Plant
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Morphological and molecular study of sex determination in *Cannabis sativa* L.

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Abstract

Cannabis sativa L. is an important plant with various uses in pharmaceutical and paper production industries. Due to the higher priority of female and male *Cannabis* plants for pharmaceutical uses and fibre industry, respectively, reduction of the quality of products after pollination, and also for detection of better genotypes before pollination for breeding purposes, early determination of sex in *Cannabis* is one of the major concerns of researchers and farmers. Seeds of 26 accessions from different regions of Iran along with one accession from Afghanistan were planted in the field based on randomized complete block design with 10 replications. Five female and five male plants were sampled from each accession for molecular and morphological analyses. Thirteen ISSR primers and two SCAR markers were used. Also, morphological differences, which possibly related to sex were measured in the *Cannabis* populations under study. The results showed significant differences among the accessions within female and male plants for plant height, number of days to flowering, days after full bloom and height of the first flowering node. Male plants were generally, but not always, taller than female plants and had a shorter life cycle. The highest expected heterozygosity was found in the ISSR3 primer and the lowest in the UBC825 primer. In total, 143 polymorphic bands and one polymorphic band were amplified using ISSR and SCAR primers, respectively. Out of 143 polymorphic ISSR bands, only 10 markers had significant relations with the gender of this plant. MADC6 SCAR marker was monomorph across the accessions. On the other hand, MADC5 was a polymorphic marker and showed a significant relation with gender. These markers, especially MADC5, have the potential to be used in sex determination of *C. sativa*.

Keywords: *Cannabis*; Female; Male; Molecular markers; Morphological markers; Sex determination.

Introduction

Approximately 7% of flowering plant species are dioecious (Dellaporta and Calderon-Urrea 1993) and distributed among 75% of plant families (Thomas and Vince-Prue 1997); however, only a few number of species have cytogenetic and/or molecular evidence for sex chromosomes (Divashuk *et al.* 2014). Sexual dimorphism is common in dioecious plant species (Pérez-Llorca and Sánchez Vilas 2019). A large variability in genetic mechanisms of sex determination has been reported within dioecious plant species (Heikrujam *et al.* 2015). Chromosomes with different morphology have been reported in several plants, including the species within the *Cannabis* genera (Parker 1990).

The dioecious phenomenon facilitates breeding programs in plant species, but the sex of an individual plant is difficult to determine before flowering (Milewicz and Sawicki 2013). Sex determination before flowering can accelerate selection process in breeding programs and, as a result, bring significant savings in time and economic resources (Hormaza *et al.* 1994).

Cannabis sativa L. is a diploid plant (2n = 2x = 20), consisting of nine pairs of autosomal chromosomes and one pair of sex chromosomes (X and Y). Female and male plants are characterized by XX and XY chromosome pairs, respectively (Rode *et al.* 2005). There is a small difference between the genome size of male and female plants (1683 and 1636, respectively), therefore, Y chromosome is larger than X (Hoffman 1961; Sakamoto *et al*. 1998).

Similar to a typical dioecious variety in Italy (Moliterni *et al.* 2004), in the field conditions of Iran, the life cycle of most dioecious varieties of *C. sativa* has a duration of around six months, and the earliest flowers appear after three to four months. The flowering date in females is, generally, two to four weeks later than males in the same cultivar. The gender of seedlings is not determined before the flowering phase, when male plants show a distinct elongation of last internodes causing them to become taller than female plants (Moliterni *et al*. 2004).

In *C. sativa*, females contain higher THC (delta-9-tetrahydrocannabinol) than male plants and mostly used as a drug source (Andre *et al*. 2016). Due to the higher priority of female and male *Cannabis* plants for pharmaceutical uses and fibre industry, respectively, the reduction of the quality of products after pollination, and also for elimination of undesirable male plants before pollination (Moliterni *et al.* 2004), early determination of sex in *Cannabis* is important for farmers and scientists.

Morphological markers have been used to describe differences among plant organisms. Although the measurement of morphological markers is time-consuming and they are not always readily available for analysis, these markers have been used widely in the research programs because they do not mainly need destructive analysis and are easy to measure. On the other hand, genetic marker systems are widely used for genetic mapping, evolutionary studies and disease diagnostics, and they can be more reliable than morphological markers, especially in the study of sex determination in dioecious plants (Samantaray *et al*. 2012). Molecular markers have been used to resolve the problem associated with dioecism to a certain extent (Heikrujam *et al*. 2015). Some authors have introduced several gender-related markers in *Cannabis*, such as RAPD (Sakamoto *et al*. 1995), SCAR (Mandolin *et al*. 1999; Torjek *et al*. 2002), AFLP (Peil *et al*. 2003; Flachowsky *et al*. 2008) and SSR (Rode *et al*. 2005).

In the present investigation, we studied the possibility of using ISSR and SCAR molecular markers, and also several morphological markers to distinguish between the female and male plants of *C. sativa* during the growing period.

Material and Methods
Plant material and DNA isolation
This study was performed in the experimental field of the University of Tehran, Karaj, Iran. Seeds from 26 accessions, collected from different regions of Iran along with one accession from Afghanistan were planted under field conditions based on randomized complete block design with 10 replications. The measured traits were as follows: plant height, number of days to flowering, days after full bloom and height of the first flowering node. For the molecular and

morphological analyses, 10 plants (five males and five females) were sampled within each accession.

Total DNA was isolated from *C. sativa* seedling leaves according to Doyle (1990) method and DNA quality and quantity were assessed by spectrophotometer and electrophoresis on agarose gel. Two different markers of ISSR and SCAR were used for sex determination. The number and sequence of the primers are shown in Tables 1 and 2.

ISSRs analysis

In this study, 13 ISSR primers were used for identification. The annealing temperatures were optimized for each primer (Table 1). Amplification reactions were accomplished in a Bio-Rad thermocycler C1000 Touch. The reaction volume was 15 µl and consisted of 3 µl genomic DNA, 2 µl primer, 2.5 µl injection water and 7.5 µl master mix (dNTP, MgCl2, *Taq* DNA polymerase, PCR buffer). PCR conditions were as follows: 1 cycle of initial denaturation at 94 ◦C for 3 minutes; 35 cycles of denaturation at 94 ◦C for 1 minute, annealing at the corresponding temperature for 50 seconds (Table 1) and extension at 72 ◦C for 1 minute; 1 cycle of final extension at 72 ◦C for 10 minutes. The amplification products (bands) were separated by size with a standard horizontal electrophoresis on 1.5 % agarose gels in 1X TBE buffer stained with ethidium bromide, visualized by UV illumination and photographed.

SCAR analysis

SCAR analysis was carried out with two markers. PCR amplification reactions were performed in 15 µl reaction mixtures containing 3 µl genomic DNA, 1 µl forward primer, 1 µl reverse primer, 2.5 µl injection water and 7.5 µl master mix (dNTP, MgCl2, Taq DNA polymerase, PCR buffer). PCR conditions were as follows: 1 cycle of initial denaturation at 94 ◦C for 2 minutes; 40 cycles of denaturation at 94 ◦C for 10 seconds, annealing at the corresponding temperature for 30 seconds (Table 2) and extension at 72 ◦C for 1 minute; 1 cycle of final extension at 72 ◦C for 10 minutes.

Data analysis

The ISSRs markers were scored as 1 and 0 for presence and absence of the bands, respectively. Number of effective alleles (Ne) (Hill 1973), expected heterozygosity (He) (Nei 1973), unbiased expected heterozygosity (uHe) (Nei 1978) and Shannon's information index (I) (Shannon 1948) were calculated by the following formulae, averaged over all loci:

$$Ne = 1 / (p^2 + q^2)$$
$$He = 2pq$$
$$uHe = (2N / (2N-1)) * He$$
$$I = - ((p \, Ln(p) + q \, Ln(q))$$

Where, p is the frequency of the presence of the band, q is the frequency of the absence of the band and N is the number of individuals.

The SCAR markers were assayed based on the genotype. After analysis of variance for morphological data, means were compared by the least significant difference test. All statistical analyses for molecular markers and morphological traits were performed using Gen Alex 6.5, NTSYSpc 2.01, SAS 9.1 and SPSS 19 software.

Results and Discussion

Significant differences were observed among

males and among female accessions for plant height, number of days to flowering, days after full bloom and height of the first flowering node (Tables 3 and 4). The beginning of the flowering in female plants ranged from 83.6 (Saqez accession) to 133 (Shiraz accession) (Table3), and in male plants ranged from 81 (Saadat Shahr accession) to 101.3 (Qazvin1 accession) (Table 4) days after seed germination. The flowering date in females was 1 to 5 weeks later than males in most of the accessions under study. Days after full bloom in female and male plants varied from 93.6 to 140 and from 91.6 to 119.3 days, respectively. Our study showed that the male plant life cycle is shorter than female plants in most of the accessions. Based on Hoffmann (1947), the ripening period of female plants was 2-4 weeks later than male plants.

The range of female and male plant heights were 70-217.3 and 77.3-243 cm, respectively. Although male plants were generally taller than female plants, but this was not true for all accessions and in some accessions the average plant height of female plants was higher than the male plants. This inconsistency was more pronounced for the height of the first flowering nodes. Although, the male plants showed a distinct elongation of the last internodes and the height of the first flowering nodes varied from 10.3 to 94.3 cm in female plants and from 21 to 123.6 cm in male plants, but in almost 40% of the accessions, average height of the first flowering nodes in male plants was not higher than the female plants. De Meijer and Keizer (1994) also reported that male plants are usually taller and slender and have a shorter life cycle, while the female plants have more branching and shorter height.

In female plants there was a mild and significant positive correlation between flowering time and plant height (r= 0.42; p-value= 0.034), but in male plants the correlation was low and didn't reach the 5% significance level (r= 0.35; p-value=

Table 1. ISSR primers used for sex determination in *Cannabis sativa* L.

Primer	Annealing temperature (∘C)	Sequence	Primer	Annealing temperature (∘C)	Sequence
ISSR-1	62	$(AGC)^6$-G	ISSR-16	55.5	$(CA)^8$-GC
ISSR-2	61	$(ACC)^6$-G	UBC-817	52	$(CA)^7$-C
ISSR-3	62	$(AGC)^6$-C	UBC-825	50	$(AC)^8$-T
ISSR-5	56.3	$(GA)^9$-C	UBC-834	52.5	$(AG)^8$-YT
ISSR-8	56.3	$(AC)^9$-G	UBC-842	55	$(GA)^8$-YG
ISSR-12	60.5	$(GACAC)^4$	UBC-845	50	$(CT)^8$-RG
ISSR-15	55.5	$(CA)^8$-AG			

Table 2. SCAR primers used for sex determination in *Cannabis sativa* L.

Primer	Annealing temperature (∘C)	Sequence	Reference
SCAR323 (MADC5)	55	Forward: GAGCGGACATCATTGCCT Reverse: ATCACCCCACCGTTTAGG	(Torjek *et al.* 2002)
SCAR119 (MADC6)	52	Reverse: GAGGCCGATAATTGACTC Forward: TCAAACAACAACAAACCC	(Torjek *et al.* 2002)

Table 3. Means of morphological traits of female plants for the *Cannabis sativa* L. accessions under study.

Accession	Plant height (cm)	Height of the first flowering node (cm)	Days after full bloom	Number of days to flowering
Abhar	161.3[c-h]	46.3[c-h]	106.3[i-m]	98[h-k]
Orumieh	154.6[hi]	86.6[ab]	122[d]	114[c]
Samen	137.6[hi]	29.3[f-i]	107[h-m]	99[h-k]
Dezful1	162.3[c-h]	66.3[a-d]	112[e-h]	103[e-h]
Dezful2	182.6[b-f]	52.3[c-g]	105.6[k-m]	95.6[jk]
Sanandaj	171[c-g]	88.3[a]	116[e]	107[de]
Qazvin 1	155.3[f-h]	54[c-g]	123.3[cd]	116[c]
Qazvin 2	159[e-h]	38[d-i]	109.3[g-k]	101[g-j]
Shiraz	186.6[b-d]	35.3[f-i]	140[a]	133[a]
Esfahan	147.6[hi]	10.3[i]	115.3[e]	107[d-f]
Sirjan	161.3[c-h]	94.3[a]	114.6[e-g]	107
Sero	81[j]	18.6[hi]	106[i-m]	96.6[i-k]
Bam	170[c-g]	37[d-i]	112[eh]	103[e-h]
Kermanshah	142[hi]	37[d-i]	103[m]	95.6[jk]
Arak	172.6[c-g]	65.3[a-e]	115[ef]	105[d-g]
Afghanistan	155[gh]	26.3[g-i]	128[bc]	115[c]
Saadat Shahr	174.3[c-g]	57.6[b-f]	104[m]	95.3[jk]
Ramhormoz	204.3[ab]	20[c-hi]	131.3[b]	123[b]
Ardebil	187.3[bc]	45.3[c-h]	103[m]	94.3[k]
Save	162[c-h]	38[d-i]	103.3[m]	95[k]
Dashtmoghan	217.3[a]	54.6[-g]	111.3[e-j]	101[f-i]
Nahavand	160[c-h]	39.6[d-i]	111.6[e-i]	102[e-i]
Malayer	127.3[i]	22[e-i]	122.6[cd]	110[cd]
Zahedan	159.3[d-h]	36.3[e-i]	109.6[f-k]	102[e-i]
Boshruyeh	183.6[b-e]	69.3[a-c]	122.6[cd]	114[c]
Saqez	70[j]	26.3[g-i]	93.6[n]	83.6[k]

In each column, means with different letters are significantly different at 0.05 probability level based on the least significant difference (LSD) test.

0.08). De Meijer and Keizer (1994) has also indicated that early-flowering accessions were shorter than the late ones.

Our results showed that plant height or number of days to flowering are not reliable markers for sex determination before flowering, because there were some over lapping between male and female plants for these traits and genetic differences existed among accessions. Moliterni *et al.* (2004) have also indicated that plant height and time until flowering, are more flexible and influenced by environmental and genetic variation.

According to Moliterni *et al.* (2004), sexual differentiation in *Cannabis* naturally occurs late during its lifetime nearly after the completion of vegetative phase, immediately before the production of the unisexual flowers. These scientists stated that investigation of the early stages of sexual differentiation requires a method for the precise identification of the sex. Therefore, they proposed a rapid molecular method for the early sex discrimination, based on the PCR amplification of a male-specific SCAR marker directly from a tissue fragment.

Results of analysis of ISSR and SCAR markers are presented in Tables 5-6. The highest number of effective alleles were observed in ISSR3 and ISSR5 primers and the lowest in UBC825 (Table 5). Polymorphic level of primers can be assessed by the comparison of their

Table 4. Means of morphological traits of male plants for the *Cannabis sativa* L. accessions under study.

Accession	Plant height (cm)	Height of the first flowering node (cm)	Days after full bloom	Number of days to flowering
Abhar	216[b-c]	123.6[a]	96.6[j-m]	88.3 [gi]
Orumieh	161.6[h-j]	49[e-g]	104.6[c-h]	96[b-d]
Samen	133[k]	32.3[f-g]	100.6[g-j]	91[e-g]
Dezful1	153.3[i-j]	22.6[f-g]	101.3[e-j]	91.3 [d-g]
Dezful2	217[ab]	55.6[c-f]	96.3[j-m]	85.3[h-k]
Sanandaj	191.3[b-g]	76[b-e]	104.3[d-h]	96.6[a-c]
Qazvin1	206[b-e]	46[e-g]	110.6[b]	101.3[a]
Qazvin2	204.3[b-f]	50.6[d-g]	108.6[b-d]	99[a-c]
Shiraz	172.3[g-j]	46.3[e-g]	104.6[c-h]	97.6[a-c]
Esfahan	184[e-h]	30.6[fg]	106[b-f]	97.6[a-c]
Sirjan	186[e-h]	89[bc]	104[d-i]	96[b-d]
Sero	77.3[l]	39.6[fg]	91.6[m]	83[jk]
Bam	213.6[b-d]	37[fg]	106.3[b-e]	95.6[b-e]
Kermanshah	173[g-j]	46.6[e-g]	94.3[l-m]	84.6[jk]
Arak	189.6[d-g]	75.3[c-f]	99[j-l]	90[fg]
Afghanistan	172[g-j]	31.3[fg]	109.6[bc]	94.6[c-f]
Saadat Shahr	204[b-f]	44.3[e-g]	93.3[m]	81[k]
Ramhormoz	243[a]	55.3[c-f]	109[b-d]	100.3[ab]
Ardebil	183.3[e-h]	49[e-g]	94.6[k-m]	86.6[h0j]
Save	181.6[e-h]	84[b-d]	105.6[b-g]	96.6[a-c]
Dashtmoghan	190.6[c-g]	31fg	101[f-j]	89.3[gi]
Nahavand	178.3[f-i]	31.6[fg]	99.6[h-j]	88.6[gi]
Malayer	160.3[h-j]	32fg	119.3[a]	97.6[a-c]
Zahedan	179.3[f-h]	46.6[e-g]	100.3[h-j]	90.6[fg]
Boshruyeh	212.6[b-d]	101.3[ab]	103.3[e-i]	96[b-d]
Saqez	147.3[jk]	21[g]	94[lm]	83[jk]

In each column, means with different letters are significantly different at 0.05 probability level based on the least significant difference (LSD) test.

expected heterozygosity. The highest expected heterozygosity was seen in ISSR3 and the lowest amount was observed in UBC825. High level of heterozygosity was not unexpected due to the cross-pollination nature of *C. sativa.* Also, the highest and the lowest Shannon's information index found in ISSR3 and UBC825 primers, respectively (Table 5). Based on our results it can be said that ISSR3 primer had the highest efficiency in population discrimination. Overall, 143 polymorphic loci and one polymorphic locus were detected in ISSR and SCAR markers, respectively. The number of loci per primer ranged from five (ISSR5) to fifteen (ISSR12, ISSR15, UBC825). In order to find markers that associated with the gender in the *C. sativa* plant, the χ^2 test

was performed for each pair of marker and gender data (Table 6). Out of 143 polymorphic ISSR markers, only 10 markers had significant relations with gender. The name of the markers is shown in Table 6. These markers may be potentially used for making specific SCAR markers to determine sex in *C. sativa*. The SCAR marker MADC6 was monomorphic over all the studied populations (Figure 1). On the other hand, the MADC5 marker showed polymorphism, and displayed significant relation with sex at 5% probability level (Table 6, Figure 1).

Several authors have reported molecular markers on sex chromosomes in *C. sativa.* Flachowski *et al*. (2001) introduced several AFLP markers for the Y chromosome of the male plants.
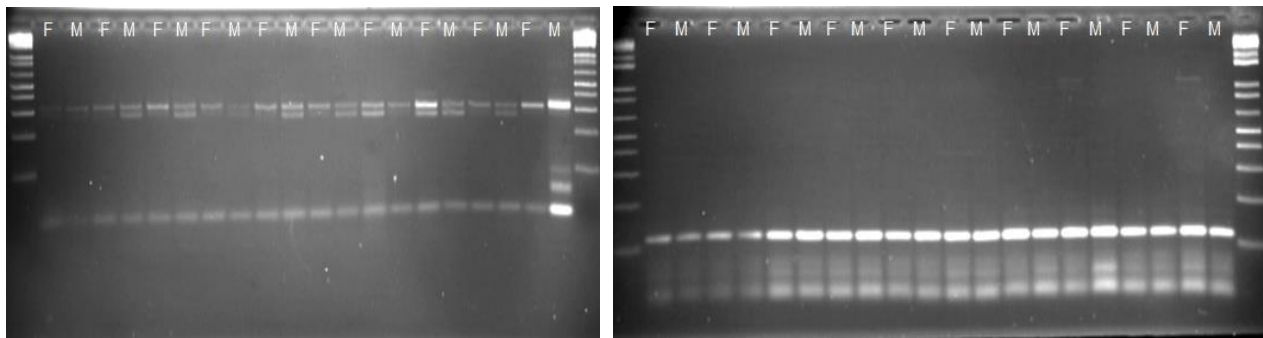
Figure 1. The SCAR markers produced by the primers of MADC5 (left) and MADC6 (right) in *Cannabis sativa* L. accessions (F: female, M: male).

Table 5. Expected heterozygosity, Shannon's information index and number of effective alleles for each ISSR marker.

Markers	uHe	He	I	Ne	Markers	uHe	He	I	Ne
ISSR1	0.22	0.20	0.28	1.37	ISSR16	0.18	0.16	0.24	1.29
ISSR2	0.22	0.18	0.28	1.30	UBC817	0.13	0.20	0.17	1.20
ISSR3	0.27	0.24	0.36	1.41	UBC825	0.10	0.09	0.14	1.15
ISSR5	0.26	0.23	0.35	1.41	UBC834	0.13	0.12	0.18	1.21
ISSR8	0.22	0.20	0.29	1.37	UBC842	0.22	0.19	0.28	1.35
ISSR12	0.18	0.17	0.26	1.31	UBC845	0.22	0.20	0.29	1.35
ISSR15	0.19	0.16	0.25	1.30					

Ne = number of effective alleles = $1 / (p^2 + q^2)$ (Hill 1973).
He = expected heterozygosity = $2pq$ (Nei 1973).
uHe = unbiased expected heterozygosity= $(2N / (2N-1)) * He$ (Nei, 1978).
I= Shannon's information index = $-1 ((pLn (p) + qLn (q))$ (Shannon 1948).
Where, p is frequency of the band present, q is frequency of the band absent and N is the number of individuals.
All parameters were calculated by the above formulae after averaging over all loci.

Table 6. Association among markers and sex in *Cannabis sativa* L.

ISSR	χ^2	ISSR	χ^2	SCAR	χ^2
UBC8251	4.52*	ISSR124	4.58*	MADC5	5.04*
UBC8254	3.7 *	ISSR122	5.37*		
UBC81712	7.6**	ISSR81	6.63**		
UBC81713	3.6 *	UBC8451	4.28*		
ISSR151	5.14*	ISSR152	5.14*		

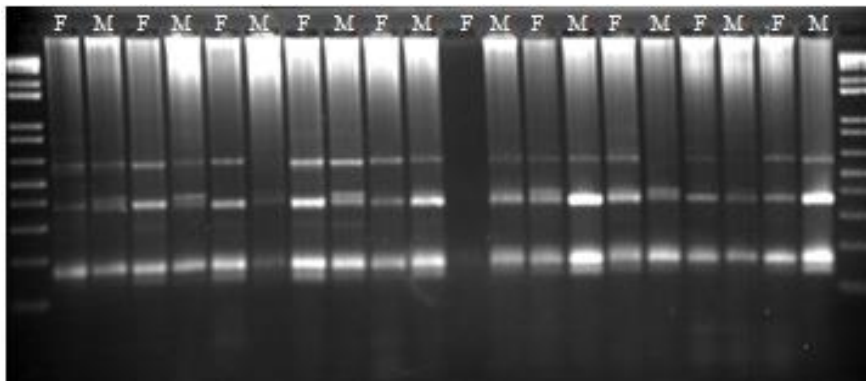*,**significant at 0.05 and 0.01 probability levels, respectively.

Figure 2. Polymorphic pattern of the primer of UBC845 in *Cannabis* accessions (F: female, M: male).

In another report, Sakamoto *et al.* (1995) identified two polymorphic RAPD markers linked to male plants in *Cannabis*. Rode *et al.* (2005) introduced several gender-related SSR markers. Three alleles were identified in two markers that showed polymorphism not only between X and Y, but also on different X chromosomes. In addition, they detected several sex-linked RAPD markers in one population. Peil *et al.* (2003) reported five AFLP markers on both chromosomes X and Y chromosomes. Mandolin *et al.* (1999) converted a male-specific RAPD marker to an SCAR marker. Torjak *et al.* (2002) also studied two RAPD markers, which were specific for male plants, and converted them to SCAR markers of MADC5 and MADC6. Our result about MADC6 marker was not consistent with the result obtained by Torjek *et al*

(2002). In our investigation, MADC6 marker was not polymorphic and didn't relate to the male gender. However, similar to Torjek *et al.* (2002), the MADC5 marker was significantly associated with the sex chromosomes.

In conclusion, morphological indicators, such as plant height and number of days to flowering, were not reliable markers for sex determination in *C. sativa*, because of a considerable overlapping between male and female plants and significant genetic variability among accessions within each sex group. On the other hand, we showed that 10 ISSR markers and one SCAR marker (MADC5) were significantly related to male plants. These markers, especially MADC5, can be possibly used for sex determination in *C. sativa*.

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مطالعه مورفولوژیکی و مولکولی تعیین جنسیت در شاهدانه

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چکیده

شاهدانه (.Cannabis sativa L) یک گیاه مهم با کاربردهای مختلف در صنایع دارویی و تولید کاغذ است. با توجه به این که اغلب گیاهان ماده در صنایع دارویی و در گیاهان نر در تولید فیبر حایز اهمیت بیشتری هستند و گردهافشانی گیاه کیفیت فرآوردههای تولیدی را کاهش میدهد، همیشه یکی از دغدغههای محققان و کشاورزان شناسایی زودهنگام جنسیت در شاهدانه بوده است. بذرها از ۲۶ جمعیت از مناطق مختلف ایران به همراه یک جمعیت از افغانستان به صورت طرح بلوکهای کامل تصادفی در ۱۰ تکرار کشت شدند. پنج گیاه نر و پنج گیاه ماده از هر جمعیت برای تجزیههای مورفولوژیکی و مولکولی نمونهبرداری شدند. از ۱۳ آغازگر ISSR و دو SCAR استفاده شد. تفاوت مورفولوژیکی مرتبط با جنسیت در جمعیتهای شاهدانه مورد مطالعه مشاهده شد. نتایج نشان داد که در برخی از صفات بین جمعیتها در درون هر دو جنس تفاوت معنیداری وجود دارد. گیاهان نر عمدتا بلندتر و باریکتر از گیاهان ماده بودند و چرخه زندگی کوتاهتری نسبت به گیاهان ماده داشتند. بالاترین میانگین هتروزیگوسیتی در آغازگر ISSR3 و کمترین آن در آغازگر UBC825 مشاهده شد. بهطور کلی ۱۴۳ جایگاه پلیمورفیک و یک جایگاه پلیمورفیک به ترتیب در آغازگرهای ISSR و SCAR شناسایی شد. از بین ۱۴۳ جایگاه پلیمورفیک در ISSR تنها ۱۰ نشانگر رابطه معنیداری با جنسیت داشتند. آغازگر MADC6 SCAR مونومورف بود. از طرف دیگر آغازگر MADC5 SCAR پلیمورفیسم نشان داد و رابطه معنیداری با نوع جنس داشت. بنابراین، به نظر میرسد که این نشانگرها، به ویژه MADC5، دارای ارزش بالقوه برای تعیین جنسیت در C. sativa هستند.

واژههای کلیدی: تعیین جنسیت؛ شاهدانه؛ ماده؛ نر؛ نشانگرهای مورفولیژیکی؛ نشانگرهای مولکولی.