Differentiating Marijuana from Hemp using an NGS Panel and Rapid Genotyping Assays Targeting Cannabinoid Synthase Genes

Ya-Chih (Jessica) Cheng, MS; Rachel Houston, PhD

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

INTRODUCTION

With the legalization of hemp and marijuana cultivation in the United States, there has emerged a pressing need for reliable methods to distinguish between these two crop varieties. Since cannabinoid synthase genes play a crucial role in determining the chemical composition or chemotype of a particular cultivar, genetic markers on the cannabinoid synthase genes could offer a promising solution for accurate crop type determination. However, the cannabis genome contains multiple copies of cannabinoid synthase genes, including pseudogenes with high nucleotide identity to functional synthase genes [1]. Ignoring pseudogenes in synthase-gene targeting approaches or conducting tests with a restricted sample range may yield inaccurate outcomes [2,3]. To address these challenges, a custom next-generation sequencing (NGS) panel was previously developed to target the three synthase genes: tetrahydrocannabinolic acid synthase (THCAS), cannabidiolic acid synthase (CBDAS), and cannabichromene acid synthase (CBCAS), as well as their pseudogenes. The panel was applied to a diverse set of *C. sativa* samples and a novel differentiation flowchart was proposed based on the sequencing results [4]. This study aimed to confirm the differentiating capability of the four markers outlined in the flowchart. These markers were subsequently utilized to create and validate a SNaPshot™ assay, which has the potential to serve as a valuable tool for law enforcement agencies in distinguishing legal hemp from illicit marijuana.

MATERIALS AND METHODS

THCAS, THCAS-like gene | **CBDAS, CBDAS-like gene** | **CBCAS gene**

Align published sequences to find primer binding sites specific to target sequences

MiSeq NGS Sequencing

Sequencing reference MJs and hemps (n=30), commercial hemp flowers and seed (n=44) , Seized MJ extract (n=61), CBG hemp extract (n=13)

NGS Data Analysis

- Geneious Prime (Normalization, de novo assembly, mapping)
- Presence of synthase genes, pseudogenes, polymorphism

Identification Flow Chart and Confirmation

- PCR, agarose gel and Sanger sequencing to confirm
- **Updated identification flow chart**

SNaPshot™ Assay Development

- Using markers from updated identification flow chart
- Primer and SBE primer designing and testing

SNaPshot™ Assay Validation

- Validated with 8 hemp categories, 7 MJ categories, 1 hops (N=132)

RESULTS AND DISCUSSION

- PCR results showed slight inconsistencies with NGS findings due to primer specificity, updated flowchart presented in **Figure 1**.
- Psedo-THCAS gene can be present in both hemp and marijuana but can be separated with a SNP at position 486 **(Figure 1)**.
- Both THCA SNPs at positions 366 and 1179 effectively differentiate hemp seeds from marijuana. SNP 1179 was chosen over SNP 366 due to primer design challenges arising from the proximity of SNP 366 to SNP 486 on the pseudo-THCA gene.
- A 4-plex SNaPshot™ assay was developed and validated with 132 samples (**Figure 2**).
- Interpretation of the SNaPshot™ data is explained in **Table 1** with few example showed in **Figure 3**.
- The assay was not able to separated marijuana with THC<1% from hemp sample (**Table 1 & Figure 3**).

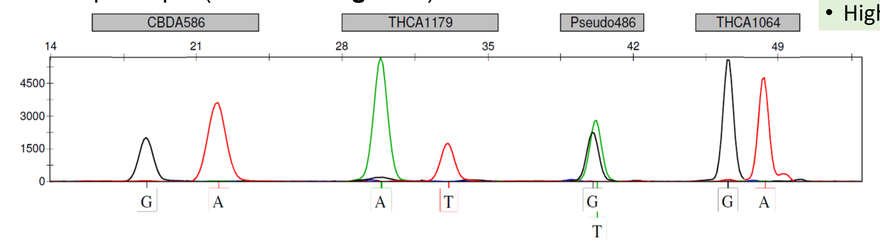

Figure 2. Four-plex SNaPshot™ assay with all possible alleles

Table 1. Interpretation of the SNaPshot™ genotypes.
Dark gray box indicates the SNP of that marker is not important in that genotype. CBDA marker in genotype 3 can provide CBD information of the marijuana sample. Genotype 5 needs peak height ratio (PHR) to determine the strains.

Genotype	CBDA 586	THCA 1179	Pseudo 486	THCA 1064	Identification
1				A	THC<1% Cannabis or Hemp Seed (n=56)
2				Abs.	
3	CBD info.		G	G	Marijuana (n=69)
4	G	T	G	G	Hemp Seed(n=2)
5			G and T	G	*PHR (1064G/486G)

Humulus lupulus (Hops) | *PHR>1: Marijuana | *PHR<1: THC<1% Cannabis

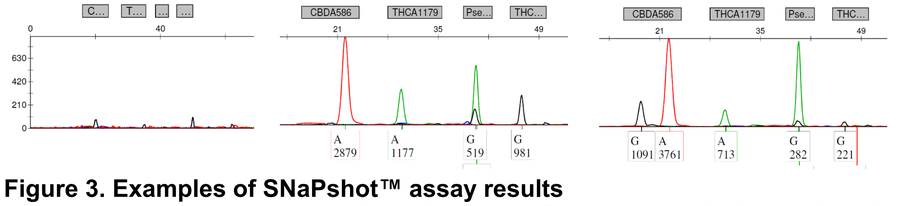

Figure 3. Examples of SNaPshot™ assay results
Hops was used to test species specificity of the assay. Genotype 5 with PHR (1064G/486G) >1 can be classified as marijuana, <1 as cannabinoid sample with THC<1%

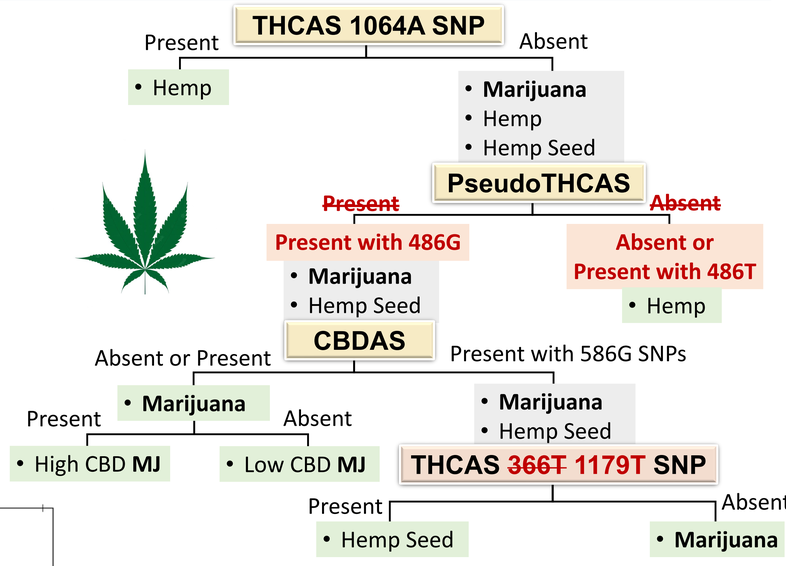

Figure 1. Updated cannabis differentiation flow chart

CONCLUSION

- An updated identification flowchart for distinguishing between hemp and marijuana was proposed.
- The SNaPshot™ assay effectively discriminated between all eight hemp categories and seven marijuana categories, with the exception of marijuana samples containing less than 1% THC.
- The synthase pseudogene can play a pivotal role in distinguishing between hemp and marijuana.

ACKNOWLEDGEMENTS

Disclaimer: This project was funded by an award to Rachel Houston, PhD at Sam Houston State University through the Center for Advanced Research in Forensic Science (CARFS), an NSF-funded IUCRC at Florida International University (Award # 1739805). The opinions, finding, conclusions, or recommendations expressed in this presentation are those of the authors.
The authors would like to thank the National Institute on Drug Abuse (NIDA), National Institute of Standards and Technology (NIST). U.S. Customs and Border Protection LSSD Southwest Regional Science Center for providing marijuana specimens for this project. Additionally, the authors would like to thank the Policia de Investigaciones in Chile, Alejandra Figueroa, and University of Mississippi for their kind donation of marijuana DNA extracts.

REFERENCES

[1] B. Hurgobin et al., Recent advances in Cannabis sativa genomics research, New Phytol 230(1) (2021) 73-89.
[2] T. Yamamuro et al., Rapid identification of drug-type and fiber-type cannabis by allele specific duplex PCR, Forensic Sci Int 318 (2021) 110634.
[3] M.G. Roman et al., Evaluation of tetrahydrocannabinolic acid (THCA) synthase polymorphisms for distinguishing between marijuana and hemp, J Forensic Sci 67(4) (2022) 1370-1381.
[4] Y.C. Cheng, R. Houston, Development of a NGS Panel Targeting Cannabinoid Synthase Genes to distinguish between marijuana and hemp, proceedings of the American Academy of Forensic Science, 2023.