APLICATION OF SEX MOLECULAR MARKERS IN HEMP PLANT (*Cannabis sativa* sp.)

Zala KOLENC[35] and Andreja ČERENAK[36]

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Abstract
Hemp (*Cannabis sativa* sp.) is important annual crop plant with increasingly developing market. It is predominantly dioecious, but the varieties intended for fiber production are bred to be monoecious. Sex expression is considered to be one of the important factors for practical implications during cultivation and during the breeding process. In this study, the three already developed markers have been tested on seven different hemp varieties grown in Slovenia in 2017. The performed Polymerase Chain Reaction (PCR) analyses indicated that the molecular marker MADC2 enables us the most reliable identification of sex in hemp plants. On the other hand, the molecular markers SCAR119 and SCAR323 are also very useful in practice. To conclude, the application of sex molecular markers in hemp plants was successful, we anticipate it for practical use during our future experiments.
Keywords: hemp, sex determination, molecular markers

UPORABA MOLEKULSKIH MARKERJEV ZA DOLOČANJE SPOLA PRI KONOPLJI (*Cannabis sativa* sp.)

Izvleček
Konoplja (*Cannabis sativa* sp.) je pomembna enoletna kmetijska rastlina s hitro-rastočim trgom. Je predvsem dvodomna rastlina, vendar so sorte, namenjene za pridelavo stebel, žlahtnjene na enodomnost. Izražanje spola je ena pomembnejših lastnosti rastlin pri pridelavi ali med samim procesom žlahtnjenja. V naši raziskavi smo preizkusili tri že objavljene molekulske markerje pri sedmih različnih sortah konoplje, ki so rastle na isti parceli v Sloveniji v letu 2017. Opravljene analize verižne reakcije s polimerazo (PCR) so pokazale, da je molekulski marker MADC2 zelo primeren za določanje spola pri konoplji. Po drugi strani sta molekulska markerja SCAR119 in SCAR323 prav tako zelo uporabna za praktično uporabo. Aplikacija molekulskih markerjev pri konoplji je bila uspešna, v prihodnje pa predvidevamo uporabo preizkušenih markerjev v nadaljnjih poskusih.
Ključne besede: konoplja, določanje spola, molekulski markerji

[35] Dr. Inštitut za hmeljarstvo in pivovarstvo Slovenije, Cesta Žalskega tabora 2, 3310 Žalec, Slovenija, e-pošta: zala.kolenc@ihps.si
[36] Doc. dr., prav tam, e-pošta: andreja.cerenak@ihps.si

1 INTRODUCTION

Hemp is a herbaceous, wind-pollinated annual crop, which is commonly cultivated to gain long and strong fibers or qualitative seeds (Salentijn et al., 2015). Hemp and marijuana are both strains of the same species (*Cannabis sativa* L.), despite the fact that they exhibit different Δ^9-tetrahydrocannabinol (THC) content (Aiello et al., 2016). The market of hemp products is increasingly developing, considering the multi-purpose use of the plant. Hemp is used for stalk (for textiles, building materials, paper …) or for seed production (for food purposes - seed cake is rich with proteins, energy (ethanol production) and environment purposes (phytoremediation), seed oil and cosmetics). Hemp leaves and inflorescences are useful as well because of their medical indications, anti-microbe purposes, agro-chemical effects, etc. (Vonapartis et al., 2015; Salentijn et al., 2015).

It is generally dioecious plant, but hemp varieties intended for fiber production have been bred to be monoecious (Srivastava and Yadav, 2013). Monoecious hemp varieties are also used when both seed production and stem harvesting are performed. On the other hand, there is the disadvantage of monoecious hemp varieties because of the occurrence of self-pollination, which could result in a lower stem yield and in the slower rate of genetic progress (Mandolino et al., 1999). So far it is known that flowering and senescence of male plants occur faster compared to female plants. As a consequence, it is difficult to make the harvesting for fiber in optimal time. In contrast, harvesting for fiber is much easier when we use monoecious varieties. To take into account all these knowledge, sex expression is considered to be one of the important factors for practical implications not only during cultivation but also during breeding process (Faux et al., 2016). During the breeding process it is essential to determine sex before flowering, to prevent unwanted pollination of female plants.

Hemp is diploid (2n=20) species with heteromorphic sex chromosomes and is characterized by sexual dimorphism. In dioecious hemp, the male plants have XY chromosomes and the female hemp plants have XX sex chromosomes, and monoecious hemp has homomorphic sex chromosomes (XX) as well (Faux et al., 2016). Molecular markers linked to male sex in hemp were identified using randomly amplified polymorphic DNA (RAPD) technology. From molecular marker OPA8, developed by RAPD technology, the primer MADC2 (Male-Associated DNA from Cannabis) was obtained with a total length of 371 bp. The obtained molecular marker should be sustainable for a precise, early and rapid identification of male plants during breeding programs of dioecious and monoecious hemp (Mandolino et al., 1999). The abundant number of potential molecular markers was obtained with amplified fragment length polymorphism (AFLP) technology for sex determination in hemp (Flachowsky et al., 2001). Subsequently, a research by testing 20 decamer RAPD primers, obtained from different species and linked to male sex, was applied. The results obtained 2 novel

male-specific molecular markers (MADC5 and MADC6) which were converted into sequence-characterized amplified region (SCAR) markers (SCAR 119 and SCAR 323) and were successfully correlated with male sex (Törjék et al., 2002). Besides of those obtained molecular markers, three of them have been tested in the latest published study (Mendel et al., 2016). This study was performed on different varieties of industrial hemp and medical cannabis and results show that primers should be successfully used for sex determination in both subspecies. So far, genetic mapping has also been carried out by using AFLP markers and genetic determination of sex expression has been investigated with quantitative trait loci (QTL) analysis (Faux et al., 2016).

Heretofore, molecular markers for sex determination in dioecious plants have been developed in *Humulus lupulus* (Poley et al., 1997; Jakše et al., 2008; Čerenak et al., 2015), *Carica papaya* (Deputy et al., 2002), *Asparagus officials* (Gao et al., 2007), *Calamus guruba* Buch.-Ham. (Sinha et al., 2017), *Simmondsia chinensis* (Heikrujam et al., 2014), *Garcinia gummi-gutta* (Joseph et al., 2014), *Ficus fulva* (Parrish et al., 2004) by RAPD-derived SCAR markers, sequence tagged site (STS) markers, Simple Sequence Repeats (SSRs), Diversity Arrays Technology (DArT) or AFLP markers.

The objective of presented study was testing three developed molecular markers for sex determination in different hemp varieties, grown in 2017 on the same field of Slovenian Institute of Hop Research and Brewing at the same technology.

2 MATERIALS AND METHODS

2.1 Plant material

Hemp samples from 7 genotypes (4 from EU Variety List: Kompolti hibrid TC, KC Dora, Tiborszallasi, Carmagnola and 3 Slovenian selections) (Table 1) were obtained in the fields of Slovenian Institute of Hop Research and Brewing. The hemp leaves were sampled and total genomic DNA was extracted from fresh plant material. The extraction was done according to the Kump and Javornik (1996) with minor modifications. The concentration of DNA was measured with the fluorometer (Qubit 3.0) and the DNA was diluted to 4 ng/µl.

2.2 PCR reaction

In PCR reaction 20 ng of diluted DNA was used in 10 µl PCR solution containing 1 x PCR buffer, 1.25 mM $MgCl_2$, 0.2 U of KAPA3G Plant DNA polymerase and primers in the concentration of 0.5 µM (Table 2). According to the used primer, the PCR amplifications were carried on as it is described in Table 2. All PCR products were separated on 2 % agarose gel in TBE buffer and electrophoresis (Biometra) lasted for 1 hour at 165 V.

***Table 1**: Hemp genotypes involved in research.*

Hemp genotype	Sex*	DNA concentration (ng/µl)	Label**
Kompolti hibrid TC	M	171	1
Kompolti hibrid TC	Ž	95	2
KC Dora	M	80	3
KC Dora	Ž	107	4
Tiborszallasi	M	123	5
Tiborszallasi	Ž	152	6
Carmagnola	M	160	7
Carmagnola	Ž	126	8
Fukal	M	154	9
Fukal	Ž	100	10
Gorička Simba	M	102	11
Gorička Simba	Ž	133	12
Helena	M	138	13
Helena	Ž	147	14

* phenotypic sex determination in the field
** label on the gel electrophoresis figures

***Table 2**: Primers used, their concentrations, and PCR conditions.*

Primer	Amplification product	Reference	PCR conditions
SCAR119_F SCAR119_R	The product of male sex-linked DNA is 113 bp long.	Törjék et al., 2002	95 °C for 15 minutes, 40 cycles on 94 °C for 30 seconds, 55 °C for 1.5 minute, 72 °C for 1.5 minutes and the reactions were completed by incubating at 72 ° for 8 minutes
SCAR323_F SCAR323_R	The product of male sex-linked DNA is 323 bp long.	Törjék et al., 2002	95 °C for 15 minutes, 10 cycles on 94 °C for 30 seconds, 67 °C – 1 °C for 30 seconds, 72 °C for 1.5 minutes, following by 30 cycles on 94 °C for 30 seconds, 57 °C for 30 seconds, 72 °C for 1.5 minute and the reactions were completed by incubating at 72 ° for 8 minutes
MADC2_F MADC2_R	The product of male sex-linked DNA is 390 bp long.	Mandolino et al., 1999	95 °C for 15 minutes, 40 cycles on 94 °C for 30 seconds, 60 °C for 1.5 minutes, 72 °C for 1.5 minutes and the reactions were completed by incubating at 72 ° for 8 minutes

3 RESULTS AND DISCUSSION

The method validation was carried out for each used primer. The tested primer SCAR119 was applied on hemp sample set and the presented band 119 bp was absolutely linked to male sex (Figure 1).

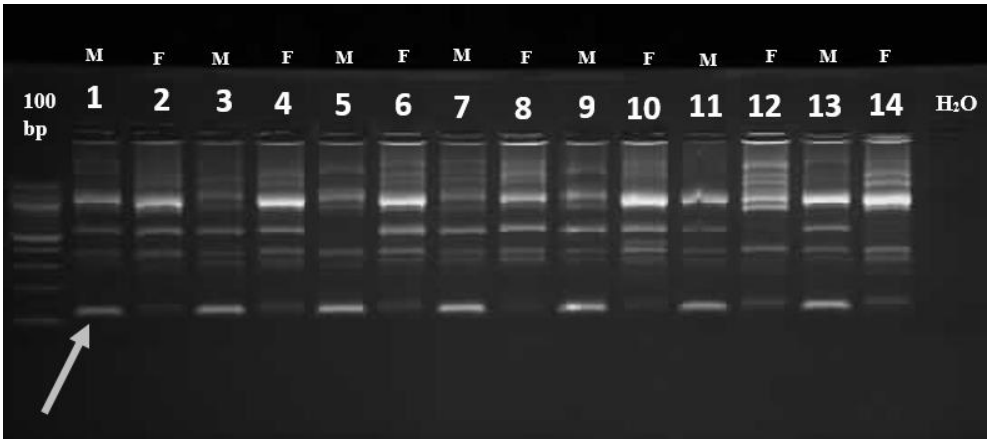

Figure 1*: The agarose gel with the marked band of molecular marker SCAR119. The numbers on the picture are described in Table 1 (label). M is mark for male and F for female plants, both determined as phenotypic traits.*

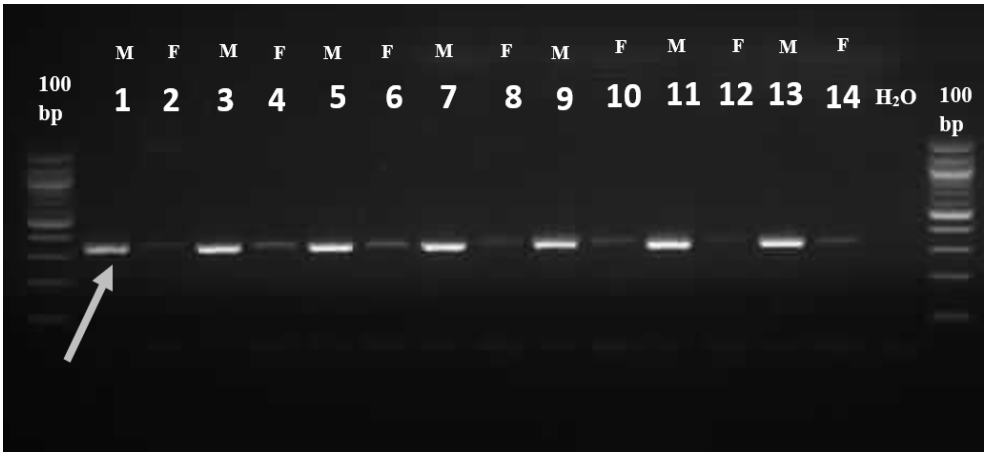

Figure 2*: The agarose gel with the marked band of molecular marker SCAR323. The numbers on the picture are described in Table 1 (label). M is mark for male and F for female plants, both determined as phenotypic traits.*

The results of PCR amplification in case of SCAR119 are partly in accordance to result of Mendel et al. (2016) who wrote that the molecular marker SCAR119 is the most reliable to detect male plants in species *Cannabis sativa*. Results of PCR amplification with the SCAR119 molecular marker are also in correlation to data of Törjék et al. (2002) who revealed that in male plants the band 119 bp was presented. In our case, the marker was amplified also in female plants, but the presence of 119 bp long band was less intensive and the sex could be easily determined and completely coincided with phenotypic results. On the other hand, in the Figure 1 longer non – specific fragments, compared to molecular marker SCAR119 (119 bp), were multiplied as well.

The tested primer SCAR323 was applied on the same hemp sample set and the presented band 323 bp was linked to male sex. When primer SCAR323 was used the presence of the band 323 bp was also multiplied in samples of female plants, but it was much less intensive (Figure 2) comparing to the male plants.

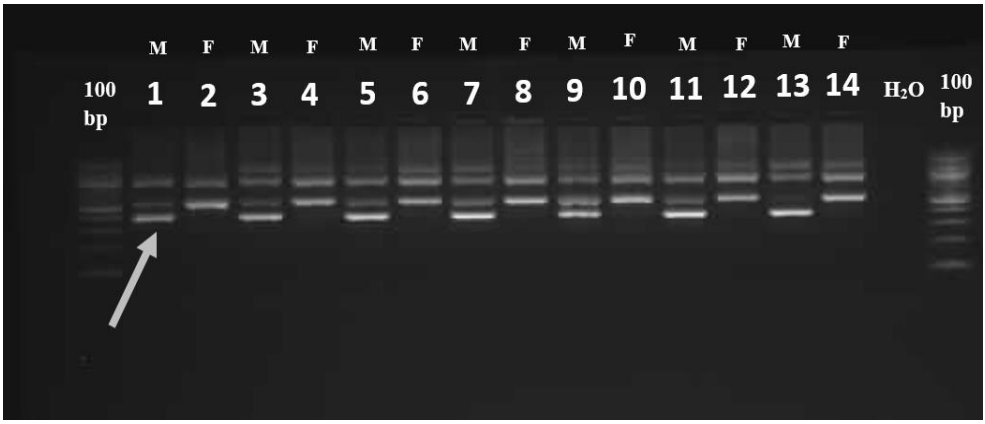

***Figure 3**: The agarose gel with the marked band of molecular marker MADC2. The numbers on the picture are described in Table 1 (label). M is mark for male and F for female plants, both determined as phenotypic traits.*

Mandolino et al. (1999) developed primer MADC2 and results of their research revealed that band at 390 bp is presented in male plants, while in female plants there is no evidence of the 390 bp band. Mandolino et al. (1999) concluded that MADC2 is probably non-coding genome region but they could not confirm if the sequence is a part of the genes for sex determination or not. Our results are in contrast to the Mendel et al. (2016) results, who concluded that MADC2 sequence is not located on the male chromosome because in their multiplication 390 bp band multiplied besides in male plants also in phenotypically female plants. On the other hand, in our case, the primer MADC2 gave us the clearest results from all 3 tested

markers (Figure 3) with no amplifications in female plant samples. On the other hand, there are also some non-specific bands, which are longer than MADC2.

4 CONCLUSIONS

All three used primers (SCAR119, SCAR323, MADC2) were applied on same hemp sample set, including 7 different genotypes with male and female plants. The results confirm that all primers are reliable and results completely coincide with phenotype scores. In our case, the MADC2 marker appeared to be the most reliable, because the band appears only in male plants, without any less intensive amplification in female plants, as it is possible for SCAR119 and SCAR323 markers. Our study confirmed that developed markers are useful tool to distinguish male and female hemp plants. We plan to use tested markers in further research on hemp plant.

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