Differentiation of drug and non-drug *Cannabis* using a single nucleotide polymorphism (SNP) assay

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A B S T R A C T

Cannabis sativa is both an illegal drug and a legitimate crop. The differentiation of illegal drug *Cannabis* from non-drug forms of *Cannabis* is relevant in the context of the growth of fibre and seed oil varieties of *Cannabis* for commercial purposes. This differentiation is currently determined based on the levels of tetrahydrocannabinol (THC) in adult plants. DNA based methods have the potential to assay *Cannabis* material unsuitable for analysis using conventional means including seeds, pollen and severely degraded material. The purpose of this research was to develop a single nucleotide polymorphism (SNP) assay for the differentiation of "drug" and "non-drug" *Cannabis* plants. An assay was developed based on four polymorphisms within a 399 bp fragment of the tetrahydrocannabinolic acid (THCA) synthase gene, utilising the snapshot multiplex kit. This SNP assay was tested on 94 *Cannabis* plants, which included 10 blind samples, and was able to differentiate between "drug" and "non-drug" *Cannabis* in all cases, while also differentiating between *Cannabis* and other species. Non-drug plants were found to be homozygous at the four sites assayed while drug *Cannabis* plants were either homozygous or heterozygous.

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1. Introduction

Cannabis sativa is one of the world's most prevalent illicit drugs with an estimated 143–190 million people using *Cannabis* during 2007 [1]. The value of the illicit trade in *Cannabis* in New Zealand alone has been estimated at NZ$131–190 million per year [2,3]. *Cannabis* is also, however, a potentially valuable legal crop which can be grown for fibre, seed oil production and bioremediation [4–6]. Differentiation between legitimate "non-drug" *Cannabis* and illicit "drug" *Cannabis* is an important facet of the regulation of the growth of *Cannabis* as a legal crop [7].

Tetrahydrocannabinol (THC) is the principle psychoactive compound present in *Cannabis* [8,9]. There are a number of additional cannabinoids found in *Cannabis*, the major cannabinoid components include cannabigerol (CBG), cannabidiol (CBD), cannabichromene (CBC) and cannabinol (CBN) [10–12]. Non-drug *Cannabis* is typically defined on the basis of THC content; for example in the European Union hemp must have a THC content below 0.2% [7]. In New Zealand the requirement is for THC content to be below 0.35% [13]. Cannabinoid content may be affected by the age or size of the plant tested and the environmental conditions in which it was grown, and this may in turn affect the accurate determination of *Cannabis* chemotype [11].

Although the methods currently available for identification of drug *Cannabis* are reliable and well established [14], a DNA assay able to discriminate between drug and non-drug *Cannabis* would have additional strengths. Foremost among them is the identification of drug *Cannabis* from material unsuitable for analysis using conventional assays for THC content. This may include juvenile plants, seeds, small leaf fragments, pollen, decaying material, partially burnt material and root material [15].

A number of studies have developed DNA assays to identify *Cannabis* samples, without distinguishing between drug and non-drug *Cannabis* [16–18]. Additionally, de Meijer et al. [19] reported a sequence characterised amplified region or SCAR marker able to differentiate between drug and non-drug *Cannabis* that has been developed from a randomly amplified polymorphic DNA (RAPD) marker associated with high THC in *Cannabis*. This marker was associated with THC/CBD phenotype rather than intrinsically linked to THC synthesis and was not able to unambiguously classify all samples tested [19].

The synthesis of THC in *Cannabis* involves the conversion of a number of precursors by a series of synthase enzymes. The final step in the synthesis of THC is the conversion of cannabigerolic acid (CBGA) into tetrahydrocannabinolic acid (THCA) catalysed by the enzyme THCA synthase [20,21]. THCA is then decarboxylated to THC [21]. This process is mirrored by the conversion of CBGA to cannabidiolic acid (CBDA) and cannabichromenic acid (CBCA) by CBDA synthase and CBCA synthase respectively, followed by subsequent decarboxylation to cannabidiol (CBD) and cannabi-

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chromene (CBC) [22,23]. Drug strains of *Cannabis* are typically high in THC. Oil and fibre strains of *Cannabis* are typically dominated by CBD and occasionally cannabigerol (CBG), the decarboxylated form of CBGA [24]. CBC is found at high levels in juvenile *Cannabis* plants and in strains with a persistent juvenile state [25].

Kojoma et al. [26] sequenced the THCA synthase genes of six drug and seven fibre strains of *Cannabis*. Comparison of these sequences revealed two distinct forms of the THCA synthase gene, one found in the six drug strains the other found in the seven fibre strains. There were a total of 63 nucleotide substitutions differentiating all six drug strain sequences from the seven fibre strain sequences, these corresponded to 37 amino acid substitutions in the THCA synthase gene product. Kojoma et al. [26] considered these divergent THCA synthase sequences to represent alleles coding for an active and an inactive form of the THCA synthase enzyme.

Kojoma et al. described a set of PCR primers used to amplify a 1.2 kb fragment of the proposed active THCA synthase sequence found in the six drug strains [26]. A 1.4 kb fragment of the ribulose bisphosphate carboxylase gene (rbcL) was amplified as a positive control. The principal drawback of this THCA synthase marker is the length of the fragment amplified which may make amplification more difficult, particularly from degraded samples such as those likely to be encountered at crime scenes [27–30].

The aim of this study was to develop a single nucleotide polymorphism (SNP) assay more suited to crime scene samples capable of discriminating between high and low THC *Cannabis* varieties based on sequence variation in the THCA synthase gene and to test this assay on drug and non-drug varieties of *Cannabis*.

2. Materials and methods

2.1. Primer design

The SNP assay was designed based around the single base extension (SBE) protocol of the ABI SNaPshot™ multiplex kit.

A 399 base pair (bp) fragment of the THCA synthase gene was amplified from both drug and non-drug *Cannabis* using the primers C and E of Kojoma et al. [26] with two modifications: two degenerate bases were added to primer C to account for differences between the active and inactive forms of the THCA synthase sequence and the terminal T was removed from primer E to bring the melting temperature closer to that of primer C. These modified primers are referred to as C2 and E2 (Table 1). Primer C2 binds 738 bp from the start of the 1653 bp THCA synthase sequence, while primer E2 (reverse) is located 516 bp from the end of the sequence as shown in Fig. 1.

Extension primers were designed to target four non-synonymous polymorphisms within the THCA synthase gene (Table 1). The active and inactive THCA synthase sequences of Kojoma et al. [26] are differentiated by 63 SNPs that differed in state between the 6 drug type strains and 7 non-drug strains sequenced. The majority of the 63 single nucleotide differences between the active and inactive forms of THCA synthase were not suitable for use as markers for THCA phenotype on the basis of synonymy, similarity with the closely related THCA synthase gene, suitability of flanking primer binding sites and restrictions on amplicon size.

SNP markers were selected based on the following criteria: (1) selected SNPs were non-synonymous (i.e. corresponded to amino acid differences between the active and inactive THCA sequence protein products); (2) the nucleotide state in the drug form was not shared with that in the published sequence of the closely related CBDA synthase gene; (3) transversions (C-A, C-G, T-A, T-G) were preferred to transitions (C-T, A-G); given that transversions are statistically less likely to occur [31], back mutation to the original state is considered to be less likely.
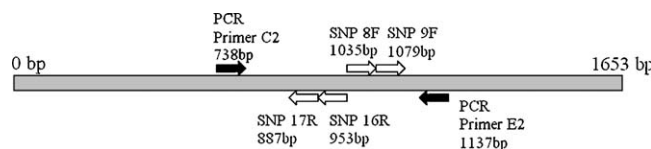

Fig. 1. Binding position of SNP primers on the THCA synthase gene.

These criteria left a set of 20 SNPs for which the forward and reverse sequences were considered during primer design. Four SNP primers were selected to be used in the analyses, all four SNPs had melting temperatures less than 1° either side of 57 °C, a separation of at least five base pairs in length between each primer for ease of analysis.

In addition to the requirements stated above the SNPs selected and their extension primer binding sites had to fall within a single readily amplified PCR amplicon. An assay based on the amplification of the entire THCA synthase gene was considered impractical from a forensic perspective as samples may be of poor quality with potentially degraded DNA. The four markers selected for the final assay fell within the 399 bp fragment amplified by primers C and E of Kojoma et al. [26].

SNPs 8F and 9F were assayed with forward primers, SNPs 16R and 17R were assayed with reverse primers.

SNP 17R is a transition located 887 bp from the beginning of the THCA synthase sequence. The active or drug form of the THCA synthase gene carries an adenine (A) at this locus while the inactive form carries a guanine (G). As the extension primer for this SNP is a reverse primer a red labelled thymine (T) is incorporated during the mini-sequencing reaction for the active form of THCA synthase and a yellow labelled cytosine (C) is incorporated for the inactive form of THCA synthase, giving rise to a red peak for the active form of THCA synthase and a yellow peak, displayed in black during analysis, for the inactive form of THCA synthase.

SNP 16R is a transversion at 953 bp; the polymorphism is an adenine in the active form of the THCA synthase gene and a thymine in the inactive form of the THCA synthase gene. It corresponds to a histidine residue in the active form of THCA synthase and a leucine residue in the inactive form of THCA synthase. As the extension primer for this SNP is a reverse primer a red labelled thymine is incorporated during the mini-sequencing reaction for the active form of THCA synthase and a green labelled adenine is incorporated for the inactive form of THCA synthase, giving rise to a red peak for the active form of THCA synthase and a green peak for the inactive form of THCA synthase.

SNP 8F is a transversion at 1035 bp with a thymine in the active/drug form and a guanine in the inactive/non-drug form, corresponding to a phenylalanine residue in the active form and a tyrosine in the inactive form. As the extension primer for this SNP is a forward primer a red labelled thymine is incorporated during the mini-sequencing reaction for the active form of THCA synthase and a blue labelled guanine for the inactive form of THCA synthase.

SNP 9F is a transversion at 1079 bp with a thymine in the active/drug form and an adenine in the inactive/non-drug form, corresponding to a lysine residue in the active form and an arginine in the inactive form. As the extension primer for this SNP is a forward primer a red labelled thymine is incorporated during the mini-sequencing reaction for the active form of THCA synthase and a green labelled adenine for the inactive form of THCA synthase.

2.2. Sample collection

A total of 79 drug-type *Cannabis* plants and 15 non-drug *Cannabis* plants were analysed. An additional five non-*Cannabis* plant species were analysed to test potential cross-species amplification. *Humulus lupulus* (Common Hop), *Celtis sinensis* (Chinese Hackberry), *Ficus macrophylla* (Moreton Bay Fig) and *Ulmus procera* (English Elm) were selected as relatives of *Cannabis* [32]. *Nicotiana tabacum* (Cultivated Tobacco) was selected on the basis that it may be mixed with *Cannabis* for drug use [33].

Drug-type *Cannabis* samples were obtained from seized materials received at the Institute of Environmental Science and Research (ESR). Hemp samples were obtained from material submitted for cannabinoid testing at ESR, with permission from the suppliers. THC levels were quantified by gas chromatography mass spectrometry (GCMS) for all hemp samples and 51 of the 79 drug-type *Cannabis* samples received.

Table 1
Table of amplification and extension primers used, including oligonucleotide sequence.

Primer	Primer sequence (5′ to 3′)	Primer length	Start position	Direction
PCR primer C2	CAAACTKGTTGYTGTCCCATC	21 bp[a]	738 bp[b]	Forward
PCR primer E2	CGTCTTCTTCCCAGCTGATC	20 bp	1137 bp	Reverse
Extension primer 8F	GAGTTGGGTATTAAAAAAACTGATTGCAAAGAATT	35 bp	1035 bp	Forward
Extension primer 9F	CAACCATCTTCTACAGTGGTGTTGTAAATT	30 bp	1079 bp	Forward
Extension primer 16R	TCRACTAGACTATCCACTCCACCA	24 bp	953 bp	Reverse
Extension primer 17R	TACTGTAGTCTTATTCTTCCCATGATTATCTGTAATATTC	40 bp	887 bp	Reverse

[a] Length of oligonucleotide in base pairs.
[b] Distance from start of sequence in base pairs.

All hemp samples were confirmed as being less than 0.35% THC in accordance with New Zealand law. THC concentrations in the drug samples ranged from 4.1% to 18.15% with an average of 10.7% THC. Where THC concentration was not quantified the presence of THC was confirmed using thin layer chromatography (TLC).

A further 11 samples were provided as blind samples by Julia Wenzel at the Bundeskriminalamt (BKA) Kriminaltechnisches Institut in Wiesbaden. DNA from these samples was extracted in Wiesbaden, Germany and then sent to ESR in Auckland, New Zealand where they were assayed using the SNP markers developed.

2.3. Extraction and amplification

DNA was extracted from dried plant material using the DNeasy® plant mini kit (Qiagen #69104) according to the manufacturer's instructions with the following modification to the tissue disruption portion of the protocol: samples were finely chopped using a scalpel and then ground in a 2 ml tube with an Eppendorf micropestle (Eppendorf 0030 120.973) using a 19 V cordless drill in the presence of 500 ml buffer AP1 and 4 μl RNAse A.

All thermocycling was performed on an Eppendorf epGradient S thermocycler. PCR amplification was performed using Sigma Extract-N-Amp™ PCR ReadyMix™ (Sigma–Aldrich #E3004). The PCR parameters used were as follows: 94 °C for 5 min then 35 cycles of: 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min. This was followed by an additional final extension of 72 °C for 5 min. PCR products were visualised alongside a 1kb+ ladder (Invitrogen #10787-018) on a 1.5% agarose-TBE gel stained with ethidium bromide (Invitrogen #15585-01).

Prior to mini-sequencing 10 μl of PCR product was incubated in a thermocycler with 5 units of antarctic phosphatase (New England Biolabs #M0289S) and 2 units of exonuclease I (New England Biolabs #M0293S) at 37 °C for 30 min to remove unincorporated primers and dNTPs. The enzymes were then inactivated by heating to 75 °C for 15 min.

2.4. Minisequencing

SNP primer extension reactions were performed using 5 μl of SNaPshot™ Multiplex Ready Reaction Mix (ABI Prism® SNaPshot™ Multiplex Kit #4323151), 4 μl of amplified product diluted to one in ten with sterile water and 1 μl of a mix of the four extension primers shown in Table 1. The concentrations of each primer in the final reaction were 0.1 μM primer 16R, 0.2 μM primer 8F, 0.2 μM primer 9F and 0.4 μM primer 17R. The mini-sequencing reactions consisted of 25 cycles of: 10 s at 95°, 5 s at 57.4° and 30 s at 60°. All extension primers were tested individually prior to multiplex amplification.

Following the minisequencing reaction, products were incubated in a thermocycler for 60 min at 37 °C with 1 unit of antarctic phosphatase (New England Biolabs #M0289S) to remove unincorporated fluorescently labelled nucleotides, followed by 15 min at 75 °C to inactivate the enzyme. Minisequencing analysis was performed on an Applied Biosystems 3130 genetic analyser, loading 0.5 μl of minisequencing product with 0.25 μl GeneScan™ 120 LIZ® size standard (ABI #4324287) and 9.25 μl Hi-Di™ Formamide (ABI #4311320), SNP profiles were analysed using Peak Scanner v1.0™ (ABI #4381867).

3. Results and discussion

3.1. Amplification

A DNA fragment of approximately 400 bp was successfully amplified in 94 Cannabis samples using the C2 and E2 primers. No detectable amplification products were observed from the other five species tested.

3.2. Minisequencing

For all four SNPs targeted the extension products observed were, approximately, of the expected length. The expected extension product lengths were 1 bp longer than the extension primer lengths shown in Table 1. The product lengths observed were 1–2 bp longer than expected (Figs. 2–4). This is likely to have been due to the effect of fluorescent dyes on DNA mobility during electrophoresis [34].

Analysis of the drug and non-drug Cannabis samples revealed three different SNP genotypes: drug Cannabis plants homozygous for the active form of THCA synthase (21 individuals, <0.4% THC), characterised by the presence of active THCA synthase extension products only (Fig. 2), drug Cannabis plants heterozygous for the active and inactive forms of the THCA synthase gene (58 individuals, average THC content 10%, range 4.1–14.9% THC) featuring both active and inactive THCA synthase extension products (Fig. 3) and non-drug Cannabis plants homozygous for the inactive form of THCA synthase (15 individuals, average THC content 10.9%, range 4.6–18.2%) with inactive THCA synthase extension products only (Fig. 4).

An additional extension product was observed in all samples investigated with the SNP multiplex. At 25 bp in length this product was close to the expected size of the extension product obtained using the 16R extension primer. The electropherogram peak produced was relatively low and was labelled yellow indicating an incorporated cytosine nucleotide. This extension product was not observed during individual trials of the SNP extension primers, but was observed in negative control reactions involving all four extension primers (Fig. 5). Peak heights (fluorescence) for this extension product did not vary with the height of the diagnostic peaks. As a result of these observations the 25 bp extension product was hypothesised to be a result of primer–primer interaction rather than an extension product from the 16R primer. Given that the nucleotides expected from a Cannabis sample for this SNP are thyamine (red) for drug Cannabis or adenine (green) for non-drug Cannabis, the appearance of this peak does not affect the accurate identification of drug Cannabis.

3.3. Blind test

The 11 blind samples provided by the Kriminaltechnisches Institut in Wiesbaden were assigned putative phenotypes based on
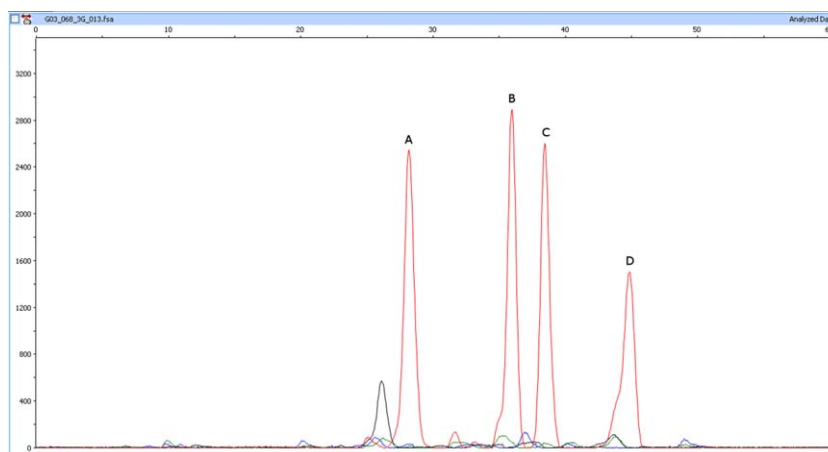

Fig. 2. Drug Cannabis homozygous for the active form of the THCA synthase gene, extension products from the active form of THCA synthase labelled A–D. Fragment lengths: A 28.19 bp, B 35.98 bp, C 38.42 bp, D 44.83 bp. Y-axis in relative fluorescence units (rfu), X-axis in base pairs (bp).
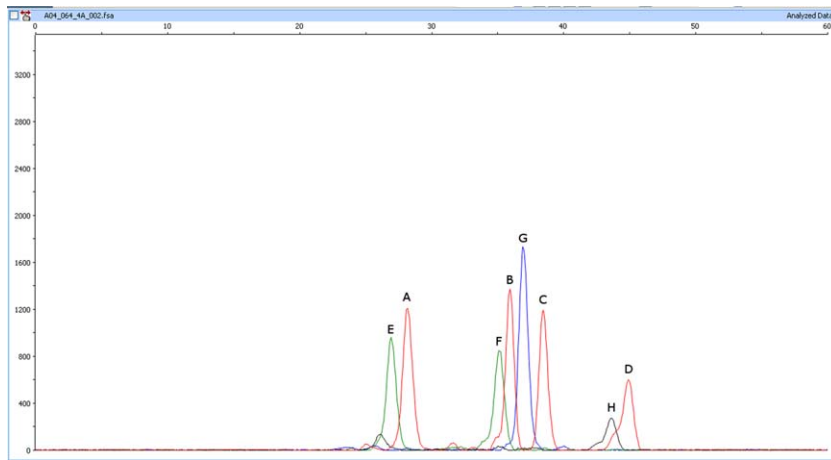
Fig. 3. Drug *Cannabis* heterozygous for the active and inactive forms of THCA synthase, characterised by the presence of both extension products associated with both the active (A–D) and inactive (E–H) forms of the THCA synthase gene. Fragment lengths: A 28.16 bp, B 35.92 bp, C 38.44 bp, D 44.87 bp, E 26.91 bp, F 35.07 bp, G 36.9 bp, H 43.66 bp. *Y*-axis in relative fluorescence units (rfu), *X*-axis in base pairs (bp).
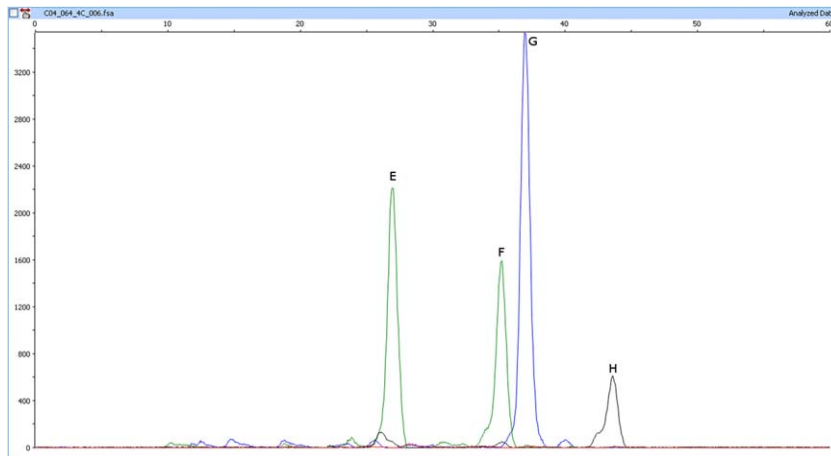

Fig. 4. Non-drug *Cannabis* homozygous for the inactive form of the THCA synthase gene. Fragment lengths E 26.93 bp, F 35.21 bp, G 36.95 bp, H 43.57 bp. *Y*-axis in relative fluorescence units (rfu), *X*-axis in base pairs (bp).
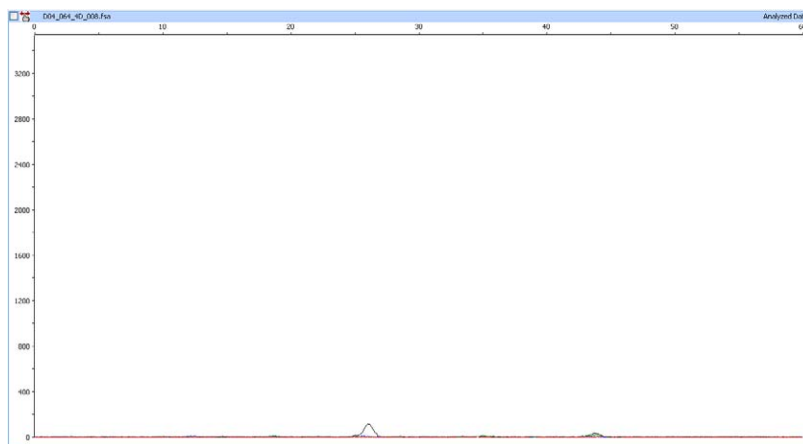

Fig. 5. Electropherogram of a negative control reaction showing a single yellow labelled extension product at 25 bp (A). *Y*-axis in relative fluorescence units (rfu), *X*-axis in base pairs (bp).

the SNP profiles observed. Seven were identified as non-drug samples and three as drug samples, all three drug samples were heterozygous. An eleventh sample was identified as a probable negative control. These results were later confirmed as being consistent with the known identities of the samples by the Kriminaltechnisches Institut in Wiesbaden.

4. Conclusions

The SNP assay developed in this study was able to identify drug *Cannabis* samples with a 100% success rate while excluding all non-drug *Cannabis* samples tested. Of the five other species tested none produced a result which could be interpreted as either drug or non-

drug *Cannabis*. Although the study presented here is a preliminary study the assay developed could be expected to make a valuable contribution subject to a full validation.

The majority of drug *Cannabis* samples analysed in this study were found to be heterozygous for the active form of the THCA synthase gene, indicating that only a single copy of the active form of the THCA synthase gene is necessary to catalyse the conversion of CBGA to THCA. The effect of heterozygosity on the level of THCA synthesised in the plant relative to levels found in plants homozygous for the active form of THCA is currently unknown. The difficulties of separating inherited variation in cannabinoid content from environmental variation [19,35] puts this matter outside of the scope of this paper, although it may be a useful avenue for future research. From a forensic perspective the presence of a copy of the active form of the THCA synthase gene appears to reliably identify plants with a drug *Cannabis* phenotype.

Acknowledgements

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