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ORIGINAL ARTICLE

Molecular cytogenetic analysis of monoecious hemp (*Cannabis sativa* L.) cultivars reveals its karyotype variations and sex chromosomes constitution

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Abstract Hemp (*Cannabis sativa* L., 2n=20) is a dioecious plant. Sex expression is controlled by an X-to-autosome balance system consisting of the heteromorphic sex chromosomes XY for males and XX for females. Genetically monoecious hemp offers several agronomic advantages compared to the dioecious cultivars that are widely used in hemp cultivation. The male or female origin of monoecious maternal plants is unknown. Additionally, the sex chromosome composition of monoecious hemp forms remains unknown. In this study, we examine the sex chromosome makeup in monoecious hemp using a cytogenetic approach. Eight monoecious and two dioecious cultivars were used. The DNA of 210 monoecious plants was used for PCR analysis with the male-associated markers MADC2 and SCAR323. All monoecious plants showed female amplification patterns. Fluorescence in situ hybridization (FISH) with the subtelomeric CS-1 probe to chromosomes plates and karyotyping revealed a lack of Y chromosome and presence of XX sex chromosomes in monoecious cultivars with the chromosome number 2n=20. There was a high level of intra- and intercultivar karyotype variation detected. The results of this study can be used for further analysis of the genetic basis of sex expression in plants.

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Introduction

Hemp (*Cannabis sativa* L., *Cannabaceae*) is economically important and one of the earliest known cultivated crops used in textiles, cordage, canvas, oil, paper, "green composite" materials, cosmetics, and various other applications (van der Werf et al. 1996; Struik et al. 2000; Shahzad 2012). It has probably been used for at least 10,000 years (Schultes et al. 1974). There has recently been an increased need to identify alternative crops, and hemp can also be used as a biofuel source (Gonzalez-Garcia et al. 2012).

C. sativa is a dioecious and obligate outbred species with a complex genetic constitution and heredity. The genetic complexity may explain the phenotype variability, sex expression, polymorphism, and the substantial biological plasticity of this species (Schultes et al. 1974; Kohiyouma et al. 2000). Dioecy is the ancestral state of family *Cannabaceae*, and members of this family may have one of the oldest sex chromosome systems (Divashuk et al. 2014).

Hemp has a diploid genome (2n=20) with a karyotype composed of nine autosomes and a pair of sex chromosomes (X and Y) (Sakamoto et al. 1998; Divashuk et al. 2014). Female plants are homogametic (XX) and male plants are heterogametic (XY). In these plants, sex expression is controlled by an X-to-autosome (X/A) balance system (Shephard et al. 2000; Vyskot and Hobza 2004). The plants with X/A=1 are female, while X/A=0.5 plants are male (Westergaard 1958; Parker and Clark 1991; Ming et al. 2011). It was previously shown that sex expression also depends on different environmental factors and can be reversed by the application of plant growth regulators (Mohan Ram and Jaiswal 1972; Chailakhyan and Khryanin 1978; Truta et al. 2007). The

plasticity of the *C. sativa* L. sexual phenotype often leads to the differentiation of hermaphrodite flowers or bisexual inflorescences on genetically male or female plants (monoecious phenotype) (Moliterni et al. 2004). Monoecy can also be expressed in diploid genetically male or female plants under some environmental conditions or can be induced by the application of plant growth regulators (Schaffner 1921; Heslop-Harrison 1956; Chailakhyan and Khryanin 1978; Moliterni et al. 2004). In *Humulus lupulus* (*Cannabaceae*) and *C. sativa*, the plants which carry a triploid or aneuploid chromosome numbers with an X/A chromosome ratio of 0.66, are also monoecious (Nishiyama et al. 1947; Haunold 1971; Skof et al. 2012). These plants are sterile and normally have no generations.

Due to the dioecious nature of *C. sativa*, the male and female plants are characterized by sexual dimorphism with respect to plant size and precocity, which results in unsynchronized maturity and problems with mechanized harvesting (Amaducci et al. 1998; Faux et al. 2014). To overcome these inconveniences, genetically monoecious diploid (2n=20) hemp cultivars have been developed that offer several agronomical advantages, including higher seed yields, higher crop homogeneity, and easier mechanical harvest compared to dioecious cultivars (Moliterni et al. 2004; Mandolino and Carboni 2004; Faux et al. 2014). The sex chromosomes that determine the monoecious forms of hemp are unknown. In 1952, Hoffmann assumed the existence of XX, XY, and YY forms in monoecious hemp plants (Hoffmann 1952). However, Menzel (1964) observed only XX chromosomes. In Menzel's study, the karyotype was limited to the cultivar "Kentucky" (Menzel 1964). The genome sizes of female and male plants differ, and this difference has been attributed to the large long arm of the Y chromosome (Sakamoto et al. 1998; Divashuk et al. 2014). A recent study utilizing flow cytometry revealed that the genome size of monoecious plants was same to that of females but significantly smaller than that of males (Faux et al. 2014). The application of the male-specific MADC2 DNA marker to monoecious plants revealed a female-associated amplification pattern (Mandolino et al. 1999; Faux et al. 2014). This indirect evidence supports the possibility that monoecious hemp cultivars likely have XX sex chromosomes.

To understand the evolution of sex determination in hemp, the sex chromosomes of genetically monoecious cultivars must be identified. There are currently no specific reports on the sex chromosomes and karyotype analysis using modern cytogenetics in monoecious hemp. This study aimed to identify the sex chromosomes of monoecious hemp by evaluating the karyotype of plants from distinct monoecious cultivars. To evaluate the sex chromosomes, we characterized the karyotype of eight monoecious hemp cultivars from different origins using DAPI/C-banding and FISH with the CS-1 subtelomeric DNA probe in mitotic cell preparations. This probe is highly useful for hemp karyotype analysis and for the sex chromosomes identification (Divashuk et al. 2014). The karyotyping of monoecious cultivars revealed a lack of Y chromosomes, and the results suggest an XX sex chromosome pattern with a chromosome number of 2n=20. Additionally, high levels of inter- and intracultivar karyotype polymorphisms were detected.

Materials and methods

Plant materials

The following dioecious hemp cultivars with different origins were used: dioecious cv 'Zenitsa', monoecious cv 'Maria', 'Kubanka' (P.P. Lukyanenko Krasnodar Research and the Development Institute of Agriculture, Krasnodar, Russia), dioecious cv 'Igorkin' and monoecious cv 'Gentus', 'Diana', 'Ingreda', 'Margo', 'Tzivilsky Skorospeliy' and 'Rigs' (Chuvashian Research and the Development Institute of Agriculture, Tsivilsk, Russia).

Mitotic chromosome preparation

Actively growing root tips approximately 1.5–2.0 cm long were harvested separately from at least nine young hemp seedlings of each cultivar. The harvested root tips were immediately pre-treated with a 2-mM aqueous solution of 8-hydroxyquinoline for 4 h at 20 °C. A 3:1 ethanol/glacial acetic acid (*v*/*v*) mix was used for fixation. Meristems 2 mm long were cut from the fixed root tips and digested in a 10-μl enzyme solution (0.5 % cellulase Onozuka R-10 (Serva, Germany) and 0.5 % pectolyase Y-23 (Seishin Corp., Japan)) in 10 mM citrate buffer (pH=4.9) for 1.5 h at 37 °C. The suspended cells were used for chromosome preparation as described by Henegariu et al. (2001) and Kato et al. (2004) with modifications by Kirov et al. (2014).

DNA isolation

The DNA isolation was performed as described by Doyle and Doyle (1990) with some modifications. The extracting buffer contained 100 mM Tris–HCl (pH=8.0), 20 mM EDTA (pH=8.0), 2 M NaCl, 1.5 % CTAB, 1.5 % PVP, and 0.2 % β-mercaptoethanol. A 15-mM ammonium acetate solution in 75 % ethanol was used for DNA washing.

PCR test for plant sex identification

A PCR test with the sex-associated molecular markers MADC2 (F: 5′GTGACGTAGGTAGAGTTGAA3′, R: 5′GTGACGTAGGCTATGAGAG3′) and SCAR323 (F: 5′GAGCGGACATCATTGCCT3′, R: 5′ATCACCCCACCGTTTAGG3′) was used for sex identification. The primers and protocol used were previously described (Mandolino et al. 1999; Torjek et al.

2002). The modified program for the MADC2 primers contained the following steps: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min and a final step of 72 °C for 5 min. The modified program for the SCAR332 primers contained the following steps: 94 °C for 2 min followed by 35 cycles of 94 °C for 10 s, 62.5 °C for 30 s, 72 °C for 1 min and a final step of 72 °C for 2 min. At least 10 seedlings from each of the monoecious cultivars were tested. In total, DNA of 210 monoecious plants was used.

Fluorescent in situ hybridization

To assist karyotype analysis, CS-1 probe (*C. sativa* subtelomeric repeat (JX402748) was made by nick translation protocol using Dig-Nick translation mix with the digoxigenin-11-dUTP according to the manufacturer's instruction (Boehringer, Germany) and used for fluorescence in situ hybridization (FISH). FISH experiments were performed as described by Divashuk et al. 2014 and Divashuk et al. 2011. The chromosomes were counterstained with 1 μg/ml DAPI and mounted in Vectashield (Vector Laboratories, UK). An AxioImager M1 fluorescent microscope (Zeiss) was used to observe the chromosome preparations. The metaphase plates with fluorescent signals were photographed with a monochrome AxioCam MRm CCD camera and visualized using Axiovision software (Zeiss).

Results

PCR analysis with the sex-specific DNA markers

The MADC2 primers (Mandolino et al. 1999) amplified the expected male-associated band of 390 bp in the male plants of the dioecious cultivars 'Igorkin' and 'Zenitsa'. In all female plants of these cultivars, the male-associated band was absent. Moreover, the male-associated band of 390 bp was absent from all of the plants belonging to eight monoecious hemp cultivars. The same results were obtained for the SCAR323 marker. The male plants had the expected male-associated band of 323 bp. All of the female plants belonging to monoecious hemp cultivars did not show the amplification of male-associated marker (Table 1). As an example, results of PCR analysis with the sex-specific DNA markers on seven plants of monoecious cultivar 'Maria' and on the male and female plants of the dioecious cultivar 'Igorkin' are presented in Fig. 1.

FISH and karyotype analysis

The karyotypes for male and female plants of dioecious cultivars were developed in the previous study by Divashuk et al. 2014. The FISH results using the CS-1 subtelomeric probe

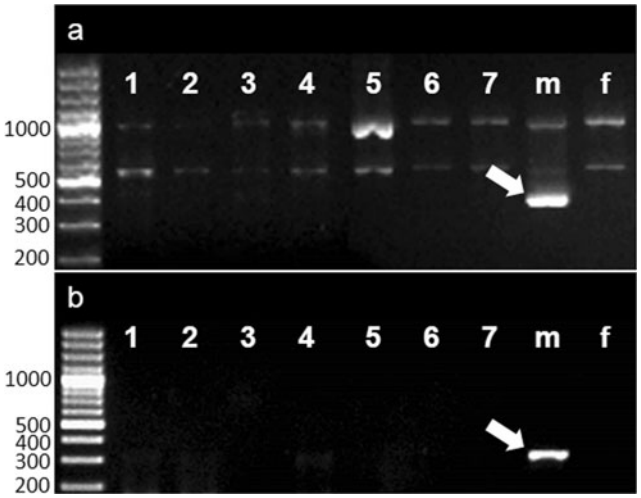

Fig. 1 PCR products obtained with the male associated DNA markers MADC2 (**a**) and SCAR 323 (**b**). *Lines 1–7*: individual plants of monoecious hemp cultivar 'Maria'. *m* male plant and *f* female plant of the dioecious hemp cultivar 'Igorkin'. The *arrows* indicate the male specific bands

and C-banding/DAPI can identify sex chromosomes in plants of *C. sativa*. The X is the largest chromosome of all and carries CS-1 subtelomeric repeats on both arms. The Y chromosome is larger than the autosomes and has a fully heterochromatic DAPI-positive arm and carries CS-1 subtelomeric repeats only on the euchromatic arm (Divashuk et al. 2014). Here, we used the CS-1 probe and DAPI/C-banding to study the karyotypes of individual plants of two dioecious and eight monoecious hemp cultivars. Six to nine plants were karyotyped from each cultivar. In total, 64 monoecious plants were analyzed. In the dioecious cultivars 'Zenitsa' and 'Igorkin', the chromosome number is 2n=20 for male and female plants. The Y chromosome was detected in all studied male plants, and the sex chromosome makeup was XY (Fig. 2a). All female plants of the dioecious cultivars had XX sex chromosomes (Fig. 2b). Our analysis of eight monoecious hemp cultivars failed to detect Y chromosomes in any studied plants, and all monoecious cultivars had XX chromosomes with a chromosome number of 2n=20 (Fig. 2 c–l).

The FISH analysis of metaphase chromosomes using the CS-1 probe in dioecious and monoecious hemp cultivars revealed a high level of inter- and intracultivar karyotype polymorphisms. The previously described hybridization sites for the CS-1 probe showed variations between individual plants. The polymorphism was attributed to the position of the CS-1 FISH probe on autosomes (chromosomes 2 and NOR-bearing chromosome 9). A comparison to standard karyotypes in some cultivars revealed that the CS-1 signal was not only subtelomeric but also in a pericentromeric position on chromosome 2 and chromosome 9 (Fig. 2). The pericentromeric signal on chromosome 2 was detected in one out of the six studied plants of cultivar 'Ingreda' only (Fig. 2d, indicated by asterisk). The pericentromeric and subtelomeric signals on

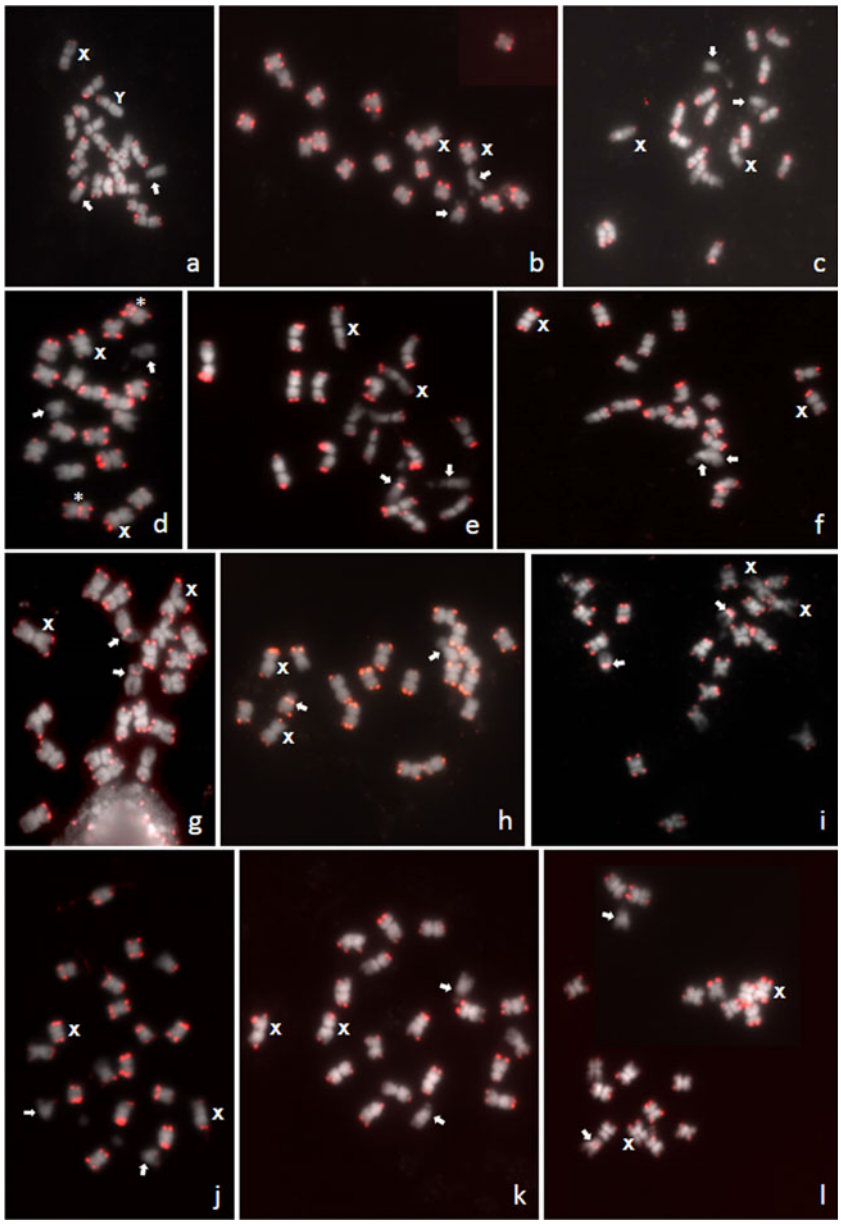

Fig. 2 Fluorescence in situ hybridization with CS-1 subtelomeric repeat (*red signal*) to metaphase chromosomes of male (**a**) and female (**b**) of dioecious cultivar 'Igorkin' and of monoecious cultivars 'Gentus' (**c**), 'Ingreda' (**d**–**f**), 'Diana' (**g**), 'Kubanka' (**h**), 'Margo' (**i**), 'Maria' (**j**), 'Tzivilsky Skorospeliy' (**k**), and 'Rigs' (**l**). Chromosome 9 is indicated by *arrows*, chromosome 2 by *asterisk*, and sex chromosomes by X and Y

chromosome 9 were present/absent in one or both homologous chromosomes, depending on the individual plants studied (Fig. 2a–l, chromosome 9 indicated by arrow). Karyotype analysis of dioecious and monoecious cultivars reveal in total ten cytotypes (Table 1). In monoecious 'Diana', 'Rigs', 'Kubanka' and 'Ingreda' at least three cytotypes were detected in each cultivar. In dioecious 'Igorkin' four cytotypes were revealed: two for males and two for females (Table 1).

Discussion

In hemp, the genetically monoecious plants evolved as a natural mutation that was selected for use in breeding programs (Moliterni et al. 2004). The male or female origin of maternal monoecious plant is unknown. In this study, the karyotype of monoecious hemp cultivars was studied using modern cytogenetics. The cytogenetic analysis of 64 plants from eight monoecious cultivars revealed a diploid nature (2n=20). No Y chromosomes were detected in any monoecious plants from the different cultivars studied. This result agrees with the data obtained by flow cytometry (Faux et al. 2014). These results showed there is the same amount of DNA per nuclei in diploid female plants and monoecious plants. The sex-specific DNA markers on monoecious plants in this study revealed female-specific patterns of amplification. These findings strongly support that monoecious hemp has the same sex chromosomes as the female plants of dioecious hemp, i.e., XX chromosomes.

Table 1 Cytotypes of dioecious and monoecious hemp cultivars revealed by FISH with use of the CS-1 subtelomeric repeat probe

Cultivar	Sex	Cyto-type	Sex chromo-somes	Male-specific PCR products	Chromosome 2	Chromosome 9 (NOR-bearing chromosome)
Igorkin	Male	1	XY	+		
		2	XY	+		
	Female	3	XX	–		
		4	XX	–		
Zenitsa	Male	5	XY	+		
	Female	6	XX	–		
Maria, Tzivilsky Skorospeliy	Monoecious	6	XX	–		
Gentus	Monoecious	3	XX	–		
Diana, Rigs, Kubanka	Monoecious	7	XX	–		
	Monoecious	8	XX	–		
	Monoecious	3	XX	–		
Margo	Monoecious	3	XX	–		
	Monoecious	4	XX	–		
	Monoecious	9	XX	–		
Ingreda	Monoecious	3	XX	–		
	Monoecious	7	XX	–		
	Monoecious	10	XX	–		

Additionally, the results confirm the cytological observations made in the 'Kentucky' cultivar by Menzel (1964), where an XX sex chromosome constitution was assumed.

FISH with subtelomeric CS-1 repeat detected many variations on the inter- and intracultivar levels. These variations were attributed to autosomes because the presence or absence of FISH signals for the CS-1 repeat was observed. In some cases, the presence of subtelomeric repeats in the pericentromeric position was detected. We did not find a correlation between chromosome polymorphisms and the manifestation of monoecy in

hemp. The chromosomal polymorphisms are often detected in different species and have no visible effects on plant morphology or sex expression (Badaeva et al. 1994; Grabowska-Joachimiak et al. 2015). The presence of subtelomeric DNA sequences at the pericentromeric position can be explained by chromosome reorganization caused by translocations, inversions, or transpositions. However, the chromosome polymorphisms suggest a dynamic karyotype and can explain the plasticity of this species. In *C. sativa*, a high degree of polymorphism has also been noted (Faeti et al. 1996; Forapani et al. 2001). Furthermore, hemp has a highly variable sexual phenotype. In a study by Faux et al. 2014, the authors demonstrate the expression in monoecious hemp varies quantitatively and significantly among cultivars. This variation can be attributed to the karyotype polymorphisms detected in our study.

A study by Schabelny (2010) included an analysis of hybrids between the dioecious female and monoecious (as a male parent) plants. The data showed that the generation of monoecy is a recessive feature. All F1 plants were dioecious females, and F1BC1 caused the segregation of dioecious female and monoecious plants. Based on the results of this study, it is difficult to establish if dioecy is localized on sex chromosomes or autosomes. The present study showed the absence of the MADC2 of SCAR323 markers in plants obtained from eight monoecious hemp cultivars. These results indicate the absence of large translocations of the Y chromosome to either the X chromosome or autosomes. Our cytogenetic analysis did not reveal such translocations. However, a small translocation from the pseudoautosome region of the Y chromosome onto the X chromosome or autosomes is possible. Alternatively, the monoecious state of genetically female plants (XX) can be determined by one or several mutated genes influencing sex expression through different mechanisms, such as changes in the level of endogenous plant hormones or changes in the sensitivity to hormones. The sexual reversal in dioecious plants and the bipotency of sexually predetermined plants through hormonal manipulation has been demonstrated in previous studies (Heslop-Harrison 1956; Chailakhyan and Khryanin 1978). Treatment with masculinizing or feminizing chemical agents leads to the formation of reproductive organs for the opposite sex. In addition, silver thiosulfate (inhibitor of ethylene) is commonly used in the marijuana industry to produce male flowers on genetically female plants (Hall et al. 2012).

In conclusion, the present study provides new insights and establishes fundamental information for further studies on the sex determination of monoecious hemp. The reversion of dioecious to monoecious plants make this plant an attractive model for use in sex evolution studies in *Cannabaceae*.

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Conflict of interest The authors declare that they have no conflict of interest.

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