



Novel male-specific molecular markers (MADC5, MADC6) in hemp

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Summary

Decamer RAPD primers were tested on dioecious and monoecious hemp cultivars to identify sex-specific molecular markers. Two primers (OPD05 and UBC354) generated specific bands in male plants. These two DNA fragments were isolated, cloned and sequenced. Both markers proved to be unique, since no sequence with significant homology to OPD05₉₆₁ and UBC354₁₅₁ markers were found in databases. These markers were named MADC3 (OPD05₉₆₁) and MADC4 (UBC354₁₅₁) (Male-Associated DNA from *Cannabis sativa*). The markers were converted into sequence-characterized amplified region (SCAR) markers. The SCAR markers correlated with the sex of the segregating F₂ population and proved the tight linkage to the male phenotype. Results of F₂ plant population analysis suggest these markers are to be linked to the Y chromosome.

Abbreviations: BSA – Bulk Segregant Analysis; MADC – Male-Associated DNA from *Cannabis sativa*; RAPD – Random Amplified Polymorphic DNA; SCAR – Sequence-Characterized Amplified Region

Introduction

While in most animals dioecy commonly occurs in the plant kingdom, however, dioecy is found in only 4% of the angiosperms (Yampolsky & Yampolsky, 1922). The sex of most dioecious plants can be reliably determined only at the time of flowering. The significance of separating male and female plants at seedling stage lies in the fact that in many dioecious plants gender influences economic value, breeding schemes and opportunities for commercial use of genetically modified materials. Such a test would allow a significant reduction in time for evaluating this trait, permit the rapid and separate evaluation of plants of both sexes during breeding processes, and considerably reduce the amount of labour and field space.

The presence of sex chromosomes has been claimed for dioecious angiosperms, but in only a few cases has been documented (Westergaard, 1958; Lewis & John, 1968; Parker & Clark, 1991). Examples

include *Silene latifolia* Poirét, *Asparagus officinalis* L., *Humulus lupulus* L., *Rumex acetosa* L. and *Cannabis sativa* L. (Westergaard, 1958; Duran & Duran, 1990).

Genetic marker systems based on direct analysis of the genomic DNA have been widely used for genetic mapping, disease diagnostics and evolutionary studies, and they can be also very useful in the study of sexual determination and identification in dioecious plants. Michelmore et al. (1991) described an application of the random amplified polymorphic DNA (RAPD) technique termed bulked segregant analysis (BSA) to identify molecular markers linked to a trait of interest. They used RAPD analysis to compare two groups of pooled DNA from individuals of a segregating population originating from a single cross involving parents that differed in only a single discrete character. By using this approach, markers for male plants in *Silene latifolia* Poirét (Mulcahy et al., 1992; Zhang et al., 1998), *Humulus lupulus* L. (Polley et al., 1997)

and *Actinidia chinensis* Planch. (Harvey et al., 1997) and for female plants in *Pistacia vera* L. (Hormaza et al., 1994), *Actinidia chinensis* Planch. (Harvey et al., 1997) and *Salix viminalis* L. (Alstrom-Rapaport et al., 1998) were identified.

Cannabis sativa L. is a dioecious plant with two heteromorphic sex chromosomes (Hirata, 1924). The diploid number of chromosomes is 20. Male plants have one X and one Y chromosome, while female have two X chromosomes. Chromosome Y is much larger than X and autosomes (Yamada, 1943; Sakamoto et al., 1998). These differences are also reflected in the genome sizes determined by flow cytometry: 1636 Mbp and 1683 Mbp for diploid female and male genome, respectively (Sakamoto et al., 1998). According to Warmke & Davidson (1944) autosomal genes are not involved in sex determination, suggesting a typical active Y system, which is yet to be confirmed, while other authors do not exclude the role of autosomal genes in sex determination (Migal, 1986).

No morphological markers have been found which would consistently identify the sex of a plant before flowering. Little is known about the hemp genome through RAPD based molecular approaches to the taxonomy of the genus have already been attempted (Jagadish et al., 1996; Faeti et al., 1996), resulting in identification of male-specific markers (Sakamoto et al., 1995; Mandolino et al., 1999). Recently Flachowsky et al. (2001) have shown application of AFLP for the detection of sex-specific markers in hemp.

The aim of our study was to test decamer RAPD primers on dioecious and monoecious hemp cultivars and to look for additional specific RAPD markers linked with the sex phenotype.

Materials and methods

Plant material

The experiments were performed with four Hungarian dioecious (Uniko B, KFF, Fleischmann and Tiborszálási), one monoecious (Fibrimon) and one South-African dioecious (SA) hemp (*Cannabis sativa* L.) cultivars. Plants were grown under natural light and photoperiod in an experimental field at the Agricultural Research Institute, Kompolt (Hungary). After the sex identification of flowering plants individual leaf samples were collected (about 1 g) and stored at -70°C . Identified female and male plants of each cultivar were collected for bulked analysis. SCAR

markers were used for the analysis of F_2 plants derived from the self cross of the F_1 generation, which was produced by the cross of the Fleischmann (dioecious line, ♀) \times Fibrimon (monoecious line, pollen donor). The material used is listed in Table 1.

DNA extraction

Genomic DNA was extracted from 100 mg freeze-dried, finely ground leaves according to the modified protocol of Benito et al. (1993) modified by Stift (T. Lelley, personal communication). The fine powder was mixed with 240 μl of Extraction Buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl), 60 μl of 10% SDS and 4 μl of 10 mg/ml proteinase K and then incubated at 65°C for 20 minutes. To eliminate RNA, 4 μl of 10 mg/ml RNase was added to the mixture and incubated at room-temperature for 10 minutes followed by adding 160 μl of 5 M Potassium-acetate, pH 5.3, and incubated at 0°C for 20 minutes. After centrifugation (13000 rpm, 4°C , 20 minutes) DNA was precipitated from the supernatant by adding 1/10 volume of Na-acetate (pH 5.2) and 2 volume of 96% ethanol for 5 minutes at room-temperature, then the DNA was collected by centrifuge, dried and resuspended in $T_{10}E_1$ buffer.

Polymerase chain reaction conditions

RAPD analysis was performed with 20 decamer primers (Table 2). PCR amplification reactions were carried out in 25 μl reaction mixtures containing 10–50 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl pH 9.0, 2 mM MgCl_2 , 300 μM dNTPs, 12 mM primer, 1U Taq DNA polymerase (Promega). Cycling was carried out in Perkin-Elmer 9600 thermocycler: 1 cycle of 94°C for 2 minutes; 40 cycles at 94°C for 10 sec, 36°C for 30 sec, 72°C for 1 minutes; 1 cycle of 72°C for 2 minutes. SCAR analysis was performed under the same conditions, but the annealing temperature was increased to 52°C and 55°C for SCAR₁₁₉ and SCAR₃₂₃, respectively.

Gel electrophoresis and recovery of DNA fragments

Electrophoresis was performed in 1.8% agarose (FMC) gels. Band size was estimated by comparison to a 100-bp ladder DNA standard (Fermentas). The polymorphic DNA fragments were resolved on 1.2% low-melting-point agarose according to Sambrook et al. (1989).

Table 1. Hemp germplasm used for sex identification by RAPD and SCAR methods

Cultivars and crosses	Plants analysed	Male plants	Female plants	monoecious plants
Sa	12	7	5	0
Unikó	11	6	5	0
Kff	10	4	6	0
Fleischmann	9	5	4	0
Tiborszálási	10	5	5	0
Fibrimon	4	0	0	4
Fleischmann ♀ × Fibrimon / x				
Fleischmann ♂	75	31	42	2
Total	131	58	67	6

Table 2. RAPD primers used for PCR in hemp

Name	Sequence	Sex	Plant species	References
OPA03	AGTCAGCCAC	male	<i>Silene latifolia</i> Poiret	Mulcahy et al., 1992.
OPD05	TGAGCGGACA	male		
OPD12	CACCGTATCC	male		
OPP08	GTCCCGTTAC	male		
OPO08	CCTCCAGTGT	female	<i>Pistacia vera</i> L.	Hormaza et al., 1994.
No. 8	ATCCGCGTTC	male	<i>Cannabis sativa</i> L.	Sakamoto et al., 1995.
No. 11	ACGGCATATG	male		
OPA07	GAAACGGGTG	male	<i>Humulus lupulus</i> L.	Polley et al., 1997.
OPJ09	TGAGCCTCAC	male		
OPU08	GGCGAAGGTT	male		
OPAL20	AGGAGTCGGA	female	<i>Actinidia chinensis</i> Planch.	Harvey et al., 1997.
OPAI21	CACGCGAACC	male		
UBC354	CTAGAGGCCG	female	<i>Salix viminalis</i> L.	Alstrom-Rapaport et al., 1998.
OPA09	GGGTAACGCC	male female	<i>Silene latifolia</i> Poiret	Di Stilio et al., 1998.
OPB07	GGTGACGCAG	male	<i>Silene dioica</i> L.	Zhang et al., 1998.
OPK02	GTCTCCGCAA	male	<i>Silene latifolia</i> Poiret.	
OPQ14	GGACGCTTCA	male		
OPX11	GGAGCCTCAG	male		
OPX18	GACTAGGTGG	male		
OPA08	GTGACGTAGG	male	<i>Cannabis sativa</i> L.	Mandolino et al., 1999.
OPD05	TGAGCGGACA	male		Mandolino et al., 1998.

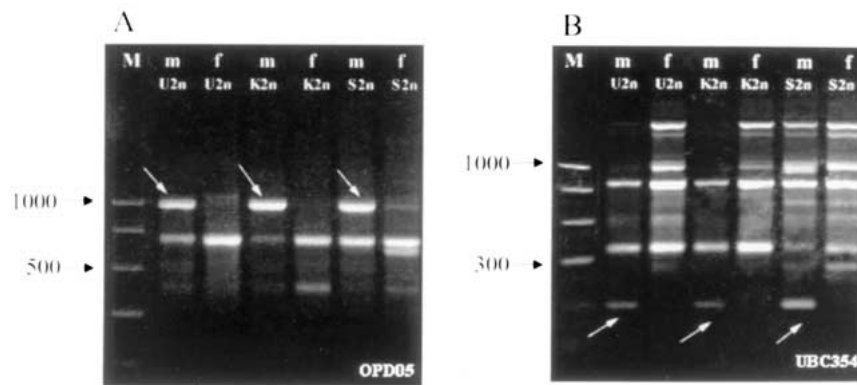


Figure 1. Male-specific DNA fragments produced by PCR with OPD05 (A) and UBC354 (B) primers in different pools of hemp. Arrows indicate sex linked fragments. M = molecular weight marker, m = male pools, f = female pools, U2n = Unikó, K2n = KFF, S2n = SA. On the left, the marker sizes are indicated in basepairs.

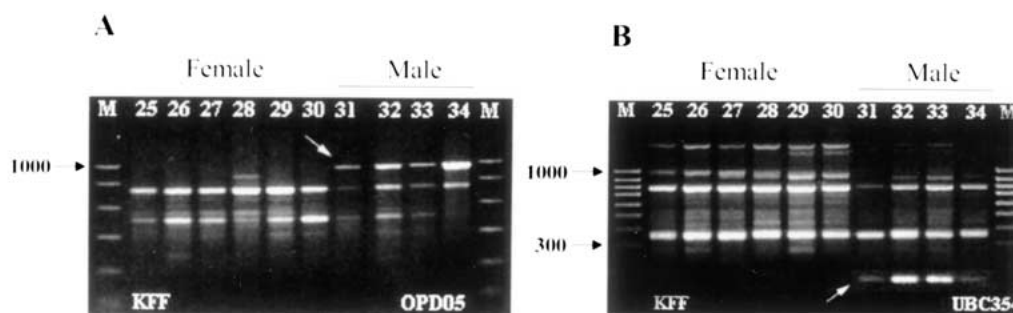


Figure 2. Male-specific markers in individual plants of hemp cultivar KFF generated with OPD05 (A) and UBC354 (B) primers. Arrows indicate sex linked fragments. M = molecular weight marker, 25–30 = female plants, 31–34 = male plants. On the left, the marker sizes are indicated in basepairs.

Cloning and sequencing of RAPD markers

The purified products of recovered DNA were cloned into the pCR^R2.1 vector (Invitrogen). The cloned fragment was recovered by *Eco*RI digestion. The DNA sequencing was performed in the Biological Research Center (Szeged) of Hungarian Academy of Science using universal primers. The database search was made using the BLAST algorithm.

Southern analysis of RAPD amplification products

RAPD amplification products were fractionated on 1.0% (w/v) agarose gel and transferred to nylon membrane (Boehringer-Mannheim, Germany). The cloned fragments were labelled with digoxigenin (Boehringer-Mannheim, Germany) and hybridized to RAPD products according to the manufacturers recommendations.

Southern analysis of genomic DNA

Ten micrograms of genomic DNA isolated from individual male and female plants were separately digested with restriction endonucleases (*Eco*RI, *Hind*III and *Dra*I). The digests were fractionated on a 1.0% (w/v) agarose gel and transferred to nylon membrane (Amersham) as recommended by the manufacturer. Southern analysis was performed according to Bucherna et al. (1999).

Results

RAPD analysis

All tested 10-meric oligonucleotids gave a reproducible RAPD pattern. The number of amplification products varied from 2 (OPD12) to 19 (No. 8). The fragment sizes ranged from 150 to 2500 bp. RAPD

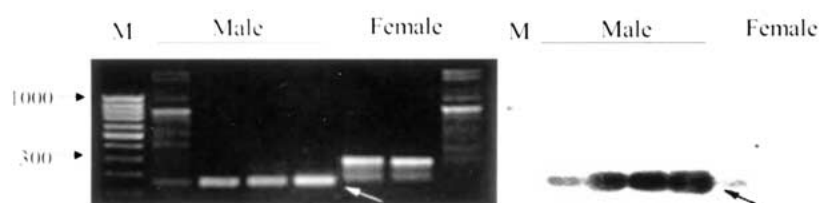


Figure 3. Southern blot analysis of RAPD profiles with the UBC354₁₅₁ fragment in hemp cultivar KFF individuals. Arrows indicate the male-linked fragment. M = molecular weight marker. RAPD patterns left, autoradiogram right. On the left, the marker sizes are indicated in basepairs.

analyses with 20 primers of DNA pools of 4–7 individuals of each unambiguously identified sex, gave no sex-specific products in the case of 18 primers. Primers OPD05 and UBC354 produced male-specific RAPD fragments in bulk analyses as it can be seen in Figure 1.

In order to prove that these differences are reproducibly male-specific, individual DNA analyses were carried out from 4–7 plants of each sex. The results of these reactions confirmed the sex specificity of these primers (OPD05 and UBC354) since the characteristic bands were present in all reaction products of male plants but were missing from the female samples (Figure 2). The estimated sizes of the obtained male specific fragments were about 1000 bp and 150 bp in the case of primers OPD05 and UBC354, respectively. Individual male and female samples giving these sex specific results with OPD05 and UBC354 primers were checked with male specific SCAR markers identified by Mandolino et al. (1999). The SCAR₃₉₀ primer pairs produced the expected DNA band of 390 bp (data not shown) exclusively in the same male individuals, as in the case of OPD05 and UBC354 primers, confirming their male-marker characteristics.

Southern analyses

To verify homology of the UBC354₁₅₁ and OPD05₉₆₁ markers in individual plants after cloning into pCR2.1 plasmid these markers were DIG-labelled and used in a hybridization study as a probe. Southern analysis indicated that male specific UBC354₁₅₁ (Figure 3) and OPD05₉₆₁ markers are homologous in all male plants. Hybridization signals have appeared also in amplified DNA of female plants, but with much lower intensity.

The cloned male specific fragments were also radiolabelled with ³²P and used for hybridization to Southern blots. Genomic DNA of randomly selected female and male individuals were digested with 3 enzymes (EcoRI, HindIII or DraI). The female and male

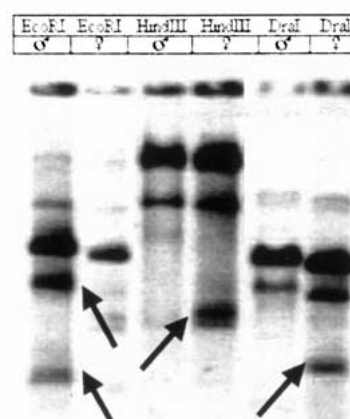


Figure 4. Southern blot analysis of the [³²P]-labelled UBC354₁₅₁ fragment to hemp cultivar SA DNA from one male and one female plant restricted with EcoRI, HindIII and DraI.

hybridization patterns with UBC354₁₅₁ probe differed from each other in one or two fragments (Figure 4). While EcoRI digestion resulted in two additional fragments in male sample, HindIII and DraI produced one-one additional band in female sample. No differences in hybridization signals were obtained with OPD05₉₆₁ probe (data not shown).

Sequence analysis

In addition to hybridization, plasmid clones harbouring the male-specific fragments were applied for sequencing (Figure 5). Homology searching revealed no significant similarity either at nucleotide or deduced amino acid sequence level to any sequence in the database. Only limited homologies (50–55% identity) were found with the retrotransposon elements from different plants.

The markers were named MADC5 (OPD05₉₆₁) and MADC6 (UBC354₁₅₁) (Male-Associated DNA from *Cannabis sativa*), according to the nomenclature

A

tgagcggacatcattgcctcgggtggaggatcggtatcaaaagtacctctttaggagggga
 acaggatttagcctacatcaaggcccaagttgctgctgagagaacccttcggatagaag
 tgcagaaagaatgggcgcctctcggcctaagatgcttctcctcgggtccccaacactggg
 gctcttgatgccagcacatccggatccggtaagttcccttaatgggtccatcatgttcc
 ttctgctcaagattattcccgggtgtaggcctatagaatttgacgaacgtctccttagat
 ttagaatgctcccctaaacgggtggggtgatcatgccaaatgcttttatttttccaactta
 ggtcatttttctaggcgggtcccgaatgggggtccggacctcatgaacctattcctctana
 tcattcagcttatctgtcttccaactggtttctccctcagacagctttcttggggatg
 acaaagctagtattatagtacaagactttgctagggtgaattagatagtcattggtagg
 actagttttacgggctatgttttctgtgtgcatattctctcgtgggtcacttntgagactt
 gatgattttgttcttntgaagcaactaggccaatggaggagctcttggcgactctt
 gcccgacctatagtcagactctgctcttccntgatgcctgatcagatgggtgacccc
 agggagatcttcttctagcatcccttagggccgaatcgctcttaattgacgaagacaggg
 aggtcaaggaggaagacgaaggcaagatcctgggccccttggaacaaaaacgcaagggga
 aaaatgggtggaggccaagtcttctaaaaggcctaggtggattgatactccgggtgccaa
 ctctaactcggggattcaacctgaagcagaggaatacactattcctctcgacatcatcc
 ttacacgtgtccgctca

B

ctagaggccgtggacgcggcggaggacgatcaaacaacaacaaaccgatatgtcagctt
 tgcagcagacctgggcatatagcttcaaaatgttaccaccagtttgacatctcatttca
 agctccagggttcagtcattatcggcctctag

Figure 5. The nucleotide sequence of the MADC5 (A) and MADC6 (B) markers, GenBank accession No. AF364954 and AF364955, respectively. The underlined sequences represent the site of designed SCAR primers.

Table 3. SCAR markers constructed from two sex specific sequences in hemp

RAPD primer	SCAR marker	Sequence 5'–3'	Annealing
OPD05 ₉₆₁	SCAR323F	GAGCGGACATCATTGCCT	55 °C
	SCAR323R	ATCACCCACCGTTTAGG	
UBC354 ₁₅₁	SCAR119F	TCAAACAACAACAAACCG	52 °C
	SCAR119F	GAGGCCGATAATTGACTG	

proposed by Sakamoto et al. (1995) and Mandolino et al. (1999).

SCAR markers

Cloning and sequencing of both male-specific RAPD fragments made it possible to convert them into SCAR markers (Table 3, Figure 5). The designed SCAR markers (SCAR₃₂₃ – MADC5 and SCAR₁₁₉ – MADC6), corresponding to our expectations, were efficient in the identification of male plants. These spe-

cific primers were able to amplify a single DNA band in both cases (a 323 bp and 119 bp fragment) in all male plants tested (Figure 6). Amplification product of the same length was seen in some female plants too, but the intensity of the bands was much lower compared with this product of male-origin. Further proof of applicability of these two SCAR markers was provided by the results of analysis in F₂ plants.

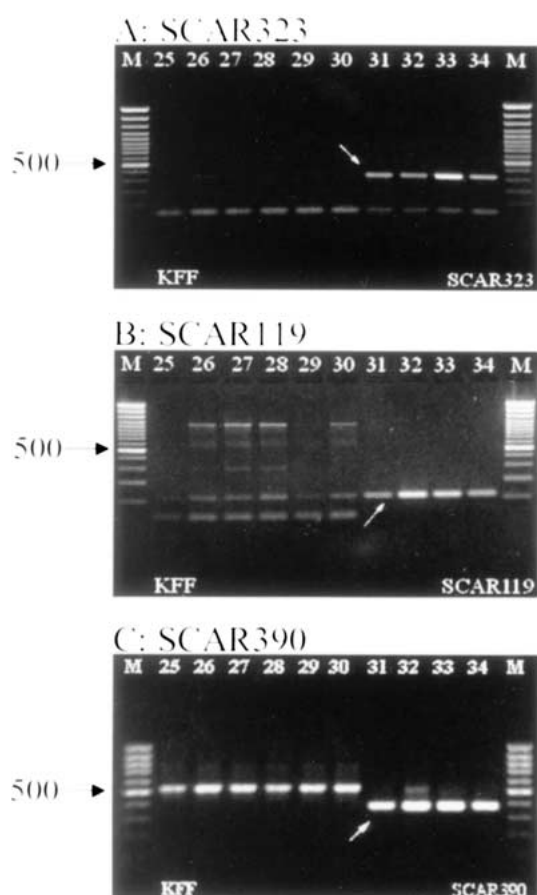


Figure 6. Individual patterns of DNA fragments produced by PCR with SCAR323 (A), SCAR119 (B) and SCAR390 (C) primers. Arrows indicate the sex linked fragments. M=molecular weight marker, 25–30 = female plants from hemp cultivar KFF, 31–34 = male plants from hemp cultivar KFF.

Analysis of F_2 plants

The two recently developed new SCAR markers and the SCAR marker described by Mandolino et al. (1999) were used to analyse individual plants of F_2 population, which derived from of the F_1 generation [Fleischmann (dioecious line, ♀) × Fibrimon (monoecious line, pollen donor)]. These crosses produced theoretically 45–55% female, 35–45% male and several percent of monoecious plants (Bócsa, 1998). From the randomly chosen 75 F_2 plants, 42 were female, 31 were male and 2 were monoecious. All of the 3 SCAR markers correlated with the sex of the plants, with the exceptions of only 2 male plants from which these markers were missing.

Discussion

Since sex determination in hemp is male heterogametic, it was presumed that all polymorphisms between sexes were due to the presence of the Y chromosome. In our experiments out of 20 RAPD primers 2 proved to be sex-specific. Our results are similar to those found in hemp by Sakamoto et al. (1995, 2 specific markers) and by Mandolino et al. (1999, 1 specific marker). Our results are also similar to those previously obtained by other groups (Flachowsky et al., 2001) who also found a relatively large number of tightly linked male-specific DNA markers. Sakamoto et al. (1998) showed that the difference between the nuclear DNA content of female and male hemp was approximately 47 Mbp. This value is about 2.8% of the total genome size of the male plants, which could correspond to the large long arm of the Y chromosome (Sakamoto et al., 1998). This difference may be reflected in the relatively high frequency of male-specific markers in hemp.

Two male-specific fragments (MADC5 and MADC6) were detected in *Cannabis sativa* L. by bulk analysis and RAPD technique (Figure 1). To check the possibility that the MADC5 and MADC6 were linked to the male phenotype, in the first step a group of 56 plants belonging to 6 cultivars (5 dioecious and 1 monoecious) were screened for the presence or absence of these markers (Figure 2). The presence of the MADC5 and MADC6 markers was evident in all male plants, as was the absence of these markers in female and monoecious plants. In hemp, OPD05 primer was successfully applied for sex identification by Mandolino et al. (1998). They determined a 900 bp long male-specific RAPD product in bulk analysis. Individual plants were not analysed by them with this primer. Our results with primer OPD05 identified a longer marker (MADC5). It is not yet known whether these two products are homologous with each other, because the 900 bp product (Mandolino et al., 1998) has not been recorded in any sequence databases.

Our study confirmed that the decamer RAPD primers described as sex-specific markers in different plants are not universal – as it was predicted – in all dioecious plants. However, in some cases a certain extent of homology may be assumed since the primer OPD05 producing sex-specific DNA fragment in *Silene latifolia* Poir (Mulcahy et al., 1992) and UBC354 produced sex-specific DNA fragment in *Salix viminalis* L. (Alstrom-Rapaport et al., 1998) generated male specific markers in *Cannabis sativa*

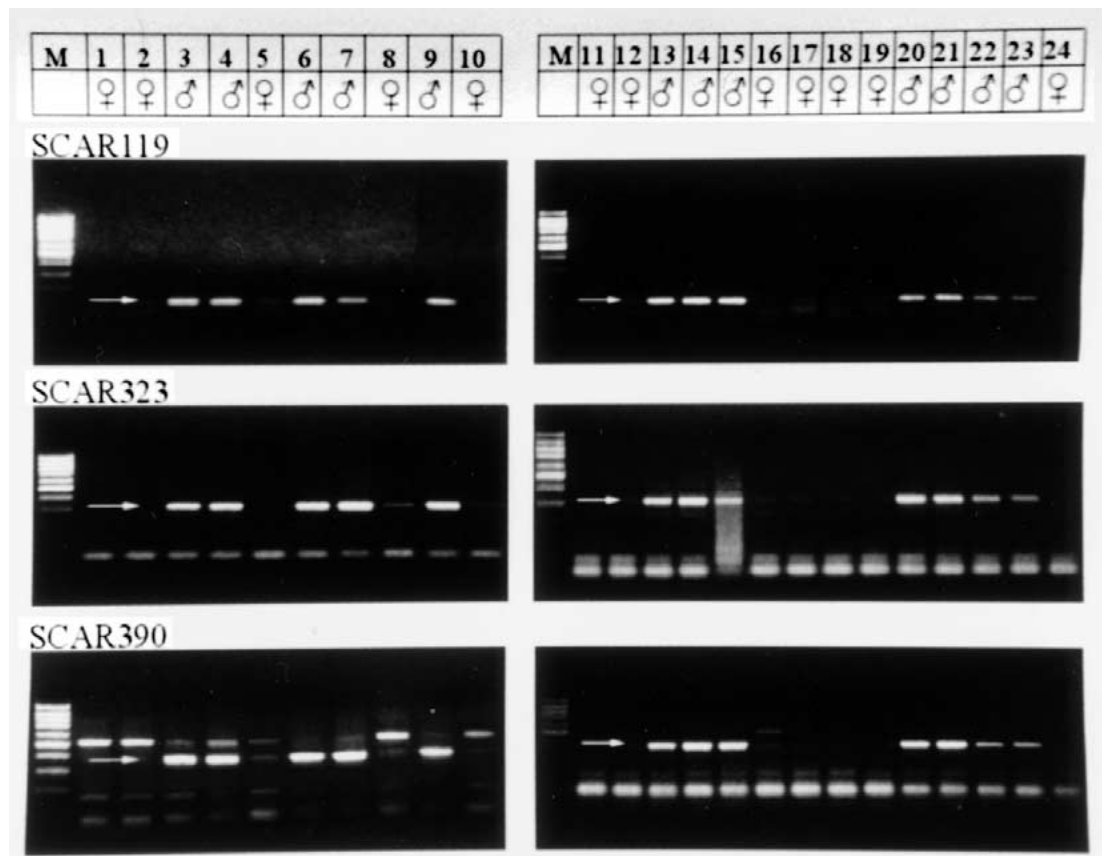


Figure 7. Analysis with the SCAR₁₁₉, SCAR₃₂₃ and SCAR₃₉₀ specific primers of F₂ individual hemp plants. The male-linked bands (119, 323 and 390 bp fragments) are present in the males (♂) but not in the females (♀). M = molecular weight marker. On the left, the marker sizes are indicated in basepairs.

L., too. With the primer UBC354 a female-specific marker was identified in *Salix viminalis* L. (Alstrom-Rapaport et al., 1998), while in our study this primer corresponded to a male-specific marker in hemp. The MADC6 marker is shorter than the one in *Salix viminalis* L. As the sequence of the female-linked marker in *Salix viminalis* L. is still unknown, presumable homology between these two markers should be checked.

MADC5 and MADC6 sequences did not show homology with other dioecious plant species in the databank. The highest similarity was found with retro-transposon sequences of different plant species, in agreement with the results obtained with sex-linked markers by other authors (Sakamoto et al., 2000). The length of MADC6 is 151 bp and taking the possible reading frames into account it contains only a few stop codons. So the presence of open reading frames cannot be excluded, but the length of the sequences is short.

The size of the clone MADC5 is 961 bp, and it can contain open reading frames of 400–600 bp.

While the MADC5 sequence gave no differences in Southern analysis of the randomly chosen female and male genomic DNA, the MADC6 probe resulted in variation in hybridization signals. While EcoRI digestion resulted in two additional fragments in male samples, HindIII and DraI produced one-one additional band in female samples (Figure 4). Further investigations are necessary to be able to explain for this. Southern analysis of more female and male plants are planned in order to answer, whether the MADC6 sequence results in consequently appearing inequalities in the two genders. Sex-specific pattern could be demonstrated in papaya (Parasnis et al., 2000) and asparagus (Reamon-Büttner et al., 1998) by Southern analysis, so far but not in hemp (Sakamoto et al., 1995; Mandolino et al., 1999).

As the RAPD reactions with the sex-specific markers proved to be highly reproducible in our analysis, two primer pairs were designed based upon the two cloned and sequenced sex-specific sequences after cloning. The current trend is to develop reliable SCAR markers from sequenced RAPD markers (Paran & Michelmore, 1993). The SCAR markers (SCAR₃₂₃ and SCAR₁₁₉) were efficient in identification of all male plants and were able to amplify a single DNA band in both cases (Figure 6). The same length amplification product was seen in some female plants too, but the intensity of the bands was much lower compared with the male-originated fragment. According to Sakamoto et al. (1995) who found less intensive female bands in southern hybridization, similar sequences might be present in the female sex. This may explain why male flowers develop on female plants under extreme conditions (Clarke, 1997).

The results of analyses of F₂ plants (Figure 7) suggest that these markers are linked with the Y chromosome. Only two male plants were found in which markers described in this study are missing. Mandolino et al. (1999) found before that some female plants in which their male-specific marker was present. They speculated on genetic recombination between the sex locus and the marker, which may also be a possible explanation for our results.

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