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SHORT COMMUNICATION

Are B-chromosomes responsible for the extraordinary genome size variation in selected *Anthoxanthum* annuals?

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Abstract Nuclear genome size is strongly influenced by the number, size and morphology of chromosomes, and there is often a good correlation between genome size and total chromosome length within a karyotype. Because aneuploidy or the presence of accessory chromosomes has repeatedly been reported within the *Anthoxanthum aristatum/ovatum* complex (Poaceae), both phenomena have to be considered as potential sources of genome size variability within this group. This variability in nuclear genome size reaches 40 %, not only within the complex but also within single populations. Genome size variation, however, does not necessarily correlate positively with the number of chromosomes, as our data also indicate. Although our karyological survey revealed the presence of at least one B-chromosome in 44 % of individuals, we found almost no correlation between the number of B-chromosomes and genome size variability. The presence of B-chromosomes usually increases individual genome size, but does not affect substantially the extent of variability within the complex or population regardless of whether individuals with accessory chromosomes are included. These findings indicate that changes mainly in A-chromosomes are responsible for a huge fraction of genome size variability in the *A. aristatum/ovatum* complex.

Keywords *Anthoxanthum* · B-chromosomes · Genome size · Intraspecific variation

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Introduction

Intraspecific variation in genome size of flowering plants has been studied for decades (see more in Šmarda and Bureš 2010). It can arise due to changes in chromosome numbers (polyploidy, aneuploidy, presence of accessory or sex chromosomes), various chromosomal rearrangements or hybridization following or preceding polyploidization. Mutational processes at the molecular level (activity of transposable elements, length polymorphism in various repeat sequences, genomic duplications, etc.) can also be responsible for significant variation in genome size.

However, the accuracy of many studies documenting intraspecific variation is doubtful, mostly because of the methodology used, and some studies have even been refuted (e.g. Greilhuber 1998). The study of intraspecific variation in genome size regained popularity when the methodology for detecting it was improved (Greilhuber 2005) and when flow cytometry became widely adopted as an analytical method (Doležel and Bartoš 2005; Doležel et al. 2007a; Greilhuber 2008).

Our previous paper (Chumová et al. 2015) reports findings of huge intraspecific variation in genome size of up to 64.8 % within the *Anthoxanthum aristatum/ovatum*

complex ($2n = 10$). We decided to subject this complex of annual grasses to a detailed study and to reveal the sources of genome size variation within it. B-chromosomes (Bs) are known as possible contributors to genome size variation and can (e.g. Rosato et al. 1998), which have been found in the karyotypes of this complex (Östergren 1942, 1947; Fernandes and Queirós 1969; Valdès 1973). Among flowering plants, B-chromosomes are more likely to occur in outcrossing species than in inbred ones. They are not more frequent in polyploids than in diploids and not even in species with multiple ploidies (Palestis et al. 2004). B-chromosomes differ from A-chromosomes in inheritance and are not required for normal growth and development of the organism. Due to their dispensable nature, B-chromosomes may or may not be present in certain individuals of the same population and species. It is widely accepted that B-chromosomes are derived from A-chromosomes, sex chromosomes or both. However, there is also evidence suggesting that B-chromosomes can be spontaneously generated in response to the novel genomic conditions following interspecific hybridization (for reviews, see Jones and Rees 1982; Jones and Houben 2003; Houben et al. 2013). Our main objective was to determine the potential impact of B-chromosomes on intraspecific variability correlated with variability caused by A-chromosomes (As).

We addressed the following questions: (1) What is the extent of genome size variation within and among *A. aristatum/ovatum* populations? (2) How is this variation related to chromosome-number variation? and (3) What is the role of A- and B-chromosomes in determining intraspecific variation in genome size?

Materials and methods

Plant material

Ripe caryopses were collected from seven *A. aristatum/ovatum* populations (we treated *A. aristatum* Boiss. and *A. ovatum* Lag. as a single species complex, based on the results of Pimentel et al. 2007, 2010) in France, Portugal and Spain during the years 2008–2009 (Table 1). They were sown in 2013 and 2014, and allowed to grow under controlled conditions in growth chambers and a greenhouse at the CEITEC centre (Kamenice 753/5, Brno, Czech Republic). Herbarium specimens are deposited in the PRC herbarium. A total of 38 individuals of appropriate size and developmental stage was used in the study. In addition, 20 of the accessions were selected for a detailed analysis of fluorescence intensities of chromosomes on metaphase plates.

Flow cytometry

Holoploid genome size (Greilhuber et al. 2005) was estimated by means of propidium iodide flow cytometry. From each plant, a 1-cm-long section of one young, intact leaf was chopped along with an appropriate amount of an internal reference standard using a new razor blade in a Petri dish containing 0.5 ml of ice-cold Otto I buffer (0.1 M citric acid, 0.5 % Tween 20; Galbraith et al. 1983; Otto 1990; Doležel et al. 2007b). The resulting suspension was filtered through a 42-μm nylon mesh and incubated at room temperature for at least 5 min. After incubation, the suspension was stained with the intercalating fluorescent dye propidium iodide supplemented with RNAse IIA (both at the final concentrations of 50 μg/ml) and β-mercaptoethanol (2 μl/ml), dissolved in 1 ml of Otto II buffer (0.4 M $Na_2HPO_4 \cdot 12\ H_20$). The samples were stained for 5 min at room temperature and analysed using a Partec CyFlow cytometer (Partec GmbH., Münster, Germany) equipped with a 532-nm diode-pumped solid-state laser (Cobolt AB, Solna, Sweden) as the source of excitation light. Fluorescence intensity of 5000 particles was recorded, and the data were analysed using Partec FloMax Software version 2.4d. *Vicia faba* 'Inovec', $2C = 26.60$ pg (Chumová et al. 2015), served as the reference standard. All samples were measured at least two times to avoid diurnal fluctuation in flow cytometry estimation. If two measurements differed by more than 2 %, a third measurement was carried out.

Chromosome preparation and analysis

Young, actively growing roots were harvested from the cultivated plants, pre-treated with ice-cold water for 12 h, fixed in freshly prepared fixative (ethanol:acetic acid, 3:1) overnight and stored in 70 % ethanol at −20 °C until use. Chromosome spreads were prepared as described by Chumová et al. (2015). Chromosomes were stained with 4′,6-diamidino-2-phenylindole (2 μg/ml) in Vectashield anti-fade (Vector Laboratories), photographed using an Olympus BX-61 epifluorescence microscope equipped with a CoolCube camera (Metasystems, Altlussheim, Germany) and counted from the captured images. At least 20 mitotic chromosome spreads were counted for each accession analysed. Chromosome numbers were determined for all 38 plants analysed. The length and fluorescence of chromosomes were measured in three mitotic chromosome spreads for each accession using Image J software (US National Institutes of Health, Bethesda, MD, USA). The level of fluorescence was corrected for total fluorescence (CTF) and evaluated as CTF = Integrated Density − (Area of selected chromosomes × Mean

Table 1 List of analysed *Anthoxanthum aristatum/ovatum* populations and their basic characteristics—short description supplemented by coordinates and altitude; collector's abbreviation; number of analysed plants; genome size (2*C* value) and its variation; observed karyotype arrangement

Population code	Locality	Coordinates	Altitude (m a.s.l.)	Collector	Number of plants	Genome size		Chromosome number
						Mean ± SD (pg)	Variation (%)	
FR10	France, Corse. Giuncheto	N41.5544; E8.8853	52	PT	2	8.04 ± 0.31	5.7	10, 10 + 1B
FR11	France, Corse. Macinaggio	N42.9704; E9.4529	12	PT	4	7.38 ± 0.24	8.1	10, 10 + 2B
FR12	France, Corse. Bonifacio	N41.4071; E9.2131	4	PT	2	8.15 ± 0.39	6.9	10
PT07	Portugal, Beira Litoral. Coimbra. Parrozelos	N40.2086; W7.8996	864	PS	1	6.80	–	10
ES06	Spain, Doňana. Mazagon	N37.1447; W6.8109	35	PT, JK, JZ	9	7.88 ± 0.50	19.0	10
ES07	Spain, Doňana. Almonte	N37.2132; W6.4426	35	PT, JK, JZ	9	7.56 ± 0.32	14.2	10, 10 + 1B, 10 + 2B
ES09	Spain, Monfragüe. Rio Tietar	N39.8412; W5.9659	258	PT, JK, JZ	11	8.06 ± 0.89	37.8	10, 10 + 2B, 10 + 3B, 10 + 5B, 10 + 6B

Collectors: *JK* Jana Krejčíková, *JZ* Jaroslav Zahradníček, *PS* Paulo Silveira, *PT* Pavel Trávníček

fluorescence of background readings; Burgess et al. 2010; Potapova et al. 2011).

Data analysis

Genome size variation at all levels was calculated according to the following simple equation: (maximum_GS/minimum_GS − 1) × 100 %. Levene's test for homogeneity of variance (Levene 1960; function 'leveneTest', package 'car', R) was used to compare variances of two datasets (particularly variances between total and A-chromosome-allocated genome size).

The proportion of genome size allocated to A- vs B-chromosomes was assessed by comparing corrected fluorescence of A- and B-chromosomes (measured on metaphase plates) and corresponding total genome size estimated by flow cytometry (Table 2). The length of chromosomes was used as supporting information for clear separation of A- and B-chromosomes.

All data analyses were performed in R version 3.2.2 (R Development Core Team 2015).

Results

The number of chromosomes and holoploid genome size (2*C* value) were determined for all 38 individuals of the *A. aristatum/ovatum* complex. The presence of A-chromosomes only ($2n = 10$) was revealed in 25 (66 %) individuals. The remaining individuals (13 individuals, 44 %) possessed supernumerary B-chromosomes that were smaller than all the remaining chromosomes and of different length in different populations (more details below). Accessions with B-chromosomes were found to have the following karyotypes: $2n = 10 + 1B$ (two individuals), $2n = 10 + 2B$ (five individuals), $2n = 10 + 3B$ (three individuals), $2n = 10 + 5B$ (one individual) and $2n = 10 + 6B$ (two individuals; see overview in Table 2; Fig. 1 and Online Resource 1 for details). Various compositions of karyotypes were found at the population level (Table 1). Some populations possessed solely A-chromosomes (e.g. FR12, ES06), while others comprised almost the entire spectrum of karyotypes (e.g. ES09).

The genome size (2C nuclear DNA content) of all 38 individuals varied from 6.80 to 9.44 pg (mean 7.80 ± 0.63 pg DNA), representing up to 39 % of intraspecific variation (Fig. 2). Variability among plants without B-chromosomes ranged from 6.80 to 8.70 pg (27.9 % variation) and among plants with at least one B-chromosome from 6.85 to 9.44 pg (37.8 % variation). Genome size variability within subsets of individuals with particular chromosome arrangements is summarized in Table 3 and Fig. 3. Although there was a positive correlation between genome size and the number of B-chromosomes (Fig. 3), only three individuals with a high number of B-chromosomes ($2n = 10 + 3B$ or $10 + 6B$) exceeded the DNA content of individuals with solely A-chromosomes (Table 2; Fig. 2).

Table 2 Basic characteristics of all accessions included in the study

Accession code (population − number of accession)	Code of the karyotype in Fig. 1 and ESM (Fig. 5)	2*n*	Mean chromosome length (μm)			Corrected total fluorescence		Genome size 2*C* value (pg)		Bs contribution to individual GS (%)
			A's	B's	A/B	Whole complement	B's	Total GS ± SD	A allocated GS	
FR10-1	a	10	9.577			32615.79	–	7.82 ± 0.02	7.82	–
FR10-2	b	10 + 1B	11.008	6.115	1.80	29933.01	1341.88	8.26 ± 0.02	7.89	4.5
FR11-1	c	10	10.003			20777.81	–	7.13 ± 0.00	7.13	–
FR11-2		10						7.32 ± 0.01	7.32	–
FR11-3		10						7.38 ± 0.02	7.38	–
FR11-4	d	10 + 2B	11.973	4.697	2.55	54944.88	3141.28	7.70 ± 0.00	7.26	5.7
FR12-1		10						7.87 ± 0.01	7.87	–
FR12-2	e	10	11.919			35124.74	–	8.42 ± 0.13	8.42	–
PT07-1	f	10	12.630			30598.24	–	6.80 ± 0.06	6.80	
ES06-1	g	10	12.408			27748.31	–	7.31 ± 0.10	7.31	–
ES06-2		10						7.43 ± 0.09	7.43	–
ES06-3		10						7.50 ± 0.03	7.50	–
ES06-4		10						7.50 ± 0.07	7.50	–
ES06-5		10						7.68 ± 0.03	7.68	–
ES06-6		10						8.21 ± 0.21	8.21	–
ES06-7		10						8.24 ± 0.12	8.24	–
ES06-8		10						8.34 ± 0.13	8.34	–
ES06-9		10						8.70 ± 0.02	8.70	–
ES07-1	h	10	11.281			31066.61	–	7.16 ± 0.11	7.16	–
ES07-2		10						7.34 ± 0.02	7.34	–
ES07-3		10						7.37 ± 0.06	7.37	–
ES07-4		10						7.41 ± 0.16	7.41	–
ES07-5		10						7.44 ± 0.01	7.44	–
ES07-6		10						7.53 ± 0.06	7.53	–
ES07-7		10						8.18 ± 0.04	8.18	–
ES07-8	i	10 + 1B	7.802	5.098	1.53	38266.40	2056.37	7.63 ± 0.09	7.22	5.4
ES07-9	j	10 + 2B	11.252	6.237	1.80	19419.84	1999.48	7.95 ± 0.08	7.14	10.3
ES09-1		10						6.86 ± 0.02	6.86	–
ES09-2	k	10	14.024			47514.21	–	7.94 ± 0.14	7.94	–
ES09-3	l	10 + 2B	11.059	7.640	1.45	29354.80	2919.56	6.85 ± 0.09	6.17	9.9
ES09-4	m	10 + 2B	12.154	9.660	1.26	46947.41	6312.65	7.25 ± 0.46	6.28	13.4
ES09-5	n	10 + 2B	13.051	8.729	1.50	39841.25	4185.72	7.47 ± 0.01	6.69	10.5
ES09-6	o	10 + 3B	12.052	8.348	1.44	25298.14	3831.06	8.08 ± 0.09	6.86	15.1
ES09-7	p	10 + 3B	9.926	7.527	1.32	41817.60	6701.01	8.22 ± 0.07	6.90	16.0
ES09-8	q	10 + 3B	8.137	5.516	1.48	27248.27	2838.28	9.00 ± 0.05	8.06	10.4
ES09-9	r	10 + 5B	6.517	3.994	1.63	14282.01	3266.82	8.44 ± 0.17	6.51	22.9
ES09-10	s	10 + 6B	7.146	4.926	1.45	20763.95	5301.66	9.07 ± 0.07	6.75	25.5
ES09-11	t	10 + 6B	5.856	3.794	1.54	15000.18	2766.93	9.44 ± 0.03	7.70	18.4

The following information is provided: population membership (accession code); references to corresponding karyotypes in Fig. 1 and Online Resource 1; chromosomal arrangement (2*n*); mean chromosome length for A-chromosomes, B-chromosomes and their ratio; corrected total fluorescence separately for whole DNA and for B-chromosomes; genome size (2*C* value) for whole nuclei and DNA content allocated in A-chromosomes; and relative contribution of B-chromosomes to individual genome size

Fig. 1 Karyotypes assembled from micrographs of somatic metaphase chromosomes of all individuals under detailed investigation. Karyotypes are ordered by the following scheme: (1) satellite-bearing chromosome pairs, (2) other A-chromosome pairs ordered by their size and (3) B-chromosomes. Particular parts of figure correspond to samples as follows: FR10-1 (*a*), FR10-2 (*b*), FR11-1 (*c*) d) FR11-4 (*d*), FR12-2 (*e*), PT07-1 (*f*), ES06-1 (*g*), ES07-1 (*h*), ES07-8 (*i*), ES07-9 (*j*), ES09-2 (*k*), ES09-3 (*l*), ES09-4 (*m*), ES09-5 (*n*), ES09-6 (*o*), ES09-7 (*p*), ES09-8 (*q*), ES09-9 (*r*), ES09-10 (*s*), ES09-11 (*t*). The *letters in parentheses* refer to detailed information provided in Table 2

The highest range of intrapopulation variation (37.8 %) was observed in population ES09 (Fig. 2); in the other five populations, it ranged from 5.7 to 19 % (Table 1).

Total length and fluorescence intensity of chromosomes were measured in twenty individuals, including all individuals with B-chromosomes and selected individuals with A-chromosomes only, covering the entire range of variability in their genome size (Table 2). Our screening of the *A. aristatum/ovatum* complex has shown that B-chromosomes are significantly shorter than A-chromosomes and that they lack homologues (Table 1; Fig. 1, Online Resource 1).

The total genome size variability in the complex (irrespective of the chromosome arrangement) was assessed to be 38.8 %, and the variation in genome size borne by A-chromosomes was calculated to be 41 % (6.17–8.70 pg; mean 7.43 ± 0.59 pg). The same comparison applied to a subset of individuals with supernumerary chromosomes shows 37.8 and 30.6 % (6.17–8.06 pg; mean 7.03 ± 0.59 pg) variation for total and A-chromosome-

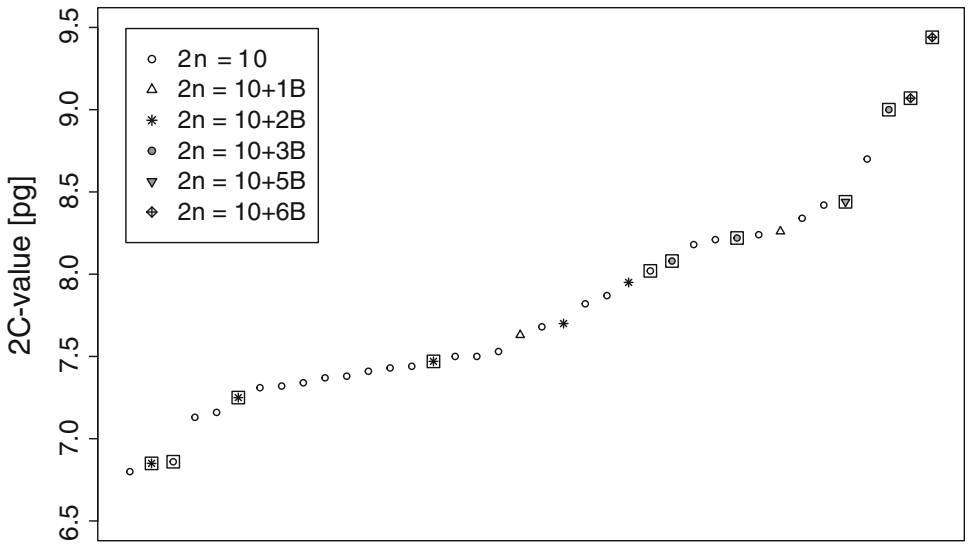

Fig. 2 Variation in holoploid genome sizes of all plants of the *A. aristatum/ovatum* complex with known karyology (sorted according to increasing 2*C* value; total variation 38.8 %). All karyotype arrangements are shown as *different symbols* according to the *inset* legend. Points belonging to the most variable population ES09 are indicated by *squares*

Table 3 Genome size (2*C* value) and its variation for all karyotype's arrangements investigated in the study

Karyotype arrangement	*N*	Genome size	
		Mean ± SD (pg)	Variation (%)
10	25	7.64 ± 0.49	27.9
10 + 1B	2	7.95 ± 0.45	8.3
10 + 2B	5	7.45 ± 0.42	16.1
10 + 3B	3	8.43 ± 0.50	11.4
10 + 5B	1	8.44	–
10 + 6B	2	9.25 ± 0.26	4.1

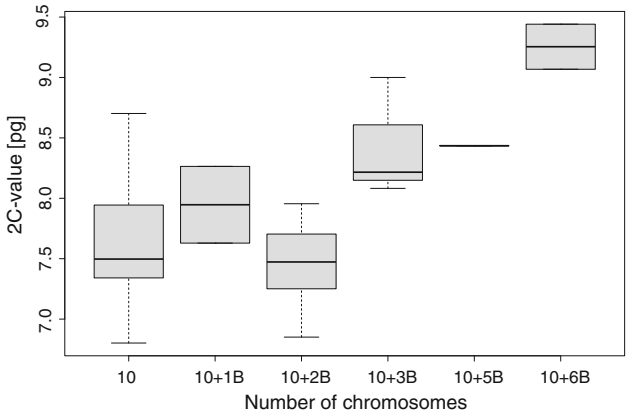

Fig. 3 Box-and-whisker plots showing holoploid genome size (2*C* values) variation of six karyotype arrangements with *different numbers* of B-chromosomes

allocated genome size, respectively (Table 2; Fig. 4). Levene's test for homogeneity of variance of total and A-chromosome-allocated genome sizes does not show any

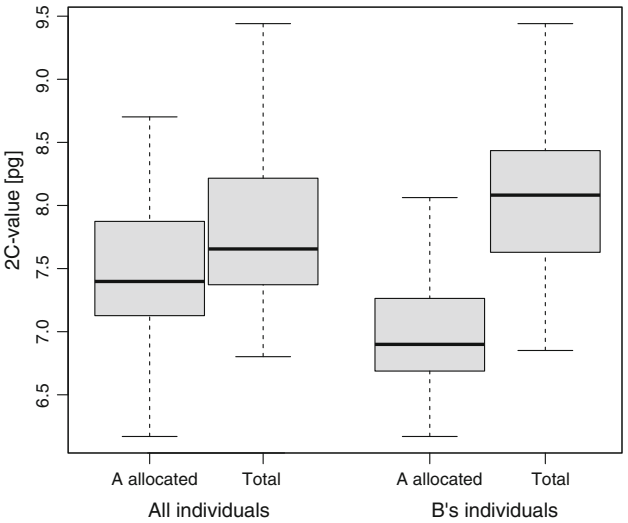

Fig. 4 Box-and-whisker plots showing variation in A-chromosome-allocated and total holoploid genome size (2*C* values) for the set of all individuals and the subset of individuals with supernumerary chromosomes

difference both for the set of all individuals ($df = 1$, $F = 0.1823$, $p = 0.671$) and the subset of individuals with B-chromosomes ($df = 1$, $F = 0.5443$, $p = 0.468$).

Discussion

Extraordinary genome size variability reaching 40 % has been detected in the *A. aristatum/ovatum* complex. We therefore focused on examining the role that chromosome number variation plays in this genome size variability. Several authors have reported aneuploidy and the presence of supernumerary chromosomes within the complex

(Östergren 1942, 1947; Fernandes and Queirós 1969; Valdès 1973). We identified 0–6 supernumerary chromosomes in the karyotypes of 44 % of individuals analysed, which is similar to what has been reported in studies of other plant groups (usually 0–4.5 supernumerary chromosomes, Jones et al. 2008). Our comparison of fluorescence intensities found B-chromosomes to be responsible for up to 25 % of genome size increases within individuals. A similar result has been reported for *Zea mays* (Jones and Rees 1982; Michaelson et al. 1991; Rosato et al. 1998). On the other hand, several other studies found no effect of B-chromosomes or aneuploidy on increases of genome size within individuals (Poggio et al. 1998; Leong-Škorničková et al. 2007; Leitch et al. 2009). There is a strong positive correlation between genome size and the presence of B-chromosomes (Trivers et al. 2004; Levin et al. 2005), but the relative contribution of B-chromosomes to genome size is small. Plants with larger genomes are therefore less sensitive to changes of their total DNA amount due to supernumerary chromosomes. Our data also show that a very large fraction if not the whole of the intraspecific or population variability is caused by A-chromosomes alone and that B-chromosomes are responsible for only marginal increases of individual genome size and have almost no effect on the overall genome size variability within the *A. aristatum/ovatum* complex. This finding contradicts the detailed study of Rosado et al. (2009), who found A-chromosomes to have consistent genome size among individuals of one inbred line of *Zea mays* and a strongly positive correlation between total genome size and the number of Bs. However, this contradiction might be caused by heterogeneity of the material under investigation because our data come from populations of two weakly differentiated taxa, *A. ovatum* and *A. aristatum*, that might be of hybrid origin and whose taxonomic complexity is as yet unresolved (e.g. Pimentel et al. 2010). On the other hand, a relatively high degree of intraspecific genome size variability in other members of the genus *Anthoxanthum* has been found to be associated with A-chromosomes only (Chumová et al. 2015). Therefore, the uncertain origin of populations of the *A. aristatum/ovatum* complex is not very likely to have biased our finding that A-chromosomes are the bearers of the main part of genome size variability within the complex.

Moreover, our data also show that FCM-based data without karyological knowledge could not have revealed a true picture of chromosomal arrangements (e.g. the presence of Bs) or the sources of genome size variability. Data on genome size alone cannot be used to draw conclusions about chromosomal arrangements, especially within plants with a high degree of genome size variation. In many biosystematic studies, ploidy is estimated by flow cytometry without any chromosome count data to confirm the assigned ploidy levels (Hulquist et al. 1996). Even if such ploidy level estimates are referred to as DNA ploidy level estimates (following Suda et al. 2006), the data may still be biased by aneuploidy or the presence of accessory chromosomes.

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Compliance with ethical standards

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

Information on Electronic Supplementary Material

Online Resource 1. Fig. 5 Micrographs of somatic metaphase chromosomes of all individuals under detailed investigation. B-chromosomes are indicated by arrows

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