. The STR loci identified in marijuana should be very useful in the future for establishing forensic linkages between source and evidentiary samples.

Comparative Databases

In order to give significance to the meaning of a random match, comparative databases need to be constructed. When constructing such databases, it is important to consider the sampling strategy and the final purpose of the database. If estimating the level of genetic diversity for evolutionary purposes, a wide distribution of genetically distinct individuals can be screened. If determining a random match probability for marijuana seizure samples, it is important to have a database of seizure sample profiles for comparison. To date, one of the great difficulties in developing tests to individualize marijuana has been acquiring access to adequate numbers of marijuana samples. Nationwide (U.S.) and Connecticut State marijuana databases are under construction (62) and may be used for both establishing the extent of genetic diversity within and between seizure samples and for estimating the expected frequency of a random DNA match.

Conclusion

In the near future, marijuana DNA analysis may be performed in conjunction with chemical identification methods to extend the current capabilities for casework identification on root and seed samples of marijuana. The ability to individualize marijuana samples will further extend the role of DNA in establishing forensic linkages by using plant evidence to link homicides and other types of cases where marijuana samples may be present. The individualizing techniques being developed for marijuana may allow for the identification of a geographic source to aid in the investigation of major marijuana growers and distributors. In particular, cloned marijuana networks may be easily tracked and distributors identified through the common DNA profiles of the seizure samples (21,22,65). In addition, since marijuana samples and drug-generated funds are associated with a wide variety of criminal activities, the applications for marijuana DNA-based tests extend far beyond the obvious use for narcotics enforcement. The success of marijuana DNA typing methods could also become the foundation for using other forms of botanical evidence (grass or tree species) in criminal and civil casework (63-65).

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