Genome size in liverworts

Velikost genomu játrovek

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Liverworts are poorly represented in the record of DNA C-values. Data for not more than nine species are reported in the literature. Here we present flow cytometric measurements of genome size for 32 foliose and 11 thallose species from 22 out of 83 families. The main method used in this study was flow cytometry using propidium iodide as the DNA stain. Feulgen densitometry was applied as a supplementary method but it proved less suitable because the rigid cell walls of liverwort tissue are resistant to maceration and apparently often inhibit the diffusion of reagents, which results in low estimates of DNA content. The precise or approximate number of chromosomes were counted, where possible. Among the thallose liverworts, the lowest 1C-value was recorded for *Marchantia polymorpha* (0.293 pg) and the highest for diploid *Pellia epiphylla* (7.401 pg). Haploid *P. epiphylla* (1C = 3.803 pg) had the largest genome among the haploid thalloid liverworts. Among the foliose liverworts, *Lejeunea cavifolia* with a value of 0.211 pg (1C) was ranked the lowest and *Mylia taylorii*, a haploid with 7.966 pg (1C) and a large amount of dense heterochromatin, concentrated in one big spherical chromocentre, the highest. This 38-fold variation covers the extremes of the whole sample and exceeds the ca 12-fold variation recorded in mosses (0.174–2.160 pg, 1C). This variation is nevertheless low compared to the 2000-fold interspecific variation found in angiosperms. Several instances of intraspecific variation in DNA ploidy (x and 2x) were found – in *Radula complanata*, *Pellia epiphylla* and *Metzgeria furcata*. In *Lophocolea heterophylla,* accessions differed 3.37-fold in C-value at haploid chromosome number. This points to cryptic taxonomic differentiation and warns against premature statements about ploidy levels based only on DNA measurements. Significant intraspecific intraploidal variation in C-value was also observed in certain instances. In *Frullania dilatata*, female plants with two large heterochromatic sex-chromosomes have a 1.35-fold higher C-value than male plants with only one sex chromosome. In most other cases of intraspecific variation the role of sex differences remains to be clarified.

Keywords: C-value, Cx-value, chromosome numbers, Feulgen densitometry, flow cytometry, genome size, liverworts

Introduction

The DNA content per holoploid genome (C-value; Greilhuber et al. 2005) varies approximately 2000-fold among species of land plants (*Embryophyta*). The record-holders at both ends of the scale are angiosperms. At the upper end of the scale is *Fritillaria assyriaca* (*Liliaceae*) with 127.4 pg or 124.6 Gbp (1C) (McLeish & LaCour 1971, pers. comm. in Bennett & Leitch 2005), a tetraploid with 2n = 48 chromosomes. At the lower end of the scale are some species of *Lentibulariaceae* with very small genomes, much below 100 Mbp, e.g. *G. aurea* with 0.065 pg or 63.5 Mbp (1C) (Greilhuber et al. 2006). This is less than half the size of the genome of *Arabidopsis thaliana,* which is 0.16 pg or

156.5 Mbp (1C) (Bennett et al. 2003), a species that for long time was considered to be the higher plant with the smallest reliably determined genome size. For other groups of land plants there is the lycophyte *Selaginella vogelii* with 0.086 pg (1C) and other *Selaginella* species with very small genomes (Little et al. 2007). As the example of the tetraploid *Fritillaria assyriaca* shows, polyploidy only plays a minor part in determining this huge variation in genome size. More important is the accumulation of retroposon-like and other repetitive elements (Bennetzen et al. 2005). The reasons and consequences for the organism of having a large or small genome are research topics of an expanding research discipline (Bennett 1998, Leitch & Bennett 2007). The nucleotype hypothesis has increased our understanding of the variation in genome size (Bennett 1971, 1972, 1998, Bennett et al. 1998, Gregory & Hebert 1999, Gregory 2001a, b, 2002a, b). It states that the phenotype of an organism is shaped not only by genotype (coding genes and regulatory elements) and environment, but also by the physico-mechanical properties of the cell nucleus (i.e. the nucleotype), in particular the quantity of DNA, via cell size and cell cycle duration (nucleotypically influenced parameters), which are adaptively significant. The rate of development of a plant is dependent on cell cycle duration, which can not exceed a certain time span, if complete development is to be achieved within a certain time frame. That is, the time available for development or certain developmental steps sets an upper limit, but not a lower limit to genome size. However, the causal connection between life style and genome size, which may seem obvious, has already been doubted. Trivers et al. (2004) found that selfers have lower C-values than outbreeders. Albach & Greilhuber (2004) report that in species of *Veronica* there are smaller genome sizes (C-values and Cx-values; Greilhuber et al. 2005) in annuals and selfers than in perennials and outbreeders. But only the correlation between small genome size and selfing was significant. The fact that the plant-based nucleotype hypothesis is also applicable to animals (Gregory 2005), in which selfing is of very limited importance, seems to confirm the explanatory power of life style, but does not exclude a role for breeding system in plants.

Genome size is of importance also in systematics. First, C-values are (within certain limits) constant within a species (Greilhuber 1998). Heterochromatin variation evidently contributes to variation in genome size, as in maize landraces (Laurie & Bennett 1985, Rayburn et al. 1985). Although it is not uncommon to find variation in the range of several percent (Šmarda & Bureš 2006, 2010, Šmarda et al. 2008) major differences between populations of a bona fide species point to some taxonomic differentiation (Greilhuber 1995, Murray 2005). *Picris hieracioides* with up to a 1.37-fold variation is an extreme example of intraspecific variation in genome size (Slovák et al. 2009). Second, in polyploid complexes, the C-values can be used to determine ploidy level (Suda et al. 2006, 2007, Ricca et al. 2008). In the genus *Sphagnum*, where chromosome counting is difficult, Cx-values vary little and C-values can provide definite clues to ploidy level (Temsch 2001, Greilhuber et al. 2003). Third, differences in genome size are also of interest at higher levels of taxonomy. According to Leitch et al. (2005), starting from small genomes, some angiosperm taxa, mostly among monocotyledons, evolved genome sizes much larger than the average for angiosperms as a whole, e.g. perennial bulbous or tuberous genera of *Liliaceae* (Leitch et al. 2007), *Alliaceae*, *Amaryllidaceae*, *Trilliaceae* and others. Large genomes occur much less frequently among eudicotyledons, with *Viscum* (*Santalaceae*) being an outlier as its genome is more than twice the size of the next largest eudicotyledon. Small genomes are characteristic also of primitive land plants, i.e. the bryophytes, of which

only the mosses have been studied in some depth (Renzaglia et al. 1995, Temsch et al. 1998, Voglmayr 2000, Košnar & Kolář 2009). Fourth, in molecular systematics knowledge of the size of the genome is often important for practical reasons, e.g. in genome sequencing (Bennett et al. 2003) and AFLP and microsatellite studies (Garner 2002, Fay et al. 2005).

After more than 50 years of genome size research, there are C-values for about 5150 plant species (Bennett & Leitch 2005), but they are not equally distributed between the different classes. There are C-values for 4427 species of angiosperm, 207 gymnosperm species, 87 pteridophyte species and 176 mosses (Bennett & Leitch 2005), but only nine liverworts (Ishida 1961a, Sparrow et al. 1972, Taylor et al. 1982, Renzaglia et al. 1995). The methods used to obtain these values are very different and the older values, in particular, require revision (Ishida 1961a, Sparrow et al. 1972). Here the gap in our knowledge of liverworts, which are considered to be sister to all other embryophytes, and thus the focus of interest in some molecular phylogenetic and genomic studies, is acknowledged (Goffinet et al. 2001, Shaw & Renzaglia 2004, Groth-Malonek et al. 2005, Mishler & Kelch 2008). Thus, the main objective of this study is to present a survey of genome sizes in liverworts. The results obtained using flow cytometry with propidium iodide as the DNA stain are compared in many instances with those obtained using Feulgen DNA image densitometry.

Material and methods

Plant material

Living plants were collected at the sites listed in Table 1. They were identified, wrapped in wet tissue and stored for up to one week at 4 °C until used for flow cytometry. From each sample material was selected and fixed in methanol:acetic acid (3:1) and herbarium vouchers (deposited in WU) prepared immediately on returning to the laboratory.

Thirty-two foliose and 11 thallose species from 22 liverwort families were included in this study (Table 2). The species selected belong to two of three classes of the Marchantiophyta (Crandall-Stotler et al. 2009). Family affiliation of the genera follows Crandall-Stotler et al. (2009). The use of the terms gametophyte and antheridiophore follow the definition in the Bryological glossary of TROPICOS – MOST (http://www.mobot.org/MOBOT/tropicos/most/Glossary/glosefr.html). The terms haploid and diploid refer to the generative ploidy levels, and haplophasic and diplophasic to the nuclear phases, characteristic of gametophyte and sporophyte, respectively (Greilhuber et al. 2005). DNA-ploidy (Suda et al. 2006) is used when chromosome numbers were not available and ploidy level is suggested from C-values.

Flow cytometry (FCM)

Young fresh gametophyte material was chopped (Galbraith et al. 1983) together with young leaves of the standard plant *Solanum pseudocapsicum* (1.295 pg DNA/1C) and exceptionally *Pisum sativum* 'Kleine Rheinländerin' (4.42 pg DNA/1C; Greilhuber & Ebert 1994, see also below) in Otto's I buffer (Otto et al. 1981, see Greilhuber et al. 2007). The resulting suspension was filtered through a 29 μm nylon mesh and double stranded RNA was digested with RNase (final concentration 0.15 mg/ml of nuclei suspension) at 37°C for 30 min in a water bath. Then it was stained with propidium iodide dissolved in

Table 1. – Origin of the material with locality code, Austrian province, district, village, transverse mercator (TM) projection square, and altitude above sea level.

Locality code	Austrian province	District	Village	TM-square	Altitude (m a.s.l.)
1	Burgenland	Oberwart	Oberwart	8662/4	400
2a	Lower Austria	Baden	Furth an der Triesting	8061/2	1000
2b	Lower Austria	Baden	Pfaffstätten	7963/3	300-400
3	Lower Austria	Bruck a.d. Leitha	Wolfsthal	7868/3	140-220
4	Lower Austria	Gänserndorf	Loimersdorf	7867/1	140
5a	Lower Austria	Lilienfeld	Kleinzell	8060/1	1000-1100
5b	Lower Austria	Lilienfeld	St. Ägyd am Neuwalde/Ahornboden	8159/3	700
5c	Lower Austria	Lilienfeld	St. Ägyd am Neuwalde/Lahnsattel	8259/1	940
6a	Lower Austria	Mödling	Kaltenleutgeben	7863/3	400-500
6b	Lower Austria	Mödling	Perchtoldsdorf	7863/3	400
6c	Lower Austria	Mödling	Wienerwald/Sittendorf	7962/2	400-440
6d	Lower Austria	Mödling	Wienerwald/Sulz im Wienerwald	7862/4	460
7	Lower Austria	Neunkirchen	Würflach	8262/1	430
8	Lower Austria	Scheibbs	Lunz am See	8156/3	770
9	Lower Austria	St. Pölten	Traismauer	7660/2	185
10a	Lower Austria	Wiener Neustadt	Muggendorf/Myrafälle	8061/4	500
10b	Lower Austria	Wiener Neustadt	Muggendorf/Unterberg	8061/3	700-900
10c	Lower Austria	Wiener Neustadt	Wiesmath	8463/2	600
11a	Lower Austria	Zwettl	Bärnkopf	7656/3	910
11b	Lower Austria	Zwettl	Sallingberg	7457/4	660
11c	Lower Austria	Zwettl	Schönbach	7556/1	660
12	Salzburg	Tamsweg	Muhr	8846/3	1900
13	Styria	Bruck a.d. Mur	Mariazell	8158/3	960
14a	Styria	Mürzzuschlag	Altenberg an der Rax	8359/2	1500
14b	Styria	Mürzzuschlag	Neuberg an der Mürz	8359/1	720-800
14c	Styria	Mürzzuschlag	Spital am Semmering	8360/3	800-1307
15	Styria	Weiz	Rettenegg	8460/4	900-1200
16	Tirol	Lienz	Tristach	9142/4	840
17a	Upper Austria	Braunau	Braunau	7744/1	350
17b	Upper Austria	Braunau	Munderfing	7945/1	550
17c	Upper Austria	Braunau	Neukirchen an der Enknach	7743/3	350
18a	Vienna		Vienna/Botanical Garden, University of Vienna	7874/1	170
18b	Vienna		Vienna/trading goods		
Outside Austria					
19	Germany	Bavaria	Rottal-Inn	7644/3	420
20	Tenerife		Chinobre hill near Chamorga		

Otto's buffer II for about one hour in a refrigerator (final concentration 50 µg/ml; Greilhuber et al. 2007). Measurements were done on a CyFlow ML flow cytometer (Partec, Münster, Germany) equipped with a green laser (100 mW, 532 nm, Cobolt Samba; Cobolt AB, Stockholm). 5000 particles were measured per run and three runs per isolate. Coefficients of variation (CV) of G0/G1 peaks were usually below 3% and only exceptionally higher than 5%. There were five instead of three runs on the samples with CVs above 3% .

Table 2. – Liverwort DNA 1C-values. Genera are listed alphabetically. Taxon (species or cytotype) mean 1C-values are presented only for flow-cytometric (FCM) data. They are given as megabase pairs (Mbp) and pg of DNA with standard deviation (S.D.). Individual accessions are given with locality code (see Table 1), chromosome number, method used (FCM, flow cytometry; FDM, Feulgen densitometry), 1C-values and their standard deviation in pg of DNA, number of runs (N) (with FCM), or slide pairs (with FDM) and the ratio of FDM to FCM values. The calculation of 1C-value in Mbp follows Doležel et al. (2003), i.e. 1 pg = 978 Mbp.

Taxon (family)	Taxon means (FCM)			Accessions						
	1C (Mbp)	1C (pg)	S.D. (pg)	Locality	Chromosome number[a]	Method	1C (pg)	S.D. (pg)	N	Ratio FDM/FCM
Apometzgeria pubescens (*Metzgeriaceae*)	557.3	0.570	0.001	14b		FCM	0.569	0.002	5	
				14b		FCM	0.571	0.001	3	
				14b		FDM	0.557	–	1	0.975
Barbilophozia floerkei (*Scapaniaceae*)	562.4	0.575		14c		FCM	0.575	0.002	3	
				14c		FDM	0.559	–	1	0.973
Barbilophozia lycopodioides (*Scapaniaceae*)	818.8	0.837		14c		FCM	0.837	0.004	3	
				14c		FDM	0.747	–	1	0.892
Bazzania trilobata (*Lepidoziaceae*)	928.9	0.950	0.032	10c		FCM	0.948	0.003	3	
				11b		FCM	0.983	0.005	4	
				17b		FCM	0.919	0.003	3	
				17b	n = x = 9	FDM	0.841	0.010	3	0.915
Blepharostoma trichophyllum (*Pseudolepicoleaceae*)	521.6	0.533	0.005	5c		FCM	0.534	0.002	5	
				5c		FCM	0.539	0.002	5	
				15		FCM	0.531	0.001	3	
				11c		FCM	0.529	0.003	5	
				11c		FDM	0.453	–	1	0.856
Calypogeia azurea (*Calypogeiaceae*)	739.9	0.757	0.003	13		FCM	0.755	0.005	3	
				14c		FCM	0.759	0.006	3	
				15		FDM	0.554	0.007	2	0.733
Calypogeia muelleriana (*Calypogeiaceae*)	747.7	0.765		14c		FCM	0.764	0.008	5	
				19	n = x = ca 9	FDM	0.496	0.035	3	0.649
Calypogeia neesiana (*Calypogeiaceae*)	463.0	0.473	0.005	5c		FCM	0.470	0.004	5	
				14c		FCM	0.471	0.001	3	
				15		FCM	0.479	0.002	3	
				15		FDM	0.255	0.002	2	0.532
Chiloscyphus pallescens (*Lophocoleaceae*)	797.1	0.815	0.004	10b		FCM	0.818	0.005	3	
				15		FCM	0.812	0.011	5	
				15	n = 2x = ca 15	FDM	0.664	0.045	2	0.818
Conocephalum conicum (*Conocephalaceae*)	672.0	0.687	0.031	14c		FCM	0.667	0.004	3	
				11c		FCM	0.722	0.013	2	
				11c		FCM	0.672	0.005	5	
				11c	n = x = 8+m[b]	FDM	0.663	0.008	3	0.986
Diplophyllum albicans (*Scapaniaceae*)	464.0	0.474		11c		FCM	0.474	0.000	3	
				11c	n = x = 9	FDM	0.459	0.025	3	0.968
Frullania dilatata (male) (*Frullaniaceae*)	540.5	0.553	0.007	3		FCM	0.548	0.004	5	
				10b	[c]	FCM	0.557	0.002	5	
Frullania dilatata (female) (*Frullaniaceae*)	732.2	0.749	0.002	6c		FCM	0.747	0.001	3	
				9		FCM	0.750	0.004	3	
				10b	[c]	FCM	0.749	0.002	3	

Taxon (family)	Taxon means (FCM)			Accessions						
	1C (Mbp)	1C (pg)	S.D. (pg)	Locality	Chromosome number[a]	Method	1C (pg)	S.D. (pg)	N	Ratio FDM/FCM
Jungermannia leiantha (*Jungermanniaceae*)	774.7	0.792		5c		FCM	0.792	0.007	4	
Lejeunea cavifolia (*Lejeuneaceae*)	206.2	0.211		11c		FCM	0.211	0.002	5	
				11c		FDM	0.198	0.011	3	0.941
Lepidozia reptans (*Lepidoziaceae*)	652.5	0.667	0.005	11c		FCM	0.661	0.003	3	
				17b		FCM	0.671	0.002	3	
				11b		FCM	0.670	0.000	3	
				11b		FDM	0.592	0.033	3	0.884
Lophocolea bidentata[d] (*Lophocoleaceae*)	448.2	0.458	0.0836	10b		FCM	0.500	0.005	3	
				14c		FCM	0.405	0.005	5	
				15		FCM	0.553	0.019	5	
				17b		FCM	0.375	0.004	3	
				17b		FDM	0.358	0.014	3	0.954
Lophocolea heterophylla (a)[e] (*Lophocoleaceae*)	239.8	0.242	0.006	9		FCM	0.246	0.004	5	
				14c		FCM	0.238	0.004	5	
Lophocolea heterophylla (b)[e] (*Lophocoleaceae*)	797.1	0.815	0.009	14b		FCM	0.808	0.007	3	
				17a		FCM	0.822	0.004	4	
				17a	n = x = ca 9	FDM	0.736	0.011	3	0.895
Lophozia incisa (*Scapaniaceae*)	1962.9	2.007	0.024	5c		FCM	2.024	0.004	3	
				15		FCM	1.990	0.002	3	
				15		FDM	1.968	0.048	3	0.989
Lophozia ventricosa (*Scapaniaceae*)	1505.4	1.539	0.014	15		FCM	1.549	0.002	3	
				15		FCM	1.529	0.004	3	
				15	n = x = ca 9	FDM	1.367	0.036	3	0.894
Lunularia cruciata (*Lunulariaceae*)	651.5	0.666	0.006	17a		FCM	0.670	0.011	5	
				18a		FCM	0.662	0.005	5	
				18a	n = x = 8+m	FDM	0.679	0.009	3	1.019
Marchantia polymorpha (*Marchantiaceae*)	286.7	0.293	0.033[f]	14c		FCM	0.316[f]	0.005	5	
				14c		FDM	0.259	0.021	3	0.819
				18a		FCM	0.270[f]	0.004	5	
				18a		FDM	0.264	0.007	4	0.976
Metzgeria conjugata (*Metzgeriaceae*)	1439.3	1.472	0.063	8		FCM	1.533	0.036	3	
				14c		FCM	1.408	0.023	5	
				14b		FCM	1.475	0.009	3	
				14b		FDM	1.536	0.062	3	1.041
Metzgeria furcata (x?) (*Metzgeriaceae*)	544.8	0.557	0.006	5c		FCM	0.562	0.009	5	
				10b		FCM	0.562	0.004	5	
				17c		FCM	0.556	0.001	3	
				1		FCM	0.549	0.009	5	
				1		FDM	0.514	–	1	0.936
Metzgeria furcata (2x?)[g] (*Metzgeriaceae*)	987.3	1.010	0.006	6c		FCM	1.005	0.010	3	
				6c		FCM	1.014	0.026	4	
Monosolenium tenerum (*Monosoleniaceae*)	356.4	0.365		18b		FCM	0.364	0.008	5	
				18b	n = x = ca 9	FDM	0.328	0.005	3	0.901

Taxon (family)	Taxon means (FCM)			Accessions						
	1C (Mbp)	1C (pg)	S.D. (pg)	Locality	Chromosome number[a]	Method	1C (pg)	S.D. (pg)	N	Ratio FDM/FCM
Mylia taylorii (*Myliaceae*)	7790.7	7.966	0.029	5c		FCM	7.987	0.022	3	
				5c		FCM	7.945	0.060	3	
				5c	n = x = 9	FDM	5.040	–	1	0.634
				12		FDM	6.479	0.214	5	
Nardia scalaris (*Jungermanniaceae*)	388.6	0.397	0.003	14c		FCM	0.399	0.002	3	
				14c		FCM	0.394	0.002	3	
				15		FCM	0.400	0.001	3	
				15		FDM	0.263	–	1	0.657
Nowellia curvifolia (*Cephaloziaceae*)	212.6	0.217		5c		FCM	0.217	0.001	4	
				5c		FDM	0.212	–	1	0.974
Pellia borealis (*Pelliaceae*)	7238.3	7.401	0.434	11a		FCM	7.862	0.104	3	
				11b		FCM	7.000	0.052	3	
				11b		FCM	7.342	0.058	3	
Pellia endiviifolia (*Pelliaceae*)	3364.0	3.440	0.104	5b		FCM	3.575	0.022	3	
				5b		FCM	3.308	0.015	3	
				14a		FCM	3.490	0.005	3	
				14c	[h]	FCM	3.454	0.085	3	
				17c		FCM	3.372	0.005	3	
Pellia epiphylla (*Pelliaceae*)	3719.2	3.803	0.539	10a		FCM	3.333	0.076	4	
				5a		FCM	3.468	0.024	4	
				17b		FCM	3.876	0.037	3	
				17b		FDM	3.686	0.062	3	0.951
				11c		FCM	4.534	0.008	3	
Plagiochila asplenioides (*Plagiochilaceae*)	1640.9	1.678	0.082	5b		FCM	1.775	0.008	3	
				7		FCM	1.553	0.003	3	
				11b		FCM	1.732	0.019	5	
				11c		FCM	1.652	0.003	3	
				14c		FCM	1.728	0.023	3	
				17b		FCM	1.627	0.003	3	
				17b	n = x = 8+m	FDM	1.326	0.004	3	0.815
Plagiochila porelloides (*Plagiochilaceae*)	1536.6	1.571	0.083	10c		FCM	1.512	0.002	5	
				17c		FCM	1.630	0.007	3	
				17c		FDM	1.604	0.067	2	0.984
Porella cf. *platyphylla* (*Porellaceae*)	1295.6	1.325		20		FCM	1.325	0.002	3	
Porella platyphylla (*Porellaceae*)	1062.6	1.087	0.036	2b		FCM	1.075	0.005	3	
				3		FCM	1.065	0.006	5	
				6c		FCM	1.070	0.003	3	
				7		FCM	1.151	0.005	5	
				6a		FCM	1.073	0.002	3	
				6a	n = x = ca 9	FDM	0.984	0.024	3	0.918
				6b	n = x = ca 9	FDM	1.046	0.005	3	
Preissia quadrata (*Marchantiaceae*)	828.7	0.847		17a		FCM	0.847	0.013	3	
				17a	n = x = ca 7–9	FDM	0.802	0.038	3	0.947
Ptilidium ciliare (*Ptilidiaceae*)	1146.3	1.172		15		FCM	1.172	0.004	3	
				15		FDM	0.948	0.041	3	0.809

Taxon (family)	Taxon means (FCM)			Accessions						
	1C (Mbp)	1C (pg)	S.D. (pg)	Locality	Chromosome number[a]	Method	1C (pg)	S.D. (pg)	N	Ratio FDM/FCM
Ptilidium pulcherrimum (*Ptilidiaceae*)	1233.8	1.262	0.014	5c		FCM	1.268	0.003	3	
				13		FCM	1.245	0.002	3	
				15		FCM	1.272	0.005	3	
				15	n = x = ca 9	FDM	1.201	0.055	3	0.944
Radula complanata (x)[i] (*Radulaceae*)	352.8	0.361	0.005	2b		FCM	0.366	0.004	5	
				2b		FCM	0.363	0.002	3	
				3		FCM	0.352	0.008	6	
				6a		FCM	0.362	0.003	3	
				6c		FCM	0.360	0.002	4	
				9		FCM	0.366	0.002	3	
				2a	n = x = 8	FCM	0.357	0.001	5	
				2a	n = x = 7–8	FDM	0.325	0.006	3	0.900
Radula complanata (2x)[i] (*Radulaceae*)	740.5	0.757	0.006	1	n = 2x = ca 16	FCM	0.763	0.007	5	
				5c		FCM	0.754	0.007	3	
				6c		FCM	0.764	0.002	4	
				6d		FCM	0.761	0.003	3	
				10b		FCM	0.751	0.002	3	
				14c		FCM	0.751	0.003	3	
Radula complanata s.l. (*Radulaceae*)	730.3	0.747		16	[j]	FCM	0.747	0.011	5	
Riccia fluitans s.l.[k] (*Ricciaceae*)	1086.5	1.111	0.084	18a		FCM	1.052	0.006	3	
				4		FCM	1.170	0.009	3	
				4	n = 2x = 16	FDM	1.129	0.023	11	0.965
Scapania aequiloba (*Scapaniaceae*)	361.0	0.369		14c		FCM	0.369	0.001	3	
Scapania cf. *irrigua* (*Scapaniaceae*)	277.7	0.284		11c		FCM	0.284	0.004	5	
Scapania nemorea (*Scapaniaceae*)	310.9	0.318	0.039	14c		FCM	0.291	0.002	5	
				11c		FCM	0.345	0.003	5	
				11c		FDM	0.353	0.019	3	1.021
Trichocolea tomentella (*Trichocoleaceae*)	1851.6	1.893		5c		FCM	1.893	0.013	3	
				5c	n = x = ca 9	FDM	1.923	0.016	3	1.016
Tritomaria quinquedentata (*Scapaniaceae*)	1169.2	1.196		11c		FCM	1.196	0.003	5	

[a] m = microchromosome

[b] *Conocephalum conicum*: first count for Austria. Fritsch (1991) reports chromosome counts ranging from n = 8 to n = 9+m; there is no sex chromosome heteromorphism in this species (Tatuno 1957).

[c] *Frullania dilatata*: male individuals have n=8 chromosomes with one large heterochromatic chromosome, of which females have two (n = 9). Lorbeer (1934) records a female:male chromosome volume ratio of 1:1.57, which is close to the 1:1.355 DNA content ratio found in a joint preparation of male and female individuals (Fig. 3D).

[d] *Lophocolea bidentata*: the data obtained from four accessions fell into two distinct groups with a 1.35-fold difference. Whether this is due to differences in ploidy is unclear and further work is necessary.

[e] *Lophocolea heterophylla*: exhibits two contrasting cytotypes ('a' and 'b') with 3.37-fold variation in C-value. This large difference points to a taxonomical differentiation that clearly needs to be clarified.

[f] *Marchantia polymorpha*: the reason for the 1.17-fold difference between the two locations is unclear. Fluorescence vs scatter light scattergrams were particularly strongly right hand distorted indicating 'debris coatings' on the nuclear surface (Loureiro et al. 2007).

[g] *Metzgeria furcata*: a DNA diploid cytotype deviating from the only known haploid level (Fritsch 1991) was found in one accession of *M. furcata* with a 1.813-fold greater C-value compared to the haploid samples.
[h] *Pellia endiviifolia*: although it was not possible to count the chromosomes, its C-value compared with that of *P. epiphylla* DNA-haploid indicates it is haploid (compare Kuta & Ochyra 1988).
[i] *Radula complanata*: the C-value ratio of 1:2.12 deviated slightly from the expected 1:2 ratio indicating a certain amount of genomic differentiation between the two ploidy levels (see Fig. 3A).
[j] *Radula complanata* s.l.: this sample, initially identified as *R.* cf. *lindenbergiana*, was found to be DNA-diploid compared with *R. complanata* although the chromosome numbers listed in Fritsch (1991) are n = x = 8. Here, we list the sample as *R. complanata* s.l. pending further clarification.
[k] *Riccia fluitans* s.l.: this sample is probably conspecific with *R. duplex*.

To calculate the 1C-value of the samples, the ratio of sample/standard G1-peaks was multiplied by the 2C-value of the standard (NB: bryophyte gametophytes are haplophasic, whereas angiosperm sporophytes are diplophasic).

Standard

The primary standard *Solanum pseudocapsicum* was calibrated against the secondary standards *Zea mays* 'CE-777' (2.59 pg DNA/1C), *Hordeum vulgare* 'Ditta' (4.83 pg DNA/1C), *Pisum sativum* 'Kleine Rheinländerin' (4.42 pg DNA/1C) and *Raphanus sativus* 'Saxa' (0.53 pg DNA/1C) and calculated according to Yokoya et al. (2000). The C-values of the secondary standard species were taken from Vilhar et al. (2001). The 1C-value of 1.295 pg DNA for *S. pseudocapsicum* is very similar to the Feulgen densitometry derived value of 1.35 pg DNA/1C given by Bennett & Smith (1991) for this species.

Feulgen densitometry (FDM)

The procedure followed closely the recommendations given in Greilhuber & Temsch (2001) with essential steps being the hydrolysis of the 3:1-fixed material for 60 min in 5N HCl at 20.0°C, staining and squashing, simultaneously for both the standard and liverwort species. Unlike angiosperm meristems, stem tips of liverwort are not macerated during hydrolysis and spreads cannot be made in the same way. Technical problems caused by the rigid nature of the cell walls, even of the youngest cells, are discussed below.

In several instances the amount of DNA in herbarium specimens was measured. Tips of branches were incubated for 90 min in 4% phosphate buffered formaldehyde (pH 7) together with radicles obtained from dry mature seeds of *Pisum sativum* ´Kleine Rheinländerin´. After a thorough wash in acetic methanol and finally in distilled water, the Feulgen reaction was conducted according to Greilhuber & Temsch (2001).

Liverwort and standard cells were squashed onto one slide. Liverwort nuclei suitable for measurement were present in variable numbers. The preference was to measure mitotic nuclei, if present. More nuclei of the standard, preferably in late telophase or early prophase, were measured than of the liverworts.

For measuring the integrated optical density (IOD) of the nuclei, the Cell Image and REtrieval System (CIRES; Kontron, Munich, Germany) was used (e.g. Vilhar et al. 2001). A 1C-value for the liverwort species was calculated for each slide from the IOD ratio liverwort/standard and the known standard 2C-value. Further details are given in Table 2.

Results and discussion

Establishment of the Solanum pseudocapsicum standard

Solanum pseudocapsicum is not a commonly used standard, but proved useful because of its narrow CVs, continuous availability and suitable genome size for analyzing the range of genome sizes encountered in liverworts. It has 2n = 24 chromosomes.

Four established secondary standard species were used to estimate the *S. pseudocapsicum* C-value. The 1C-values (pg) of these were averages taken from the data sets in the densitometric study by Vilhar et al. (2001) in which *Zea mays*, *Hordeum vulgare* and *Raphanus sativus* were calibrated against *Pisum sativum*, which is estimated to possess 1C = 4.42 pg DNA by Greilhuber & Ebert (1994). According to the regression based approach of Yokoya et al. (2000), the 1C-value for *S. pseudocapsicum* is 1.295 pg (Table 3).

Table 3. – The secondary standards used to determine the C-value of the internal standard *Solanum pseudocapsicum*, their averaged 1C-values (after Vilhar et al. 2001) and the G1 peak ratios of the standards vs *S. pseudocapsicum*. For explanation see Material and methods. Regression equation: y = 1.2781x+0.0165 = 1.2946 (R^2 = 0.99)

Secondary standards	1C-values (DNA pg)	Ratios vs *Solanum pseudocapsicum*
Zea mays 'CE-777'	2.590	1.997
Hordeum vulgare 'Ditta'	4.833	3.827
Pisum sativum 'Kleine Rheinländerin'	4.420	3.388
Raphanus sativus 'Saxa'	0.525	0.414

FCM and FDM measurements of DNA content

Forty-three liverwort species were evaluated, seven using only FCM and 36 by both methods (Table 2). Often both methods gave similar values (r^2 = 0.94), but there were exceptions, some of which were alarming (Table 2). The genome sizes estimated using FDM were on average 14% less than those obtained using FCM. The species with the largest deviation between the results obtained using the two methods were *Nardia scalaris* (34% less), *Calypogeia azurea*, *C. muelleriana* and *C. neesiana* (27, 35 and 47%) and *Mylia taylorii* (37%). In 10 further species the FDM values were 20 to 10% less than the FCM values. Remarkable is *Marchantia polymorpha*, for which the FCM gave values for two different accessions that differed by 1.17-fold, but the analysis of one accession (loc. 18a) using developing antheridia and both FCM and FDM, gave similar results.

The correlation of C-value with the deviation between the FDM and FCM measurement was not significant (r^2 = 0.054). Therefore, the size of the genome has no influence on the discrepancy in the measurements obtained using the two methods.

Liverworts have rigid cell walls and crystalline deposits on the surface of the thallus and even the very youngest parts of a plant are barely suitable for such measurements using FDM. Young antheridia are more suitable but are only occasionally found. It seems that the cell walls are a barrier to chemical reagents, so that in differentiated cells, the nuclei are often poorly stained and practically invisible, especially when the size of the genome is small. All this indicates that Feulgen-based data for liverworts must be interpreted with the utmost care and it is best that they are only considered in parallel with FCM measurements, which are unaffected by mechanical barriers, because in this case the nuclei are stained after isolation.

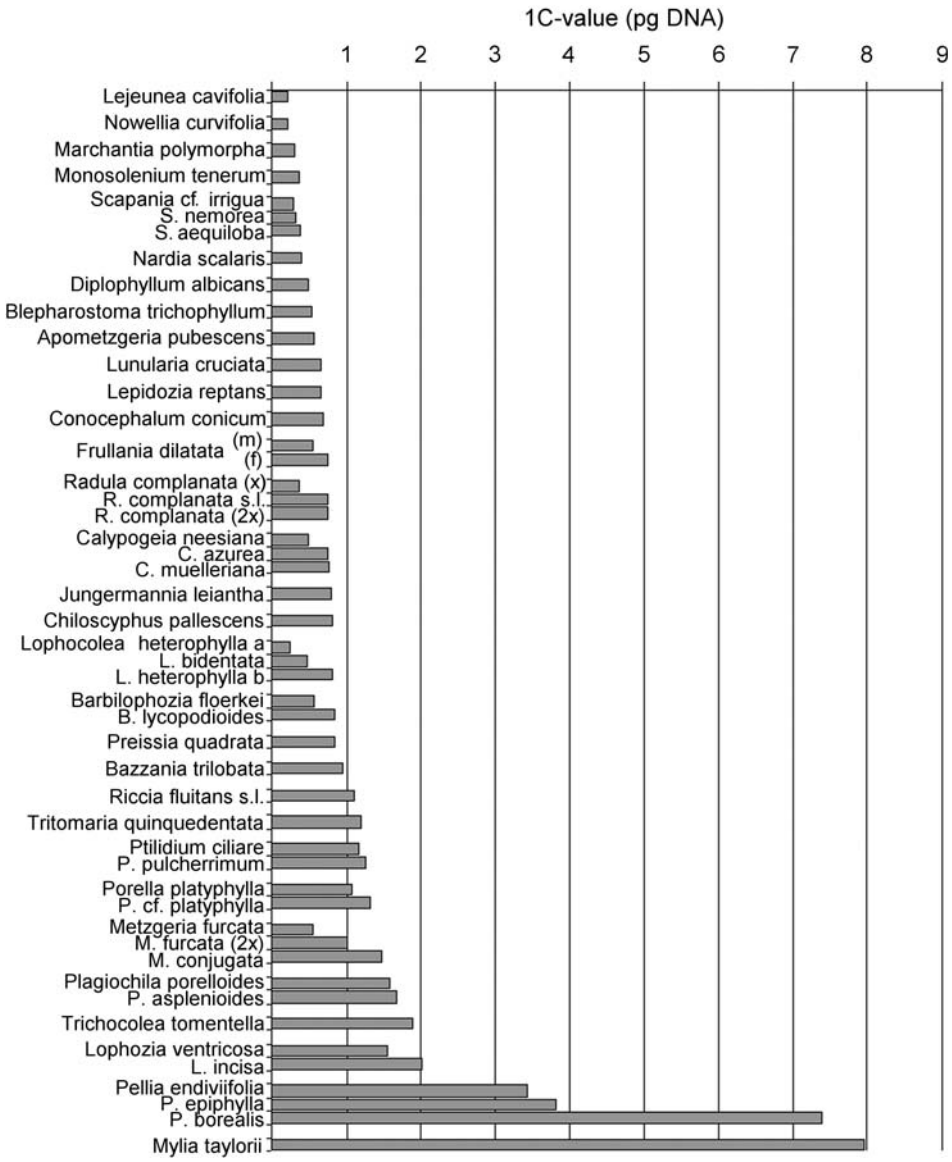

Fig. 1. – Mean 1C-values for 32 foliose and 11 thallose species of liverworts, determined by propidium iodide flow cytometry, ordered according to the largest C-value in the genus.

Flow cytometric measurements

The results are presented in Table 2, and include values obtained using both FCM and FDM and chromosome counts or ploidy levels when chromosome numbers could not be precisely established. The FCM derived measurements are regarded as valid and the

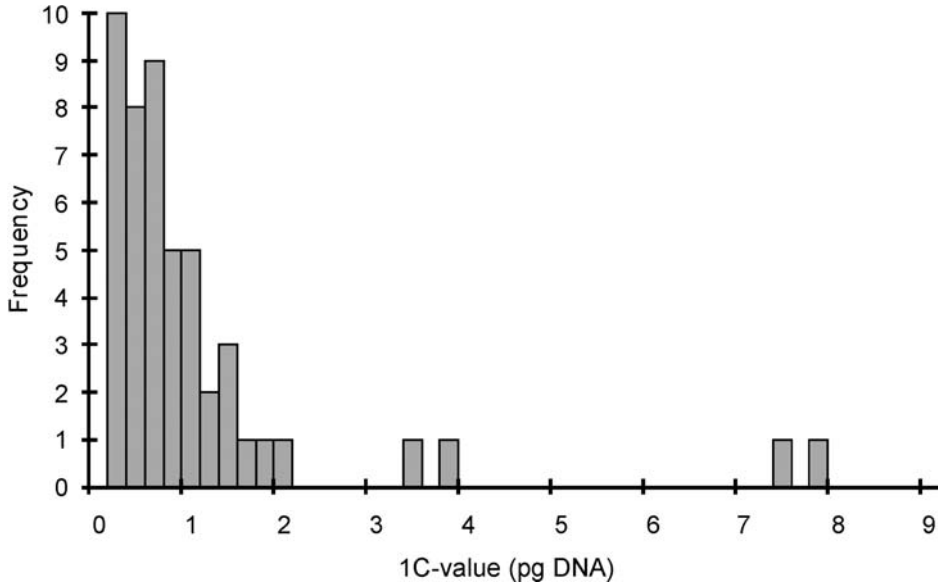

Fig. 2. – The frequency distribution of mean 1C-values (determined by flow cytometry) of the species or cytotypes within species of 32 foliose and 11 thallose liverworts. Class width is 0.2 pg.

Feulgen-based data as secondary information. The distribution of the 1C-values ordered by size and frequency distribution of all the genome sizes are shown in Figs. 1 and 2, respectively. Remarks pertaining to the species are listed as footnotes to Table 2, representative FCM histograms are shown in Fig. 3.

Comparison of the results obtained using flow cytometry and Feulgen densitometry

The data presented show that FCM is more suitable than FDM for measuring the DNA content of liverworts. The essential advantage of FCM is that isolated nuclei are stained, while the cell walls of even the youngest cells can act as barriers, with an unpredictable effect on staining, when FDM is used. So FDM on its own is not recommended for measuring the DNA content of plants belonging to this plant group. In contrast, there are many examples that show that thin cell walls, typical of higher plant meristems, do not affect stoichiometric Feulgen staining (e.g. Doležel et al. 1998). In *Sphagnum*, FDM and FCM yield very similar results (Temsch et al. 1999, Melosik et al. 2005) and in mosses there is a good correlation between the results obtained using both methods (Voglmayr & Greilhuber 1999).

Nevertheless, a major benefit of Feulgen stained slides is that they also provide karyological information. If chromosomes are visible, their number can be counted or the level of ploidy estimated. The frequency of replicated nuclei can be checked, which may be important for the interpretation of flow cytometric peaks, because a high frequency of nuclei in G2 may lead to misinterpretations if only FCM is used. For instance, a very low proportion of nuclei in G1 is typical for *Physcomitrella patens* gametophytes (Schween et al. 2003). The amount of heterochromatin present can also be assessed using FDM. This is important because heterochromatin consists of highly repeated sequences, which are evolutionarily unstable components of a genome with less systematic significance at

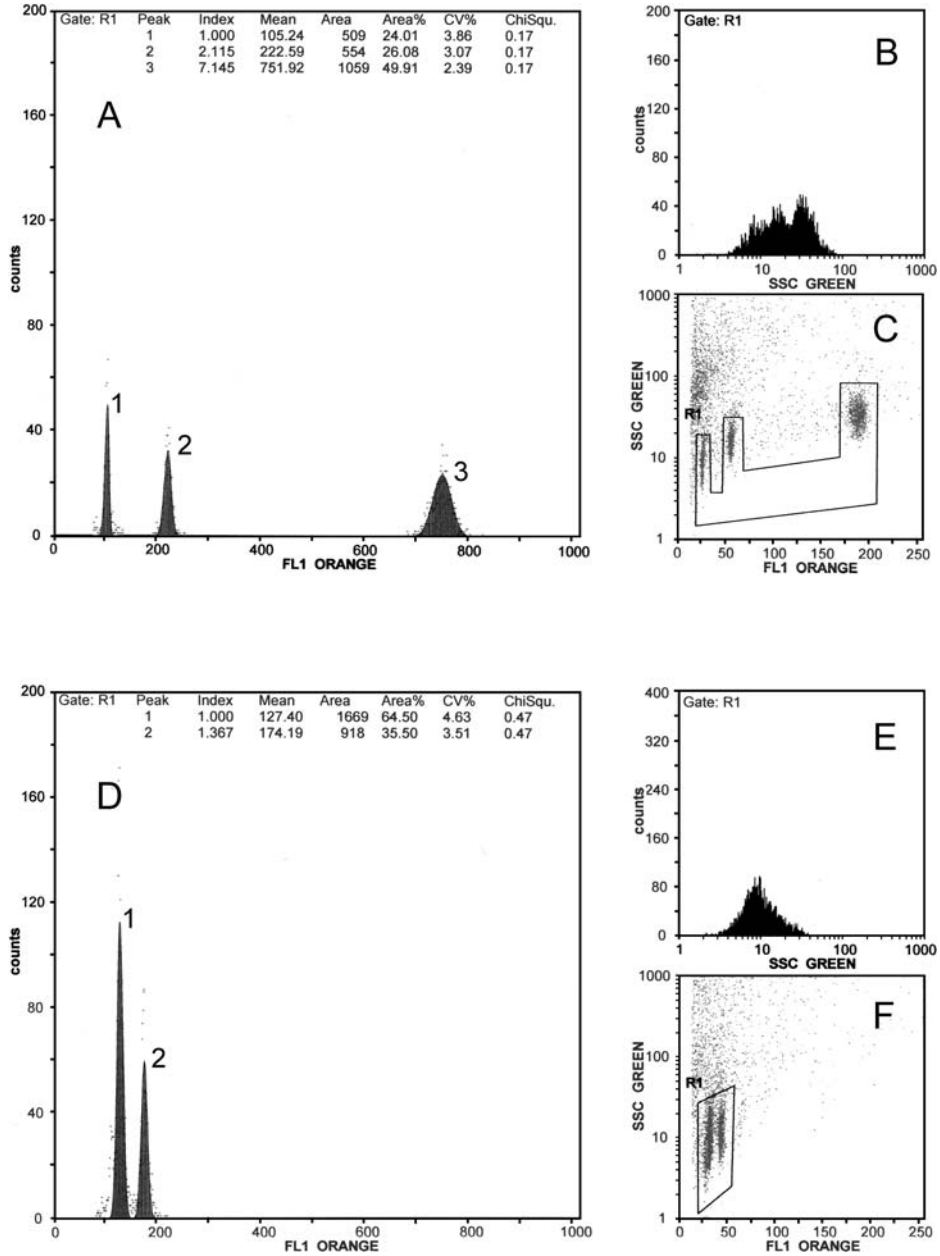

Fig. 3. – (A) Co-chopped haploid (1) and diploid (2) gametophytes of *Radula complanata* plus *Solanum pseudocapsicum* (3); (B) side scatter (SSC) histogram; (C) cytogram fluorescence (FL1) vs side scatter, gating indicated. (D) co-chopped male (1) and female (2) gametophytes of *Frullania dilatata*; (E) side scatter histogram; (F) cytogram fluorescence vs side scatter, gating indicated. Nuclei of analyzed plants were isolated, stained with propidium iodide and simultaneously measured by flow cytometry.

higher taxonomic levels. Higher taxonomic significance can be ascribed to the amount of DNA in the euchromatic fraction of the genome (Greilhuber & Ehrendorfer 1988).

Interspecific variation in ploidy and genome size

Very small genome sizes are recorded for liverworts, hornworts, mosses and among the primitive tracheophytes with *Selaginella* as a good example (Little et al. 2007). Tracheophytes are often regarded as the sister group of hornworts (Groth-Malonek et al. 2005). Small genome sizes thus can be regarded as plesiomorphic in land plants and tracheophytes. For liverworts there are too few data for a meaningful discussion of the changes in genome size in a phylogenetic framework. Worldwide, there are ~ 5000 species of liverworts distributed in 83 families and 391 genera (Crandall-Stotler et al. 2009). The 43 species of 31 genera studied make up 0.86 % of the species and 7.9 % of the genera and at the family level 26.5 %. The range in genome sizes is similar for thallose and foliose liverworts, 0.293–7.401 pg/1C vs 0.211–7.966 pg/1C, with medians of 0.687 pg vs 0.757 pg and means of 1.709 pg vs 1.023 pg. For the whole sample the corresponding 1C-values range from 0.211 to 7.966 pg, with a median of 0.757 pg and mean of 1.205 pg. There are only two genera in our sample that significantly exceed the upper limit of the remaining liverwort taxa and mosses (Renzaglia et al. 1995, Temsch et al. 1998, Voglmayr 2000, Greilhuber et al. 2003). These are *Pellia*, a thalloid liverwort and *Mylia*, a foliose liverwort. The large genomes in these species appear to be independently derived rather than primitive. There is a large amount of dense heterochromatin in *Mylia taylorii*, which is not present to a similar degree in *Pellia epiphylla* (Newton 1985). Thus, it is likely that the main mechanism of genome size increase in these two species is different. In *Pellia* it may be predominantly by the accumulation of dispersed repetitive elements (compare also Heitz 1928) while in *Mylia* an extensive accumulation of tandemly arranged sequences (satellite DNA?) seems more plausible.

The limited variation in genome size in bryophytes as a whole compared to vascular plants is plausibly explained by Renzaglia et al. (1995) as a consequence of the weight of the cell nucleus limiting the motility of biflagellate sperm cells. This is a unique example of the nuclear mass or weight restricting genome size. In most other instances, in which a nucleotypic explanation of the variation in genome size is proposed, it is thought to affect the duration of the cell cycle (Bennett 1971, 1972, Leitch & Bennett 2007). Species such as *Lejeunea cavifolia* and *Mylia taylorii,* with up to a 38-fold difference in their 1C-values, may provide material for studying the role of sperm weight on sperm performance in liverworts.

Intraspecific variation in ploidy and Cx-values

Radula complanata is chromosomally haploid and diploid (Fig. 3A) with a 1.06-fold Cx-value in the diploid accession. Here, an increase in genome size with polyploidization is reported, which is not as frequent as a decrease in genome size (Leitch & Bennett 2004).

There are haploid and diploid forms of *Pellia epiphylla*. The chromosome numbers of our accessions were not counted, but include both DNA-haploid and -diploid forms, with remarkable differences in DNA content within ploidy levels. The average Cx-value of the diploids almost approaches that of the haploids (1: 0.973).

In *Metzgeria furcata* there are both DNA-haploid and -diploid forms with a 0.906-fold Cx-value in the diploid accession. This is an example of a decrease in genome size.

Lophocolea heterophylla was found to have two genome variants differing in size by a factor of 3.37-fold with the haploid chromosome count associated with the higher DNA level. This example points to a cryptic taxonomic differentiation and is at the same time

a warning against premature conclusions about ploidy levels based on the amount of DNA in the absence of chromosomal evidence (Suda et al. 2006).

Intraspecific variation in *Frullania dilatata* can be attributed to sex-related chromosomal heteromorphism. Female plants with two large heterochromatic sex-chromosomes (n = 9) have a 1.35-fold higher C-value than male plants with only one sex chromosome (n = 8) (see also Fig. 3D).

Comments on the measurements of other authors

Ishida (1961b) used chemical methods and reports a value of 0.48 pg DNA/cell for *Marchantia polymorpha* gametophytes (probably approximating the 1C-value), which is 1.6-fold greater than the value reported here. However, Ishida (1961a) reports no positive nuclear Feulgen staining in situ. Sparrow et al. (1972) cite 1C = 0.80 pg DNA for *M. polymorpha* and 1C = 4.28 pg DNA for a *Riccia* species based on the nuclear volume method. Both these values are 3 to 4-times higher than those reported here, which clearly demonstrates that nuclear volume measurements give values that are less reliable than those based on measurements of DNA content. Taylor et al. (1982) cite 1C = 0.99 pg DNA for *Conocephalum conicum* based on Feulgen cytophotometry, which is somewhat higher than the value of 0.697 pg reported here. The discrepancy may be because the material was standardised against chicken red blood cells (Taylor et al. 1982), the suitability of which for use with plant material is questioned (Johnston et al. 1999, Doležel & Bartoš 2005). Renzaglia et al. (1995) report Feulgen densitometry based 1C-values of 1.03 pg for *Bazzania trilobata*, 0.49 pg for *Blasia pusilla*, 1.58 pg for *Dumortiera hirsuta*, 0.73 pg for *Lophozia capitata*, 4.05 pg for *Pellia epiphylla* and 1.31 pg for *Riccardia multifida*. Of these species, only *Bazzania trilobata* with 0.950 pg and *Pellia epiphylla* with 3.803 pg were investigated in our study and our results are reasonably close to values given by this author. Renzaglia et al. (1995) used chicken red blood cells as a standard with an assumed DNA amount of 2.5 pg (2C). If for chicken a 2C-value of 2.33 pg (Galbraith et al. 1983) is used, which is certainly not too low (Greilhuber et al. 1983, 2007, Bennett et al. 2003), the genome sizes reported in these two studies are very similar, differing by less than 1%.

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Souhrn

Dosavadní znalosti o velikosti genomu játrovek jsou velmi kusé – literární prameny udávají množství jaderné DNA jen u 9 druhů. Tento článek představuje první cílenou studii věnovanou velikosti genomu játrovek a shrnuje data pro 32 foliózních a 11 frondózních druhů řazených do 22 (z celkových 83) čeledí. Velikost genomu všech druhů byla stanovena pomocí průtokové cytometrie s využitím DNA-selektivního fluorochromu propidium jodidu. Část vzorků byla též analyzována Feulgenovou densitometrií, která se však ve srovnání s průtokovou cytometrií ukázala méně vhodná. Důvodem je obtížná macerace pletiv játrovek a jejich pevné buněčné stěny, což často znesnadňuje pronikání použitých chemikálií do buněk a vede k výrazně nižším naměřeným hodnotám velikosti genomu. Pokud to kvalita preparátu dovolila, u studovaných vzorků byly též stanoveny přesné nebo alespoň přibližné počty chromozómů. Nejmenší genom (1C-hodnota) u frondózních játrovek byl zjištěn u druhu *Marchantia polymorpha* (0,293 pg), největší u diploidní *Pellia epiphylla* (7,401 pg). Mezi haploidními frondózními druhy měla největší genom haploidní *P. epiphylla* (1C = 3,803 pg). *Lejeunea cavifolia* (1C = 0,211 pg) se vyznačuje nejmenším genomem mezi

foliózními játrovkami, zatímco haploidní *Mylia taylorii* (1C = 7,966 pg) má genom největší (obsahuje velké množství heterochromatinu). Celkově se tedy játrovky liší ve velikosti genomu téměř 38-násobně, což je výrazně větší rozpětí nežli variabilita zjištěná u mechů (zhruba 12-násobná, rozmezí 1C-hodnot 0,174–2,160 pg). Nicméně tato variabilita je nepatrná ve srovnání s přibližně 2000-násobným rozdílem velikostí genomu u krytosemenných rostlin. U některých druhů játrovek (*Radula complanata*, *Pellia epiphylla* a *Metzgeria furcata*) byla pozorována vnitrodruhová ploidní diferenciace (haploidní a diploidní jedinci). Sběry *Lophocolea heterophylla* s haploidním počtem chromozómů se lišily 3,37-násobně ve velikosti genomu. Tato variabilita ukazuje na existenci kryptických taxonů a dokládá problematické stanovení ploidního stupně jen na základě měření obsahu jaderné DNA (bez znalosti počtu chromozómů). V některých případech byla odhalena též vnitrodruhová variabilita ve velikosti genomu. Samičí rostliny druhu *Frullania dilatata* se dvěma velkými heterochromatinovými pohlavními chromozómy měly přibližně 1,35-násobně větší genom než rostliny samčí, které nesou jen jediný pohlavní chromozóm. Role pohlavních chromozómů jako možného zdroje vnitrodruhové variability u ostatních játrovek vyžaduje další podrobné studium.

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