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ORIGINAL PAPER

Genetic individualization of *Cannabis sativa* by a short tandem repeat multiplex system

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Abstract *Cannabis sativa* is the most frequently used of all illicit drugs in the USA. Cannabis has been used throughout history for its stems in the production of hemp fiber, seed for oil and food, and buds and leaves as a psychoactive drug. Short tandem repeats (STRs) were chosen as molecular markers owing to their distinct advantages over other genetic methods. STRs are codominant, can be standardized such that reproducibility between laboratories can be easily achieved, have a high discrimination power, and can be multiplexed. In this study, six STR markers previously described for *C. sativa* were multiplexed into one reaction. The multiplex reaction was able to *individualize* 98 cannabis samples (14 hemp and 84 marijuana, authenticated as originating from 33 of the 50 states of the USA) and detect 29 alleles averaging 4.8 alleles per loci. The data did not relate the samples from the same state to each other. This is the first study to report a single-reaction sixplex and apply it to the analysis of almost 100 cannabis samples of known geographic origin.

Keywords Short tandem repeat multiplex · Marijuana · *Cannabis sativa* · Genetic identification · DNA typing

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Introduction

Cannabis is the most frequently used of all illicit drugs [1]. Plants that contain high Δ^9-tetrahydrocannabinol (THC) content are described as marijuana and are used as a psychoactive drug [2]. Those that have low THC content are described as hemp and are cultivated for fiber from the stalk or oil from the seeds [1]. There are several hundred strains of cannabis owing to selective breeding to increase THC content [1]. Marijuana is a Controlled Substance Act Schedule 1 drug, meaning it has high potential for abuse and no currently accepted medical use [3]. There are currently 70 cannabinoids [4] found only in marijuana and THC accounts for virtually all the psychoactive effects.

Many branches of the biological sciences aim to identify, individualize, and determine genetic relatedness among and within different species, populations, and individuals. The data generated by different polymorphic DNA markers have been used in taxonomy, phylogeny, ecology, genetics, plant breeding, and forensics [5–8]. With the introduction of the polymerase chain reaction (PCR) [9] a wide variety of PCR-based markers were developed to detect genetic variation. PCR is widely used in forensic analysis because

of its sensitivity, speed, and amenability to minute and even degraded DNA [10].

The most widely used molecular markers for DNA typing are short tandem repeats (STRs) [11], also known as microsatellites [12], or simple sequence repeats [13]. They are DNA sequences of six or fewer bases that are repeated multiple times in tandem arrays and are flanked by unique sequences [14]. Once highly polymorphic STR markers have been identified, they provide an excellent method to assess genetic variation owing to their high information content, ease of genotyping, codominancy, high discriminatory power, and reproducibility [15–18]. These advantages make STRs ideal for several techniques, including genetic mapping [5], marker-assisted selection [19], genetic relatedness studies [7], and DNA typing [8].

Previous typing methods used to analyze *Cannabis sativa* have included restriction fragment length polymorphism [20], random amplified polymorphic DNA [20–27], inter-simple sequence repeat [28], and amplified fragment length polymorphism [29, 30]. Three research groups have reported the development of cannabis STR markers and have used them to DNA-type cannabis [31–34]. This includes a study that used ten STR markers in four separate multiplex reactions to analyze cannabis plants and to create a database of cannabis genotypes in Australia [35].

The objective of the current study was to use an independently developed, single-reaction STR sixplex to genetically identify and individualize cannabis plants known to have originated in the USA.

Methods

Samples

DNA was isolated at the University of Mississippi, National Center for Natural Products Research from 100 cannabis samples from materials of known origin. Out of the 100 samples, 17 were identified as mixtures and were not included in the sample set (Table S1). The 98-sample set also included a marijuana sample donated by a law enforcement agency in south Florida (safe DNA) and 14 hemp DNA samples provided by Tariq Mahmood of the Alberta Research Council in Alberta, Canada.

Cannabis DNA extraction

Cannabis genomic DNA was extracted with the QIAGEN (Valencia, CA, USA) DNeasy plant kit according to the manufacturer's protocol. The high molecular weight DNA obtained was quantified using an Eppendorf™ BioPhotometer (Bio-Rad, Hercules, CA, USA). All samples were diluted to a working stock of 20 ng μl^{-1}. For the sensitivity study, the DNA was diluted to concentrations between 2 and 120 ng μl^{-1}. The DNA used for the interference study was obtained from Florida International University Forensic DNA Profiling Facility.

Primer compatibility for multiplex

To ensure that the primers were compatible, the forward and reverse sequences were analyzed with the software program AutoDimer [36] and screened for potential primer–dimer and hairpin structures. Two primers, ANUCS303, and ANUCS305, were from Gilmore and Peakall [31] and the four other primers, E07-CANN1 (P17), B05-CANN1 (P19), D02-CANN1 (P24), and H06-CANN2 (P25), were from Alghanim and Almirall [34] (Table 1). Trinucleotide primers were chosen by design to avoid any complications with interpreting dinucleotide repeats. The melting temperatures were calculated for each forward and reverse primer using AutoDimer [36] and used to calculate the average annealing temperature for the PCR cycling parameters.

Multiplex PCR amplification

The primers were fluorescently labeled (Table 1) and prior to being multiplexed all were tested in singleplex reactions to ensure all loci were amplified correctly and were identical in the multiplex. The singleplex reactions were analyzed using samples hemp 3, hemp 8, DNA 29, DNA 49, DNA 69, and DNA 80. To optimize the PCR multiplex reagent concentrations, a $MgCl_2$ gradient was performed as well as an annealing temperature gradient. The final PCR reagent concentrations for the multiplex were 1× AmpliTaq Gold™ PCR buffer, 2.5 mM $MgCl_2$, 10 mM concentration of each dNTP (Promega Madison, WI, USA), 0.5U AmpliTaq Gold™ DNA polymerase (Applied Biosystems, Foster City, CA, USA), 0.1% bovine serum albumin (fraction V) (Fisher Scientific, Fair Lawn, NJ, USA), 1 ng μl^{-1} DNA template, and diethylpyrocarbonate-treated water

Table 1 The six short tandem repeat primers used in the multiplex, the repeat region, the allele range, and the fluorochromes used to label the forward primers

Primer	Repeat	Allele base pair range	Fluorochromes
ANUCS303	$(GTG)_7$	142–151	6-FAM
ANUCS305	$(TGG)_{10}$	142–160	VIC
P17	$(CTA)_9$	103–121	VIC
P19	$(TTG)_9$	233–248	6-FAM
P24	$(GTT)_7$	106–115	NED
P25	$(ACG)_7$	269–275	NED

P17 E07-CANN1, *P19* B05-CANN1, *P24* D02-CANN1, *P25* H06-CANN2

to a final volume of 20 μl. The final concentrations of the forward and reverse primers used in the multiplex were as follows: ANUCS303 at 0.15 μM, ANUCS305 at 0.1 μM, P17 at 0.015 μM, P19 at 0.15 μM, P24 at 0.05 μM, and P25 at 0.08 μM. For the singleplex reactions, the reagents were the same except only one primer set was used at a concentration of 0.1 μM for both forward and reverse primers. To confirm the 17 samples were mixtures, they were run individually and with fungal internal transcribed spacer primers at a final concentration of 0.5 μM for forward and reverse primers.

Sensitivity experiments were performed on serial dilutions of hemp 3 DNA ranging from 0.1 to 6 ng μl^{-1} of genomic DNA. An interference study was also conducted to determine if the presence of other genomic DNA would interfere with the amplification of cannabis DNA and included human (male), fungi (*Agaricus bisporus*), bacteria (*E.coli*), cycad (*Dioon mejiae*), St. Augustine grass (*Stenotaphrum secundatum), and hops plant (Humulus lupulus).*

Multiplex PCR thermal cycling conditions

The PCR parameters were as follows: denaturing at 94 °C for 11 min, followed by 35 cycles of 30 s at 94 °C, annealing at 53 °C, and elongation at 72 °C with a final extension of 1h at 72 °C. The PCRs were carried out using a 9700™ thermocycler (Applied Biosystems, Foster City, CA, USA).

Fragment analysis and determination of allelic sizes

The PCR products were electrophoresed in a capillary electrophoresis instrument, ABI Prism™ 310 Genetic Analyzer, using Performance Optimized Polymer 4 (POP-4™), matrix DS-31, and filter set D (6-FAM™, VIC™, NED™, and ROX™ all from Applied Biosystems, Foster City, CA, USA). Each sample was prepared with 0.5 μl PCR product, 9.4 μl of a Hi-Di™ formamide, and 0.1 μl of GeneScan™ 500 ROX™ size standard (Applied Biosystems, Foster City, CA, USA). Samples were electrokinetically injected at 15 kV for 5s and were run at 60 °C for 27min. The data were imported into GeneMapper™ ID version 4 (Applied Biosystems, Foster City, CA, USA) for allele size determination.

Statistical analysis

To discriminate and show relationships between the 98 cannabis samples, the data were analyzed using HW-QuickCheck and PRIMER version 6 [37, 38]. HW-QuickCheck was used to determine the allele frequencies, observed heterozygosity, expected heterozygosity, and the number of alleles per locus. The effective number of alleles per locus (n_e) was calculated according to Morgante's formula [39]:

$$n_e = \left(\Sigma p_i^2\right)^{-1}.$$

The probability of identical genotypes (PI) was calculated according to Paetkau et al. [40] using the following equation:

$$\mathrm{PI} = \Sigma \mathrm{P}_\mathrm{i}^4 - \Sigma\Sigma\left(2P_iP_j\right)^2.$$

PI represents the average probability of a match for any genotype in a given population. p_i is the frequency of the ith allele at a locus, and P_j equals the frequency of the (i+1)th allele. The PI over multiple loci is calculated as a product of the individual loci.

An analysis of molecular variance (AMOVA) was also performed to estimate the genetic variation among and within the hemp and marijuana samples using GenAlEx [41]. To analyze the samples using PRIMER version 6, each allele was treated as a separate entity and scored as a discrete variable using 1 to indicate presence, and 0 for absence. Cluster analysis was performed using Euclidean distance as defined by the following equation:

$$D_1 = \sqrt{\sum_i \left(y_{i1} - y_{i2}\right)^2}$$

Results and discussion

DNA extraction

The extraction was successful for all samples, most of which were 3 years of age or younger (stored as dried leaf or bud material at room temperature) and one of which was at least 7 years old. The DNA recovered concentrations ranged from 21 ng μl^{-1} to as much as 304 ng μl^{-1} and all samples were successfully amplified.

PCR multiplex

After analyzing the primer sequences using AutoDimer, we detected no primer–dimer or hairpin structures for six of the primer sets selected. Primer ANUCS307 reverse was found to form a primer–dimer with primer P24 reverse; therefore, the ANUCS307 primer set could not be incorporated into the multiplex.

PCRs were optimized by using primers at various concentrations and were adjusted after each run until all six loci were being amplified correctly and no locus was preferentially amplified. Temperature and $MgCl_2$ gradients were performed and the necessary adjustments were made until the optimal PCR conditions were met. The best results

for all loci were achieved at 2.5 mM $MgCl_2$; therefore, this concentration was used for all multiplex and singleplex reactions. The annealing temperature gradient proved that the amplification was successful at all annealing temperatures. The annealing temperature of 53 °C was used since this temperature provided the most reproducible results and was very close to the annealing temperature calculated using AutoDimer. Thirty-five cycles provided the optimal resolution as an increased number of cycles produced nonspecific amplification. A final extension of 60 min was used to alleviate the nontemplate adenylation observed with some of the primers in the multiplex.

A sensitivity study was performed with the STR multiplex to determine the upper and lower limits of DNA template concentrations that can be successfully amplified. Reproducible allele peaks were observed with DNA concentrations as low as 0.1 ng μl^{-1}, while DNA concentrations as high as 6.0 ng μl^{-1} produced repeatable peaks that were not as sharp, with some split peaks observed (Fig. 1). The optimal starting DNA template concentration selected was 1 ng μl^{-1}.

An interference study was conducted using non-cannabis DNA to resolve any issues with species specificity with the multiplex,. The interference study showed some cross-species amplification of fungi and hops (Fig. 2). The other DNA tested produced peaks with very low signals (70–300 relative fluorescent units) but there were no allele overlaps. Hops DNA was amplified for allele P25 within the marker range but clearly different from a cannabis allele (Fig. 2).

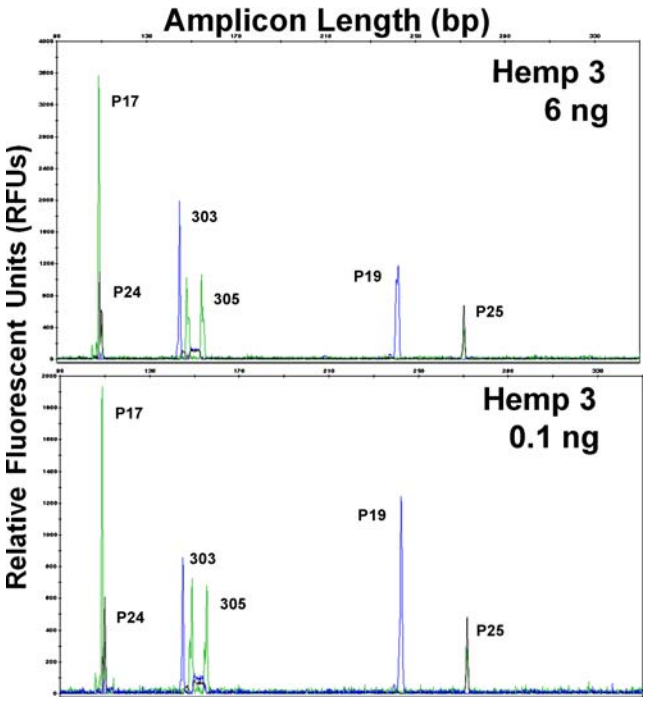

Fig. 1 Sensitivity test with sample hemp 3 DNA at concentrations of 6 ng μl^{-1} (*top*) and 0.1 ng μl^{-1} (*bottom*) both analyzed at the same conditions for the thermocycler and the capillary electrophoresis. The *x*-axis is the amplicon length in base pairs (*bp*) of the alleles from 100 to 300 bp. The *y*-axis is the intensity of the allele peaks in relative fluorescence units. The allele peaks are labeled with their corresponding loci. *P17* E07-CANN1, *P19* B05-CANN1, *P24* D02-CANN1, *P25* H06-CANN2, *303* ANUCS303, *305* ANUCS305

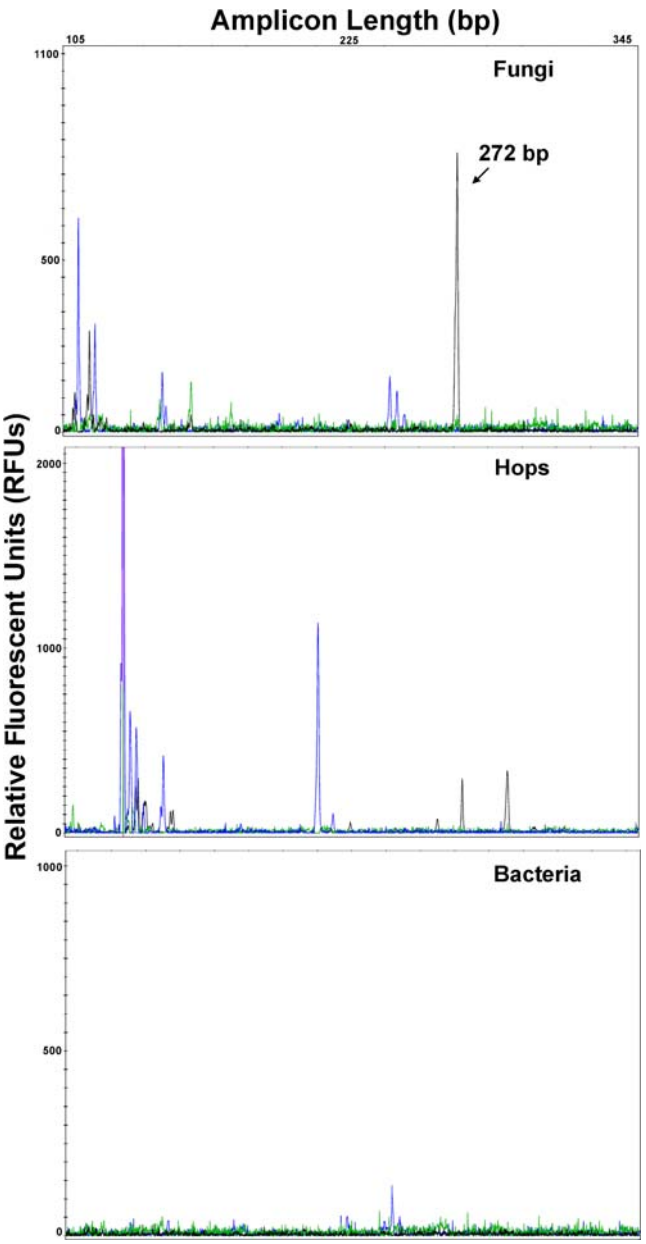

Fig. 2 Interference study with fungi, hops, and bacteria DNA. The *x*-axis and the *y*-axis are the base amplicon length of the alleles and the intensity of the allele peaks in relative fluorescence units, respectively. The electropherograms show hops DNA was amplified the most but there was no allele overlap with the cannabis alleles. The allele peak for fungi DNA at the P25 locus was found to overlap with the cannabis allele at 272 bp

Fungi DNA was also amplified for primer P25, producing the only cannabis allele observed (272bp) at high intensity (Fig. 2). Therefore, fungi DNA, if present, could be a possible interferent when amplifying cannabis DNA with the multiplex.

The allele calls amplified with the multiplex (Fig. 3a) were identical to the alleles in the singleplex (Fig. 3b) reactions for all the samples. Figure 3 illustrates that the allele peaks for cannabis sample DNA 80 are identical in the singleplex and multiplex. This confirmed the conservation of the allele calls in the six-primer multiplex, justifying the use of the six-primer multiplex in place of six separate singleplex reactions which was robust, sensitive, and species-specific. A blind study was also conducted which confirmed that the same results could be obtained from the multiplex regardless of the laboratory technician handling the samples or data.

One hundred marijuana samples believed to be individual plants were donated for this study. However, it is not unusual to encounter more than one plant contributor (mixture) when dealing with dried marijuana leaf and bud material. This sixplex method was able to identify 17 mixtures out of the 100-sample set. The samples were identified as mixtures owing to the presence of more than two alleles per locus and a peak imbalance of greater than 30% for heterozygous alleles. To confirm that these samples were in fact mixtures and not interference, the 17 mixtures were analyzed individually and amplified with fungal primers (since this proved to be the only detectable interference). The individual runs were identical to those of the multiplex for all samples and the fungal primers only amplified within the P19 marker allele range but there was no allele overlap.

STR profiles

The six-primer multiplex was sufficient to distinguish all 98 cannabis samples (Table S2). The alleles differed by the expected three base pairs (trinucleotide repeats) and the heterozygous alleles had a peak balance within 30%. The DNA samples that were extracted from the bud material generally contained higher THC content than the DNA samples extracted from the leaf (Table S1); this is expected according to the literature [3, 5]. Even though there was no uniformity in extracting DNA from the leaves or buds with all the samples (with two exceptions, DNA 73 and DNA 86) all the samples with a THC content of 10% or greater were grown indoors. The samples with the highest THC content were not restricted to any particular region (state); however, four of the 12 samples with a THC content greater than 10% were collected from Florida.

Genetic identifications and relationships

There were a total of 29 alleles detected across all six loci. The number of alleles per locus ranged from three at locus P25 to seven at locus ANUCS305, averaging 4.8 alleles and 2.67 effective alleles per locus (Table 2). Allele diversity was evaluated using expected heterozygosity, which ranged from 0.53 to 0.75 and averaged 0.61. The observed heterozygosity ranged from 0.40 to 0.55 and averaged 0.47. Since cannabis plants are clonally propagated, this increases the likelihood of finding identical genotypes. Taking both cloning and selective breeding into consideration, one would expect a decrease in heterozygosity (genetic variation) in cannabis as is seen in other eukaryotes with similar manipulation [42, 43]. This

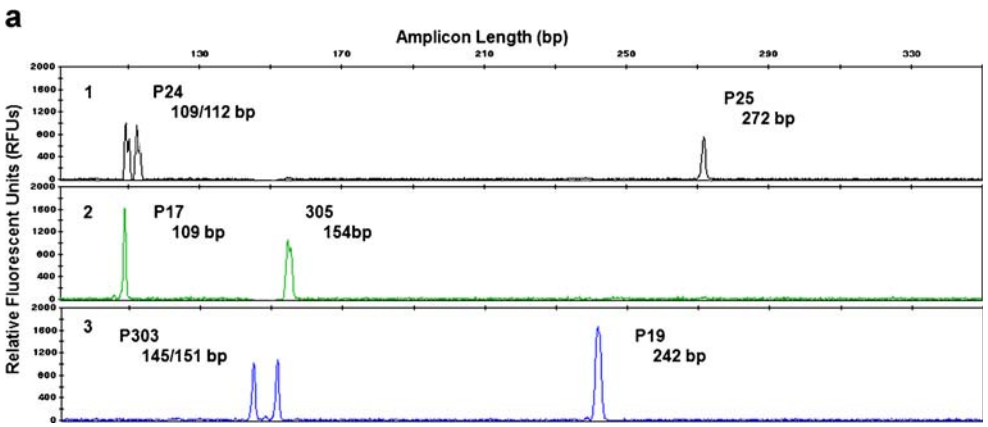

Fig. 3 Individual allele peaks (**a**) and the multiplex allele peaks (**b**) for the sample DNA 80 are shown in the electropherogram. The *x*-axis and the *y*-axis are the amplicon length and relative fluorescence unit intensities of the allele peaks, respectively. These are separate electropherograms grouped together, six for the individual runs (one for each locus) and one for the multiplex with three panels, one for each of the three dyes: *1* is NED for P24 and P25, *2* is VIC for P17 and ANUCS305, and *3* is 6-FAM for ANUCS303 and P19. All the allele calls were identical for both individual and multiplex runs

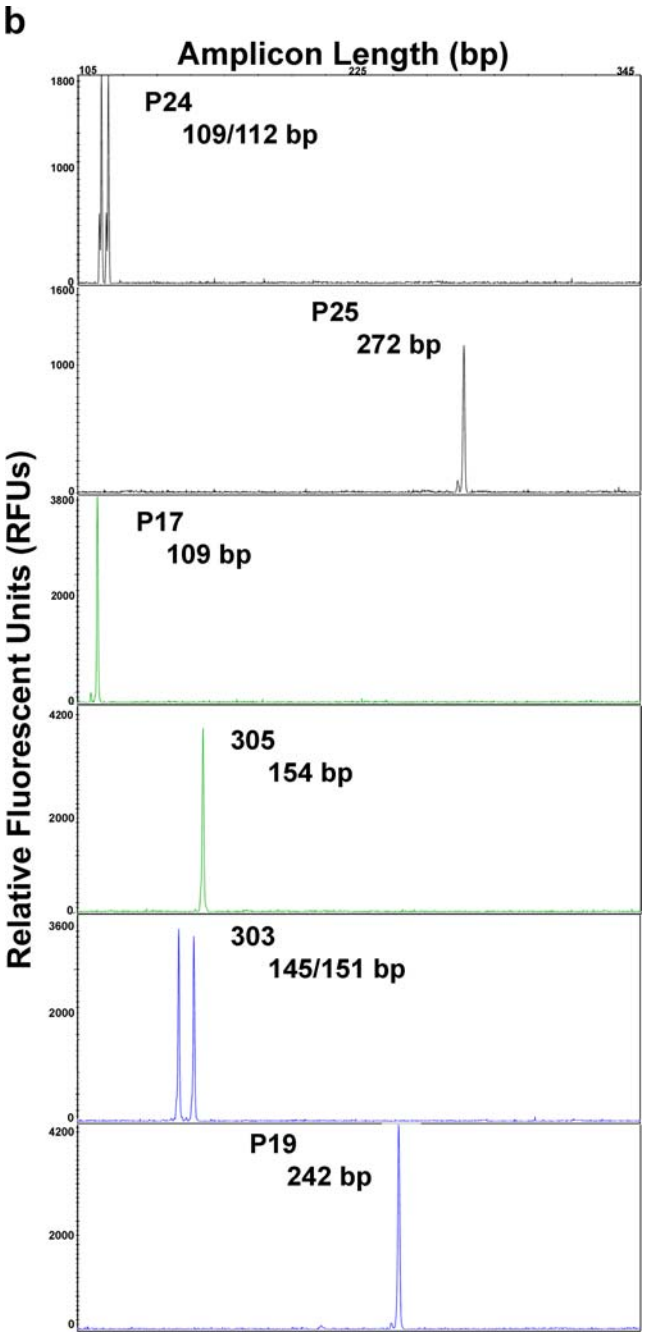

Fig. 3 (continued)

selective breeding is also known to promote linkage disequilibrium in many crop plants [44] and it may also be applied to cannabis for the same reasons.

The frequencies for the alleles found for the 98 samples at each locus are shown in Table 3. There were four alleles (233 and 248 at locus P19, 106 at locus P24, and 269 at locus P25) unique to marijuana samples and two alleles unique to hemp samples (160 at locus 305 and 121 at locus P17). Using this six-primer STR multiplex, we calculated the PI to be 1.10×10^{-4}, or one in 9,090. This PI is high enough to not expect unrelated samples to have the same multilocus genotype. Identical genotypes are more likely to be a result of clonal propagation than by chance alone. According to the results from HW-QuickCheck, several of the loci vary from Hardy–Weinberg (HW) expectations. It is difficult to assume HW equilibrium for cannabis plants since they are routinely manipulated by several methods. They are selectively bred to increase their THC content,

Table 2 The statistical data of the cannabis samples, including the observed heterozygosity (*Ho*), expected heterozygosity (*He*), number of alleles (*N*) and effective alleles (n_e), probability of identical genotypes (*PI*) per locus, and probability of identity for all six loci

Locus	Ho	He	*N*	n_e	PI
ANUCS303	0.48	0.64	4	2.78	0.19
ANUCS305	0.55	0.75	7	4.00	0.11
P17	0.49	0.62	6	2.63	0.23
P19	0.46	0.55	5	2.22	0.29
P24	0.45	0.56	4	2.27	0.26
P25	0.40	0.53	3	2.13	0.29
Average	0.47	0.61	4.83	2.67	1.10×10^{-4}[a]

[a] Product of the PI across all loci

Table 3 Alleles and their frequencies for each locus

Locus	Allele	Frequency
ANUCS303	142	0.2194
	145	0.5153
	148	0.0459
	151	0.2194
ANUCS305	142	0.2908
	145	0.0408
	148	0.0765
	151	0.0102
	154	0.2959
	157	0.2806
	160	0.0051
P17	103	0.0204
	106	0.102
	109	0.4388
	112	0.4286
	115	0.0051
	121	0.0051
P19	233	0.0051
	239	0.0153
	242	0.5612
	245	0.3622
	248	0.0561
P24	106	0.0051
	109	0.5816
	112	0.2959
	115	0.1173
P25	269	0.2296
	272	0.6378
	275	0.1327

resulting in the possible isolation of the THC gene [1]. Previous studies have shown that the genetic variation of the areas near a selected locus can be altered [45]. This could be an explanation for why the loci do not conform to HW expectations. The AMOVA results show that with this STR sixplex there was a 10% variance between the marijuana and hemp samples, which explains why they could not be distinguished genetically. Using PRIMER with both geographic region (state) and THC content as factors for analyzing the samples, we found no groups were distinguishable (i.e., the samples from the same state did not group together nor did samples with high or low THC content).

These results were found to be in good agreement with those of similar studies [31, 34]. For example, for locus ANUCS305, seven alleles were detected in this and previous studies and locus P17 previously produced three alleles and this study found seven. The improvements over previous studies may be due to the fact that only a few markers from each group were selected (Table 2) purposefully because they were better suited. It may also be that this study encompasses a larger sample set than either previous study, 98 samples compared with 48 [31] and 41 [34] samples. The total number of markers used for this study was less (six) than in either study (15 and 11), which contributes to the lower PI. A recently published study [35] reports the use of four more markers but at a cost of having to perform three more PCRs, thereby significantly increasing the analysis time. This is the first one-reaction STR sixplex reported for cannabis DNA typing.

Conclusions

The STR sixplex was successfully able to differentiate each of the 98 cannabis samples as individuals (and detect a mixture when present). The probability of finding the same genotype in a population of unrelated individuals was calculated to be one in 9,090 cannabis samples.

Marijuana and hemp samples are too genetically similar and cannot be distinguished on the basis of the STR genotypes using this sixplex. Many methods were attempted to try to group samples of similar origin and percentage THC content together but they were all unsuccessful.

The STR sixplex described was found to be reproducible, simple, efficient, and cost-effective. The ability to individualize marijuana samples to such a degree could serve as a forensic tool by using plant evidence in criminal casework and potentially aid in the identification of clonal marijuana, linking the major marijuana growers and distributors. The success of cannabis DNA typing illustrates how botanical evidence could be an added tool for criminal and civil casework.

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