Optimization of Single Nucleotide Polymorphism (SNP) SNaPshot™ Multiplexes to Distinguish Fiber and Drug-type *Cannabis sativa*

Natalia Czado, MSFS*, Sheree Hughes, PhD, Rachel Houston, PhD

Department of Forensic Science, Sam Houston State University, Huntsville, TX 77340

MEMBER THE TEXAS STATE UNIVERSITY SYSTEM

INTRODUCTION

Cannabis sativa is a highly trafficked and cultivated plant, mainly for its intoxicant properties. The plant can be classified into two forms, fiber-type (hemp) or drug-type (marijuana). In the U.S., drug-type *C. sativa* remains federally classified as a Schedule I drug under the Controlled Substances Act. In contrast to drug-type *C. sativa*, hemp production and sale have been legalized since the passage of the 2018 Agricultural Improvement Act. For *C. sativa* to be classified as hemp in the U.S., it must contain less than 0.3% THC by weight. This established threshold has made it difficult for law enforcement agencies to prosecute drug cases due to most seized drug labs lacking a validated quantitation method. Furthermore, detection of the presence of THC does not provide information on the origin of the plant material; therefore, law enforcement cannot effectively mitigate illegal drug trafficking.

Due to the limited intelligence provided by chemical analysis, researchers have been investigating molecular methods to distinguish and associate *C. sativa* samples [1-5]. Recently, a study described a 23 SNP panel that was able to differentiate crop-type and provide biogeographical information [6]. The panel used a custom KASP assay to examine 22 nuclear SNPs and one mitochondrial SNP [6]. Although the assay is useful for determining the genetic diversity and cluster association of *C. sativa*, it requires separate reactions for each SNP. This requires the consumption of larger volumes of DNA extract, which may not always be possible. Therefore, it would benefit the forensic community to convert the assay to a technique that requires less input and utilizes the traditional instrumentation found in crime laboratories, such as a SNaPshot assay, which utilizes capillary electrophoresis. In this study, DNA sequences provided by Henry et al. [6] were used to design PCR and single-base extension (SBE) primers to create a 3-multiplex SNaPshot assay.

MATERIALS & METHODS

Samples

Commercial hemp (n=46) and NIST and NIDA *C. sativa* (n=15) samples previously extracted were used to evaluate the SNaPshot assay. All extracts underwent chemical analysis to ensure correct crop-type designation.

Primers

PCR and SBE primers were designed using Primer3 software according to DNA sequences provided by Henry et al. [6]. Primer sequence interactions were evaluated using AutoDimer Software, and species specificity was confirmed using Primer-BLAST. Primers were separated into two multiplexes based on cross-reactivity and optimal annealing temperature (**Table 1**).

RESULTS & DISCUSSION

- 61 samples were amplified with two SNaPshot™ multiplexes.
- One pair of markers in each multiplex exhibited some degree of overlap: SVIP14 and SVIP 21 in Multiplex 1 (**Fig. 1**) and VSSL_digi12 and VSSL_digi2 in Multiplex 2.
- One locus, VSSL_A250D, exhibited low peak heights and dropout. Dropout was not restricted to either crop-type. Higher concentrations of the marker's primers did not increase locus amplification (**Fig. 2**). Due to inconsistent amplification of this marker, it was excluded from further analysis. Locus VSSL_digi2 also exhibited low peak heights but was amplified in all samples.
- When multiplexing, green artifacts were observed around loci SW6 and VSSL_digi19. Artifacts in VSSL_digi19 were indistinguishable from expected alleles. Therefore, this marker was also excluded from further analysis.
- Three SNPs were genotyped at two loci: SVIP19 and VSSL_A250D (**Fig. 3**). Amplification of a third SNP was not restricted to either crop-type. Two samples amplified three SNPs at more than one locus.
- Principal component analysis did not indicate the ability to differentiate *C. sativa* crop-type using the thirteen SNPs in the two multiplexes.

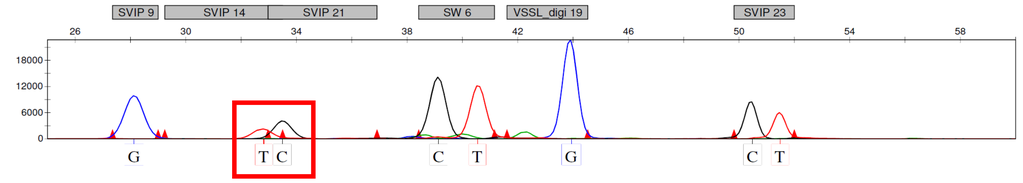

Figure 1. Representative example of SNaPshot™ results of marijuana using Multiplex 1 primers. Overlap of SNPs between SVIP 14 and SVIP21 was seen, indicating the need for primer redesign.

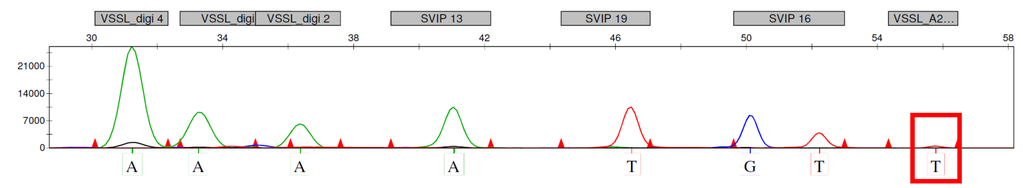

Figure 2. Representative example of SNaPshot™ results of commercial hemp using Multiplex 2 primers. Although the primer concentration of VSSL_A250D was increased, peak heights for the marker remained low in samples with successful amplification.

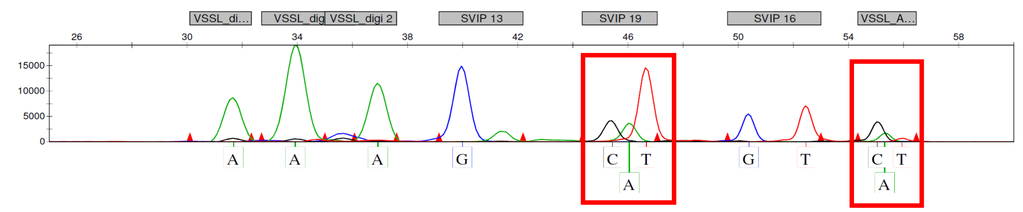

Figure 3. SNaPshot™ results of a marijuana sample with three SNPs genotyped at two loci in Multiplex 2. In addition to the amplification of expected SNPs, C/T, in a subset of samples, A was amplified at both SVIP19 and VSSL_A250D.

MATERIALS & METHODS

DNA Amplification

Initial amplification was performed using the Type-it® Microsatellite PCR kit (QIAGEN, Germantown, MD) in 12.5 µL reactions containing: 6.25 µL Type-it Multiplex PCR Master Mix, 1.25 µL Q solution, 1.25 µL primer mix, 2.35 µL diH_2O, 0.4 µL BSA (8mg/mL), and 1 µL of DNA (0.2 ng/µL). Subsequent single-base extension of purified DNA products was performed using the SNaPshot™ Multiplex Kit (ThermoFisher Scientific, Waltham, MA) in 5 µL reactions containing: 2.5 µL SNaPshot Multiplex Ready Reaction Mix, 1 µL diH_2O, 0.5 µL primer mix, and 1 µL of PCR product.

Separation, Detection, and Analysis

Amplified DNA was separated and detected on the ABI 3500 Series Genetic Analyzer (ThermoFisher Scientific). SNP profiles were analyzed using GeneMapper® ID Software v5.0 (ThermoFisher Scientific). Principal component analysis was completed using PAST software v4.03.

Table 1. Composition of two SNaPshot™ multiplexes evaluated in this study. Primers were assigned to multiplexes based on optimal annealing temperature. Final concentrations of primers in the PCR and SBE primer mixes are given.

Multiplex 1 (53°)			Multiplex 2 (56°)		
SNP Marker	PCR Primers (µM)	SBE Primers (µM)	SNP Marker	PCR Primers (µM)	SBE Primers (µM)
SW6	0.5	2	VSSL_A250D	5	2
SVIP9	2	2	SVIP13	2	2
SVIP21	3	2	SVIP16	2	2
SVIP14	3	2	SVIP19	2	2
SVIP23	2	2	VSSL_digi2	4	2
VSSL_digi19	2	2	VSSL_digi4	0.3	2
			VSSL_digi12	1	2

CONCLUSIONS

- Balanced profiles were difficult to achieve due to the inconsistent amplification of markers VSSL_digi2 and VSSL_A250D. Furthermore, overlap of two pairs of markers complicated genotyping. Primer redesign is needed.
- Further investigation into the amplification of more than two SNPs at several loci is warranted.
- Our results demonstrate that the loci used in this study do not have strong discriminating power. There was no clear distinction between fiber-type and drug-type *C. sativa* using the first two multiplexes. Amplification using the third multiplex, comprising 10 additional SNPs, may be able to differentiate *C. sativa* crop-type, and provide biogeographical information.

REFERENCES

1. Kojoma, M., et al., *DNA polymorphisms in the tetrahydrocannabinolic acid (THCA) synthase gene in "drug-type" and "fiber-type" Cannabis sativa L.* Forensic science international, 2006. **159**(2-3): p. 132-140.
2. Rotherham, D. and S. Harbison, *Differentiation of drug and non-drug Cannabis using a single nucleotide polymorphism (SNP) assay.* Forensic science international, 2011. **207**(1-3): p. 193-197.
3. Piluzza, G., et al., *Differentiation between fiber and drug types of hemp (Cannabis sativa L.) from a collection of wild and domesticated accessions.* Genetic Resources and Crop Evolution, 2013. **60**(8): p. 2331-2342.
4. Houston, R., et al., *Evaluation of a 13-loci STR multiplex system for Cannabis sativa genetic identification.* International journal of legal medicine, 2016. **130**(3): p. 635-647.
5. Cascini, F., et al., *Highly predictive genetic markers distinguish drug-type from fiber-type Cannabis sativa L.* Plants, 2019. **8**(11): p. 496.
6. Henry, P., et al., *A single nucleotide polymorphism assay sheds light on the extent and distribution of genetic diversity, population structure and functional basis of key traits in cultivated north American cannabis.* Journal of Cannabis Research, 2020. **2**(1): p. 1-11.

ACKNOWLEDGEMENTS

The authors would like to thank the Department of Forensic Science at Sam Houston State University for funding this project. They would also like to thank the National Institute on Drug Abuse (NIDA) and the National Institute of Standards and Technology (NIST) for providing marijuana samples for this study.