Use of embryos extracted from individual *Cannabis sativa* seeds for genetic studies and forensic applications

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ABSTRACT: Legal limits on the psychoactive tetrahydrocannabinol (THC) content in *Cannabis sativa* plants have complicated genetic and forensic studies in this species. However, *Cannabis* seeds present very low THC levels. We developed a method for embryo extraction from seeds and an improved protocol for DNA extraction and tested this method in four hemp and six marijuana varieties. This embryo extraction method enabled the recovery of diploid embryos from individual seeds. An improved DNA extraction protocol (CTAB3) was used to obtain DNA from individual embryos at a concentration and quality similar to DNA extracted from leaves. DNA extracted from embryos was used for SSR molecular characterization in individuals from the 10 varieties. A unique molecular profile for each individual was obtained, and a clear differentiation between hemp and marijuana varieties was observed. The combined embryo extraction-DNA extraction methodology and the new highly polymorphic SSR markers facilitate genetic and forensic studies in *Cannabis*.

KEYWORDS: forensic science, *Cannabis sativa*, marijuana, seed embryo, DNA extraction, simple sequence repeats

Cannabis sativa L. is native to Central Asia and is one of the oldest domesticated plants (1). *Cannabis sativa* is unique in containing cannabinoids, which are C_{21} terpenophenolic compounds with bioactive (medicinal and/or psychoactive) properties (2). Divergent selection for fiber, food and oil in some types (hemp; *C. sativa* var. *sativa*) and for psychoactive properties due to high contents of Δ-9-tetrahidrocannabinol (THC) in others (marijuana; *C. sativa* var. *indica*), have resulted in different plant typologies (1,3). Usually, hemp plants are tall and have long stems (fiber use), produce large quantities of seeds (food use), or both (dual use) and have low THC contents; marijuana plants are generally small and have a high THC content (up to 20-25%) in the glandular trichomes of female inflorescences (3,4). Most populations and cultivars of *C. sativa* are dioecious, and sex is determined by heteromorphic chromosomes, with males being XY and females XX (5); however, some hemp cultivars are monoecious, which is a trait particularly important for cultivars bred for seed production. In the case of marijuana, male plants are of little interest for drug production. Therefore, sex reversion using silver thiosulfate, silver nitrate or aminoethoxyvinylglycine is used to obtain functionally male flowers in genetically female plants with 100% X male gametes with which to fertilize X female gametes, resulting in an offspring of 100% female plants (5). These feminized varieties are currently the most popular in marijuana cultivation (6).

Marijuana cultivation is forbidden in most countries of the world (7). The legal distinction between drug and non-drug types is generally based on the THC content. For example, in the European Union, a limit of 0.2% THC in dried material was enforced in 2001, and the cultivation of plants and possession of *Cannabis* plants or its parts with THC concentrations above this limit is forbidden (4). The prohibition of the cultivation of drug-type *Cannabis* complicates the study of genetic diversity and crop evolution as

well as forensic research in this species because the leaves and inflorescences of marijuana plants have THC concentrations above legal limits (8). However, *Cannabis* kernels (botanically achenes, but for convenience called seeds) present very low levels of THC even in drug types with maximum values below 0.02% (9). Thus, the possession, use, and commercialization of marijuana seeds are legal in many countries. Therefore, using seeds instead of plants for DNA extraction may overcome the legal difficulties of cultivating drug-type *Cannabis* plants to obtain leaf samples for standard DNA extraction from leaves (10).

Seeds have three genetically distinct components: the embryo, endosperm and seed coat (11). In *Cannabis*, the botanical seed is covered by the pericarp, which is firmly attached to the seed coat. The embryo is 2n and contains maternal (n) and paternal (n) material; the endosperm is 3n and is genetically identical to the embryo with the exception of the ploidy level of the maternal material, which is 2n. The seed testa and pericarp are 2n and are composed of somatic maternal genetic material (11). Given that *Cannabis* is an allogamous plant with a high degree of heterozygosis (5,12), DNA extraction of whole *Cannabis* kernels would result in a mixture of genetic material from the zygotic embryo with genetically distinct somatic maternal material. Thus, the extraction of DNA from seed embryos would provide DNA from diploid zygotic *Cannabis* individuals without the need to grow plants. In addition to the interest in genetic diversity and evolution studies, the DNA extraction from individual seed embryos would also be of interest to forensic studies that seek to quickly identify the *Cannabis* type in seized seed samples.

DNA of a sufficient quality and concentration is required for successful and repeatable genotyping with molecular markers. Most standard DNA extraction protocols have been developed for young leaf tissue (10). However, *Cannabis* seeds

(and therefore their embryos) contain high concentrations of substances that can negatively interfere with DNA extraction, such as protein (approximately 25%), carbohydrates (>25%), and fats (>35%) (13), as well as significant amounts of phenolic compounds (14). Therefore, specific protocols may be required for the extraction of sufficient quantities of quality DNA from *Cannabis* embryos.

Here, we present a method for the rapid extraction of embryos from *C. sativa* seeds and evaluate several protocols for DNA extraction, and we applied this method to determine the relationships among varieties of hemp and marijuana. The aim was to develop and test a methodology that enables rapid and efficient molecular marker studies from *C. sativa* embryo tissue from both hemp and marijuana types. This methodology may contribute to enhancing molecular genetics research in *Cannabis*, especially for studies in drug-type materials.

Materials and Methods

Plant material

Seeds of 10 varieties of *C. sativa* were used (Table 1). Four of these varieties correspond to hemp (*C. sativa* var. *sativa*) and six to marijuana (*C. sativa* var. *indica*). The hemp varieties were obtained from four different sources, and the marijuana varieties originated from two companies (Table 1). The hemp varieties were either dioecious or monoecious, whereas the marijuana varieties were feminized (i.e., 100% females) varieties (Table 1). To compare the quality of embryo-extracted DNA and young leaf-extracted DNA, seeds of hemp accession BSC002 were germinated, sown in

commercial growing substrate and grown in a climatic chamber. Young leaf tissue was obtained from young plants with a height of approximately 15 cm.

Embryo extraction from seeds

The following procedure was developed to extract the embryos from *C. sativa* seeds (Fig. 1):

1. Achenes (seeds) are placed on a Petri dish with moistened hydrophilic cotton covered by a layer of filter paper.
2. When the radicle begins to emerge (1-2 mm) from the swollen seeds, which typically occurs after 1-3 days at room temperature, a perimetral incision into the pericarp and seed coat is made beginning from the point of emergence of the radicle to the other end using a scalpel.
3. The pericarp, testa and endosperm are removed with forceps to expose the embryo.
4. The embryo, with no observable traces of tissue from the endosperm, testa, or coat, is transferred with a forceps to an Eppendorf tube and immediately frozen with liquid N and stored at -80ºC until analyzed.

DNA extraction protocols

Embryos of variety BSC002 were used to test six DNA extraction protocols. Four of the protocols were based on the cetyltrimethylammonium bromide (CTAB) method (10), and the two others consisted of commercial kits. The CTAB methods

included a conventional CTAB method (CTAB0) and three modifications of the CTAB0 method: a) the addition of 1% PVP40 to the extraction buffer (CTAB1), b) the addition of 1% proteinase K to the extraction buffer (CTAB2), and c) the addition of phenol:chloroform:isoamyl alcohol (25:24:1) prior to washing with chloroform:isoamyl alcohol (24:1) (CTAB3). Extractions using the DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany; KIT1) and NucleoSpin® Plant II (Macherey-Nagel, Düren, Germany; KIT2) commercial kits were performed according to the manufacturers' instructions.

For the CTAB-based DNA extraction protocols, the procedure included the following steps (modifications are indicated in bold for each of the CTAB1-CTAB3 protocols):

1. Frozen individual embryos are placed in a 1.5 mL microtube and ground under liquid nitrogen in a mixer mill using two metal balls.
2. Immediately after maceration, 700 μL of extraction buffer and 1.4 μL of β-mercaptoethanol are added.
3. The samples are incubated at 65ºC for 30 min and are shaken gently at regular intervals every 5 min.
4. Subsequently, 700 μL of 25:24:1 of phenol:chloroform:isoamyl alcohol are added to the microtubes (**this step only for CTAB3**).
5. The microtubes are centrifuged at 11.000 rpm for 5 min.
6. The supernatant is recovered and transferred to a new microtube.
7. A volume of 700 μL of 24:1 of chloroform:isoamyl alcohol is added to the microtubes, which are immediately shaken.
8. The microtubes are centrifuged at 11,000 rpm for 5 min.

9. The supernatant is recovered and DNA precipitated with 700 μL of absolute ethanol.

10. The microtubes are left for 10 min in a freezer at -20ºC.

11. The microtubes are centrifuged at 11,000 rpm for 5 min, and the supernatant is discarded.

12. The pellet is washed with 1 mL of cold 70% ethanol.

13. The supernatant is discarded and the pellet is air-dried.

14. The pellet is resuspended in 50 μL of TE buffer.

The extraction buffer consisted of 2% CTAB, 1.42 M NaCl, 100 mM Tris-HCl, 20 mM EDTA (**all CTAB methods**), 1% PVP40 (**only for CTAB1**), and 1% proteinase K (**only for CTAB2**). In all cases, the pH was adjusted to 8.

The absorbance of the extracted DNA was measured at 260 nm with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The DNA concentration was calculated using the NanoDrop nucleic acid application module. DNA purity was assessed based on 260/280 and 260/230 absorbance ratios. DNA integrity was evaluated via 0.8% agarose gel electrophoresis.

To compare the results of the extraction from the seed embryos and those from leaves, the conventional CTAB0 protocol together with the best protocol among the other five tested were evaluated for the extraction of DNA from 100 mg of young leaves of plantlets of hemp accession BSC002.

SSR characterization

For simple sequence repeat (SSR) characterization, 11 genomic SSRs (Table 2) were developed *de novo* from the genome of *C. sativa* var. *indica* (variety Purple Kush) (15) using the SciRoKo software (16). These SSRs were used for the amplification of DNA extracted from one embryo of each of the ten *C. sativa* varieties using the selected extraction protocol.

The PCR reaction consisted of 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.04 units of *Taq* DNA polymerase (Roche, Basel, Switzerland), 0.05 μM forward primer, 0.25 μM reverse primer, 0.2 μM M13 fluorescent-labeled primer, 10 ng of DNA and dH_2O in a 10 μL total reaction volume. The amplifications were carried out in an Eppendorf thermocycler with an initial step at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 58 °C for 45 s, 72 °C for 1 min and a final 10-min extension at 72 °C. The PCR products were separated in an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, California, USA). The analysis was performed using GENSCAN and Genotyper software (Applied Biosystems).

For each SSR locus, the number of alleles (N_a) and frequency of the predominant allele (f) were determined. The polymorphism information content (*PIC*) was calculated as $PIC = 1 - \sum_{i=1}^{n} p_i^2 - \sum_{j=i+1}^{n} 2p_i^2 p_j^2$, where *n* is the total number of alleles detected, p_i is the frequency of the *i*th allele, and p_j is the frequency of the *j*th allele. Additionally, the observed heterozygosity (H_o) and expected heterozygosity (H_e, calculated as $H_e = 1 - \sum_{i=1}^{n} p_i^2$ were determined. Nei and Li genetic similarities were calculated and used to graphically represent the genetic relationships among accessions via principal coordinate analysis (PCoA) using GenAlEx 6.5 software (17).

Results

Embryo extraction from seeds

We extracted complete embryos from *C. sativa* seeds for DNA extraction (Fig. 1). Seed imbibition and the initiation of germination softens the pericarp and seed testa and facilitates the release of intact complete embryos from the seed. The most critical step in the protocol is the perimetral incision into the pericarp and seed coat with a scalpel. Care must be taken to avoid making a cut that is too deep (which may damage the embryo) or too shallow (which may complicate the extraction). Embryos extracted from seeds using this protocol have a neat appearance and present neither traces of other seed or pericarp tissue nor browning due to mechanical damage to the embryo during extraction (Fig. 1). Immediate freezing in liquid nitrogen ensures that the embryo tissues are conserved under optimum conditions for DNA extraction. This protocol does not require special technical skills (with the exception of the commonly used security measures when using the scalpel). Using this protocol, we were able to extract more than 30 embryos/h per person.

DNA extraction

The highest embryo DNA yields were obtained using the CTAB methods (Table 3). The highest yield was obtained with CTAB3 with an average concentration of 614 ng/μL, followed by the CTAB method without modifications (CTAB0) with an average concentration of 377 ng/μL. The two other CTAB methods (CTAB1 and CTAB2) gave lower values but were significantly higher than those obtained with the commercial kits (Table 3). The best purity of DNA from extracted from embryos was obtained using the CTAB3 method (Table 3). The extraction of DNA from young leaf tissue with the

conventional CTAB0 and CTAB3 protocols revealed that high concentrations of DNA were obtained with average values greater than 750 ng/μL in both cases (Table 3). A comparison of the DNA concentration and purity results obtained with the CTAB3 protocol from embryo extractions with the CTAB0 and CTAB3 protocols for young leaf tissue revealed that the quality of embryo DNA extracted with this protocol was similar to that of DNA extracted from young leaf tissue.

SSR characterization

The combinations of primers for the 11 genomic SSRs produced successful amplification products as discrete bands that were effectively resolved. One of the primer combinations (G3) amplified two loci, which we denominated CSG3a and CSG3b, resulting in a total of 12 SSR loci that were scored (Table 4). In each of the individuals, amplified SSR loci revealed either one (homozygous) or two (heterozygous) alleles.

A total of 64 alleles were detected for the 12 loci with an average number of alleles per locus (N_a) of 5.33 and a range between two (CSG03b and CSG14) and 11 (CSG24) (Table 4). The frequency of the predominant allele (*f*) ranged from 0.20 (CSG20 and CSG24) and 0.86 (CSG14) with an average value of 0.49. The average value for *PIC* was 0.59; however, the *PIC* value of individual SSR loci ranged between 0.21 (CSG14) and 0.87 (CSG24). The mean value for the observed heterozygosity (H_o; 0.23) was lower than that for the expected heterozygosity (H_e; 0.63). The values for H_o ranged between 0 (five loci) and 0.70 (CSG24), whereas those for H_e ranged between 0.24 (CSG14) and 0.88 (CSG24). For all loci, the H_o value was lower than the H_e value (Table 4).

All individuals tested presented a unique SSR profile and, with the exception of marijuana variety Buddha Purple Kush, which was homozygous for all loci, all individuals presented several loci in heterozygosis. No SSR alleles were specific and universal to all hemp or marijuana varieties.

The first and second principal coordinates of the PCoA analysis accounted for 34.5% and 16.5% of the total variation, respectively. The representation of the varieties in the PCoA graph showed that the *C. sativa* var. *sativa* and *C. sativa* var. *indica* accessions were plotted in different sections of the graph (Fig. 2) and were separated by the first component. The hemp varieties had positive values for the first principal component, whereas marijuana varieties had negative values. The hemp varieties presented a wider dispersion in the second component than the marijuana varieties (Fig. 2). When considering the origin of the marijuana varieties, the materials from Hemp Trading plot together; those from Automaris are more dispersed (Fig. 2).

Discussion

Although methods for DNA extraction from whole *Cannabis* seeds are available (18), the seeds contain distinct genetic components, including 2n maternal genetic material in the seed testa (and in the pericarp of the achene in the case of *Cannabis*), 2n maternal (n) and paternal (n) material in the embryo, and 3n genetic material identical to that of the embryo (with the exception for the ploidy level of the maternal parent (2n)) (11). Therefore, DNA extraction from seeds may result in a mixture of two genetically distinct individuals, the maternal parent and the new zygotic individual represented by the embryo, which may complicate the interpretation of genotyping data. DNA extraction from embryos represents an alternative.

We developed and validated a method for the extraction of DNA from *C. sativa* seed embryos that is suitable for SSR molecular marker analysis. Our method has the advantage of making a 2n zygotic individual available for DNA extraction from a source (seed) that contains very low levels of psychotropic THC both in hemp and marijuana varieties (9). In contrast with whole plants, the possession and commercialization of marijuana seeds is legal in many countries because their THC content is below legal limits (9). Therefore, this method enables the genotyping of *C. sativa* individuals without the need to grow plants and may overcome legal issues related to the prohibition of marijuana plant cultivation (7) and the possession of *Cannabis* material with THC levels above the legal limits, which makes it difficult to perform genetic studies in this species. Additionally, by avoiding the need to grow plants, this method accelerates research because there is no need to wait for seed germination and plant growth. This approach also reduces the costs associated with plant cultivation (19).

Mature dry *C. sativa* achenes (seeds) are hard, and the mechanical extraction of embryos is impractical. To facilitate embryo extraction, seeds are moistened to trigger germination, which softens the achene pericarp and seed coat. In *Cannabis*, the embryo occupies the entire seed cavity, and the endosperm is represented by only a thin layer immediately below the testa (20), which is easily removed. Similar to non-germinated seeds, the THC levels of germinating seeds of *Cannabis* remain very low (8). Using our method, a large number (>30 embryos/h) of embryos without any other seed tissue remains can be obtained for DNA extraction. Furthermore, the embryos do not sustain mechanical injuries from the extraction process, which avoids the oxidation of polyphenols and other reactions that can interfere with DNA extraction. Although seeds

of *C. sativa* var. *sativa* are usually somewhat larger than those of *C. sativa* var. *indica*, (21) the embryo extraction protocol works well in both botanical varieties.

Most standard protocols for DNA extraction, including that for *Cannabis* (22), are based on fresh leaf tissue (10). However, seeds present a composition very different from young leaves and in the case of *Cannabis* have a high content of compounds that may interfere with DNA extraction, such as proteins, carbohydrates, fats, and phenolics (13,14). The standard CTAB method as well as commercial kits did not enable the extraction of large quantities of high-quality DNA for molecular studies from the seed embryos. The CTAB method (10) with a modification consisting of the additional 25:24:1 phenol:chloroform:isoamyl alcohol prior to the first centrifugation step was shown to be the most adequate method among those tested. Compared with the other methods, this modification of the protocol likely results in the recovery of a great proportion of DNA by disrupting cell membranes and allowing the release of DNA (23). Our protocol allows for the acquisition of high-concentration and good-quality DNA with values close to the ideal 1:2:1 for the proportions between absorbances at 230, 260 and 280 nm (24). The quantity and purity of the obtained DNA are similar to those obtained with CTAB protocols from young leaves and are, therefore, appropriate for molecular methods that require high-quality DNA in abundant quantity. This method also represents an addition to other available methods for DNA extraction from other *Cannabis* samples, including those from leaves (22) and resin (hashish) (25).

The use of DNA from embryos extracted via the developed protocol enabled successful SSR characterization from 2n zygotes. Herein, we found that *C. sativa* presents a certain degree of heterozygosis; thus, DNA extraction from whole seeds (achenes) could theoretically result in the amplification of three alleles when the maternal plant is heterozygous and the allele carried by the male gamete is different

from the maternal alleles (26). However, we did not find any SSR profiles with three alleles, indicating a lack of contamination in the embryo tissues resulting from the presence of tissue from the seed testa or achene pericarp.

The 11 genomic SSR primers used in this study have previously been used to detect many alleles and represent an addition to the available SSR markers for the genetic characterization of *Cannabis* (12,26-30). The fact that genomic SSR markers are usually highly polymorphic compared with EST-SSRs (31) is likely a primary factor in this high degree of polymorphism and suggests that the development of further SSRs for identification purposes could benefit from the availability of the genome sequence (15). In particular, SSR loci with a high number of alleles and *PIC* values (such as CSG15, CSG20 and CSG24) may be of great utility for molecular characterization studies in *C. sativa*.

The SSR data show that, as expected, *C. sativa* generally presents a certain degree of heterozygosis due to its commonly dioecious reproductive system (4,26). Although marijuana varieties are usually highly inbred (6), we found that, with the exception of variety Purple, they are heterozygous for several of the SSR loci, suggesting that a certain degree of heterozygosis is maintained during breeding and selection. This has important implications for variety identification and the development of genetic fingerprints specific to varieties; for certain loci, it may be possible to identify individuals that are homozygous for different alleles or that are heterozygous. This heterozygosity is also an indication of a certain extent of intra-variety genetic variation.

Our molecular study is also in agreement with previous works indicating that hemp and marijuana varieties present different genetic backgrounds (21,32). In this respect, the PCoA analysis clearly delineated both types of *Cannabis* materials. This

indicates that a limited number of SSRs may be useful for a complete discrimination between both types of materials in forensic studies. However, further studies of intra-varietal diversity should be performed to confirm that these markers can be used to provide a specific varietal fingerprint and a clear-cut distinction between hemp and marijuana.

In conclusion, we present an efficient method for embryo extraction from seeds of *Cannabis sativa* and for the DNA extraction of individual embryos. The mechanical method for embryo extraction from imbibed seeds enables the extraction of clean embryos free from other genetically distinct tissues in the seed. The CTAB3 method, which uses extraction with phenol:chloroform:isoamyl alcohol prior to the first centrifugation, enables the recovery of DNA of high concentration and good quality for PCR applications, such as SSR analysis. This combined methodology, which includes newly developed highly polymorphic genomic SSR markers, enables genetic studies and forensic analyses of individual diploid zygotes in *Cannabis* without the need for leaf tissue. This may not only accelerate these studies but also does not require the cultivation of *Cannabis* plants, which may require legal authorization when THC levels are above certain limits.

Acknowledgements

We thank Emilio Martín, Cristina Casanova and Mireya Martínez for their help in data collection. Automaris provided the seeds of *Cannabis sativa* varieties Callipso, Critical#Chiva and Gitane.

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