Analysis of Published PCR Primers to Determine Sex of Industrial Hemp Seedlings

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Background

Industrial hemp refers to non-intoxicating strains of *Cannabis sativa* that produce high levels of cannabidiolic acid (CBDA), the precursor to cannabidiol (CBD). CBD has known health benefits and is being tested for a wide range of potential medicinal applications. When growing hemp for CBD, unfertilized female plants are desired as they produce high amounts of CBDA in their flower organs. Identification and elimination of male hemp plants is imperative and allows cultivators to spend fewer resources on plants that will not produce flower and could possibly pollinate female plants. It has been reported that male cannabis plants can be identified weeks before any visual sex features using the polymerase chain reaction (PCR), however the results from reported studies have been inconsistent in identifying male plants. Here, genomic DNA from 13 hemp seedlings was analyzed using male-DNA-specific PCR primer pairs described in the research literature. Three different primer pairs (SCAR323, SCAR119, and MADC2) were used in the analyses. SCAR323 primers have been reported to amplify a male-specific amplicon of 323 base pairs (bp); SCAR119 are reported to amplify a male-associated amplicon of 119 bp; and MADC2 primers have been reported to amplify a male-associated amplicon of 300 bp and a female-associated amplicon of 450 bp.

Aim

To use DNA analyses to determine sex of hemp seedlings many weeks before sex traits are visible.

Methods

Hemp plant growth: Hemp seeds were germinated and grown in ProMix soil with dilute Miracle-Gro under full-spectrum fluorescent lighting using a 18/6 hour light/dark cycle. After 4 weeks of growth, the light cycle was changed to a 12/12 cycle to induce sexual development. **Isolation of Hemp genomic DNA:** PureLink® Plant Total DNA Purification Kit (Invitrogen, Inc) was used to isolate genomic DNA from 2 week-old leaf tissue (~100 mg). **Polymerase Chain Reaction (PCR)**: Hemp DNA was amplified using PCR and GoTaq reagents (Promega, Corp). Three different DNA primer pairs were used - MADC2, SCAR323, SCAR119 - to amplify purported sex-specific DNA regions. ITS2 primers were used to amplify the rRNA gene. Initial denaturation was completed at 94°C for 5 mins. PCR cycle: denaturation at 94°C for 30 seconds; annealing step 50°C for 30 seconds; extension step at 72°C for 2 minutes; cycle repeated 34 times. **Agarose Gel Electrophoresis:** a 1% agarose gel was used to analyze amplified DNA. 5 µL of PCR product was separated using 150 volts for ~40 minutes. Axygen Ladder DNA 100bp-3000bp was used as size markers. **Purification of DNA for Sequencing:** ITS2 PCR products were purified for DNA sequencing via the GeneJet™ PCR Purification Kit. DNA concentration was determined using a NanoDrop spectrophotometer, and DNA samples were mailed to ACGT, Inc. for sequencing.

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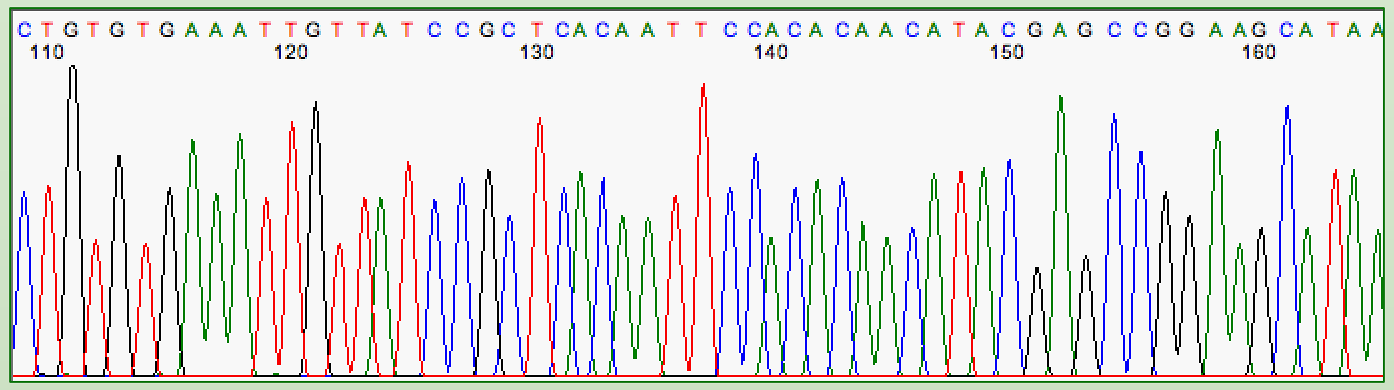
Verification of plant species using DNA sequence

The ITS2 region of the rRNA gene was amplified using PCR and sequenced. The resulting DNA sequence was compared to known sequences in the National Center for Biotechnology Information (NCBI) database, which verified that the plant species used in this study is *Cannabis sativa*.



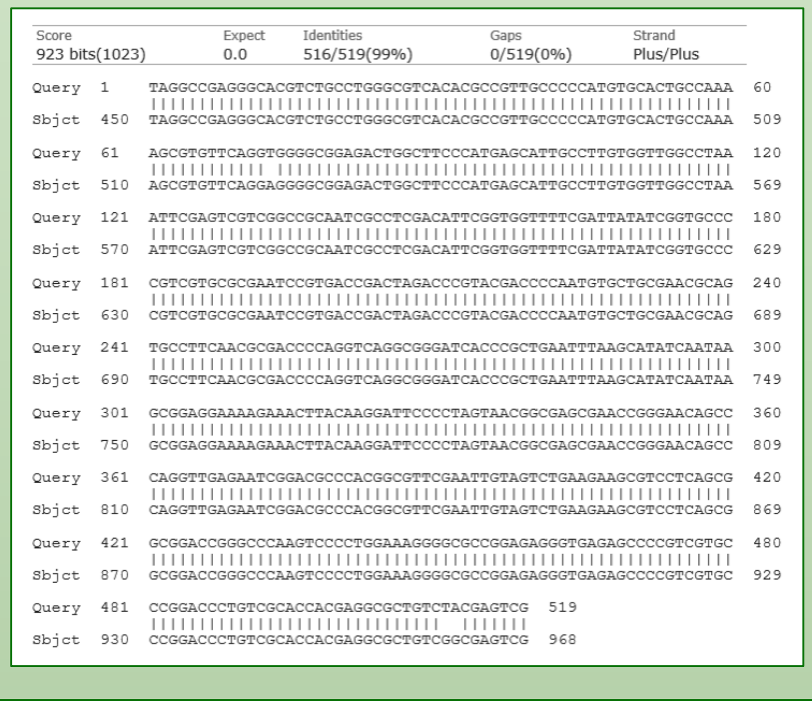


Figure 1. Alignment of our plant DNA sequence and database sequence corresponding to *Cannabis sativa* subsp. indica. The sequences are a 99% match, confirming that the hemp plants used is *Cannabis sativa*. Individual alignments were performed for all 13 plants.

Plant	Primer					
	MADC2		SCAR323	SCAR119	ITS2	M / F Phenotype
	300 (bp)	450 (bp)	323 (bp)	119 (bp)		
1	-	600	+	-	+	F
2	-	600	+	-	+	F
3	-	650	+	-	+	F
4	350	+	+	+	+	M
5	410	-	+	+	+	M
6	-	550	+	-	+	F
7	400	-	+	+	+	M
8	450	-	+	+	+	M
9	450	-	+	+	+	M
10	-	600	+	-	+	-
11	-	550	+	-	+	F
12	-	600	+	-	+	F
13	400	-	+	+	+	M

Table 1. DNA fragment presence (+/-) & size (base pairs, bp) for 13 hemp seedlings. Sex traits were determined based on presence of mature female pistils or male pollen sacs.

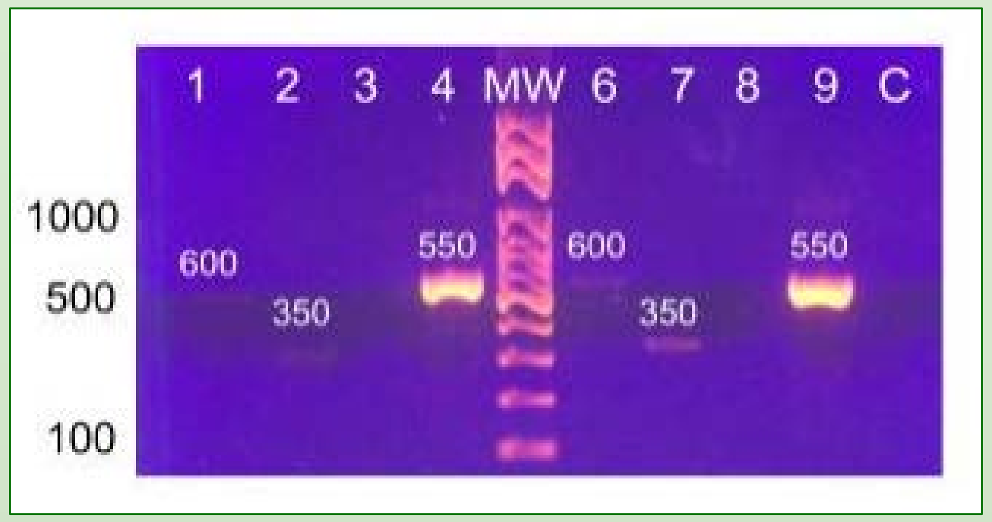


Figure 2. PCR-amplified DNA separated using agarose gel electrophoresis for two hemp seedlings. Lanes 1 & 6 present amplicons obtained using MADC2. Lanes 2 & 7 show amplicons obtained using SCAR323. Lanes 3 & 8 lack amplicons from SCAR119 primer pair. Lanes 4 & 9 present amplicons obtained using ITS2 primer pair at 550 bp. Lane C is a negative control reaction.

Figure 3. (left) Female hemp plant showing pistils; **(center)** mature female flower cluster; **(right)** mature male plant showing pollen sacs.

Results

PCR analyses showed DNA from all tested hemp seedlings produced a single SCAR323 amplicon of ~320 bp, suggesting this primer set might not be useful for sex determination. A SCAR119 amplicon of the expected size was seen using DNA from 6 of the 13 hemp seedlings. The DNA from the same 6 plants produced an amplicon of about 400 bp using the MADC2 primers, while DNA from plants that lacked SCAR119 amplicon showed a MADC2 amplicon of about 550 bp. After 10 weeks of growth, the hemp plants revealed the presence of male or female reproductive organs. Male pollen sacs correlated with the SCAR119 amplicon and the MADC2 amplicon of 400 bp, indicating these primer sets could be a useful tools for determining plant sex at the seedling stage.

Significance

Early sex determination of hemp seedlings using DNA analyses allows removal of male plants before maturity to show reduce pollination of females. Our results suggest male seedlings of *Cannabis sativa* can be identified using the SCAR119 and MADC2 primer pairs, but not the SCAR323 primers, before sexual maturity.

References

Bergamaschi, M. M., et al. (2011). Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naive social phobia patients. *Neuropsychopharmacology*, *36*(6), 1219.

Bih, C. I., Chen, T., Nunn, A. V., Bazelot, M., Dallas, M., & Whalley, B. J. (2015). Molecular targets of cannabidiol in neurological disorders. *Neurotherapeutics*, *12*(4), 699-730.

House Bill 698. (2018). *Agriculture – Industrial Hemp Pilot Program – Establishment.* [PDF file].

Klotz, K. A., Schulze-Bonhage, A., San Antonio Arce, V., & Van, J. L. (2018). Cannabidiol for Treatment of Childhood Epilepsy–a cross-sectional Survey. *Frontiers in neurology*, *9*, *9*, 731.

Lightfoot, D. A., Throgmorton, W. C., & Johnson, C. (2016). A Rapid Method for Cannabis Species Determination by DNA Sequencing. *Atlas Journal of Biology*, 292-294.

Marsicano, G., et al. (2002). The endogenous cannabinoid system controls extinction of aversive memories. *Nature*, *418*(6897), 530.

Moliterni, V. C., Cattivelli, L., Ranalli, P., & Mandolino, G. (2004). The sexual differentiation of Cannabis sativa L.: a morphological and molecular study. *Euphytica*, *140*(1-2), 95-106.

Public Law 91-513. (1970). *Comprehensive Drug Abuse Prevention and Control Act of 1970.* [PDF file].

Small, E., & Beckstead, H. D. (1973). Cannabinoid phenotypes in Cannabis sativa. *Nature*, *245*(5421), 147-148.

Small, E., & Marcus, D. (2002). Hemp: a new crop with new uses for North America. *Trends in new crops and new uses*, 284-326.

Van Bakel, H., Stout, J. M., Cote, A. G., Tallon, C. M., Sharpe, A. G., Hughes, T. R., & Page, J. E. (2011). The draft genome and transcriptome of *Cannabis sativa*. *Genome biology*, *12*(10), R102.