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Developmental Validation of a *Cannabis sativa* STR Multiplex System for Forensic Analysis

ABSTRACT: A developmental validation study based on recommendations of the Scientific Working Group on DNA Analysis Methods (SWGDAM) was conducted on a multiplex system of 10 *Cannabis sativa* short tandem repeat loci. Amplification of the loci in four multiplex reactions was tested across DNA from dried root, stem, and leaf sources, and DNA from fresh, frozen, and dried leaf tissue with a template DNA range of 10.0–0.01 ng. The loci were amplified and scored consistently for all DNA sources when DNA template was in the range of 10.0–1.0 ng. Some allelic dropout and PCR failure occurred in reactions with lower template DNA amounts. Overall, amplification was best using 10.0 ng of template DNA from dried leaf tissue indicating that this is the optimal source material. Cross species amplification was observed in *Humulus lupulus* for three loci but there was no allelic overlap. This is the first study following SWGDAM validation guidelines to validate short tandem repeat markers for forensic use in plants.

KEYWORDS: forensic science, DNA typing, *Cannabis sativa,* short tandem repeat, validation, SWGDAM, ANUCS301, ANUCS302, ANUCS303, ANUCS304, ANUCS305, ANUCS308, ANUCS501, B01-CANN1, B05-CANN1, B02-CANN2, C11-CANN1

Cannabis sativa L. has a long association with humans, with fiber and drug varieties well known. Drug varieties are typically characterized by elevated levels of psychoactive cannabinoid compounds leading to *C. sativa* being the most used illicit drug worldwide (1). In Australia, as elsewhere, organized crime syndicates are often involved in large-scale production of *C. sativa*, with the commission of other offenses related to the process of production, such as theft of electricity for hydroponics crops, firearms offenses, money laundering, and violence to enforce debts or settle disputes, being common (2,3). The ability to identify and/or link syndicates by determining the likely origin of seized drugs and to distinguish between legalized fiber crops and drug crops are highly sought by the international forensic community.

In recent studies the geographical origin of seized *C. sativa* samples has been elucidated by the analysis of isotopic ratios combined with knowledge of the elemental makeup from geographical regions (4,5). While this method enabled *C. sativa* grown in the different local regions to be distinguished, it did not provide information that could link growers. Approaches utilizing DNA information may provide even finer resolution than isotopic analysis, and as such DNA-based tools for *C. sativa* identification and population studies are being developed by multiple research groups around the world. For example, DNA markers for distinguishing *C. sativa* from other plant species have been developed (6,7) and population genetic surveys of genetic variation within *C. sativa* have been conducted using polymerase chain reaction (PCR)-based multilocus DNA fingerprinting methods (8–12). However, the dominant nature of these multilocus markers and the potential for non-*C. sativa* DNA amplification limit their application for routine forensic analysis.

Codominant short tandem repeat (STR) markers, now the standard marker in human forensic investigations (13), have recently been developed for *C. sativa* (14–17). A comprehensive study employing a subset of these STR markers provided information on *C. sativa* agronomic type, and the geographical origin of *C. sativa* drug seizures (16). However, to enable the use of *C. sativa* STR markers for routine forensic analysis, they need to be validated using standards that match those developed for human forensic DNA profiling (18). Once validated, these methods may provide a powerful new investigative tool for intelligence analysis of organized and commercially motivated criminal activity involving *Cannabis*.

This paper describes the developmental validation of a set of *C. sativa* STR markers based on the applicable guidelines established by the Scientific Working Group on DNA Analysis Methods (SWGDAM) (19). Developmental validation is a critical first step in the transfer of new research tools to the forensic laboratory. The purpose of such validation is to provide detailed assessments of the sensitivity, accuracy, and reproducibility of the DNA profiles generated by the genetic markers. Examination of the stability of various sources of DNA, including casework type samples, with respect to the production of reliable profiles also forms an important component of developmental validation. Additionally, examination of species specificity and knowledge of population variation are required. To our knowledge, this is the first paper following SWGDAM validation guidelines to validate STR markers for forensic use in plants.

Methods

Loci and Multiplex Amplification Conditions

A subset of STR loci were chosen from the set of publicly available STRs for *C. sativa* (14–16). In this initial validation study, we

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avoided loci with dinucleotide repeats as their DNA profiles can be more complicated to score. Consequently only tri- or pentanucleotide repeat loci were chosen (with the exception of a combined di- and trinucleotide repeat unit).

Due to fragment size overlap and fluorescent dye constraints, the loci were divided into four separate groups for multiplex amplification. Multiplex amplification was carried out according to the conditions described in Table 1. Prior to finalizing the PCR conditions, the effect of magnesium concentration on each PCR multiplex was examined by amplifying 10.0 ng of a *C. sativa* control DNA sample with final $MgCl_2$ concentrations of 1.5, 2.0, 2.5, 3.0, and 4.0 mM. There was a trend for reduced PCR artifacts and more uniform heterozygote balance at the higher $MgCl_2$ concentrations (data not shown). Consequently, final $MgCl_2$ concentrations (3.0–4.0 mM) were adopted for subsequent multiplex PCR (Table 1).

A touchdown PCR thermal profile was employed. This allowed us to multiplex loci effectively, eliminating the need to PCR amplify each locus individually with differing cycling conditions (20). Thermal cycling conditions were 95°C for 3 min, followed by 10 cycles of 95°C 30 sec, 66°C 30 sec (reducing by 3°C every second cycle down to 54°C), 72°C 45 sec, followed by 30 cycles of 95°C 30 sec, 50°C 30 sec, 72°C 45 sec, with a subsequent final extension at 72°C for 30 min. Reactions were held at 10°C prior to further manipulation.

Tissue Source and DNA Extraction

Cannabis sativa samples were obtained from drug seizures from within the Australian Capital Territory. DNA from different tissue sources, tissue storage methods, and the effect of DNA concentration on multiplex PCR were examined as follows.

Tissue source (air dried leaf, stem, and root) and storage method of leaf tissue (fresh, frozen at −80°C, and air dried) were examined separately in triplicate using three independent samples for each category. Plant DNA was extracted from a selection of tissues using the DNeasy® Plant Kit (QIAGEN, Hilden, Germany). This extraction method has previously been validated for forensic DNA extraction of *C. sativa* by Miller-Coyle et al. (21). DNA concentration for these validation experiments was standardized by precipitation with 0.3 M sodium acetate with subsequent resuspension following standard protocols (22). DNA samples were electrophoresed along with known DNA concentration standards in 1.5% agarose gel containing ethidium bromide. Gels were recorded using a GelDoc XR Gel Documentation System (BIO-RAD, Hercules, CA) and DNA concentration was estimated using Quantity One V5.6.2 software (BIO-RAD).

Sensitivity Study

To examine the appropriate range and limit of DNA template required for successful amplification, 10.0, 1.0, 0.1, 0.01 ng of DNA from each tissue type and tissue storage condition was assessed. Each PCR batch contained two types of negative control: DNA storage buffer (Buffer AE, QIAGEN) and sterile distilled H_2O. An additional *C. sativa*-positive DNA control (*c.* 1.0 ng) was also included. We subsequently recommend 1.0–10.0 ng of *C. sativa* DNA template as optimal; however, this was not known at this study's onset and therefore the amount of our control throughout was 1.0 ng.

Species Specificity

To assess their specificity, the chosen *C. sativa* STR loci were tested for amplification across a range of non-*C. sativa* DNA

TABLE 1—*PCR components for each multiplex group.*

	Loci	Forward Primer 5′ Label	Final Concentration (Forward and Reverse Primers) (μM)	Standard PCR Components	Multiplex Specific PCR Components	Final Reaction Volume and Dilution Factor*
Multiplex group 1	ANUCS501	FAM	0.1	1 × PCR Buffer (QIAGEN)	4.0 μg bovine serum albumin	Reaction volume: 40 μL
	C11-CANN1	VIC	0.1	0.2 mM dNTPs	3.0 mM $MgCl_2$	Dilution factor: 1:20
	ANUCS302	NED	0.1		1 unit *Taq* DNA polymerase (QIAGEN)	
Multiplex group 2	ANUCS303	FAM	0.1	1 × PCR Buffer (QIAGEN)	4.0 μg BSA	Reaction volume: 40 μL
	ANUCS305	VIC	0.1	0.2 mM dNTPs	3.0 mM $MgCl_2$	Dilution factor: 1:20
	B02-CANN2	NED	0.1		1 unit *Taq* DNA polymerase (QIAGEN)	
	ANUCS308	PET	0.15			
Multiplex group† 3	ANUCS304	PET	0.2	1 × PCR Buffer (QIAGEN)	2.0 μg BSA	Reaction volume: 20 μL
	ANUCS301	VIC	0.4	0.2 mM dNTPs	4.0 mM $MgCl_2$	Dilution factor: 1:5
					0.5 unit *Taq* DNA polymerase (QIAGEN)	
Multiplex group† 4	B05-CANN1	NED	0.05	1 × PCR Buffer (QIAGEN)	2.0 μg BSA	Reaction volume: 20 μL
	B01-CANN1	PET	0.2	0.2 mM dNTPs	3.0 mM $MgCl_2$	Dilution factor: 1:10
					0.5 unit *Taq* DNA polymerase (QIAGEN)	

Concentrations indicated are for the final reaction volume.
*Post PCR dilution factor prior to analysis on ABI PRISM® 3100 Genetic Analyzer.
†Multiplex groups were combined with dilution following PCR.

sources. This examination included species widely considered to be the most closely related to the *Cannabis* genus, *Humulus lupulus* (Hops), *Celtis australis* (Hackberry), and *Trema tomentosa* (Poison Peach). Also included were *Nicotiana tabacum* (Tobacco), a species known to be associated with *Cannabis* drug use (2) and *Homo sapiens* DNA, obtained using a BuccalAmp™ DNA Extraction Kit (EPICENTRE, Madison, WI). For this test, 10.0 ng of each DNA sample was added in duplicate to multiplex PCRs (Table 1).

Fragment Detection and Genotype Analysis

To size and score the STR fragments, the amplification reactions were diluted (see Table 1) with sterile deionized water and 1 μL of each diluted reaction was added to a 19 μL mix consisting of 18.95 μL HiDi™ Formamide and 0.05 μL GeneScan™-500 LIZ™ Size Standard (Applied Biosystems, Foster City, CA). Fragments were separated in Performance Optimized Polymer 4 (Applied Biosystems) and detected on an ABI PRISM® 3100 Genetic Analyzer using the default sample injection settings.

To enable ease of transferability among laboratories, nonoverlapping bin size ranges were designed to match the tri- or pentanucleotide repeat units with integer designations for fragment sizes and even left and right offsets.

Fragment sizes were determined using GENEMAPPER® Software V3.7 (Applied Biosystems). To ensure reliability, the genotype scoring process proceeded in two steps. First, genotype scoring was achieved by initially running the automatic scoring feature of GENEMAPPER® Software V3.7 (Applied Biosystems) with default settings. Second, the automatic genotype scoring was manually checked. Any fragments not automatically scored but occurring within designated bins were manually scored if overall peak height was above 200 relative fluorescence units (rfu) if homozygous and 100 rfu if heterozygous.

The amount of amplification product for each allele was estimated from peak area values determined by the GENEMAPPER® Software V3.7 (Applied Biosystems). Additionally, allelic stutter proportion and heterozygote balance were measured from fragment peak height determined by the GENEMAPPER® Software V3.7 (Applied Biosystems). Allelic stutter proportion was calculated as the height of the stutter peak divided by height of the associated allelic peak. Stutter peaks were only considered in either homozygous samples or heterozygous samples where the stutter pattern was not obscured by an allelic peak. Additionally, stutter peaks were only considered if peak height exceeded 100 rfu. Heterozygote balance was calculated as the height of the smaller allelic peak divided by height of the larger allelic peak.

Results

Loci Characterization

As anticipated for STR loci, the putative allele sizes only differed by the expected repeat unit length. Codominance was confirmed by the detection of no more than two alleles per sample. In most cases alleles were detected in both homo- and heterozygous states.

As is common for STR loci (23,24), there was some variation in heterozygote balance among the loci. For most heterozygous allele combinations at each locus, either PCR amplification marginally favored the shorter allele or there was very little difference in the level of amplification for each allele (Figs. 1*a*,*b* and 2*a*,*b*,*c*, Table 2). However, there were several exceptions across the loci.

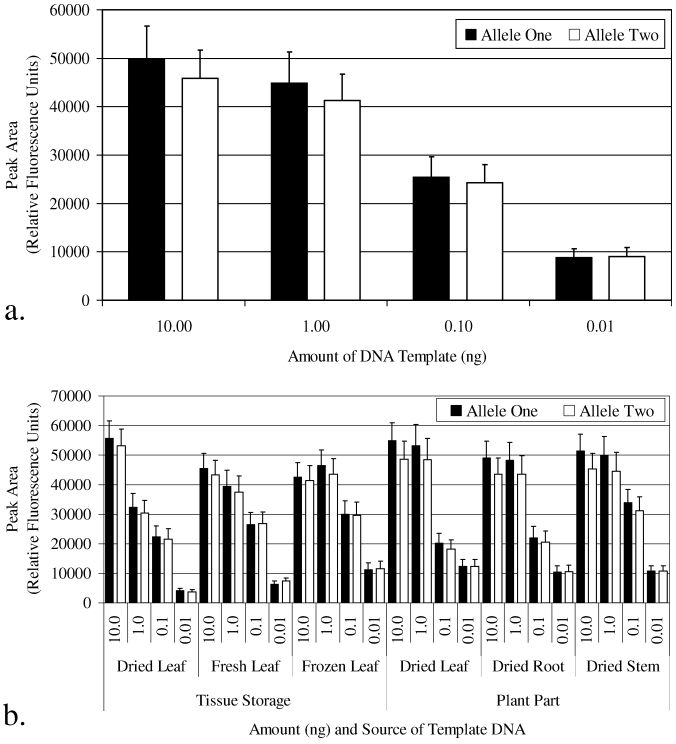

FIG. 1—*(a) Relative amounts of PCR amplification for all loci and all DNA sources combined over differing starting DNA template amounts. (b) Level of PCR amplification for differing DNA template amount and DNA source. Error bars represent standard error of the means.*

In a number of particular heterozygous allelic combinations, heterozygote balance was lower than other allelic combinations for the same locus (Table 2). In addition, some heterozygous allele combinations at the loci B02-CANN2 and C11-CANN1 exhibited PCR amplification favoring the longer allele and also lower heterozygote balance. However, at these loci, not all heterozygous allelic combinations showed this amplification pattern (Table 2).

Typical STR stutter peaks (25) were apparent at most loci (Figs. 2*b* and 2*c*). Stutter peaks were identified without ambiguity from allelic peaks by their repetitive and substantially smaller height compared to the one or two major allelic peaks (Table 2). Allelic stutter proportions showed some variation among loci, and among alleles at the same locus (Table 2). The automatic scoring by GENEMAPPER® Software V3.7 (Applied Biosystems) sometimes included these stutter peaks which required manual removal of these false allele calls. We note that there is further scope to modify the GENEMAPPER® Software V3.7 (Applied Biosystems) analysis parameters to improve automatic scoring; however, manual checking of automatic scoring will always be essential.

Sensitivity and Stability

For all DNA sources and tissue storage methods, genotypes were amplified and scored consistently for DNA template amounts of 10.0 and 1.0 ng for all but locus ANUCS308. Within the 10.0–1.0 ng DNA template range, multiplex amplification of locus ANUCS308 was inconsistent, with amplification failure occurring in *c.* 33% of samples in this DNA amount range. For the accompanying loci in Multiplex Group 2, amplification failure was not observed at the 10.0–1.0 ng template DNA range indicating that DNA quality was not responsible *per se*. Given this inconsistency of

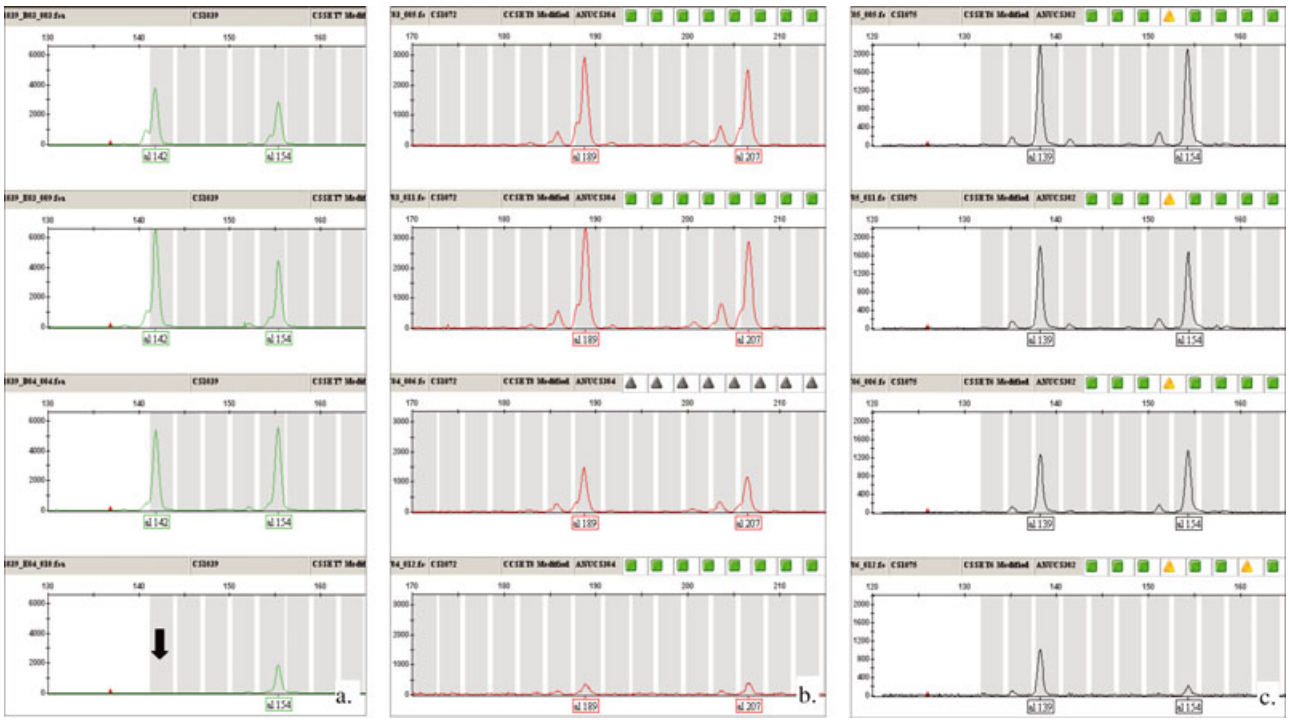

FIG. 2—*Electropherograms of three loci: (a) ANUCS305, (b) ANUCS304, (c) ANUCS302, showing levels of amplification for DNA template amounts of 10 ng (top), 1, 0.1, and 0.01 ng (bottom). An allelic dropout is evident for ANUCS305 at the 0.01 ng DNA template level.*

amplification despite adequate DNA quality and that preliminary data indicated low allelic variation for this locus, it was removed from further validation analysis.

For all 10 remaining loci, some amplification failure and allelic dropouts were detected with the lower DNA template amounts of 0.1 and 0.01 ng (Fig. 2*a*). For DNA template amounts of 0.1 and 0.01 ng, *c*. 9% and 18% of samples respectively failed to amplify, and of the amplifiable samples, 1% and 5% of samples respectively showed an allelic dropout. Additionally, a decrease in PCR amplification product was observed with decreasing amounts of template DNA across the different DNA sources and different tissue storage methods (Figs. 1 and 2). Generally there was little difference between the amount of amplification product when the PCR was initiated with 10.0 ng or 1.0 ng of template DNA for both tissue source and tissue storage method (Figs. 1*a* and 1*b*). However, DNA amplification from dried tissue was notably greater with the highest amount of template DNA (Fig. 1*b*). Multilocus genotypes were fully reproduced across the 10 loci. No unexpected genotypes were detected in the three replicates of each tissue type and tissue storage method when DNA template ranged from 10.0 to 1.0 ng.

Species Specificity

Three of the 10 loci—ANUCS303, ANUCS305, and B05-CANN1—produced discernible amplification products from *H. lupulus* DNA (Fig. 3). However, the level of amplification in *H. lupulus* was considerably lower than for *C. sativa* DNA, and all putative alleles were smaller than the range of allele sizes known for *C. sativa*. Additionally, for loci ANUCS303 and ANUCS305, the amplified *H. lupulus* fragments were not consistent with the repeat unit length of known *C. sativa* alleles. No other amplification products were detected for the non-*C. sativa* species tested.

Discussion

Following applicable SWGDAM guidelines, this developmental validation has shown that the set of 10 codominant *C. sativa* STR loci examined in this study can be routinely and reliably amplified and scored for the multiplex PCR conditions tested. This study now opens the way for internal validation studies within operational forensic laboratories. Given the expectation of some inter-laboratory variation in optimal PCR conditions (26), some minor modifications of the protocols tested here may be useful in subsequent internal validation studies. In the discussion that follows, we offer recommendations for forensic laboratories planning to adopt these STR markers for forensic analysis of *C sativa*. We also highlight some of the issues encountered when applying SWGDAM validation guidelines to plants.

In our study, consistent genotypes were obtained from DNA templates in the range of 10.0–1.0 ng, from leaf, root, and stem tissue of *C. sativa*. Despite success with root and stem tissue as a DNA source, where possible we recommend that DNA be obtained from either fresh or air-dried leaf as this tissue yielded the most consistent results. Leaf tissue is easily sampled and it is the most reliable source for morphological identification (27) if required.

As anticipated, where DNA is limited there is a risk of allelic dropout or overall amplification failure. We recommended that where possible 1.0–10.0 ng of DNA template be used for casework analysis of *C. sativa* with this multiplex system. We note that this is a larger amount of DNA that can be used in human forensics studies (28). Additionally, while there were some variations in heterozygote balance and stutter proportions among alleles and heterozygote allelic combinations across the loci, allele scoring was never compromised by this variation.

Cross-species amplification of STRs in plants is common, but typically this is restricted to only a subset of loci in closely related species (29). Cross-species amplification occurred between *C. sativa*

TABLE 2—*Average allelic stutter proportion and average heterozygote balance for each locus.*

Locus	Allele (bp)	Average Allelic Stutter Proportion* (%)	Replicates	Heterozygous Allelic Condition	Average Heterozygote Balance† (%)	Replicates
ANUCS501	88	0	18	88/93	86	3
	93	0	3	88/98	73	3
	98	0	3			
C11-CANN1	152	11	3	158/152‡	33	3
	155	9	12	158/155‡	47	9
	158	13	3	158/176	70	3
	176	5	3			
ANUCS302	139	8	9	139/145	95	3
	145	9	6	139/154	97	3
	151	6	3	145/154	94	3
	154	12	6			
ANUCS303	145	5	9	145/151	55	6
	151	8	15			
ANUCS305	142	1	9	142/154	77	9
	154	8	18			
B02-CANN2	164	2	3	167/164‡	30	3
	167	3	11	164/173	87	3
	173	5	5	173/167‡	84	3
ANUCS304	171	20	3	171/192	73	3
	189	16	3	189/207	88	3
	192	29	3	207/210	82	3
	204	23	3			
	207	25	12			
ANUCS301	226	26	6	226/232	14	3
	232	24	3	241/247	66	3
	241	19	3	244/265	32	3
	244	22	6			
	247	25	3			
	265	31	3			
B05-CANN1	236	3	3	239/242	84	6
	239	5	9	239/245	96	3
	242	5	6			
	245	7	3			
B01-CANN1	317	5	3	326/329	79	3
	326	9	9	329/332	27	3
	329	13	6			

*Measured as height of the stutter peak divided by height of the associated allelic peak from profiles generated with 10.0 ng of template DNA added to multiplex PCR.

†Measured as height of the smaller allelic peak divided by height of the larger allelic peak from profiles generated with 10.0 ng of template DNA added to multiplex PCR.

‡Heterozygotes displayed greater level of amplification for the second allele.

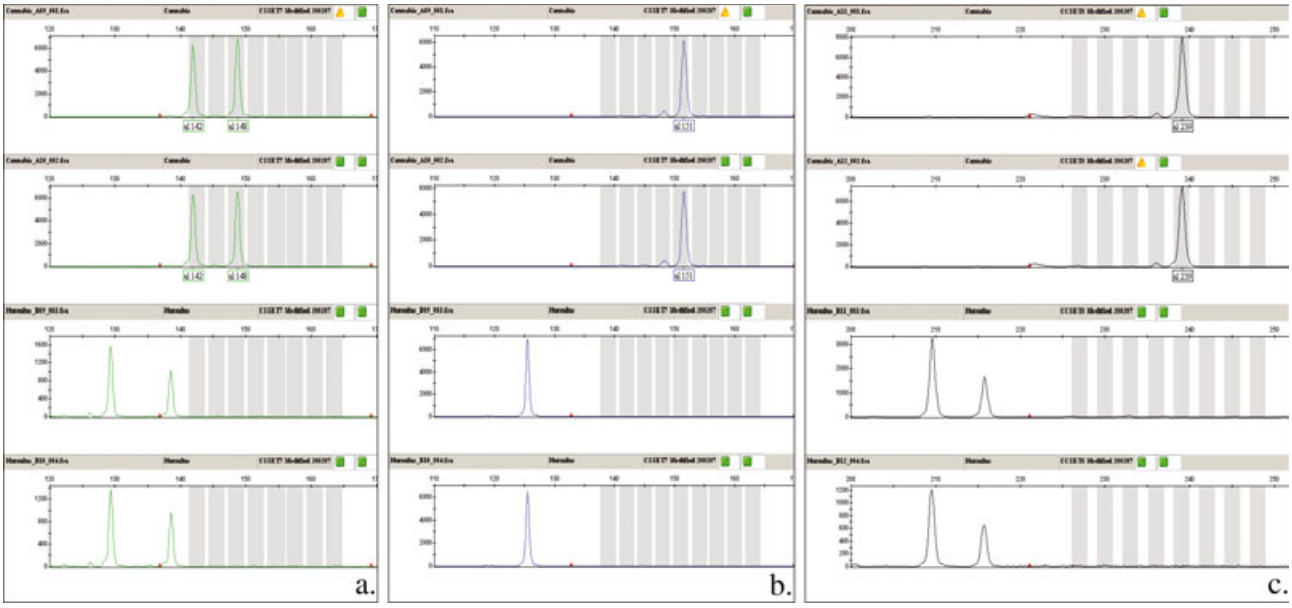

FIG. 3—*Electropherograms of three loci: (a) ANUCS305, (b) ANUCS303, (c) B05-CANN1, showing duplicate amplification products for* Cannabis sativa *(top two profiles) and* Humulus lupulus *(lower two profiles). Amplification products for* H. lupulus *fall outside the known allelic range of* C. sativa.

and its close relative *H. lupulus* for three of the 10 STR loci examined. As amplification in *H. lupulus* was poor and there was no allelic overlap between the two species, any contamination or misidentification can be easily detected. Furthermore, there are obvious macroscopic morphological differences between *C. sativa* and *H. lupulus*. We anticipate that more likely sources of DNA contamination of casework samples will be from human or tobacco DNA. Crucially, neither of these DNA types amplify under these multiplex conditions.

The high sensitivity of these validated PCR protocols demonstrates the importance of minimizing contamination from unknown sources of *C. sativa* DNA, with amplification occurring from as little as 0.01 ng of template DNA, albeit with some inconsistencies. Therefore, standard forensic procedures such as isolating PCR preparation from template DNA extraction, using sterile disposable plasticware, and avoiding aerosols carryover from pipettes (30) are recommended.

This study indicated that inter-sample amplification failure of some loci can occur. While we eliminated one locus due to its high frequency of amplification failure, some sample-specific amplification failure may occur at the remaining loci in casework samples. This may be overcome by repeating the sample in a singleplex reaction (31).

SWGDAM guidelines were specifically developed for human DNA forensic analysis (19). Due to some differences between humans and *C. sativa*, it was not possible to meet all of the SWGDAM guidelines. For example, SWGDAM guidelines recommend that inheritance and chromosomal mapping studies are completed. However, due to legal restrictions it was not possible to conduct breeding experiments with *C. sativa* in this study. Therefore, inheritance characteristics (linkage or non-Mendelian segregation) and chromosomal locations of these markers were not directly assessed. Measures of linkage disequilibrium (LD) in plants, especially species which have been domesticated, often prove unreliable for inferring linkage given that the targeted selection of some phenotypic characters often impose a bias (32). We also note that, unlike humans, *C. sativa* can be clonally propagated which avoids Mendelain segregation and results in identical genotypes between plants of clonal origin. Clonal reproduction has been shown to further bias LD estimates (32).

The SWGDAM guidelines also specify that the ability to obtain reliable results from mixed source samples should be determined (19). At least in initial forensic applications, we assume that an analysis of *C. sativa* DNA mixtures will prove to be both unnecessarily complex and likely to be of limited value to the law enforcement community. *Cannabis sativa* is commonly seized both as whole plants or highly homogenized dried fragments with the latter being possibly mixtures from several unknown and/or unlinked sources. Detecting a genotype mixture will show that the *C. sativa* sample has been mixed at some point after production; it will not provide unequivocal evidence for when it was mixed, and by whom. We propose that analysis using this marker system will be most effective when seizures provide samples from which a single piece of intact tissue is easy to obtain. DNA mixtures of genetically distinct *C. sativa* individuals were not assessed in this study, as genotype mixing at the time of seizure can be minimized in this way.

This study is part of a larger investigation including the development of an Australia-wide *C. sativa* genotype database. The allelic and genotypic diversity for this set of validated markers will be the subject of a separate publication. Meanwhile, the present successful developmental validation of this set of 10 STR markers will allow for their conversion to an operational technology for routine forensic DNA analysis of *C. sativa* drug seizures.

Acknowledgments

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