GENOME SIZE IN *HUMULUS LUPULUS* L. AND *H. JAPONICUS* SIEBOLD & ZUCC. (CANNABACEAE)

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ABSTRACT

We analysed chromosome lengths, karyotype structure, and nuclear DNA content (flow cytometry) in diploid (2n=20) and triploid (2n=30) European *H. lupulus* var. *lupulus*, American *H. lupulus* var. *neomexicanus* (2n=20) and Japanese ornamental hop, *H. japonicus* (F/2n=16; M/2n=17). Diploid female representatives of *H. lupulus* var. *lupulus* and *H. l.* var. *neomexicanus* differed in total length of the basal chromosome set (23.16 µm and 25.99 µm, respectively) and nuclear 2C DNA amount (5.598 pg and 6.064 pg) but showed similar karyotype structure. No deviation from the additivity, both in chromosome length and 2C DNA amount was evidenced in triploid monoecious *H. lupulus* (2n=30, XXY). *H. japonicus* showed different karyotype structure, smaller basal chromosome set (F/18.04 µm, M/20.66 µm) and lower nuclear DNA amount (F/3.208 pg and M/3.522 pg). There are first evaluations of nuclear genome size in diploid, not commercial representative of European *H. lupulus* var. *lupulus* and American *H. lupulus* var. *neomexicanus* and first attempt to determine the absolute male and female genome size in two *Humulus* species.

KEY WORDS: *Humulus lupulus*, *H. japonicus*, flow cytometry, DNA amount, karyotype, sex chromosomes.

INTRODUCTION

The genus *Humulus*, one of two genera in the Cannabaceae family contains three species: *H. lupulus*, *H. japonicus* (=*H. scandens* (Lour.) Merr.) and *H. yunnanensis* (Small 1978). Both *H. lupulus* (European common hop) and *H. japonicus* (Japanese ornamental hop) are dioecious, climbing plants with sex chromosomes (Winge 1929) and sex determination system based on X/autosome balance (Parker and Clark 1991; Shepard et al. 2000). There are no reports on chromosome number and sex determination in *H. yunnanensis*.

H. lupulus, a perennial species important in the brewing industry is usually diploid with 2n=20. The simplest sex chromosome system described for hop plants of European origin is XX/XY, where males are heterogametic with the Y smaller than the X chromosome (Winge 1929; Parker 1990; Parker and Clark 1991; Shepard et al. 2000). In common hop, X/autosome ratio of 0.5 or less gives male phenotype, while a ratio of 1.0 gives rise to female plants. Sporadically, at the diploid and more frequently at the polyploid level monoecious plants can be observed (Haunold 1991). Triploids (2n=3x=27+XXY, X/A=0.67) are generally of male phenotype with only a few terminal female

inflorescence within male panicles (Parker and Clark 1991; Shepard et al. 1999, 2000). Occasionally, on such monoecious triploid plants bisexual flowers are formed (Shepard et al. 2000).

Small (1978) separated wild *H. lupulus* into five botanical varieties, each of them being, in author's opinion, geographically and morphologically distinct i.e. *H. lupulus* var. *cordifolius* (Japanese indigenous), *H. lupulus* var. *neomexicanus*, *H. lupulus* var. *lupuloides* (North American indigenous), *H. lupulus* var. *pubescens* (American Midwestern) and *H. lupulus* var. *lupulus* (European indigenous).

H. japonicus is an annual decorative vine with chromosome constitution 2n=14+XX for females and 2n=14+XY_1Y_2 for males, that remains the sex chromosome system of *Rumex acetosa* (Parker and Clark 1991; Shepard et al. 1999). The sex chromosomes in *H. japonicus* are the largest in the complement, and the Ys are only slightly smaller than the X (Parker 1990; Parker and Clark 1991). It is still an unresolved question whether different karyotype organization in two *Humulus* species resulted from different organization of similar genetic material (at least in respect of nuclear DNA amount) or from the other, more substantial changes on the genomic level (chromosome duplications/deletions, dysploid reduction, etc.).

In hops, especially in *H. lupulus*, the morphological differences between mitotic chromosomes are small, thus their exact identification and classification is practically not available using standard cytogenetic methods. The sex chromosomes in these plants were predominantly recognised during meiotic prophase or metaphase I (Ono 1955; Parker and Clark 1991; Shepard et al. 1999). However, the more precise karyotype structure of *H. lupulus* based on combined data from FISH signals and DAPI banding was presented recently by Karlov et al. (2003). The authors identified the Y chromosome as the shortest and deprived of fluorescent signals, whereas the X as middle sized with characteristic interstitial DAPI band on its short arm.

The absolute size (C-value) seems to be the other very important general character of a plant genome. C-value, as a 'key diversity character with many uses' (Bennett and Leitch 2005), shows not only an effect on many traits of organisms, but also on quality and utility of genetic fingerprints (Fay et al. 2005), commonly used by plant breeders for more detail germ-plasm characterization. Moreover, the knowledge of absolute genome size is essential for the detection of interspecific and intervarietal differences, polyploid/aneuploid identification and inter-laboratory comparisons of DNA data. Unfortunatelly, information about nuclear DNA amount in *Humulus* species and varieties are scant and inconsistent. Cytometric DNA measurements were performed only in some, mainly triploid *H. lupulus* cultivars for the flow cytometric identification of putative aneuploids (Šesek et al. 2000), in vitro induced tetraploids (Roy et al. 2001) or sexually derived polyploids (Beatson et al. 2003). Absolute 2C DNA values were published by Šesek et al. (2000), for triploid synthetics only, and recently by Zonneveld et al. (2005), most probably for diploid forms, although the chromosome numbers of analysed plants were not determined by the authors. The size of basal chromosome set (DNA content of a monoploid genome, 1Cx-value, according to Greilhuber et al. 2005) of *H. lupulus* calculated from the Sesek's data (about 2.3 pg) is much lower than that mentioned by Zonneveld et al. (2005) (two accessions, *H. l.* var. *cordifolia* – 2.9 pg and *H. l.* cv. Brambling cross – 3.1 pg). The reasons for these differences in DNA estimations remain unclear. They can result from the intraspecific 2C DNA polymorphisms, and/or from the deviation from the additivity in triploid forms analysed by Šesek et al. (2000). It is well known that polyploids can show different, unexpected changes (deviations from additivity) in nuclear DNA amount (Ohri 1998).

Nuclear DNA amount in *H. japonicus* was analysed only once, by Zonneveld et al. (2005). It turned out that nuclear 2C DNA amount in this species (3.4 pg) is considerably lower than in diploid representatives of *H. lupulus* (~6 pg). Unfortunatelly, chromosome number and sex of analysed plants were not mentioned in the paper.

The knowledge of genome sizes in *Humulus* should be helpful for recognition of existing differences between basal diploid taxa belonging to this interesting genus, especially between botanical and commercial varieties of *H. lupulus* and between *H. lupulus* and *H. japonicus*, two species differing by chromosome number (2n=20 and 2n=16/17) and sex chromosome system (XX/XY and XX/XY_1Y_2). To broaden the knowledge on this field we estimated the nuclear DNA content in two botanical varieties of *H. lupulus* (European var. *lupulus* and American var. *neomexicanus*), and Japanese ornamental hop, *H. japonicus*.

MATERIALS AND METHODS

Plant material

Humulus lupulus – plants used in this study, all derived from Institute of Soil Science and Plant Cultivation, Puławy, Poland, are listed bellow:

H. lupulus cv.'Lubelski' – Polish diploid cultivar, released in 1964 as a noble aroma hop, is cultivated in Poland to date on an area of about 800 ha. Was used for crossing for its aroma character; '3/19' – triploid monoecious plants, developed in Puławy as seedling selection of cross made in 1991 between the diploid cultivar Talisman and male genotype '5/23' derived from a cross of cv. 'Lubelski' and male hop from Jugoslavia. Was used for crossing as a male parent for its early maturity and high alpha acid content in pollen; 'B1' – male wild plants, collected in 1997 from natural population near Polanczyk, Bieszczady Mts., Poland. Was used for crossing for its early maturity and moderate resistance to hop downy mildew; *H. lupulus* var. *neomexicanus* A. Nelson & Cockerell – female plants obtained from Hop Institute in Żytomierz (Ukraine), in Puławy hop collection from 1994. Very late in maturity, therefore it has never been used for crossing in Poland;

Humulus japonicus – male and female plants were grown from purchased seeds (collected in Plant Breeding Enterprise 'W. Legutko', Poland).

The young plants were grown in flower-pots, in a greenhouse (Department of Plant Breeding and Seed Science, The Agricultural University of Cracow, Poland), then they were grown in flower-pots under open- air conditions.

Chromosome analysis

The root tips were collected, pretreated with ice-cold water for 24 h, and fixed in 3:1 absolute alcohol: glacial acetic acid for 3 h. After rinsing in distilled water, fixed root tips

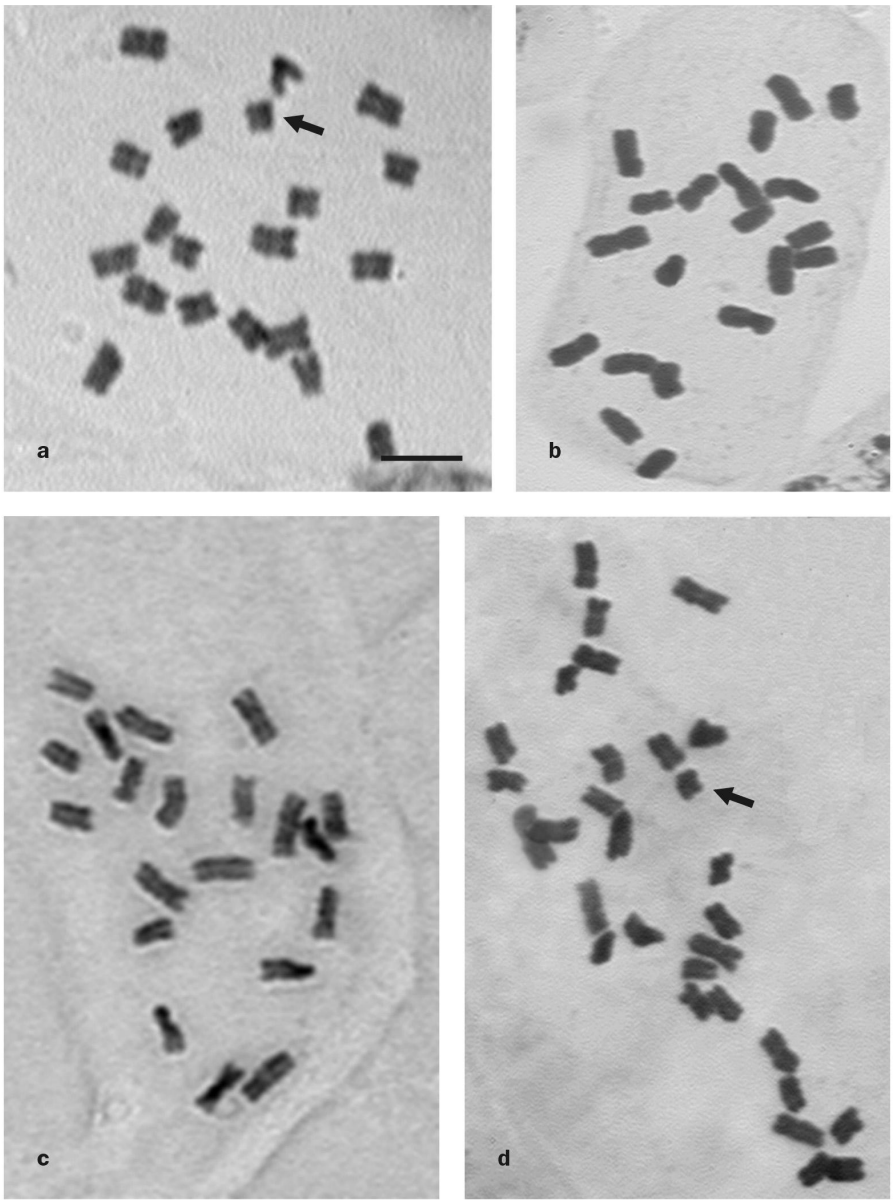

Fig. 1. Metaphase plates of *H. lupulus* var. *lupulus* (a, b, d), and *H. lupulus* var. *neomexicanus* (c); a – diploid male [B1]; b – diploid female (cv. *Lubelski*); c – diploid female; d – triploid monoecious plant [3/19]; Y chromosomes arrowed; bar – 5 µm.

were hydrolysed in 1 M HCL at 60˚C for 12 min., Feulgen-stained with pararosaniline (Sigma, St. Louis, Mo.) for 2 h at room temperature and then squashed in 45% acetic acid.

For chromosome measurements only well spreaded metaphase plates were selected. Finally, ten metaphase plates of *H. lupulus* cv. Lubelski, *H. lupulus* 'B1', *H. lupulus* var. *neomexicanus*, and *H. japonicus* (F/M) and five plates of triploid monoecious '3/19' plant were analysed. Chromosome images were captured, processed and measured using a CCD camera and Lucia G (Laboratory Imaging Ltd., Praha, Czech Republic) software.

DNA measurements

Young leaves were used for the flow cytometric analysis of DNA content estimation. Samples were prepared according to Galbraith et al. (1983), with some minor modifications. Plant tissue (of the plant in question and of the internal standard, simultaneously) was chopped with a sharp razor blade in a plastic Petri dish with 1 ml nucleus-isolation buffer (0.1 M Tris, 2.5 mM $MgCl_2 \cdot 6H_2O$, 85 mM NaCl, 0.1% v/v Triton X-100; pH 7.0), supplemented with propidium iodide (50 µg/ml) and ribonuclease A (50 µg/ml). After chopping, the suspension was passed through a 50 µm mesh nylon filter. For each sample, 5000-7000 nuclei were analysed using a Partec CCA (Münster, Germany) flow cytometer, equipped with an argon laser. For each entry 10 measurements of separate nucleus isolation from different plants were made. Histograms were analysed using a DPAC v.2.2 computer program. *Petunia hybrida* cv. P × Pc6 (2C=2.85 pg/nucleus; Marie and Brown 1993) was used as the internal standard for *H. lupulus* and *Zea mays* CEE-777 (2C=5.43 pg/nucleus; Lysák and Doležel 1998)

TABLE 1. Chromosome lengths of diploid female *H. lupulus* plants. Lub – cv. 'Lubelski', Neo – var. *neomexicanus*, x^F – basal female chromosome set (A+X), % – relative length of chromosomes calculated as a percent of x^F; bold – putative X chromosome.

	Lub		Neo	
	µm	%	µm	%
1	2.87±0.17	12.39	3.29±0.20	12.66
2	2.73±0.18	11.79	3.08±0.24	11.85
3	2.57±0.17	11.10	2.91±0.29	11.20
4	2.45±0.16	10.58	2.77±0.27	10.66
5	**2.34±0.11**	**10.10**	**2.64±0.24**	**10.16**
6	2.24±0.10	9.67	2.52±0.21	9.70
7	2.15±0.09	9.28	2.40±0.17	9.23
8	2.10±0.08	9.07	2.28±0.18	8.17
9	1.95±0.09	8.42	2.14±0.17	8.23
10	1.76±0.11	7.60	1.96±0.23	7.54
x^F	23.16±1.16	100	25.99±2.05	100

TABLE 2. Absolute (µm) and relative (%) chromosome lengths in diploid male *H. lupulus* plant from Bieszczady. x^F – basal female chromosome set (A+X), x^M – basal male chromosome set (A+Y); % – calculated as a percent of x^F; bold – putative X chromosome.

	Chromosomes	
	µm	%
1	2.94±0.11	12.51
2	2.79±0.11	11.88
3	2.63±0.16	11.20
4	2.48±0.13	10.56
5	**2.39±0.10**	**10.17**
6	2.28±0.17	9.71
7	2.20±0.17	9.36
8	2.09±0.16	8.90
9	1.94±0.20	8.26
10	1.75±0.20	7.45
Y	1.63±0.11	6.97
x^F	23.49±1.26	100
x^M	22.73±1.20	96.76

for *H. japonicus*. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions *Humulus*/internal standard, on the histogram of fluorescence intensities.

RESULTS

H. lupulus var. lupulus

By morphology, chromosomes of *H. lupulus* are hardly recognizable (Fig. 1). Because there are no clear differences in total length and/or arm ratio between the majority of adjoining chromosome pairs, chromosomes derived from each diploid metaphase plate analysed here were arranged by length, and 10 chromosome pairs (from the smallest to the largest) were distinguished arbitrarily. The average lengths of the chromosomes 1 – 10 are presented in the Tables 1 and 2.

Although *H. lupulus* is long recognized as the dioecious plant with sex chromosome system (Winge 1929; Jacobsen 1957; Westergaard 1958), its sex chromosomes are not easy to identify even in males, possessing heteromorphic sex chromosome pair. Especially the identification of X chromosomes seems to be very difficult because of the lack of the morphological difference between them and the medium-sized autosomes. Karlov et al. (2003) suggested that the X chromosome, although slightly longer, had substantially the same size and morphology as autosome 4. Thus, we identified 5th chromosome as the female sex chromosome. The single, shortest chromosome identified here both in diploid males and triploid monoecious plants was most probably the Y chromosome (Fig. 1a, d).

The absolute (in µm) and relative (calculated as the percent of total length of the basal female set, i.e. A+X) lengths of chromosomes 1 – 10 are nearly the same in analysed diploid plants (Tables 1 and 2). They show also similar nuclear DNA amount (females 5.598 pg, males 5.523 pg) (Table 3), and the observed difference (0.075 pg, i.e. 1.34%) probably resulted from the size difference between male and female sex chromosome. The detected length difference between male and female genome is 3.24% (Table 2), thus the difference on the diploid level (total karyotype length) is only 1.62%.

The measured total karyotype length of the triploid monoecious plant was 65.49 µm, i.e. about 1.5 fold of the karyotype length of diploid plants. Triploid nuclear DNA content was also about one and a half times higher than this of diploid (Table 3, Fig. 2).

H. lupulus var. neomexicanus

In comparison to the female plants of cv. 'Lubelski', the analysed female specimens of this botanical variety show higher nuclear DNA amount (6.064 pg) and slightly longer chromosome set (Tables 1 and 3). Despite of these differences, the relative chromosome lengths in two compared *H. lupulus* varietes (calculated as the percent of total length of the basal female chromosome set) were similar (Table 1).

TABLE 3. Chromosome numbers (2n) and nuclear 2C DNA amounts (pg) of analysed hops. 2n – somatic chromosome number, 2C – 2C DNA amount. Lub – cv. 'Lubelski', Bie – from Bieszczady, 3/19 – triploid monoecious plant, Neo – var. *neomexicanus*.

	Female		Male		Monoecious	
	2n	2C	2n	2C	2n	2C
H. lupulus						
Lub	20	5.598±0.044				
Bie			20	5.523±0.055		
3/19					30	8.956±0.114
Neo	20	6.064±0.048				
H. japonicus	16	3.208±0.028	17	3.522±0.117		

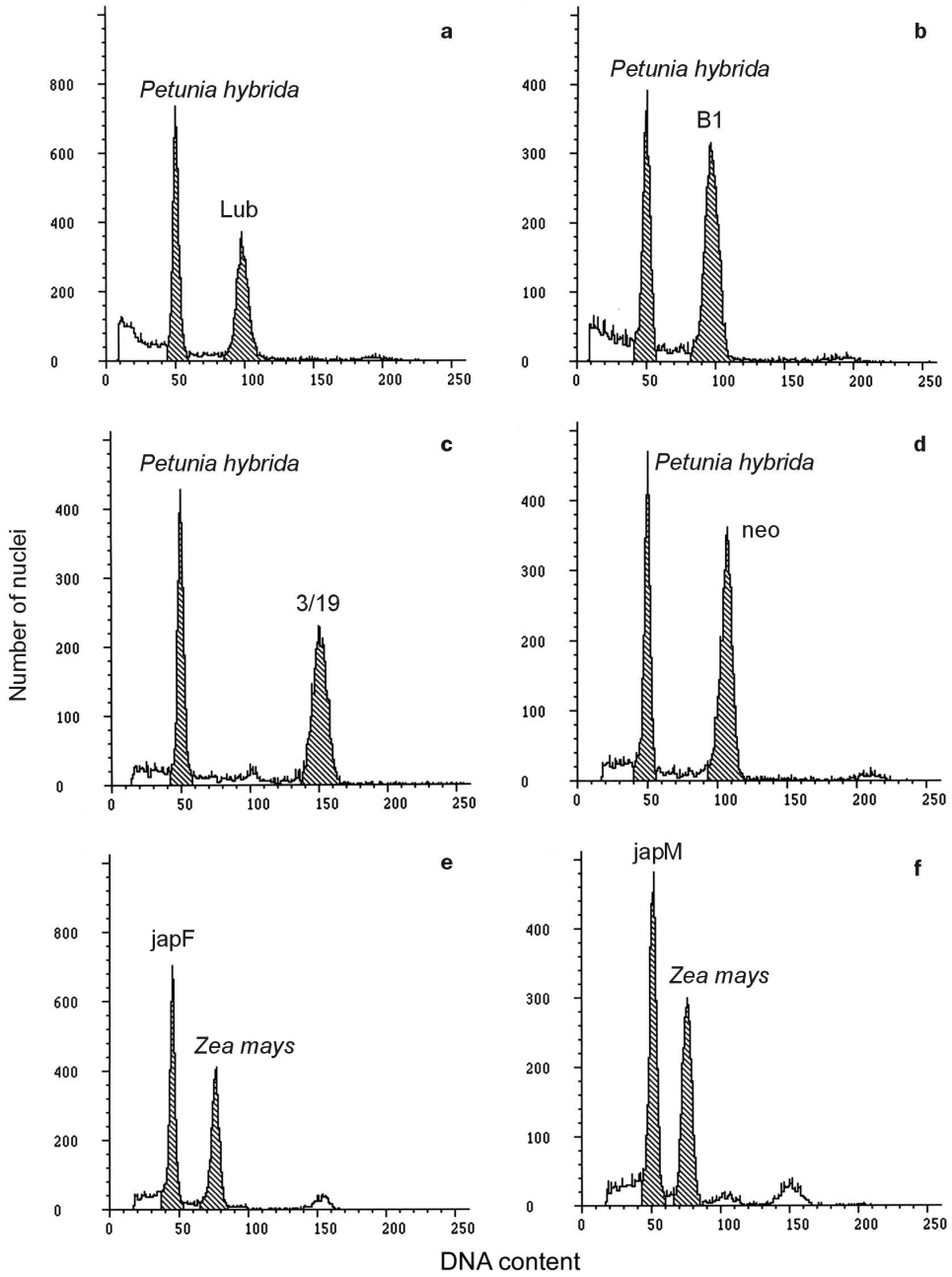

Fig. 2. DNA-histograms of nuclei isolated simultaneously from leaves of *Humulus* and internal standards; *H. lupulus* var. *lupulus* (a-c), *H. lupulus* var. *neomexicanus* (d), and *H. japonicus* (e, f); Lub – var. *Lubelski* (2n=20), B1 – male plant from Bieszczady (2n=20), 3/19 – triploid monoecious plant (2n=30), neo – var. *neomexicanus*, female plant (2n=20), japF – female plant (2n=16), japM – male plant (2n=17).

H. japonicus

H. japonicus shows a different somatic number of chromosomes (2n=16 in females and 2n=17 in males) (Fig. 3) and different sex chromosome system (XX/XY_1Y_2). The X chromosomes in this species are the largest in the complement and the Y chromosomes (Y_1 and Y_2) are larger than the largest autosome pair, however they are of similar size and shape to the X (Table 4). The ornamental hop differ from common hop not only by chromosome number but also by the genome size (Table 3, Fig. 2). Its basal female chromosome set contains less DNA (1.604 pg) than the set of *H. lupulus* var. *lupulus* (2.799 pg) and *H. lupulus* var. *neomexicanus* (3.032 pg) and is much shorter (18.04 µm) (Table 4). Because of the presence of two large Y chromosomes the basal male chromosome set in this species is much bigger than the female one. Detected length difference between basal male and female set (i.e. $A+Y_1Y_2$ and A+X) in this species is 14.52% (Table 4), thus the difference on the diploid level is 7.26%. The de-

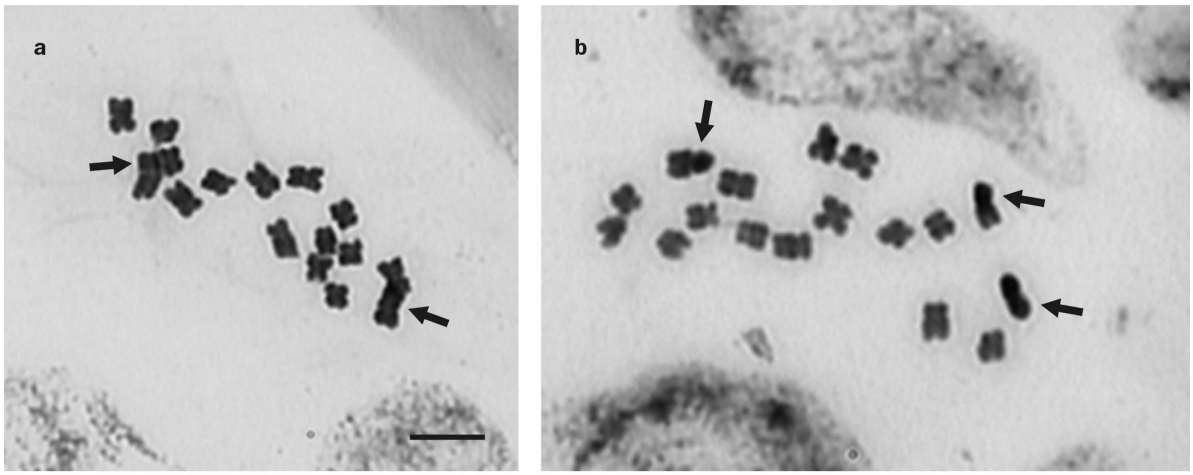

Fig. 3. Metaphase plates of *H. japonicus*; a – female (2n=16); b – male (2n=17); sex chromosomes arrowed; bar – 5 µm.

TABLE 4. Chromosome lengths of *H. japonicus*. x^F – basal female chromosome set (A+X), x^M – basal male chromosome set (A+Y_1Y_2), % – relative length of chromosomes calculated as a percent of x^F; bold – X chromosome.

	H. japonicus	
	µm	%
1	**3.11±0.29**	**17.24**
2	2.53±0.22	14.02
3	2.38±0.22	13.19
4	2.25±0.20	12.47
5	2.13±0.17	11.48
6	2.01±0.16	11.14
7	1.88±0.18	10.43
8	1.75±0.15	9.70
Y1	2.98±0.23	16.52
Y2	2.75±0.24	15.24
x^F	18.04±1.74	100
x^M	20.66±1.85	114.52

tected difference in 2C DNA content between male and female nuclei is even greater (9.79%) (Table 3).

DISCUSSION

The different sex chromosome system (XX/XY in *H. lupulus* and XX/XY_1Y_2 in *H. japonicus*), and the existing X/Y length differences make the direct comparisons of 2C DNA amounts in different hop lineages difficult. It seems, however, that the good indicator of the genome size in hops could be DNA amount in the single female chromosome set, A+X (Cx^F). The calculated Cx^Fs in diploid representatives of *H. lupulus* (2.799 pg in *H. lupulus* var. *lupulus* and 3.032 pg in *H. lupulus* var. *neomexicanus*) are considerably higher than in *H. japonicus* (1.604 pg). It suggests that the observed karyotypic difference between the two species (2n=2x=20 *vs.* 2n=2x=16/17) resulted not only from the different organization of the nuclear material.

Unfortunately, there are still no reports about nuclear genome of the third species belonging to this genus, *H. yunnanensis*. This controversial endemic form was recognized as a separate species by Small (1978) on the basis of the morphological analysis of herbarium specimens. The author suggested that this hop, although almost always mistaken for *H. lupulus*, is more closely related to *H. japonicus* than to *H. lupulus*. The chromosomal evaluation should clear this problem, but we were not able to obtain living *H. yunnanensis* plants for analysis.

Two of the analysed *H. lupulus* varieties (var. *lupulus* and var. *neomexicanus*) show a nearly identical karyotype composition (in respect to the relative lengths of particular chromosome types) and ~8% difference in the nuclear genome size (Table 1). It suggests a rather small, equal DNA change in all chromosomes, characteristic for spontaneous deletions/insertions or changes in the copy number of interspersed elements (Kubis et al. 1998; Hartl 2000; Kidwell 2002). Small differences in genome size between different botanical *H. lupulus* varieties can be also deduced from Zonneveld et al. (2005) data. The 1C DNA value (2.9 pg) estimated by authors for *H. lupulus* var. *cordifolia* is very close to *H. l.* var. *lupulus* (~2.8 pg) and *H. l.* var. *neomexicanus* (~3.0 pg).

It cannot be also excluded, that there are some differences in nuclear DNA amounts between different *H. l.* var. *lupulus* cultivars, because DNA value (Cx^F=2.8 pg) obtained for cv. Lubelski (our study) differ from value (Cx^F=3.1 pg) for cv. Bramblig cross (Zonneveld et al. 2005). All commercial plants are females, thus the observed inconsistence cannot result from sex difference. It was most probably due to the different internal standards (*Petunia hybrida vs. Agave americana*). On the other hand, *H. japonicus* 2C DNA values obtained by us (males ~3.5 pg, females ~3.2 pg) and by Zonneveld et al. (2005) (3.4 pg) are very similar despite of the use of different standards (*Zea mays vs. A. americana*).

Triploid monoecious *H. lupulus* (with karyotype formula 3A+XXY), shows about 9 pg DNA in G_1 nuclei, thus the size of basal genome of this plant is about 3.0 pg, close to estimated for diploid representatives of this species. It shows that the intraspecific triploidization in hop was not accompanied with the major changes in nuclear DNA content. Although it was recently suggested that nuclear DNA amount in majority of polyploid plants is rather less than expected (Levy and Feldman 2002; Leitch and Bennett

2004), both additive and non-additive effects on the 2C DNA level were reported for different polyploid plant taxa (Ohri 1998). Most probably the deviation from additivity depends on the difference between the parental genomes and the ploidy level (Albach and Greilhuber 2004). Thus, DNA values of autotriploids should be near the sum of the DNA values of their parents.

2C DNA value estimated by us (~9 pg) is much higher than that obtained by Šesek et al. (2000) for Slovenian triploid cultivars (~6.8 pg, i.e. about 2.3 pg per basal chromosome set). Such a large inconsistency was most probably due to the different internal standard (*Trifolium repens*) and fluorochrome used for DNA estimation. The fluorochrome used by Šesek et al. (2000) was 4',6-diamidino-2-phenylindole (DAPI), the dye showing base preference (AT), and in our study intercalating dye, propidium iodide (PI), was applied. It has been shown that the use of dyes showing base preference (e.g. DAPI) may lead to large errors in genome size estimation, thus the use of PI is recommended for flow cytometric determination of DNA amount in plants (Doležel et al. 1998; Johnston et al. 1999). Moreover, for calculation of DNA content in *H. lupulus* Šesek et al. (2000) used for internal standard the value estimated by Arumuganathan and Earle (1991), obtained with PI and not DAPI, which additionally increased the error in their genome size estimations.

It has been suggested that structurally distinct sex chromosomes have evolved from a pair of homologues (Charlesworth 1991; Liu et al. 2004). In many animal taxa Y chromosome is shorter than X and genetically degenerated (Charlesworth and Charlesworth 1998). However, in plants possessing well recognizable sex chromosomes, Y chromosome (or sum of Y chromosomes in XX/XY_1Y_2 system) is usually longer than the X (Parker 1990; Ainsworth 2000). For instance, in the model plant *Silene latifolia* and in its close relative, *S. dioica* (XX/XY sex chromosome system) the X and Y chromosome differ by factor ~1.4, both by length and DNA amount (Grabowska-Joachimiak and Joachimiak 2002). In this point, *H. lupulus* with much shorter Y chromosome (Table 2), is the only known exception to the rule. Our measurements of the mitotic chromosomes in males revealed X:Y size ratio approximated to 1.5, that is in agreement with the previous data estimated for X and Y chromosomes in male meiosis, reported by Shepard et al. (2000). The length difference between male and female chromosome complement in this species was only 1.62%. A very similar difference (1.71%) can be deduced from the data published by Karlov et al. (2003).

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