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TECHNICAL NOTE

CRIMINALISTICS

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Field Testing of Collection Cards for *Cannabis sativa* Samples With a Single Hexanucleotide DNA Marker*,†

ABSTRACT: The validity and feasibility of using DNA collection cards in the field for preservation and analysis of *Cannabis sativa* genotypes were investigated using a highly specific hexanucleotide marker. Collection cards were submitted to the National Marijuana Initiative, which selectively trained and managed the collection of specific types of samples from a variety of participating agencies. Samples collected at seizure sites included fresh marijuana leaf samples, dried "dispensary" samples, U.S. border seizures, and hashish. Using a standardized PCR kit with custom-labeled oligonucleotide primers specific to marijuana, collection cards produced eight genotypes and 13 different alleles, extremely low baselines, and no cross-reactivity with control plant species. Results were produced from all sample types with the exception of hashish. Plant DNA collection cards represent an easily implementable method for the genetic identification and relatedness of *C. sativa* street and grow site–seized samples with applications for databasing and market disruption.

KEYWORDS: forensic science, *Cannabis*, marijuana, NMI01, FTA cards, databasing, DNA, dispensary

Collection cards have been used in human DNA collection, processing, and long-term storage in forensic laboratories for many years (1–3). They have found extensive use in convicted felon databasing because DNA applied to a collection card is protected from degradation by the antimicrobial and antifungal properties of the paper, can be stored at room temperature in a space efficient manner, and processing is automatable (4–6). Despite plant material showing proven value as trace evidence (7–14), there is currently no standard method of plant DNA archival comparable to that available for human DNA samples. While it has been known for some time that plant DNA can successfully be collected, stored, and processed on collection cards for genomic research (4–6), this method has not been employed regularly as a standard and valuable tool in the forensic or toxicology laboratories (15,16).

The development of short tandem repeat (STR) analysis has streamlined human individualization and now allowed for DNA genotyping of many additional species such as cat (17,18), dog (19–24), horse (25–30), and marijuana (31–37) that have significant value as trace evidence. There are several scientific reports about STR markers in development for *Cannabis sativa* (31–37). While the usefulness of these markers is apparent, some of the primers are not species-specific, allowing for potential amplification of contaminant fungi or bacteria (32). Also, many of these markers have been tested for allele frequencies against street seizure samples of undefined origin and therefore, naturally, would be expected to show a greater degree of discrimination power than if tested against related seed or sibling samples (31,32). As a model system, we selected an STR marker previously developed by Hsieh et al. (37), which is highly specific for *C. sativa* to test the validity of storing and recovering marijuana DNA on collection cards. The STR locus, defined by primers CS1F and CS1R and referred to in this paper as NMI01, for National Marijuana Initiative (NMI), is a highly polymorphic simple sequence repeat motif of six base pairs and is the first such marker to be isolated from *C. sativa*. *C. sativa* is the most common illicit drug used in the United States and constitutes a significant proportion of plant material in police drug seizures (38,39). Of increasing concern is the rapid rise of federally illegal marijuana dispensaries on the west coast where state law enforcement is experiencing difficulty in constraining state-legislated marijuana use for the medically ill; large-scale illegal cultivation by drug dealers has become rampant in the State and Federal parks and in private communities and represents a major long-term public safety and health issue because of booby-trapped grow operations and smoking damage to the body, respectively (38–42).

Through a project funded by the NMI and the Office of National Drug Control Policy (ONDCP), we were able to obtain samples of *C. sativa* applied to collection cards by law enforcement agents during seizures of plant material. Participating agencies were provided

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with collection cards and recommendations for proper sample application by The University of New Haven Department of Forensic Science (UNH) and the NMI. Samples were then purified and amplified for fragment analysis allowing comparison between fresh and dry plant material and hashish, and evaluation of percentage genotype recovery from storage on collection cards. Obtaining these types of field samples is extraordinarily difficult for forensic study as they must be acquired through agreement between agencies that seize and prosecute drugs and agencies that monitor public land for invasive species. In addition, all parties must be trained in evidence collection and have Drug Enforcement Agency (DEA) licenses or appropriate authority to receive and ship drug samples for study. One major advantage to using collection cards is that it takes the marijuana sample from a usable drug form to a nonsmokable drug format, making research and storage at universities possible. Because of this extensive inter-agency cooperation, we were able to use STR NMI01, not previously applied to DNA collection cards, as a means of investigating the potential success of the use of collection cards for marijuana DNA in the field. The largest issues with developing DNA as a tool for databasing and subsequently linking source material to seizures relate to (i) how cooperative the field agents will be in taking extra time to collect samples on FTA cards and (ii) how successfully a laboratory can genotype from these cards. One purpose of this study was to define which types of marijuana samples (fresh leaves, dried dispensary powder or leaves, and hashish) are most likely to yield DNA results.

Materials and Methods

Sample Collection

Instructions and Whatman FTA Classic Cards (Whatman Inc., Florham Park, NJ) were sent to Tommy LaNier at the NMI for training and dispersal to law enforcement agencies. Inserts accompanying the collection cards included the following directions: (i) label the bottom of the card with date, sample, and location, (ii) use gloved fingers to press a bunched leaf onto the sample circles until visible green material adheres to the card, (iii) fill as many sample circles as available material allows, (iv) apply only one plant to a card, and (v) allow cards to air-dry, protected from sunlight and excess heat, before packaging. Collection cards of *C. sativa* material obtained during police seizures were returned to UNH and represent separate cases. One sample was applied to an individual card; multiple cards may be part of the same case for prosecution.

Sample Processing and Preparation for PCR

Sterile 3-mm punches were taken from a sample-containing circle on each FTA Classic Card and washed three times with FTA Purification Reagent (Whatman Inc.) followed by three washes with TE-1 buffer. Punches were dried thoroughly at 56°C. Negative control cards consisted of ivy, northern bamboo, and catnip applied to cards with even pressure and processed in the same manner as for the marijuana. For a positive control, a large sample of *Cannabis* was provided by the DEA so that the same batch could be processed coincident with the card samples and would provide a positive control for the PCR kit as well as a migration control for the capillary electrophoresis instrument.

PCR

Sample amplifications were performed directly on 3-mm FTA Classic Card punches in a total reaction volume of 25 μL, using 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 0.5 μL dNTP (New England BioLabs, Ipswich, MA), 2.5 μL Standard Taq reaction buffer (New England BioLabs), and 1.5 μL each forward and reverse primer (CS1F and CS1R, respectively) (Applied Biosystems). The CS1F primer was labeled with a 5′-fluorescent tag (NED™; Applied Biosystems), and PCR consisted of 32 cycles of 94°C denaturation for 60 sec, 55°C annealing for 60 sec, 72°C extension for 60 sec, and one 72°C extension for 30 min.

Fragment Analysis

One microliter of amplicons was mixed with ROX 500 size standard and Hi Di Formamide (Applied Biosystems) and heat-denatured for 3 min at 95°C. Fragment separation and detection were performed on an ABI 3130 Genetic Analyzer using Performance Optimized Polymer 7 (POP-7), Multi-capillary DS-32 (Dye Set F) (Applied Biosystems). Data were analyzed using GENEMAPPER 4.0 (Applied Biosystems), and alleles were assigned using a customized panel and bin set (Table 1) (UNH, West Haven, CT). The bin set was calculated from previously amplified *C. sativa* samples

TABLE 1—*Bin set used for marker NMI01 for allele designations with this data set.*

Bin	Start	Location	End
2	127.5	128	128.5
2.1	128.5	129	129.5
2.2	129.5	130	130.5
3	133.5	134	134.5
4	139.5	140	140.5
5	145.5	146	146.5
6	151.5	152	152.5
7	157.5	158	158.5
8	163.5	164	164.5
9	169.5	170	170.5
10	175.5	176	176.5
11	181.5	182	182.5
12	187.5	188	188.5
13	193.5	194	194.5
13.3	196.55	197.05	197.55
14	199.5	200	200.5
15	205.5	206	206.5
16	211.5	212	212.5
16.3	214.6	215.1	215.6
17.3	220.5	221	221.5
19	229.5	230	230.5
20	235.5	236	236.5
21.2	243.75	244.25	244.75
22	247.5	248	248.5
22.2	249.5	250	250.5
23	253.5	254	254.5
25	265.5	266	266.5
26	271.5	272	272.5
26.1	272.6	273.1	273.6
26.3	274.5	275	275.5
27	277.5	278	278.5
27.1	278.5	279	279.5
27.3	280.5	281	281.5
28	283.5	284	284.5
30	295.9	296.4	296.9
30.2	297.4	297.9	298.4
31	301.5	302	302.5
32	307.5	308	308.5
33	313.5	314	314.5
34	319.5	320	320.5
35	325.5	326	326.5
36	331.5	332	332.5
37	337.5	338	338.5

TABLE 2—*Alleles detected at the NMI01 locus from fresh and dried plant material and hashish after amplification from DNA collection cards.*

Sample	Material	Allele 1	Allele 2	Allele 3	Size 1	Size 2	Size 3	Height 1	Height 2	Height 3
Z 1	Dried									
Z 2	Dried									
Z 3	Dried									
Z 4	Dried									
Z 5	Dried									
Z 6	Dried									
Z 7	Dried									
Z 8	Dried									
Z 9	Dried									
Z 10	Dried									
Z 11	Dried									
Z 12	Dried									
Z 13	Dried	16.3	26.1		215.04	273.16		1457	989	
Z 14	Dried	22.2	26.1		250.17	273.18		2517	2148	
Z 15	Dried	13.3	16.3		197.55	215.11		1890	1678	
Z 16	Dried	22.2	28		250.15	284.13		2363	1992	
Z 17	Dried	26.1	27.1		273.15	279.16		866	665	
Z 18	Dried									
Z 19	Dried									
Z 20	Fresh	2.2	26.3	27.3	130	275.19	281.31	1094	1709	1609
Z 21	Fresh	2.1	17.3	30.2	129.39	220.87	298.09	1205	3579	1456
Z 22	Fresh	30			296.84			480		
Z 23	Fresh									
Positive		2.2	22.2	26.1	129.88	250.12	273.22	881	725	663
Negative										

Allele designations are based on fragment size in bases; peak heights are approximately equivalent and are in relative fluorescence units. Z1–Z6 from U.S. border seizures; Z7–Z17 from marijuana ''dispensary'' seizures; Z18–Z19 from hashish; and Z20–Z23 from fresh marijuana leaves.

(data sets not shown) by subtracting 90 bases flanking the 5′ side and 26 bases flanking the 3′ side (including the primer sequence) and dividing the repeat length by 6. The relative fluorescent unit (RFU) threshold for peak height determination was set at a minimum of 50 RFUs.

Results and Discussion

Genotype Recovery

A total of 23 collection cards were returned to UNH from which a total of eight genotypes were detected. Four cards contained rubbings from fresh plant material, 17 from dried border seizures or dispensaries, and two from hashish samples. Of the four fresh material cards, three genotypes and a total of seven alleles were detected, while of the 17 dried material cards, five genotypes and a total of 10 alleles were detected (Table 2). No genotypes were recovered from the two hashish samples. A representative electropherogram is shown in Fig. 1; peak quality and height is similar to what is observed in human identification systems. Of interest, this DNA marker often generates multiple peaks (between one and four peaks observed per sample). This could result from a duplicated locus on the same chromosome or from amplification from multiple sets of chromosomes, or from PCR primer homology to multiple sites within the genome.

Background levels of noise were exhibited in all 23 field samples, the positive control, and four negatives controls (catnip, ivy, formamide, and water); in all cases, the levels were below 25 RFUs (data not shown). The threshold for detection was placed at 50 RFUs, safely twice the background, ensuring all alleles were detected and with minimal risk of false allele calls.

Our results show that the field use of collection cards for plant material is a viable option for law enforcement agencies. With the estimated time to take a sample of this nature <5 min, easy storage and packaging requirements, and potential for STR genotyping, collection cards offer an easily implemented procedure for marijuana evidence DNA collection and preservation. The collection cards are ideal for fresh plant samples and also, surprisingly, yield a DNA result from a fair number of samples that were extremely dry and powdery in appearance. This suggests that the number of cells contained within a 3-mm card punch is not required to be very high to yield a positive marijuana STR result. For those samples that failed to genotype, this could result from (i) low initial DNA for dry or hashish samples or (ii) poor transfer of DNA to the card for dried samples.

The benefits to using collection cards for marijuana samples are numerous: (i) cards are archivable in a nonusable drug format for storage, shipping, and sample retesting and (ii) marijuana identification by DNA and databasing can be performed simultaneously. This provides a potential to eliminate the large holding cages for seized drugs and the use of DNA to identify low delta-9-tetrahydrocannabinol (THC) content samples such as seeds, roots, etc., as well as leaves and bud. When using highly specific markers such as NMI01, potential linkages between seizures can be made or refuted, providing law enforcement with data on the location and movement of *C. sativa* supplies on a local and eventually a national level (35,36). By combining the capability of marijuana-specific DNA markers with a field-friendly collection method, genetic relatedness can be established revealing similarity between different variants of material sold under street names and be used to link cloned samples together. This is a potentially valuable tool for market disruption in the global campaign against illicit *C. sativa* trafficking.

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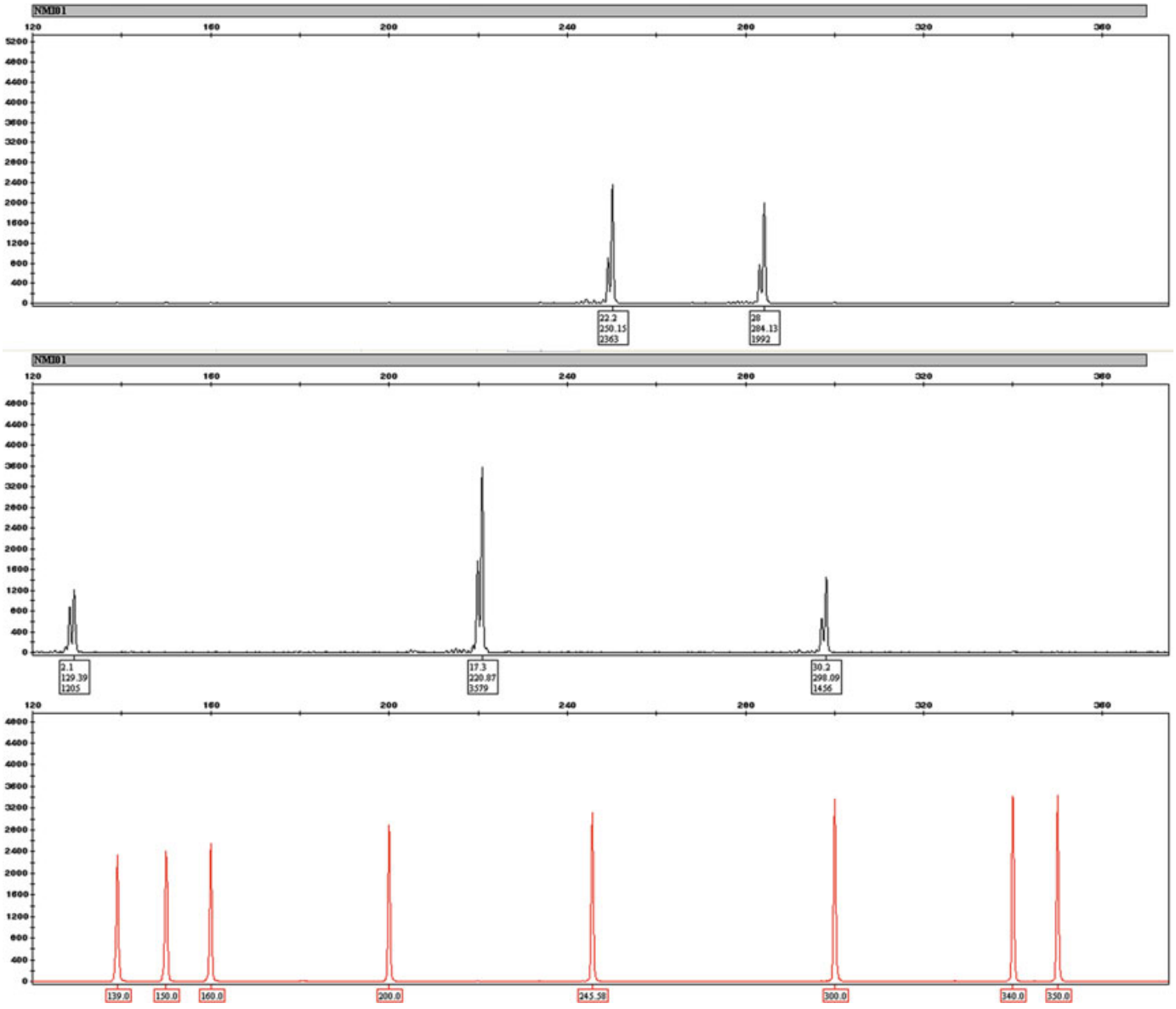

FIG. 1—*Electropherograms of samples Z16 (dried) and Z21 (fresh) marijuana PCR amplified for the NMI01 marker; data shown in the top and middle panels respectively. The ROX 500 internal size standard used to size the fragments is shown in the bottom panel. The* x*-axis is size in bases 0–350; the* y*-axis is relative fluorescence units scale of 0–5200.*

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