TRACKING CLONAL MARIJUANA USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) ANALYSIS: AN OVERVIEW

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Introduction

Cannabis sativa (marijuana) is one of the oldest cultivated crops in the world. As a plant, it is valued for both its hallucinogenic and medicinal properties. Marijuana has been used to treat a variety of ailments including pain, glaucoma, nausea, asthma, depression, insomnia and neuralgia (1). Like many cultivated crops (e.g. wheat, corn), it has been domesticated from a naturally occurring weed species and propagated to yield increasing amounts of Δ-9-tetrahydrocannabinol (THC) and for other desirable smoking traits (e.g. flavor, smoothness). Marijuana is a large cash crop in the United States and marijuana cases account for enormous asset forfeitures. These forfeited assets, in turn, are used to fund law enforcement budgets for drug eradication efforts, community-based treatment and drug prevention programs. Marijuana is a primary focus for drug intervention programs in schools and is considered by many as a "gateway" drug to introduce young users to other addictive illicit drugs such as heroin and cocaine. Most drug arrests are for crimes involving marijuana. In 1998, eighty-eight percent of seven hundred thousand drug-related cases were for marijuana possession in the United States (1).

Until the mid-1970's, most United States marijuana was imported from Mexico. When Mexico joined the United States in its marijuana eradication efforts, a domestic "home-grow" industry was born. Industrious breeders developed several superior *Cannabis* cultivars (e.g. Northern Lights, Skunk #1, California Orange and Big Bud) in the early 1980's (1). Most subsequent marijuana cultivars are thought to be derived from these few genetic lines. Constant breeding is in progress but since it is performed as an underground activity, it is difficult to determine the number of true marijuana cultivars in existence. Numerous seed catalogues are available via internet access, however, none list genetic markers for cultivar identification. The on-line seed catalogues rely on traditional morphological descriptors (e.g. red stems, foliage color) complete with photographic examples. The morphological phenotype of *Cannabis sativa* is highly influenced by environmental conditions, therefore, traits such as leaf size and shape are not truly reliable for cultivar identification.

Cannabis sativa is a dioecious, herbaceous annual plant with a four to six month growing season (2). Dioecy, by definition, means that pistillate (female) and staminate (male) flowers are presented on separate plants. Marijuana can be propagated in two ways: by seed or by cloning (2, 3). Seeds are a result of sexual reproduction between a pistillate and staminate plant and produce new individuals with recombinant genotypes. The highly prized sinsemilla marijuana results from growing pistillate plants in isolation from any source of pollen. Isolated female plants will produce prolific floral buds with a high THC content. Cloning is a form of asexual reproduction that allows for preservation of the genotype due to lack of meiotic recombination. This form of propagation is desirable to the grower because it perpetuates the unique characteristics of the parent plant. It also generates a population of nearly identical, all-pistillate, fast-growing and evenly maturing *Cannabis* plants. To propagate marijuana by cloning, a cutting is removed from the parent plant and induced to form a new root system (Figure 1) (2, 3). Root systems typically develop in three to six weeks and the clones are then ready to be transplanted into larger containers. Plant development can be accelerated by supplying excess nutrients, carbon dioxide and light. With a sudden shift from twenty-four hour daylight to a twelve hour light

regime to mimic autumn conditions, marijuana plants can be forced to flower before they are eight weeks of age (2).

Amplified fragment length polymorphism (AFLP) analysis is a method that can be used for individualizing any single source biological sample based on a DNA profile (4). This method has been routinely used for creating highly saturated genetic marker maps for identifying linked traits in plants (5-14), animals (15, 16), insects (17, 18), fungi (19) and bacteria (20-23). Only recently has AFLP analysis been applied to a species in a forensic context (24, 25). AFLP analysis involves the polymerase chain reaction (PCR) amplification of restriction fragments to generate a band pattern that can be used as an identifying profile for the sample. The band pattern can be "adjusted" for complexity based on the use of selective PCR primer sets in the second round of PCR amplification (Figure 2). This method is somewhat analogous to the original forensic restriction fragment length polymorphism (RFLP) DNA typing method that used multi-locus probes for human identity testing. Both AFLP analysis and multi-locus probes generate a highly discriminating and complex band pattern for individualizing biological samples (26). Scoring complex band patterns and converting them into useful data points is a challenge for the AFLP user, however, the authors have developed a system for categorizing variable peaks that simplifies AFLP analysis.

Forensic analyses involve two steps: identification and individualization of a biological sample.

1) Identification. For botanical evidence, the molecular identification of a species can be determined by cloning and DNA sequencing of the internal transcribed spacer (ITS) region of the nuclear genome and the large subunit of ribulose 1,5-bisphosphate carboxylase (rbcL) (27). For identification of marijuana, many tests are available including the microscopical examination of leaf hair morphology, the Duquenois-Levine test, and gas chromatography analyses for THC and other cannabinoid compounds (28-31). Identification of *Cannabis* by molecular methods has also been published (32, 33).
2) Individualization. Amplified fragment length polymorphism analysis is a method for individualizing marijuana samples and the authors are validating this method for profiling marijuana seizure samples. An AFLP database containing profiles from seizure samples obtained from the United States and Canada is under construction to establish the extent of genetic diversity in marijuana cultivars. AFLP analysis of marijuana seizure samples can determine if seizures from two or more geographic locations share a common profile. Our data show that AFLP profiles can provide genetic evidence for the form of plant propagation being utilized by the growers of this illicit crop. This type of evidence can be useful in providing investigative leads and for illustrating the extent of grower organizations and distribution networks.

Material and Methods

Tissue source. All samples for DNA extraction were obtained from marijuana seizures in the New England area. Fresh (100 mg) or dry (20 mg) young leaf tissue was preferred for DNA extractions, however stems and flowers were also used. When fertilized female flowers were used, any visible seeds (mature or developing) were removed. If only seeds were available, the perianth and pericarp were removed and the embryo (2-5 mg) was processed.

DNA extraction and estimate of DNA yield. DNA was extracted with the QIAGEN (Valencia, CA) Plant DNeasy kit according to the manufacturer's protocol. DNA was eluted from the filter twice with AE Buffer (100 μl) and combined into one tube. DNA yields were estimated by comparing to known mass standards co-electrophoresed with the samples on either a 1.0% or 1.5% agarose gel in 1X TAE buffer. Gels were stained with 0.4 ng/ml ethidium bromide to visualize the DNA. If the DNA was degraded but a high molecular weight (MW) band was visible, that band was used to estimate the quantity. If no high MW band was visible, the DNA was used if the following criteria were satisfied: 1) the majority of the fragments were > 3 Kb and 2) the smear was clearly visible under ultra-violet (UV) light (302 nm) when the extract (2 μl) was loaded into a $4.5 \times 1 \times 5$ mm well (gel dimensions, 14.5 cm length x 11 cm width x 0.75 cm height) and subjected to

electrophoresis (100 V, 1Hr). If high MW DNA was less than 4 ng/μl, or if degraded DNA was not visible, it was concentrated up to 10-fold using a Microcon YM-100 centrifugal filter (Millipore; Boston, MA) and re-quantified.

AFLP analysis. AFLP analysis was performed according to the AFLP™ Plant Mapping Kit (Applied Biosystems; Foster City, CA) manufacturer's protocol with the following modifications. Digestion-ligation reactions were prepared in batches of 20 reactions at a time. High MW DNA (approximately 20 ng) was prepared to a final volume of 5 μl in distilled water in a thin-walled 0.2 ml PCR tube and set aside while the other reagents were prepared. For degraded DNA, 5 μl of the DNA extraction was used. The adaptor pairs were annealed by heating in a 90-99˚C water bath for 5 minutes, and cooled at room temperature for 10 minutes. The enzymes used included MseI (New England Biolabs (NEB), Inc., Beverly, MA: 50 units/μl or Life Technologies Gibco BRL, Rockville, MD: 5 units/μl), EcoRI (Gibco BRL: 50 units/μl) and T4 DNA ligase (Gibco BRL: 5 Weiss units/μl or NEB: 30 Weiss units/μl). All enzymes were maintained in a –20˚C cold block while the reactions were being prepared.

In order to prevent pipeting of small volumes of reagents into individual tubes, two "pre-master" mixes were assembled (designated Enzyme Mix and Adaptor Mix) for the digestion-ligation step. The Enzyme Mix was prepared by combining 20× [1 unit MseI, 5 units EcoRI and 1 Weiss unit ligase] in the bottom of a 0.5-ml tube and placing it in a cold block. If the Enzyme Mix recipe required pipeting volumes less than 0.5 μl, then the enzymes were diluted with an appropriate Diluent Buffer (NEB) and the recipe adjusted accordingly. The Adaptor Mix was prepared by combining 20× [2.2 μl 5X (or 1.1 μl 10X) Ligase Buffer), 1.1 μl 0.5 M NaCl, 0.11 μl 5 mg/ml bovine serum albumin, 1 μl MseI adaptor, and 1 μl EcoRI adaptor]. It was then brought to a final volume equal to (20 × 6 μl minus the volume of the Enzyme Mix) with distilled water. The following two steps were carried out expeditiously to prevent the adaptors from ligating to one another. The entire Adaptor Mix was transferred to the tube containing the Enzyme Mix and the solution was pipeted gently to mix. Enzyme/Adaptor Mix (6 μl) was added to each tube containing DNA and gently mixed. The 0.2-ml tubes were incubated at 37˚C in a thermal cycler with a heated lid for 2 hours.

Pre-amplification reactions were performed in batches of 20-50 reactions plus one negative control. After diluting the digestion-ligation reactions with 189 μl $TE_{0.1}$ Buffer (20 mM Tris-HCl, 0.1 mM EDTA, pH 8), an aliquot of each reaction (4 μl) was transferred to a 0.2-ml PCR tube and set aside. The negative control tube was prepared with 4 μl $TE_{0.1}$ Buffer in lieu of the diluted digestion-ligation component. A master mix (designated Pre-amp Mix) was prepared by combining [the number of samples + 2] × [1 μl AFLP EcoRI & MseI pre-amplification primers and 15 μl AFLP Core Mix]. Pre-amp Mix (16 μl) was transferred to each of the 0.2-ml tubes and pipeted gently to mix. PCR was carried out in a Perkin Elmer 9700 model thermal cycler using the temperature profile described in the AFLP kit protocol (ramp speed set to MAX). Pre-amplification reactions were then transferred to 0.5-ml tubes and diluted with 380 μl $TE_{0.1}$ Buffer.

Selective amplifications were carried out in batches of 20-50 for each selective primer pair. AFLP profiles were generated with four selective PCR primer pairs (our designation: A1, A4, F4 and F5, where A is EcoRI-ACT FAM, F is EcoRI-AAG JOE, 1 is MseI-CAA, 4 is MseI-CAT, and 5 is MseI-CTA). The diluted pre-amplification reaction (3 μl) was transferred to 0.2-ml PCR tubes and set aside. A negative control was included with each primer pair, using $TE_{0.1}$ Buffer in lieu of diluted pre-amplification components. A master mix (designated Selective-amp Mix) was prepared by combining [the number of samples + 2] × [1 μl AFLP EcoRI primer, 1 μl AFLP MseI primer and 15 μl AFLP Core Mix]. Expeditiously, Selective-amp Mix (17 μl) was transferred to each of the 0.2-ml tubes and gently mixed. PCR was performed as described in the kit protocol.

Amplification products were separated and detected using an ABI Prism 377 DNA Sequencer. A 0.2-mm acrylamide gel (Long Ranger® Singel® Packs, BioWhittaker Molecular Applications,

Rockland, ME), a 34-well square-tooth comb, and plates with a 36 cm well-to-read distance were used to separate PCR products. A master mix (designated FLS-ROX) was prepared by mixing [the number of samples] × [4.75 μl Loading solution (deionized formamide:blue dextran 5:1) and 1 μl GeneScan-500 size marker [ROX] (ABI)]. FLS-ROX (5 μl) was added to the selective amplification product (4 μl, generated from one primer pair). These samples were heated at 95˚C for 2 minutes, quick-chilled on cold blocks, and loaded (2 μl) into each well of the gel. The gel was run for 5 hours at 1680 volts.

Data management. AFLP profiles are represented as peaks labeled with the specified fluorescent dye designated by the selective PCR primer pair. Peaks between 50 and 500 bases were sized with GeneScan® 3.1.2 software. The presence or absence of variable peaks in user designated categories was determined by alignment of 50 – 100 AFLP profiles and the data converted to binary code with Genotyper® 2.5 software. A binary code database representing each AFLP profile was established and the database was managed using Microsoft Excel software.

Results

Tissue source and presence of plant resin does not affect the AFLP profile. A single hydroponically grown marijuana plant was sampled. The tissues sampled (103 mg, fresh weight) included a female floral bud with resin, a stem (approximately ¼ inch thick, no visible resin), and a mature leaf (dark green, no visible resin). The different tissues collected from the individual marijuana plant were found to yield sufficient amounts of genomic DNA (data not shown) for AFLP analysis. Each tissue source exhibited identical AFLP profiles (Figure 3, three color Genescan overlay), thereby illustrating that AFLP typing will yield the same profile regardless of the portion of the plant from which the DNA is extracted. The presence of visible resin on the floral bud did not interfere with PCR amplification as evidenced by the ability to obtain an AFLP profile match with both stem and leaf tissues.

Genetic evidence of clonal marijuana propagation. Two marijuana grow operations were suspected to be linked based on informant information. Location #1 had plants of various sizes growing under lights in different containers. Location #2 was a high-tech hydroponic grow operation. Plants from each location were seized and the grower's labeling compared. AFLP analysis of a subset of the seized plants from each location showed that plants with similar labels had identical AFLP profiles for all four selective primer pairs (Figure 4, panels A-D, data for primer pair A1). The AFLP results provide genetic evidence for cloning and demonstrate that the two growers are sharing plant material or are obtaining it from a common source (Figure 4, panel E, primer pair A1).

Unrelated marijuana samples have different AFLP profiles. Nineteen different marijuana seizure samples obtained from adjudicated cases from the Vermont Crime Laboratory were processed to obtain AFLP profiles and the results compared. The Vermont marijuana samples were typical of street seizure material that has been observed in Connecticut and other New England states. These samples consisted of mixtures of leaves, stems, seeds and flower buds. Individual fragments were selected for processing to avoid the possibility of typing plants from two different sources. The extracted DNA from these seizure samples was partially degraded but met the criteria for AFLP processing listed in materials and methods. Six AFLP profiles from the Vermont seizure samples are illustrated in Figure 5. Twenty variable categories (designated A1-01 through A1-20) have been selected for scoring the AFLP profiles generated by PCR primer set A1 to binary code. When comparing the AFLP profiles, presence (scored as 1) or absence (scored as 0) of a peak is easily determined by visual examination of the Genotyper results. For example, when comparing the six AFLP profiles in Figure 5, all samples contain a peak in category A1-01. However, only the first profile (R13) has a peak in category A1-03. The binary codes for all twenty of the Vermont marijuana samples for two PCR primer sets (A1, A4) are listed in Table 1. A total of forty variable AFLP markers are scored for the two PCR primer sets.

Three positive control peaks are present in the profiles generated by each primer set and are useful for assessing the data are on a comparable relative fluorescence (RFU) scale for comparing two or more profiles.

Discussion

Our data show that AFLP analysis is a useful method for establishing genetic profiles from seized marijuana samples. These profiles can be utilized to provide genetic evidence that clonally propagated marijuana samples are being shared between two locations or have a common origin. This information can provide an investigative lead for law enforcement when tracking common sources of marijuana and for linking source, distributor and user. AFLP analysis performed on floral bud, stem, fresh and dried leaf tissue was successful in generating interpretable profiles. Interestingly, all of the Vermont seizure samples had different AFLP profiles suggesting they may be genetically unrelated or distantly related. Although it is thought that most marijuana cultivars are highly inbred and derived from a few superior genetic lines, AFLP markers are able to distinguish sufficient genetic diversity within the marijuana samples we have surveyed to date. Based on our current data, two AFLP primer sets (and frequently a single primer set) are sufficient to determine that two marijuana samples are not clonal in nature. AFLP analysis is a promising new method for forensic analyses of single source plant material and has the potential to be applied to other forms of non-human biological evidence and additional types of casework.

Acknowledgements

The authors would like to thank Dr. Eric Buel, The Vermont Crime Laboratory, Captain Peter Warren, and members of the Connecticut Statewide Narcotics Task Force for their assistance in providing either samples or helpful information.

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Figure 1. An example of clonally propagated *Cannabis sativa* (marijuana) plants. Photo courtesy of Dr. Gary Shutler, Royal Canadian Mounted Police, Winnipeg, Canada.

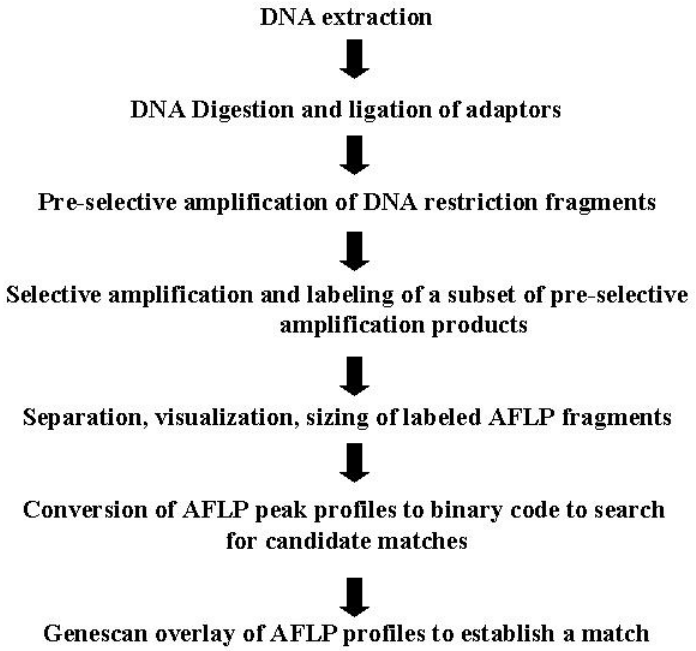

Figure 2. Schematic depicting the basic steps required to perform amplified fragment length polymorphism (AFLP) analysis on a biological sample.

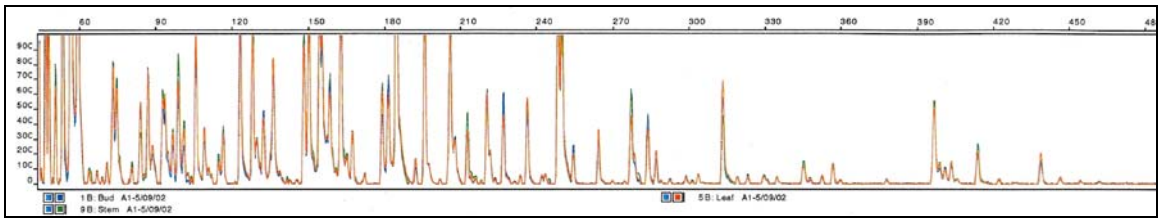

Figure 3. A Genescan overlay of three AFLP profiles generated using the selective PCR primer pair A1 from female floral bud (blue), stem tissue (green), and mature leaf tissue (orange). The overlay of the profiles is exact and illustrates that tissue source does not change the AFLP profile from an individual plant. Note that AFLP profiles generated from a floral bud containing seeds would be a mixture of both a maternal profile and those seeds representing the F1 generation.

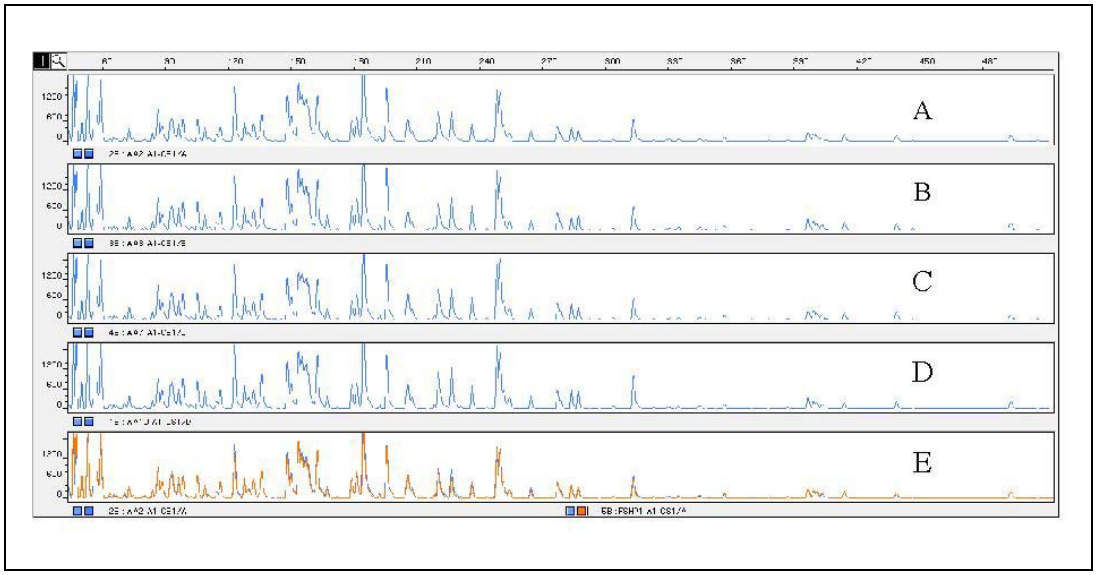

Figure 4. AFLP results for four suspected clones obtained from a New England marijuana grow operation. The samples from location #1 exhibit identical profiles (panels A – D, A1 primer set). A two-color overlay comparing AFLP profiles from commonly labeled plants from locations #1 (blue) and #2 (orange), respectively, show a match (panel E, A1 primer set) indicating clonal propagation and sharing of plant material between two separate sites.

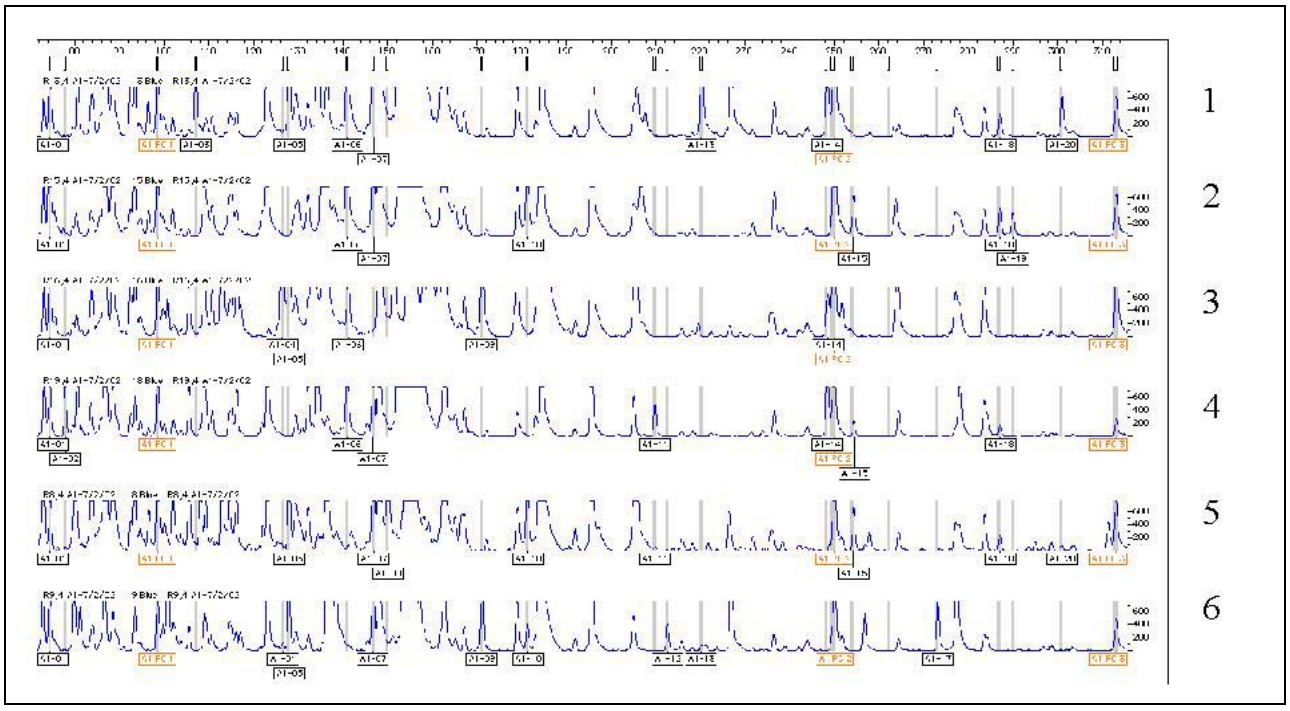

Figure 5. Six different AFLP profiles are variable for presence and absence of peaks within the designated (grey) categories. All twenty categories for primer set A1 (A1-01 to A1-20) are depicted as gray bars as well as the three control peaks (A1 PC 1, A1 PC 2, A1 PC 3).

Sample	PC 1	PC 2	PC 3	A1-01	A1-02	A1-03	A1-04	A1-05	A1-06	A1-07	A1-08	A1-09	A1-10	A1-11	A1-12	A1-13	A1-14	A1-15	A1-16	A1-17	A1-18	A1-19	A1-20	PC 4	PC 5	PC 6	A4-21	A4-22	A4-23	A4-24	A4-25	A4-26	A4-27	A4-28	A4-29	A4-30	A4-31	A4-32	A4-33	A4-34	A4-35	A4-36	A4-37	A4-38	A4-39	A4-40
R1	1	1	1	1	0	0	0	1	0	1	0	0	1	1	1	0	0	1	0	0	1	1	1	1	1	1	1	1	0	1	1	0	1	1	0	0	1	0	1	1	0	0
R2	1	1	1	1	0	0	0	1	0	1	0	0	1	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	0	1	0	1	1	1	0	0	0	0	0	1	0	1	1	0	1
R3	1	1	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	1	0	0	1	0	0	1	1	1	1	1	0	1	0	0	1	1	0	0	0	0	1	0	0	1	0	1	1
R4	1	1	1	1	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0	1	1	0	1	1	0	1	0	1	1	1	0	0	0	0	0	0	0	1	1	1	0
R5	1	1	1	1	0	0	0	1	0	1	0	0	0	0	1	0	1	1	0	0	0	0	0	1	1	1	0	1	0	1	0	0	1	1	0	0	0	0	0	1	0	0	1	1	1
R6	1	1	1	1	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0	0	0	0	1	1	1	0	1	0	1	1	0	1	1	0	0	0	0	0	0	1	0	0	1	0	1
R7	1	1	1	1	1	0	0	1	1	1	0	0	1	0	1	0	0	1	0	0	1	0	0	1	1	1	1	1	0	1	0	0	0	1	0	1	0	0	0	0	1	0	1	0	0	1
R8	1	1	1	1	0	0	0	1	0	1	1	0	1	1	0	0	0	1	0	0	1	0	1	1	1	1	1	1	0	1	0	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0
R9	1	1	1	1	0	0	1	1	0	1	0	1	1	0	1	1	0	0	0	1	0	0	0	1	1	1	1	1	0	1	1	0	1	1	0	0	0	0	0	1	1	0	0	1	0	0
R10	1	1	1	1	1	0	0	1	1	1	0	0	1	0	0	1	0	0	0	0	1	1	0	1	1	1	1	1	0	1	0	0	1	1	0	0	1	0	0	0	0	0	0	1	0	0
R11	1	1	1	1	0	1	0	1	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	1	1	1	0	1	0	0	0	1	1	0	0	0	0	1	0	1	1	0	0
R12	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	1	1	1	1	1	0	1	1	1	1	1	0	0	0	0	0	0	1	0	0	1	0	0
R13	1	1	1	1	0	1	0	1	1	1	0	0	0	0	1	1	0	0	0	1	0	0	1	1	1	1	0	1	0	1	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	1
R14	1	1	1	1	0	0	0	1	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	1	1	1	1	1	0	1	0	1	0	0	0	1	0	0	0	0	1	0	0	1	0	0
R15	1	1	1	1	0	0	0	0	1	1	0	0	1	0	0	0	0	1	0	0	1	1	0	1	1	1	1	1	0	1	1	0	1	1	0	0	1	0	1	0	0	1	0	0	1	1
R16	1	1	1	1	0	0	1	1	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	1	1	1	1	1	0	1	0	0	1	1	0	0	0	0	0	1	1	0	1	1	0	0
R18	1	1	1	1	0	0	0	1	0	1	0	0	1	0	1	1	0	0	0	0	1	1	0	1	1	1	1	1	0	1	1	0	1	1	0	0	0	0	1	1	0	1	0	0	0	0
R19	1	1	1	1	1	0	0	0	1	1	0	0	0	1	0	0	1	1	0	0	1	0	0	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	0	1	0	0	0	0
R20	1	1	1	1	0	0	0	1	1	1	0	0	1	0	0	0	1	1	0	0	0	0	0	1	1	1	1	1	0	1	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0

Table 1. Binary codes for nineteen Vermont marijuana seizure samples corresponding to the presence or absence of peaks within categories. Two primer pairs (A1, A4) are represented here with twenty scorable markers per primer pair for a total of forty markers.