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TECHNICAL NOTE

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Genetic Variation in Hemp and Marijuana (*Cannabis sativa* L.) According to Amplified Fragment Length Polymorphisms*

ABSTRACT: *Cannabis sativa* L. (Cannabaceae) is one of the earliest known cultivated plants and is important in the global economy today as a licit and an illicit crop. Molecular markers distinguishing licit and illicit cultivars have forensic utility, but no direct comparison of hemp and marijuana amplified fragment length polymorphism (AFLP) has been made to date. Genetic variation was surveyed in three populations of fiber hemp and a potent cultivar of marijuana using AFLP markers. Ten primer pairs yielded 1206 bands, of which 88% were polymorphic. Eighteen bands represented fixed differences between all fiber populations and the drug cultivar. These markers have practical utility for (1) establishing conspiracy in the cultivation and distribution of marijuana, (2) identifying geographic sources of seized drugs, and (3) discriminating illegal, potent marijuana cultivars from hemp where the cultivation of industrial hemp is permitted.

KEYWORDS: forensic science, DNA typing, amplified fragment length polymorphism, analysis of molecular variance, heterozygosity, DNA fingerprinting, sex linkage

Marijuana (*Cannabis sativa* L.) is the most abundant illegal drug of abuse in the United States, and although authorities seized more than 1200 metric tons in 2001, marijuana trafficking today is a thriving, multibillion dollar industry. Drug enforcement requires forensic tools that provide evidence for conspiracy in the cultivation and distribution of marijuana. The capacity to pinpoint the geographic origins of seized drugs and to identify *Cannabis* cultivars has further forensic utility. *Cannabis* is one of the oldest known domesticated plants and today is cultivated throughout the world for psychoactive cannabinoids, durable fiber, and nutritious seed. All *Cannabis* plants are controlled substances in the United States despite substantial variation in drug content. *Cannabis* can be separated into psychoactive and nonpsychoactive cultivars according to the ratio of Δ^9-tetrahydrocannabinol (THC), the primary psychoactive agent, and cannabidiol (CBD) (1). Hemp plants have a relatively low THC:CBD ratio compared with marijuana (2,3). Recent studies suggest that THC and CBD are derived from a common precursor, cannabigerol (4), and that the THC:CBD ratio might be controlled by a single gene affecting cannabinoid biosynthesis (5).

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Apart from differences in chemotype, hemp and marijuana are quite difficult to separate on morphological grounds. When marijuana was outlawed in the United States in 1937 (6,7), it was necessary to ban the domestic cultivation of hemp, at that time an economically important fiber crop (8). Drug enforcement efforts could benefit from DNA fingerprinting technology capable of (a) separating marijuana from hemp, (b) pinpointing geographic sources, and (c) establishing conspiracy in illicit distribution networks (9).

Recent studies identified DNA sequences that distinguish the genus *Cannabis* from its nearest relative, the genus *Humulus* (hops) (10,11), and intraspecific molecular markers for *Cannabis* have been developed. Surveys of genetic variation in *Cannabis* include randomly amplified polymorphic DNA (RAPD) (12,13), microsatellites (11,14), and amplified fragment length polymorphisms (AFLP) (15–17). RAPD markers led to the identification of a sequence-characterized, amplified region (SCAR) putatively associated with drug content (5), and AFLP markers have been used to fingerprint marijuana plants in forensic investigations (15,18). Molecular markers distinguishing cultivars have forensic utility, but a recent study using the marker of choice for human DNA, short tandem repeats (STR), failed to separate drug and fiber strains unequivocally (9).

This paper reports on the extent of AFLP differentiation between nonpsychoactive cultivars and a potent marijuana cultivar. Genetic markers separating hemp and marijuana have practical utility for drug enforcement in Canada and Europe, where hemp cultivation is permitted but marijuana cultivation is not. In the United States, where all *Cannabis* plants are controlled substances, the technique has the potential to connect marijuana samples with source populations. The repeatability of *Cannabis* AFLP profiles was also examined, an important consideration for the application of this technology in criminal casework.

Methods

Seeds of the drug variety Skunk #1 (HortaPharm B.V., Amsterdam, The Netherlands) and the hemp variety Carmen (Kenex Ltd., Chatham, Ontario, Canada) were imported under permit from the United States Drug Enforcement Administration. Thirteen Carmen plants from stock seed, six Carmen plants after a generation of inbreeding, and 12 Skunk #1 plants after a generation of inbreeding were screened for AFLP variation. In addition, seeds from two naturalized hemp populations were collected in Shakopee and Minneapolis, Minnesota. Seeds were germinated in the laboratory, and 14 plants were sampled from each population. Drug content expressed as percent THC per unit dry weight was approximately 7% in Skunk #1 (Table 1). Carmen had low-THC content (0.03% dry weight), as did Minnesota hemp ($<0.1\%$ dry weight).

DNA was extracted from primary leaves of young seedlings 2 weeks after germination and from dried inflorescences 60–160 days after harvesting. Extractions were performed using the DNeasy Plant Extraction kit (Qiagen, Valencia, CA) with genomic DNA eluted in a total volume of 120–150 μL AE buffer. DNA extracts were quantified in a Turner Quantech Fluorometer (Barnstead International, Dubuque, IA) using PicoGreen dsDNA quantitation reagent (Invitrogen, Euguene, OR). For AFLP analysis, restriction–ligation reactions (19) were performed using 30 ng of genomic DNA, × 1 DNA ligase buffer, 0.63 μg bovine serum albumin , 27 mM NaCl, 4.5 μM *Mse*I adaptor, 0.45 μM *Eco*RI adaptor, 3 U *Eco*RI, 0.6 U *Mse*I, and 0.6 U T4 DNA ligase. Each reaction was mixed, incubated for 2 h at 37°C in a thermal cycler, and held indefinitely at 4°C. Following restriction–ligation, reactions were diluted by a factor of 10 in $TE_{0.1}$. Preselective amplification reactions were performed with 9 μL AFLP amplification core mix (Applied Biosystems, Foster City, CA), 0.5 μM each primer, and 2.4 μL diluted restriction–ligation product for a total reaction volume of 12 μL. Ten primer pairs were selected from the 64 included in the AFLP kit (Applied Biosystems) following a preliminary survey of *Cannabis* AFLP variation. Ten pairs that yielded the greatest variability in preliminary screening included *Eco*RI-AAC—*Mse*I-CTT, *Eco*RI-ACA—*Mse*I-CAG, *Eco*RI-ACT—*Mse*I-CAT, *Eco*RI-ACT—*Mse*I-CTT, *Eco*RI-AGC—*Mse*I-CAA, *Eco*RI-AGC—*Mse*I-CTG, *Eco*RI-AGG—*Mse*I-CAA, *Eco*RI-AGG—*Mse*I-AAC, *Eco*RI-AGG—*Mse*I-CTA, and *Eco*RI-AGG—*Mse*I-CTT. Thermal cycling conditions for preselective amplification reactions were as follows: 94°C for 2 min, 20 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 2 min, followed by a 4°C hold. Preselective amplification products were then diluted by a factor of 20 with $TE_{0.1}$ before selective amplification. Selective amplifications were performed in 6 μL reaction volumes with 4.5 μL AFLP amplification core mix, fluorescently labeled *Eco*RI primers with three selective bases (0.01 μM), and unlabeled *Mse*I primers with three selective bases (0.25 μM), and 0.9 μL diluted preselective amplification product. Stepdown PCR conditions were as follows: initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing for 30 sec (first 10 cycles with annealing temperatures from 65 to 56°C with a -1°C step per cycle, followed by 23 cycles at 56°C), and extension at 72°C for 2 min, followed by a final extension for 30 min at 72°C, and an indefinite hold at 4°C. Fluorescent-labeled AFLP products were separated using an ABI 377 DNA sequencer (Applied Biosystems) equipped with GeneScan software. Selective amplification products (0.5 μL) were mixed with ROX-labeled size standard (0.5 μL; fragments ranging 50–500 bases in 25 bp intervals; The Gel Company, San Fransisco, CA) and formamide loading dye (1 μL). Electropherograms were sized in Genescan (Applied Biosystems) and exported to Genotyper® software where band profiles of a given length measured in base pairs were scored for presence or absence. Bands were considered different if they differed by ≥ 1 bp. Differences between bands that were considered to the same averaged 0.21 bp and ranged from 0 to 0.87 bp.

TABLE 1—*Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) content in four* Cannabis *cultivars.*

	% THC		% CBD		
	X (+SD)	Range	*X* (+SD)	Range	*N*
Skunk #1	7.08 (2.92)	3.56–12.32	0.02 (0.01)	0.01–0.03	9
Carmen	0.03 (0.03)	0.01–0.12	0.66 (0.82)	0.13–2.61	15
Shakopee	0.07 (0.03)	0.03–0.12	0.92 (0.14)	0.59–1.08	14
Minneapolis	0.11 (0.12)	0.04–0.41	1.39 (0.14)	1.13–1.61	14

The percentage of total dry weight for each compound in mature female inflorescences as measured by gas chromatography is reported. The mean and standard deviation, *X* (+SD), range, and the number of individuals sampled, *N*, is listed for each population.

Statistics

The repeatability of AFLP profiles was examined by estimating error associated with (a) restriction–ligation, (b) preselective amplification, and (c) selective amplification from *Cannabis* DNA accessions fingerprinted with a single primer pair (*Eco*RI-ACT—*Mse*I-CAT). A nested sampling design was employed, consisting of two replicate restriction–ligations, two preselective amplifications per restriction–ligation, and two selective amplifications per preselective amplification, for a total of eight replicates per accession. Banding profiles were compared between replicates and a standard for each of five DNA accessions. The number of mismatches between each replicate and the standard was calculated, where mismatches consisted of bands present in the standard but not in a replicate or vice versa. Calculations were performed with three different band intensity thresholds, the default value of 50 relative fluorescence units (RFU), and more stringent thresholds of 100 and 150 RFU. A nested analysis of molecular variance (AMOVA) was performed with restriction–ligation, preselective amplification, and selective amplification as factors to test for sources of significant variation in the AFLP procedure.

Genetic variation within and among lines was quantified in several ways. Fixed genetic differences based on diagnostic AFLP markers separating drug and fiber hemp populations were identified for Skunk #1 vs. Carmen, and Skunk #1 vs. all hemp plants including the two Minnesota populations. The total number of loci, the percentage of polymorphic loci, and heterozygosity for each population were also measured. All loci occurring in only a single individual and all markers at a frequency $\geq 95\%$ were excluded from these calculations based on the recommendation of Lynch and Milligan (20). Heterozygosity was calculated from phenotypic and genotypic frequencies under the assumption of Hardy–Weinberg equilibrium using the computer program Tools For Population Genetic Analyses version 1.3 (TFPGA, M. Miller, personal communication). AMOVA (21) was performed using Arlequin version 2.0 (22). AMOVA partitions genetic diversity within and among populations to estimate the extent of differentiation. A principal coordinates analysis (PCA) was used to graphically depict genetic variation among populations. A similarity matrix was constructed from the Dice coefficient (23) and PCA was performed using the program Numerical Taxonomy System (NTSYS-PC) Version 2.0 (24).

Results and Discussion

AFLP Repeatability

The use of AFLP in plants has grown in popularity for at least two reasons. First, AFLP markers detect more bands per unit effort than RAPD, intersimple sequence repeats (ISSR) and microsatellites (25). Second, AFLP are more highly repeatable than RAPD and ISSR markers (26). Repeatability is especially important for criminal investigations where the object is to gather evidence that is unequivocal and admissible in court (27). Studies of AFLP repeatability have focused on the selective amplification step (25) whereas factors such as DNA concentration and quality almost certainly influence band detection at the restriction–ligation and preselective amplification steps as well. This study investigated potential sources of variability at each step in the AFLP procedure and found preselective amplification to be a significant source of inconsistency in AFLP profiles under standard conditions (Table 2). Preselective amplification accounted for more variation than either restriction–ligation or selective amplification steps. Replication of the AFLP procedure with the same DNA accession at a default band intensity threshold of 50 RFU produced an average of 10.8 inconsistencies per 100 bands, yielding an error rate of 11%. This is the first report of a procedural error rate for *Cannabis* AFLP, a necessary legal consideration for the admissibility of AFLP evidence in court. Inconsistency in band scoring was associated with low-intensity bands. The mean $\pm$ standard deviation in peak height for inconsistent bands was 276 ± 292, whereas the mean peak height for consistent bands was 838 ± 564. Additionally, 95% of the bands showing inconsistencies had average peak intensities less than 500 RFU. Inconsistency could result from probabilistic differences in the abundance of rare fragments propagated during preselective amplification. It is therefore important to standardize the quantity of genomic DNA when the AFLP procedure is used in forensic investigations. The influence of DNA quality on AFLP profiles also deserves consideration given that seized marijuana could be old and degraded compared with fresh tissue.

AFLP profiles from three different primer pairs were compared between fresh leaves and dried mature inflorescences taken from each of four individuals. The percentage of band mismatches between fresh leaves and mature inflorescences stored 60–150 days under dry conditions at room temperature (12%) was not significantly different from replicated fresh leaf extractions (paired *t*-test; $t = 1.10$, $\mathrm{df} = 11$, $p = 0.29$). These findings corroborate a recent report that tissue type and the presence of cannabinoid resins do not affect AFLP profiles (15). Although the condition of plant material appears not to influence the fingerprint, inconsistency inherent in the procedure requires that multiple markers from different primer pairs be employed in forensic situations. The probability that two samples could be attributed to a common source in error is a function of the number of independent markers and the size of the population sampled. Another way to reduce inconsistency is to increase band intensity thresholds at the expense of some potentially informative markers. The overall error rate dropped to 8% when the band intensity threshold was raised from 50 to 100 RFU, while the number of fixed differences between cultivars dropped by half. There was, however, no significant improvement in error rate when the threshold was raised from 100 to 150 RFU (paired *t*-test; $t = 1.56$, $\mathrm{df} = 39$, $p = 0.13$). Analyses of genetic variation were performed at the intermediate threshold of 100 RFU to minimize inconsistency associated with the AFLP procedure while maximizing the number of markers.

Repeatability is especially important for criminal investigations of the expanding trade in clonally propagated marijuana. Drugs seized at multiple locations with matching AFLP profiles can be linked in a conspiracy because plants propagated by this method are genetically identical (28). Results indicate that AFLP profiles drawn from the same DNA source will be more than 90% similar because of a degree of inconsistency associated with AFLP detection. At first glance it would appear that AFLP lack forensic utility if identical genotypes are only 90% similar by this procedure. However, analyses of genetic variation demonstrate that the extent of AFLP differentiation between individual plants and between cultivars exceeds the procedural error rate.

Genetic Variation

Cannabis cultivars exhibit considerable morphological, chemical, and genetic variation within and among populations (3,12,13,29). Molecular genetic variation is especially useful in a forensic setting, where markers are needed to unequivocally separate licit and illicit cultivars or to attribute multiple drug seizures to a common source. This study is the first comparison of AFLP variation among *Cannabis* cultivars differing markedly in drug content. Fixed genetic differences between marijuana and nonpsychoactive hemp were identified, demonstrating the ability of AFLP to separate cultivars. Using 10 primer pairs, 1206 bands were detected, 52 of which were singletons and 178 had a frequency $\geq 95\%$. The remaining 976 polymorphic bands (88% of the total) yielded 47 fixed differences between Carmen and

TABLE 2—*Analysis of molecular variance in repeated amplified fragment length polymorphism (AFLP) profiles obtained from the same DNA sample.*

Source	df	50 RFU			100 RFU			150 RFU		
		Variance	*F*	%	Variance	*F*	%	Variance	*F*	%
Restriction–ligation	1	0.23	0.12	0.1	0.40	0.02	0.6	3.02	0.23	6.4
Preselective	1	112.23	6.21*	55.5	19.60	0.78	30.9	24.02	1.84	50.9
Selective	1	2.03	0.11	1.0	1.60	0.06	2.5	0.62	0.05	1.31
RL × PS	1	65.03	3.60	32.2	14.40	0.57	22.7	0.02	0.15	0.04
RL × S	1	1.23	0.07	0.6	1.60	0.06	2.5	5.62	0.43	11.9
PS × S	1	0.23	0.01	0.1	0.40	0.02	0.6	0.62	0.05	1.3
RL × PS × S	1	3.03	0.17	1.5	0.40	0.02	0.6	0.22	0.02	0.5
Residual	32	578.0		8.9	25.10		39.5	13.02		27.6

A nested experimental design examined inconsistency arising from each step of the AFLP procedure at two band intensity thresholds, the standard 50 relative fluorescence units (RFU) and more stringent thresholds of 100 and 150 RFU. Degrees of freedom (df), the test statistic (*F*), and the percentage of the total variance explained by each step in the procedure are shown with * indicating significance at $p < 0.05$. There were no significant interaction terms for restriction–ligation (RL), preselective amplification (PS), and selective amplification (S).

TABLE 3—*Genetic variation in four Cannabis cultivars.*

	N	*B*	*P*	H_g
Skunk #1	12	804	29.9	0.121
Carmen	13	705	53.7	0.203
Inbred Carmen	6	593	40.5	0.135
Shakopee	14	744	60.2	0.223
Minneapolis	14	745	56.7	0.207
Overall	59	976	100	0.273

The number of individuals surveyed (*N*), the number of bands present (*B*), the percentage of polymorphic loci (*P*), heterozygosity based on genotypic values (H_g) with Taylor expansion correction (20).

Skunk #1, with 28 diagnostic bands for Skunk #1 and 19 for Carmen. Four bands were present in all hemp lines including the Minnesota populations but absent in Skunk #1, whereas seven Skunk #1 bands were absent from all three hemp lines. Comparison of individual Skunk #1 and Carmen plants showing the highest pairwise similarity to one another based on the Dice coefficient (0.560) indicated that even the two most similar plants differed by at least 289 bands.

Each of the four populations surveyed had at least 700 bands, whereas the fewest bands were detected in a sample of inbred plants (Table 3). The percentage of polymorphic loci was highest in the naturalized Minnesota populations, whereas Skunk #1 and inbred Carmen lines had the lowest polymorphism. The same trend was found for heterozygosity (Table 3). Levels of polymorphism and heterozygosity based on AFLP markers were lower than levels detected with RAPD markers (13), but a major difference between these studies is that two of the four populations sampled here were inbred for at least one generation, whereas five of the six cultivars surveyed by Forapani et al. (13) were outbred. Carmen plants also show slightly lower levels of polymorphism and heterozygosity than naturalized hemp in Minnesota. Heterozygosity is likely underestimated for inbred populations that deviate from the assumption of Hardy–Weinberg equilibrium. Nonetheless, a comparison of AFLP and RAPD results restricted to outcrossed populations (13) still showed relatively lower estimates of polymorphism and heterozygosity for AFLP. More fixed differences among cultivars were detected with AFLP probably owing to the greater number of markers relative to RAPD.

Analysis of molecular variance in four *Cannabis* populations indicated that 27.2% of the genetic variation is attributable to differences between marijuana and hemp lines, 20.9% to differences among hemp varieties, and 51.9% to variation within populations (Table 4). PCA separated the four *Cannabis* populations in the first two principal coordinates (Fig. 1), further suggesting extensive genetic differentiation between these cultivars. The first coordinate explained 19% of the variation and distinguished marijuana from hemp, whereas the second coordinate explained 10% of the variation and separated the three hemp populations. Levels of population differentiation and heterozygosity detected

TABLE 4—*Nested analysis of molecular variance.*

Source	df	Sum of Squares	%
Among groups	1	53.77	27.2
Among populations	3	41.29	20.9*
Within populations	54	102.55	51.9*
Total	58	197.62	

Groups consisted of marijuana vs. hemp, and populations included Skunk #1, Carmen, Shakopee, and Minneapolis. Degrees of freedom (df) and significance at $p<0.05$ (*) are indicated.

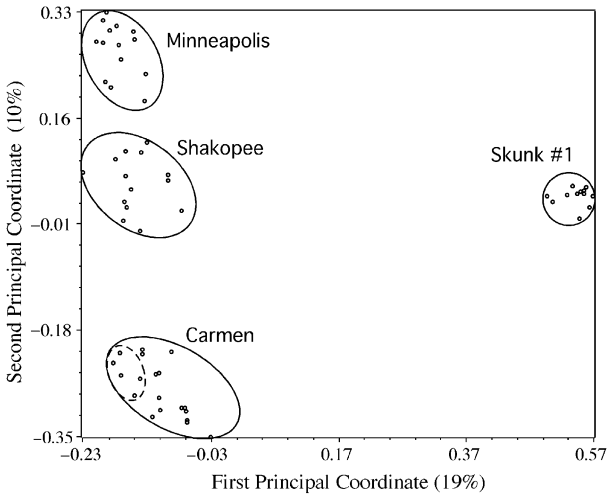

FIG. 1—*Principal coordinates analysis of amplified fragment length polymorphism (AFLP) variation in four populations of* Cannabis *sativa. Hemp and marijuana are separated by the first principal coordinate, whereas three hemp populations are distinguished by the second principal coordinate. The dashed ellipse encloses Carmen full siblings after one generation of inbreeding.*

in our study were similar to results based on RAPD markers (13). Estimates of Φ_{ST} for RAPD and AFLP markers of 0.488 and 0.481, respectively, suggest a great deal of differentiation among populations. This is not at all surprising given that modern, commercial hemp and marijuana cultivars were intensely selected for different characteristics including the opposite extremes of cannabinoid content. Our results corroborate estimates of genetic variation based on RAPD markers, which indicated that a substantial amount of the genetic polymorphism in *Cannabis* exists within populations (13). Even inbred full siblings had unique AFLP fingerprints (dashed ellipse, Fig. 1). In a forensic setting, polymorphism enhances the power of analyses to identify marijuana samples arising from a common source. Extensive AFLP polymorphism is sufficient to fingerprint individual plants, an important line of evidence for establishing conspiracy in distribution networks and the clonal propagation of indoor marijuana (28), an enormous and expanding industry. AFLP also performed better than STR markers in distinguishing cultivars (9). Because of the much larger number of markers compared with STR and RAPD, AFLP appear provide the most efficient method for the discrimination of *Cannabis* cultivars to date.

Some of the fixed genetic differences between drug and fiber lines detected in this survey could be closely linked to THC production, a possibility that can be examined in the future by a broader survey of *Cannabis* varieties. de Meijer et al. (5) developed a SCAR marker that discriminated high- and low-THC cultivars and hypothesized that the marker is closely linked to THC synthase. Quantitative trait locus mapping is proposed as the logical next step in this line of investigation to test the hypothesis that these markers are indeed linked to THC production. Molecular markers closely linked to drug content in *Cannabis* have forensic utility in that they can distinguish illicit material from licit cultivars in countries where a distinction is made.

In the field of drug enforcement, the utility of *Cannabis* DNA fingerprinting parallels that of human DNA typing. Genetic markers separating hemp and marijuana already have practical utility for drug enforcement in Canada and Europe, where hemp cultivation is permitted but marijuana is illegal. Admittedly, the sampling universe for this study was small, but the large number of

polymorphic markers and extent of AFLP differentiation among four populations suggest that AFLP hold promise as a forensic tool. Broader surveys of cultivars and seized drugs are needed to develop the capacity to routinely apply AFLP in forensic investigations, including the inference of distribution networks from seized marijuana. The capacity to attribute seized drugs to source populations based on AFLP is especially important for drug enforcement in the United States, where marijuana trafficking today is a thriving, multibillion dollar industry.

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