Evaluation of Cannabinoid Synthase Polymorphisms for Distinguishing Between Marijuana and Hemp

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INTRODUCTION

Cannabis sativa (*C. sativa*) is cultivated worldwide for its use as fiber and oil (hemp) or as a psychoactive drug (marijuana). Researchers have been interested in using genetic tools for identifying and individualizing *C. sativa* samples for decades. The cannabinoid precursor CBGA is converted into acid form of common cannabinoids, THC, CBD, or CBC, catalyzed by THCA synthase, CBDA synthase, and CBCA synthase, respectively. Since cannabinoid synthase genes and chemical composition (chemotype) are likely to be closely related, the use of polymorphisms in the synthase genes to predict C. sativa chemotype has been proposed. Kojoma et al. [1] reported two variants of the THCA synthase gene: one active found only in marijuana and one inactive present in hemp and some marijuana. Rotherham and Harbison [2] later designed a SNaPshot™ assay using four single nucleotide polymorphisms (SNPs) between the two variants proposed by Kojoma et al., resulting in 100% accuracy in their preliminary study. However, Laverty et al. [3] later identified the "inactive" THCA synthase gene as the CBCA synthase (CBCAS) gene, which indicated that existing crop type differentiation methods could lack specificity and accuracy. The purpose of this work was to re-evaluate the SNaPshot™ assay using the current knowledge and expanding the sample size and varieties. If proven accurate, this SNaPshot™ assay could provide a simple, rapid method of distinguishing between marijuana and hemp.

MATERIALS AND METHODS

DNA Extraction
10mg of plant material

Assay Optimization
- Previous SNaPshot assay **(Figure 1)**
- PCR condition, multiplex SBE

Sample Collection
- Reference MJs and hemps (n=15), commercial hemp flowers (n=17)
- Seized MJ extract (n=18), hemp seeds (n=12)

SNaPshot™ Assay (Table 2)

Sanger Sequencing (Table 1)
Only reference samples

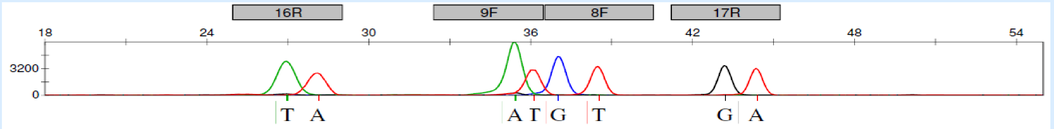
GC-MS Analysis
- Cannabinoid identification
- 1%THC decision-point

DNA and chemistry results comparison

Table 1. PCR Primer for SNaPshot and Sequencing

Primer	Primer sequence
SNaPshot PCR F primer	CAAACT**K**GTTG**Y**TGTCCCATC
SNaPshot PCR R primer	CGTCTTCTTCCCAGCTGATC
Sequencing THCAS PCR F primer	CAAACT**G**GTTG**C**TGTCCCATC
Sequencing CBCAS PCR F primer	CAAACT**T**GTTG**T**TGTCCCATC
Sequencing PCR R primer	CGTCTTCTTCCCAGCTGATC

RESULTS AND DISCUSSION



Genotype	Previous Identification [2]	Current Knowledge [3]	
ATTA	Active THCAS → Marijuana	THCAS genotype	THCAS → only in marijuana
ATTA+TAGG	Heterozygous → Marijuana	THCAS and CBCAS genotype	CBCAS → in both marijuana and hemp
TAGG	Inactive THCAS → Hemp	CBCAS genotype	

Name	Certificate of Analysis				Analyzed in this study			
	Strain	Total CBD	Total THC	CBD/ THC	CBC & CBG	THC estimate	THCA synthase genotype [2]	Sequencing
NIDA B	Marijuana	0.17%	1.9%	0.09	Present	>1%	Heterozygous*	THCA, CBCA
NIDA C	Marijuana	0.01%	3.9%	0.00	Present	>1%	Heterozygous*	THCA, CBCA
NIDA D	Marijuana	0.09%	8.0%	0.01	Present	>1%	Heterozygous*	THCA, CBCA
NIDA E	Marijuana	0.02%	6.7%	0.00	Present	>1%	Heterozygous*	THCA, CBCA
NIDA F	Hemp	3.3%	0.12%	27.50	Present	<1%	Inactive	CBCA only
NIDA G	Marijuana	0.04%	10.1%	0.00	Present	>1%	Heterozygous*	THCA, CBCA
NIDA H	Marijuana	3.7%	2.4%	1.54	Present	>1%	Heterozygous†	THCA, CBCA
NIDA I	Marijuana	3.8%	2.4%	1.58	Present	>1%	Heterozygous	THCA, CBCA
NIDA J	**Marijuana**	9.2%	0.37%	24.86	Present	<1%	**Inactive**	Ambiguous
NIST 1	Hemp	1.70%	0.11%	15.45	Present	<1%	Inactive	THCA, CBCA
NIST 2	**Marijuana**	12.65%	0.47%	26.91	Present	<1%	**Inactive**	CBCA only
NIST 3	**Marijuana**	11.90%	1.23%	9.67	Present	>1%	**Inactive**	Ambiguous
NIST 4	Hemp	6.82%	0.25%	27.28	Present	<1%	Inactive	CBCA only
NIST 5	**Marijuana**	11.61%	1.92%	6.05	Present	>1%	**Inactive**	CBCA only
NIST 6	Hemp	4.01%	0.14%	28.64	Present	<1%	Inactive	CBCA only
CBD Hemp 1	Hemp	16.60%	0.67%[1]	24.78	Present	<1%	Inactive	N/A
CBD Hemp 2	Hemp	15.35%	0.68%[1]	22.57	Present	<1%	Inactive	N/A
CBD Hemp 3	Hemp	12.96%	0.56%[1]	23.14	Present	<1%	Inactive	N/A
CBD Hemp 4	Hemp	16.61%	0.70%[1]	23.73	Present	<1%	Inactive	N/A
CBD Hemp 5	Hemp	13.63%	0.67%[1]	20.34	Present	<1%	Inactive	N/A
CBD Hemp 6	Hemp	20.54%	<LOQ	>3	Present	<1%	Inactive	N/A
CBD Hemp 7	Hemp	19.49%	<LOQ	>3	Present	<1%	Inactive	N/A
CBD Hemp 8	Hemp	21.81%	<LOQ	>3	Present	<1%	Inactive	N/A
CBD Hemp 9	Hemp	18.27%	<LOQ	>3	Present	<1%	Inactive	N/A
CBD Hemp 10	Hemp	14.80%	<LOQ	>3	Present	<1%	Inactive	N/A
CBD Hemp 11	Hemp	17.12%	<LOQ	>3	Present	<1%	Inactive	N/A
CBD Hemp 12	Hemp	18.28%	<LOQ	>3	Present	<1%	Inactive	N/A
CBD Hemp 13	Hemp	17.26%	<LOQ	>3	Present	<1%	Inactive	N/A
CBG Hemp 1	**Hemp**	<LOQ	0.21%[2]	<3	Present	<1%	**Active**	N/A
CBG Hemp 2	**Hemp**	<LOQ	0.21%[2]	<3	Present	<1%	**Active**	N/A

[1]COA indicated Total THC = THCA x 0.877 + Δ9-THC; [2] COA indicated Total THC = THCA x 0.877 + Δ9-THC + Δ8-THC; * indicates heterozygote peak imbalance with much smaller "active" peaks; † indicates heterozygote peak imbalance with much smaller "inactive" peaks

Figure 1. SNaPshot™ results for a marijuana sample with both THCAS and CBCAS genes. Using touchdown PCR method resulted in relatively balanced peaks in samples with both THCAS and CBCAS.

Table 2. SNaPshot™ results explanation. SNaPshot™ results using the Rotherham and Harbison classification compared with current knowledge about THCAS and CBCAS.

Table 3. Summary of cannabinoid concentrations from COAs, results of GC-MS analysis and genetic assays. This table only shows results from reference samples and commercial hemps. The false negative and false positive samples are highlighted in red. Samples with inconsistent SNaPshot™ and sequencing results are highlighted in yellow.

CBD/ THC	SNaPshot™ genotype	Sample	FP/FN
Low	Both synthases with bigger CBCAS	Marijuana	Possible FP-low CBD hemp
Medium	Balanced peaks or bigger THCAS	Marijuana, Hemp	FP-low CBD hemp
High	CBCAS peaks only	Marijuana, Hemp	FN-high CBD marijuana

Table 4. Relationship between CBD/THC ratio and SNaPshot™ genotype, which could explain false positives (FP) and false negatives (FN).

CONCLUSIONS

- The SNaPshot™ assay reported by Rotherham et al. had an error rate of 10.4% (FN only) if CBG hemp and seed samples were excluded **(Table 3)**.
- Marijuana with higher CBD resulted in false negatives; and hemp plants with low CBD or seed samples resulted in false positives **(Table 4)**.
- Inconsistences and ambiguous results were seen with Sanger sequencing **(Table 3)**; a more thorough approach should be considered.
- Future research should include more varieties of cannabis samples.

REFERENCES

[1] M. Kojoma, H. Seki, S. Yoshida, T. Muranaka, DNA polymorphisms in the tetrahydrocannabinolic acid (THCA) synthase gene in "drug-type" and "fiber-type" Cannabis sativa L, Forensic Sci Int 159(2-3) (2006) 132-40.
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[3] K.U. Laverty, J.M. Stout, M.J. Sullivan, H. Shah, N. Gill, L. Holbrook, G. Deikus, R. Sebra, T.R. Hughes, J.E. Page, H. van Bakel, A physical and genetic map of Cannabis sativa identifies extensive

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