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TESTING OF DNA ISOLATION FOR THE IDENTIFICATION OF HEMP

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ABSTRACT

Hemp is diploid organism (2n = 2x = 20, genome size 534 Mb) with nine pairs of autosomes plus XX (♀) or XY (♂) chromosomes. *Cannabis sativa* L. is an important economic plant for the production of food, fibre, oils, and intoxicants. Genotypes (varieties or chemovar) of hemp with low Δ^9-tetrahydrocannabinol content are used for industrial applications. Varieties with high Δ^9-tetrahydrocannabinol or high cannabidiol content are used for medicinal applications. Biochemical and molecular methods can be used for identification and classification. An important step for molecular biology methods is to obtain the matrix of the native and sufficiently pure DNA. We tested two different experimental variant of samples (20 mg and 100 mg) of seeds, oilcake and dried flowers for analysis of the Italian variety Carmagnola for analysis (harvested in 2014, Hempoint Ltd., Czech Republic). The DNeasy® Plant Mini Kit (Qiagen, GE) was used to isolate the DNA. The DNA concentration and purity was assessed by agarose electrophoresis and via a spectrophotometer. Samples of lower weight yielded lower values of DNA concentration (average 16.30 – 38.90 ng.μL^{-1}), but with better purity than samples of higher weight (ratio A_{260nm}/A_{280nm} for low-weight samples was near 1.80). To test the applicability of DNA analysis, we used two SSR markers (*CAN1347* and *CAN2913*). PCR products were separated on 1% agarose and on 8% polyacrylamide electrophoresis. DNA samples obtained from samples of higher weight exhibited less PCR amplification than samples of lower weight. We found no effect of sample weight on the formation of non-specific amplification products during the PCR reaction. Based on our results we can be recommended for practical isolation procedure using DNeasy® Plant Mini Kit with lower of sample weight (20 mg). In future work the procedure for DNA isolating from wheat-cannabis products, e. g. breads, rolls or pasta, will be optimized.

Keywords: *Cannabis*; seed; oilcake; dry flower; DNA isolation

INTRODUCTION

Hemp (*Cannabis* L.) is one of the oldest cultivated plants. It is both cultivated and grows wild around the world, and is used in diverse applications **(Gilmore and Peakall, 2003)**. *Cannabis sativa* L. has been distributed as a source of fibre, feed, oils, medicine and intoxicants **(Small and Cronquist, 1976)**. However, *Cannabis* is the botanical genus of the plant and marijuana describes *Cannabis* plants that contain high Δ^9-tetrahydrocannabinol (THC) content and are used for their psychoactive potency **(Alghanim and Almirall, 2003)**. Hemp is used to describe *Cannabis* plants that have low THC content and are cultivated for industrial applications. Therefore, there are two distinctive strains; one is generally cultivated for fibre (hemp) and the other for drug use (marijuana) **(Mechoulam, 1970)**. Historically, there were three recognized varieties of *Cannabis*: *C. sativa*, *C. indica*, and *C. ruderalis*. For many years, botanists considered each of them to be a distinct species. However, most botanists now generally agree that *Cannabis* is a genus with a single highly variable species (*C. sativa*) that has diversified into a wide variety of ecotypes and cultivated races **(Siniscalco Gigliano, 2001)**. Identification of *Cannabis* is also important for farmers and industry.

There are two main methods in most classification schemes that can be applied to hemp identification. For marijuana, both biochemical **(Debruyne et al., 1994)** and DNA tests **(Siniscalco Gigliano, 1999)** are available to identify a substance as *Cannabis*. Biochemical methods to establish geographic origin of a plant have met with variable success **(Pitts et al., 1992)**. Biochemical profiling has also successfully differentiated between resinous and textile *Cannabis* **(Debruyne et al., 1981)**. One of the most useful and widely used DNA markers is SSR, otherwise known as microsatellite, or short tandem repeat (STR) **(Alghanim and Almirall, 2003)**. Microsatellites have become well suited for a fingerprint and genotype identification **(Gregáňová et al., 2005; Musilová et al, 2013)**, seed purity evaluation and germplasm conservation **(Brown et al., 1996)**, and marker assisted selection **(Röder et al., 1998)**.

The first step for the application of DNA markers in hemp is DNA isolation. In our study we tested sources of DNA and subsequent application of the DNA for DNA fingerprinting in *Cannabis*.

MATERIAL AND METHODOLOGY

Genomic DNA was isolated from seeds, oilcake and dry flowers (Figure 1) using the isolation kit DNeasy® Plant Mini Kit (Qiagen, GE). Italian variety Carmagnola from Hempoint, Ltd. (Czech Republic), harvested in 2014, was used. Two experimental sample variants were used for

Figure 1 Matrix used for analysis. S – seeds, O – oilcake, F – dry flower.

analysis: 20 mg and 100 mg (in triplicate). The DNA concentration and purity was assessed by 1% agarose electrophoresis and a spectrophotometrically by Picopet 1.0 (Picodrop, UK). The values obtained were compared using ANOVA at $p < 0.05$.

To test the applicability of DNA analysis for identification, two SSR markers (*CAN1347* and *CAN2913*) were used, as described by Gao et al., (2014). PCR analyses were repeated twice. The reaction mixture for PCR of a total volume 25 µL contained 0.5 U *Taq* polymerase (Promega), 1× aliquot buffer, 0.1 mM of each dNTP (Promega), 0.3 M of each primer and 20 ng of template DNA; the reaction conditions of PCR in T3 cycler (Biometra) by **Gao et al., (2014)**. The PCR reaction profile comprised a 10 min incubation at 94 °C, then a cycle of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 40 s, repeated 35 times. Following cycling, the reaction was held at 72 °C for 10 min, before a final 10 °C hold. Useful step seems to be control electrophoresis on 1% agarose gel (stained with ethidium bromide) and on polyacrylamide gels. The amplification of SSR products was then visualized on 8% non-denaturating polyacrylamide (PAA) gels in TBE (Tris-borate-EDTA) buffer followed by staining with silver (0.2% $AgNO_3$).

RESULTS AND DISCUSSION

In our experiment we tested two experimental variants of cannabis weighed in three different matrices, which are commonly used in agriculture and food industry. Sample weights were used according to the manufacturer's protocol **(Qiagen, 2012)**. The obtained results show that in samples weighing 100 mg, the average DNA yield was about 2 times higher (56.80 – 68.80 ng.µL^{-1}) than that of samples weighing 20 mg (Table 1). The observed variability in the values was not dependent on the weight of the material or on the biological matrix. The observed variability could be due to human factors in the course of DNA isolation, especially during homogenisation of the sample using a mortar in the presence of liquid nitrogen. Homogenization of the matrix is one of the most critical steps of the entire DNA isolation and significantly affects the yield of the applied protocol **(Blim and Stafford, 1976)**.

For subsequent use of the DNA for molecular biology methods, however, DNA purity is a more important factor. Sufficiently pure samples satisfy the condition $A_{260nm}/A_{280nm} \geq 1.8$ **(Moeller et al., 2014)**.

Table 1 Parameters of obtained DNA.

Sample	Concentration (ng.µL^{-1}) average ±SD	v_x (%)	Purity ($A_{260nm}/A_{280\ nm}$) average ±SD	v_x (%)
S100	63.13 ±17.92	28.38	1.64 ±0.04	2.31
O100	68.80 ±6.94	10.09	1.42 ±0.04	2.67
F100	56.80 ±4.30	7.58	1.67 ±0.06	3.51
S20	19.03 ±4.82	25.31	1.77 ±0.18	10.25
O20	16.30 ±6.16	37.78	1.83 ±0.28	15.42
F20	38.90 ±16.48	42.36	1.60 ±0.06	3.82

S – seeds, O – oilcake, F – dry flower, 100 – sample weight 100 mg, 20 – sample weight 20 mg, SD – standard deviation, v_x – coefficient of variation.

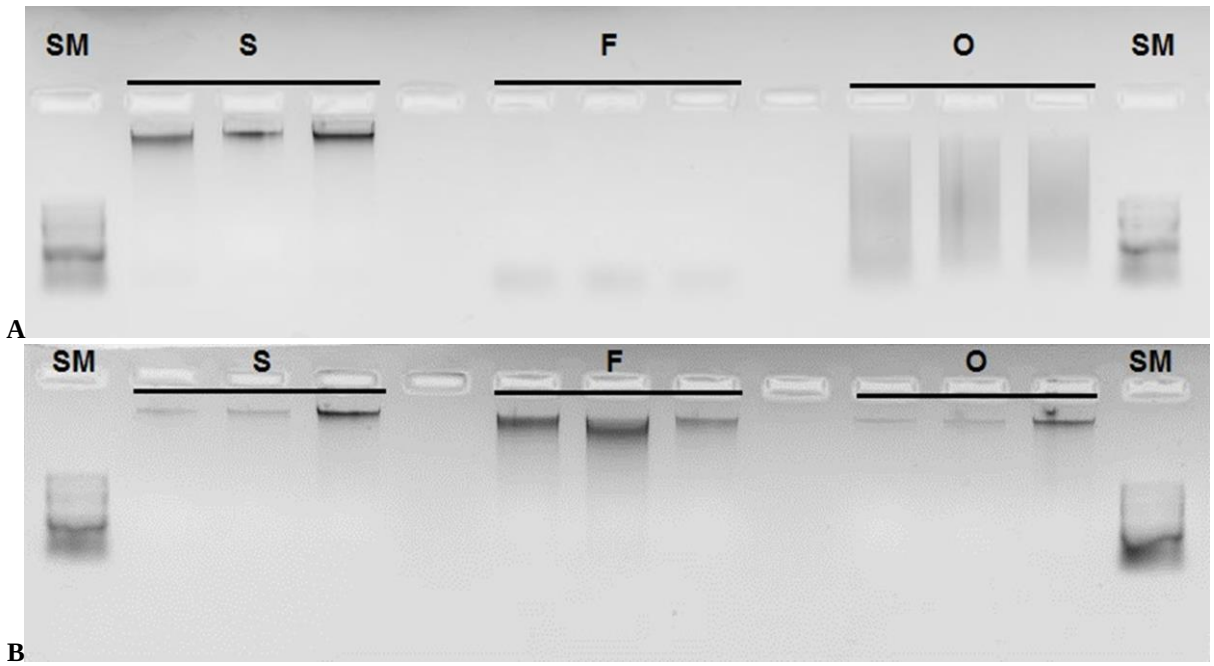

Figure 2 Control electrophoresis of DNA (1% agarose). A – sample weight = 100 mg, B – sample weight = 20 mg, SM – size marker, S – seeds, O – oilcake, F – dry flower.

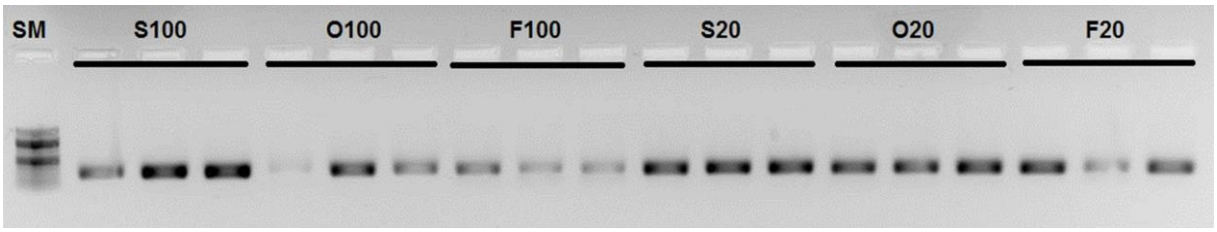

Figure 3 Agarose electroforetogram of SSR marker (*CAN2913*). SM – size marker (100 bp), S – seeds, O – oilcake, F – dry flower, 100 – sample weight 100 mg, 200 – sample weight 20 mg.

When comparing samples for analysis to achieve better parameters for weighing 20 mg with an average purity of 1.60 to 1.83 (Table 1). This may be influenced by the absorption capacity of the purification columns provided in the kit and by removing large quantities of impurities from the final DNA sample **(Qiagen, 2012)**. The visible difference in the quantity and quality of DNA obtained from 20 mg samples indicates that the lower weight samples are preferable over higher weight samples (Figure 2). During electrophoresis, the 100 mg oilcake sample exhibited distinct smears indicating a possible degradation of DNA. Although these are the products (oilcake) from cold pressing, but in the high pressure process that heats **(Small and Marcus, 2002)**, which may negatively affect the DNA and lead to its degradation. Increasing the purity of the obtained DNA is possible via purification procedures, but purification processes can reduce the final concentration of DNA in the sample **(Demeke and Jenkins, 2010)**.

Dirt and degraded DNA may negatively affect the progress of the PCR reaction **(Collard et al., 2007)**. Therefore, we decided to use two SSR for testing the effect of concentration and purity of DNA on the progress of the PCR reaction. Our results confirm the known fact **(Ning et al., 2009)**, that the level of purity has a much greater influence on the course of the PCR reaction than DNA concentration. Especially during electrophoresis with agarose gels (Figure 3), compared to polyacrylamide gels (Figure 4), were observed greater PCR amplification from 20 mg samples for analysis with a lower concentration, but higher purity in comparison with samples weighing 100 mg, where the values were reversed. The most significant negative influence on the formation of the PCR product was demonstrated at 100 mg variants - dried flowers, which could adversely exhibit high essential oil content in the flowers of hemp **(Hazekamp and Fischedick, 2012)**. Simultaneously, it was not shown toaffect the formation of non-specific amplification in the PCR reaction, which is visible on a polyacrylamide gel (Figure 4).

Previous reports indicated that the ideal concentration of DNA for analysis using SSR markers is 20 - 30 $ng.\mu L^{-1}$ **(Gregáňová et al., 2005; Musilová et al., 2013; Ovesná et al., 2014)**. Within the isolation of DNA from the portion of the lower concentration of DNA was achieved on the border of the reference value. Given this fact and the results of the analysis of SSR markers of cannabis, the DNeasy® Plant Mini Kit (f. Qiagen) can be recommended for DNA isolation of samples weighing 20 mg.

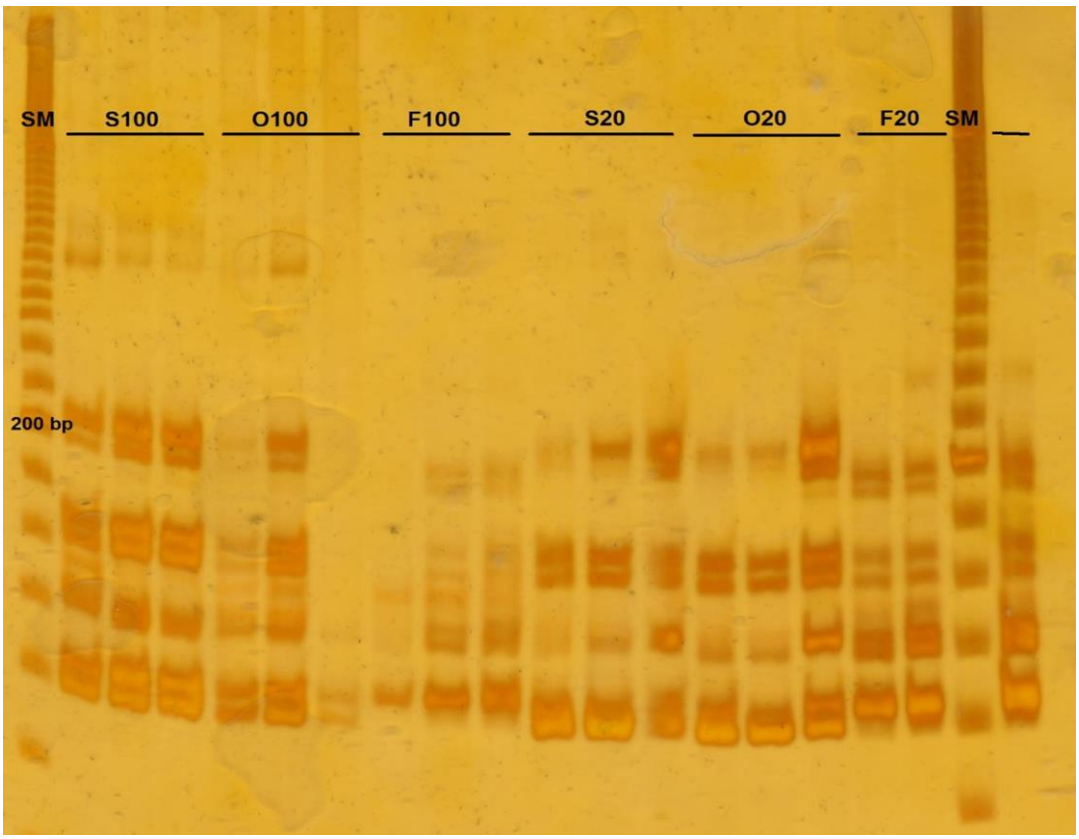

Figure 4 Polyacrylamide electroforetogram of SSR marker (*CAN2913*). SM – size marker (20 bp), S – seeds, O – oilcake, F – dry flower, 100 – sample weight 100 mg, 200 – sample weight 20 mg.

CONCLUSION

Based on our results we can recommend practical DNA isolation procedure using the DNeasy® Plant Mini Kit with sample weights of 20 mg. From this size sample, we obtained the best results for DNA quality and purity, and there was no effect on subsequent analysis of DNA variation using microsatellite markers. In future work we will optimize the procedure for DNA isolation from cannabis products and we will look for a combination of SSR markers to identify varieties of industrial hemp.

REFERENCES

Alghanim, H. J., Almirall, J. R. 2003. Development of microsatellite markers in *Cannabis sativa* for DNA typing and genetic relatedness analyses. *Analytical and Bioanalytical Chemistry*, vol. 376, no. 8, p. 1225-1233. http://dx.doi.org/10.1007/s00216-003-1984-0 PMid:12811461

Blim, N., Stafford, D. W. 1976. A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acid Research*, vol. 3, no. 9, p. 2303-2308. http://dx.doi.org/10.1093/nar/3.9.2303

Brown, S. M., Hopkins, M. S., Mitchell, S. E., Senior, M. L., Wang, T. Y., Duncan, R. R., Gonzalez-Candelas, F., Kresovich, S. 1996. Multiple methods for the identification of polymorphic simple sequence repeats (SSRs) in sorghum [*Sorghum bicolor* (L.) Moench.]. *Theoretical and Applied Genetics*, vol. 93, no. 1-2, p. 190-198. http://dx.doi.org/10.1007/BF00225745 PMid:24162217

Collard, B. C. Y., Das, A., Virk, P. S., Mackill, D. J. 2007. Evaluation of ´quick and dirty´ DNA extraction methods for marker-assisted selection in rice (*Oryza sativa* L.). *Plant Breeding*, vol. 126, no. 1, p. 47-50. http://dx.doi.org/10.1111/j.1439-0523.2006.01272.x

Debruyne, D., Albessard, F., Bigot, M. C., Moulin, M. 1994. Comparison of three advanced chromatographic techniques for cannabis identification. *Bulletin on Narcotics*, vol. 46, no. 1, p. 109-121. PMid:7866395

Debruyne, D., Moulin, M., Bibot, M. C., Camsonne, R. 1981. Identification and differentiation of resinous cannabis and textile cannabis: combined use of HPLC and high-resolution GLC. *Bulletin on Narcotics*, vol. 33, no. 1, p. 49-58. PMid:6272923

Demeke, T., Jenkins, R. G. 2010. Influence of DNA extraction methods, PCR inhibitors and quantification methods on real-time PCR assay of biotechnology-derived traits. *Analytical and Bioanalytical Chemistry*, vol. 396, no., 6, p. 1977-1990. http://link.springer.com/article/10.1007/s00216-009-3150-9

Gao, C., Xin, P., Cheng, C., Tang, Q., Chen, P., Wang, C., Zang, G., Zhao, L. 2014. Diversity analysis in *Cannabis sativa* based on large-scale development of expressed sequence tag-derived simple sequence repeat markers. *PLoS ONE*, vol. 9, no. 10, p. e110638. http://dx.doi.org/10.1371/journal.pone.0110638 PMid:25329551

Gilmore, S., Peakall, R. 2003. Isolation of microsatellite markers in *Canabis sativa* L. (marijuana). *Molecular Ecology Notes*, vol. 3, no. 1, p. 105-107. http://dx.doi.org/10.1046/j.1471-8286.2003.00367.x

Gregáňová, Ž., Kraic, J., Gálová, Z. 2005. Effectiveness of microsatellites in differentiation of elite wheat cultivars. *Biologia*, vol. 60, no. 6, p. 665-670.

Hazekamp, A., Fishedick, J. T. 2012. Cannabis – from cultivar to chemovar. *Drug Testing and Analysis*, vol. 4, no. 7-8, p. 660-667. http://dx.doi.org/10.1002/dta.407 PMid:22362625

Mechoulam, R. 1970. Marijuana chemistry. *Science*, vol. 168, no. 3936, p. 1159-1166. http://dx.doi.org/10.1126/science.168.3936.1159 PMid:4910003

Moeller, J. R., Moehn, N. R., Waller, D. M., Givnish, T. J. 2014. Paramagnetic cellulose DNA isolation improves DNA yield and quality among diverse plant taxa. *Applications in Plant Sciences*, vol. 2, no. 10. http://dx.doi.org/10.3732/apps.1400048 PMid:25309836

Musilová, M., Trojan, V., Vyhnánek, T., Havel, L. 2013. Genetic variability for coloured caryopses in common wheat varieties determined by microsatellite markers. *Czech Journal of Genetics and Plant Breeding*, vol. 49, no. 3, p. 116-122.

Ning, J., Liebich, J., Kästner, M., Zhou, J., Schäffer, A., Burauel, P. 2009. Different influences of DNA purity indices and quantity on PCR-based DGGE and functional gene microarray in soil microbial community study. *Applied Microbiology and Biotechnology*, vol. 82, no. 5, p. 983-993. http://dx.doi.org/10.1007/s00253-009-1912-0 PMid:19247649

Ovesná, J., Leišová-Svobodová, L., Kučera, L. 2014. Microsatellite analysis indicates the specific genetic basis of Czech bolting garlic. *Czech Journal of Genetics and Plant Breeding*, vol. 50, no. 3, p. 226-234. Available at: http://www.agriculturejournals.cz/publicFiles/131273.pdf

Pitts, J. E., Neal, J. D., Gough, T. A. 1992. Some features of Cannabis plants grown in the United Kingdom from seeds of known origin. *Journal of Pharmacology and Pharmacotherapeutics*, vol. 44, no. 12, p. 947-951. http://dx.doi.org/10.1111/j.2042-7158.1992.tb07072.x

Qiagen, 2012. DNeasy® Plant Handbook. [online] s.a. [cit. 2015-07-09], Available at: https://www.qiagen.com/cz/resources/resourcedetail?id=95dec8a9-ec37-4457-8884-5dedd8ba9448

Röder, M. S., Korzun, V., Wendehake, K., Plaschke, J., Tixier, M. H., Leroy, P., Ganal, M. W. 1998. A microsatelitte map of wheat. *Genetics*, vol. 149, no. 4, p. 2007-2023. PMid:9691054

Siniscalco Gigliano, G. (1999). Preliminary data on the usefulness of internal transcribed spacer (ITS) sequence in *Cannabis sativa* L. identification. *Journal of Forensic Sciences*, vol. 44, no. 3, p. 475-477. http://dx.doi.org/10.1520/JFS14497J PMid:10408103

Siniscalco Gigliano, G. (2001). *Cannabis sativa* L. – Botanical problem and molecular approaches in forensic investigations. *Forensic Science Review*, vol. 13, no. 1, p. 1-17.

Small, E., Cronquist, A. 1976. A practical and natural taxonomy for *Cannabis*. *Taxon*, vol. 25, no. 4, p. 405-435. http://dx.doi.org/10.2307/1220524

Small, E., Marcus, D. 2002. *Hemp: A new crop with new uses for North America*. p. 284-326. In: J. Janick and A. Whipkey (eds.), *Trends in new crops and new uses*. ASHS Press, Alexandria, VA, USA. ISBN 0-970756-5-5.

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