Variation in genome size in the *Valeriana officinalis* complex resulting from multiple chromosomal evolutionary processes

Variabilita ve velikosti genomu *Valeriana officinalis* jako výsledek mnohočetných evolučních procesů

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Polyploidy, aneuploidy and change in DNA content of monoploid genomes or chromosomes are the principal causes of the variation in genome size. We studied these phenomena in central-European populations of the *Valeriana officinalis* complex in order to identify mechanisms or forces driving its evolution. The complex comprises di-, tetra- and octoploid morphologically defined so-called taxonomic "types". Within the study area there are also intermediate "transitional types" the existence of which hampers the application of traditional taxonomic concepts. We thus chose AFLP genotyping and admixture analyses to identify the genetic structuring of the material studied. Di- (2x), tetra-(4x) and octoploidy (8x) were confirmed as major ploidy levels. Major genetic clusters roughly corresponded to these ploidy levels (for K = 2: 2x- and 8x-clusters, for K = 4 with nearly identical probability: 2x-, 4x-, 8x- and unspecific clusters were identified), which further more significantly differed from each other in monoploid absolute genome size (mean 1Cx for 2x = 1.48 pg, 4x 1.29 pg, 8x 1.10 pg). Several individuals of all ploidy levels were admixed, particularly tetraploids. Relative genome size (the sample: standard DAPI fluorescence) was positively correlated with the proportion of the diploid genetic cluster shared by the tetraploids, indicating that hybridization caused the variation in genome size. This result is in accordance with the significant negative correlation of the genome size of tetraploids with their geographic distance to the diploids. However, remarkable intra-ploidy variation in relative genome size was recorded for all ploidy levels (1.14-fold in diploids, 1.28-fold in tetraploids, 1.19-fold in octoploids). We identified aneuploidy as an additional source of variation in genome size in the di- and tetraploids. The contribution of extra chromosomes to absolute genome size exceeded the observed variation within euploids in the diploids, whereas it was included in the regular variability in genome size recorded for the eutetraploids. Variation in monoploid genome size was recorded in polyploids but not in diploids, indicating that polyploids experienced higher dynamics in the evolution of their genomes. Finally, 38.0–63.2% of the total intra-ploidy variation in relative genome size occurred within populations. In conclusion, the *Valeriana officinalis* complex provides an example of variation in genome size due to four principal evolutionary forces: polyploidization, change in chromosome number and in DNA content of chromosomes and (secondarily) hybridization, but their relative importance differed among ploidy levels. Although the stability in the size of the monoploid genome in species is considered to be the standard case, we found great variability within populations suggesting that genome size is variable even within narrowly defined taxa.

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K e y w o r d s: aneuploidy, AFLP, chromosome number, evolution, flow cytometry, genome size, hybridization, polyploidy, population, *Valeriana*

Introduction

Data on genome size are available for an increasing number of plant species and deemed of great evolutionary importance and taxonomic significance. Variation in genome size is associated with ecological preferences (Reeves et al. 1998, Jakob et al. 2004) or tolerances (Macgillivray & Grime 1995), generation time (Bennett 1972), cell size (Kondorosi et al. 2000), rates of speciation (Jakob et al. 2004, Soltis et al. 2009) and species limits (Ohri 1998, Soltis et al. 2007). Currently genome size is known for about 8500 species of plants (http://data.kew.org/cvalues) and varies by a factor of nearly 2400 in angiosperms (Bennett & Leitch 1995, 2011).

Variation in genome size is generated by three principal mechanisms: polyploidization, deletion or proliferation of DNA and gain or loss of single chromosomes (i.e. aneuploidy). Polyploidy refers to the presence of more than two basic chromosome sets (i.e. monoploid genomes: Greilhuber 2005b) within a single cell nucleus, either due to genome multiplication within a species (i.e. autopolyploidization) or in association with interspecific hybridization (i.e. allopolyploidization: Ramsey & Schemske 1998). Genome size in polyploids may add up to the sum of the DNA amounts of the inherited monoploid progenitor genomes (Levin 2002, Leitch & Bennett 2004). However, polyploidization has been shown to trigger changes in the size (and structure) of plant genomes frequently leading to a decrease in DNA content per monoploid genome in polyploids compared to the diploid progenitors (Leitch & Bennett 2004). Molecular mechanisms generating genome downsizing comprise transposon activation, excessive homologous pairing of chromosomes, or specific elimination of genes and DNA sequences (Soltis & Soltis 1999, Leitch & Bennett 2004, Soltis et al. 2009). Aneuploidy, a chromosome number that differs from a multiple of the base chromosome number due to non-balanced gain or loss of whole chromosomes, finally, arises as a result of chromosome missegregation (e.g. Compton 2011). Aneuploidy is also associated with the occurrence of B chromosomes (Guerra 2008), which originate and are maintained through specific evolutionary mechanisms (Camacho et al. 2000).

A fourth evolutionary force that brings about change in genome size is hybridization among individuals that differ in their nuclear DNA content. Additivity of parental genomes provides a basis for the identification of primary hybrids and later hybrid generations provided that parents are sufficiently differentiated to allow for the separation of genomes intermediate in size. Approaches applied involve reconstruction of the actual process of hybridization by comparison of genome sizes observed and expected in a given situation and evolutionary scenario (e.g. the genome size of the F1 expected from the fusion of meiotically reduced parental gametes), and correlation of genome size with data providing independent evidence for hybridization, like the morphological differentiation of parents (Ekrt et al. 2010, Vít et al. 2014).

The use of flow cytometry (FCM) has made it much faster and convenient to determine the genome size of plants (Doležel et al. 1998, Suda 2004, Shapiro 2007). However, its application requires special care to avoid methodological pitfalls (Greilhuber 2005a).

A particular challenge for the application of FCM in plants is the effect of secondary metabolites on the measurement. Endogenous metabolites such as phenolic substances (e.g. tannins, flavonoids, anthocyans, cumarins) are known to interfere with the staining of the DNA (i.e. fluorescence quenching) and may introduce serious stoichiometric errors (Greilhuber 1987, 2008).

The variation in the genome size of Eurasian *Valeriana officinalis* complex (*Caprifoliaceae*) has come about by the three described principal mechanisms. The complex thus comprises three major ploidy levels: di-, tetra- and octoploidy (Walther 1949, Titz 1964, 1969). Aneuploidy is indicated by the presence of a B chromosome in tetraploid individuals in an Italian population (Corsi et al. 1984), which contrasts with earlier studies that report solely euploidy for a large number of individuals (Titz & Titz 1979, 1980, 1981). However, karyological studies mostly comprise only single accession or a few individuals per population. Finally, monoploid genome size in di- and octoploid individuals indicates elimination of DNA in octoploids (Hidalgo et al. 2010).

Complex morphological differentiation is proposed for *V. officinalis* (Titz & Titz 1979, 1981, 1982, Titz 1984) with ploidy levels corresponding to "basic types", which are further divided into informal taxa called "types". Mixed populations consisting of different "types" and of morphologically different forms are repeatedly described, resulting in variation being rather continuous and what are now considered as "transitional types" (Keller 1973, Titz & Titz 1979, 1981). Little is known about the evolutionary history and associated mechanisms underlying the differentiation of the *V. officinalis* complex (for convenience, henceforth the complex will be referred to as *V. officinalis*), but hybridization between diploids and polyploids as well as among polyploids is assumed based on morphological evidence (e.g. Titz & Titz 1979, 1982).

An illustrative example of the intricate evolutionary relationships in *V. officinalis* is reported for western Austria (Tyrol and Vorarlberg) and adjacent mountainous regions of southern Germany, South Tyrol and Switzerland (Titz & Titz 1981, 1982). Three "types" of differing ploidy occur in this area: diploid *V. exaltata*, tetraploid *V. collina* and octoploid *V. procurrens*. The authors postulate hybridogenous relationships between the di- and tetraploid "types" (the tetraploid "transitional types" *V. vorarlbergensis* and *V. valligena*) and the tetra- and octoploid "types" (the octoploid "transitional type" *V. versifolia*), respectively. In our study we have chosen western Austria and adjacent regions (Eastern Alps) to identify the processes resulting in the variation in genome size in *V. officinalis* as a basis for understanding the relevant mechanisms underlying its evolution. Based on karyological and morphological differentiation we consider variation in the DNA content of chromosomes/monoploid genomes and variation introduced through hybridization, and change in chromosome number (i.e. aneuploidy), as putative processes causing intra-ploidy level variation. Specifically we asked (i) whether hybridization (inferred from AFLP-based admixture analysis) among cytotypes contributed through additivity of parental genomes to variation in genome size; to assess this question we further (ii) estimated whether variation in absolute monoploid genome size differs (as a result of DNA gain or loss) among ploidy levels. (iii) We reinvestigated the frequency of aneuploidy and inferred associated variation in genome size. Finally, (iv) we aim to uncover the extent of the variation in genome size within and among populations as a possible indication for "intraspecific" variation. As a methodological precondition we tested for the confounding effects of fluorescence stain inhibitors.

Material and methods

Plant material

From 5–20 individuals were collected in 2009 and 2010 from each of 47 populations, totaling 633 individuals, in the Tyrol and Vorarlberg (western Austria) and adjacent regions of Bavaria and Baden-Württemberg (Germany). Additional single individuals were collected from 38 localities, including eastern Austria (Fig. 1, Electronic Appendix 1). Plants were cultivated in 10-liter pots using a substrate consisting of 7 parts soil and 3 parts bark humus (Rindenhumus, Kranzinger, Straßwalchen, Austria) in the experimental garden of the Department of Molecular Systems Biology, University of Vienna. Leaf material and rhizomes/roots used for DNA extraction and karyological analyses, respectively, were sampled from the cultivated individuals. An overview of the analyses of the populations sampled and individuals is provided in Electronic Appendix 2. Vouchers are deposited in herbarium WUP for the purpose of permanent documentation of the study material and future morphological analyses.

DNA ploidy level and relative genome size

DNA ploidy levels (Suda et al. 2006) were inferred for all the individuals studied using a Partec PA flow cytometer (Partec, Münster, Germany) equipped with a mercury vapour lamp. Samples were prepared using the two-step Otto procedure (Otto 1990) following Doležel et al. (2007): fresh leaves of valerian and the internal standard *Pisum sativum* L. cv. Kleine Rheinländerin (Greilhuber & Ebert 1994) were cochopped with a razor blade in 500 µl Otto I buffer, the suspension filtered through a 30 µm nylon mesh (Partec Cell Trics), and 1 ml Otto II buffer containing 4 µg DAPI (4'-6-diamidino-phenylindole) added. Samples were analysed after a lag phase of at least 3 minutes. Sample and standard fluorescent peaks were manually gated using FlowMax v2.7 (Partec, Münster, Germany). Sample/standard DAPI fluorescence ratios (henceforth relative genome size) were calculated from the means of fluorescence histograms. DNA ploidies were inferred by comparison of the relative genome size of unknown samples with those of karyotyped (i.e. chromosome counted) individuals. In order to verify the differences in relative genome size within ploidy levels, individuals were coprocessed pairwise. Eleven pairs of individuals of the three major ploidy levels were analysed. Finally, the measurement error of the method was estimated by processing individuals three to six consecutive times and expressed as the 5%/95% confidence intervals calculated for each of these individuals.

Valeriana contains valepotriates, which are known DNA intercalators and may inhibit staining of the DNA (Hidalgo et al. 2010). We tested for fluorescence quenching by comparing the DAPI fluorescence intensity of the standard *Pisum* analysed alone to the intensity when cochopped with the valerian sample. The gain was held constant and the percentage difference in the fluorescence intensities of the standard obtained in the two measurements was calculated. Twelve comparative measurements were made. The differences recorded were tested for deviation from zero using the t-test in Statistica v6 (StatSoft 2002).

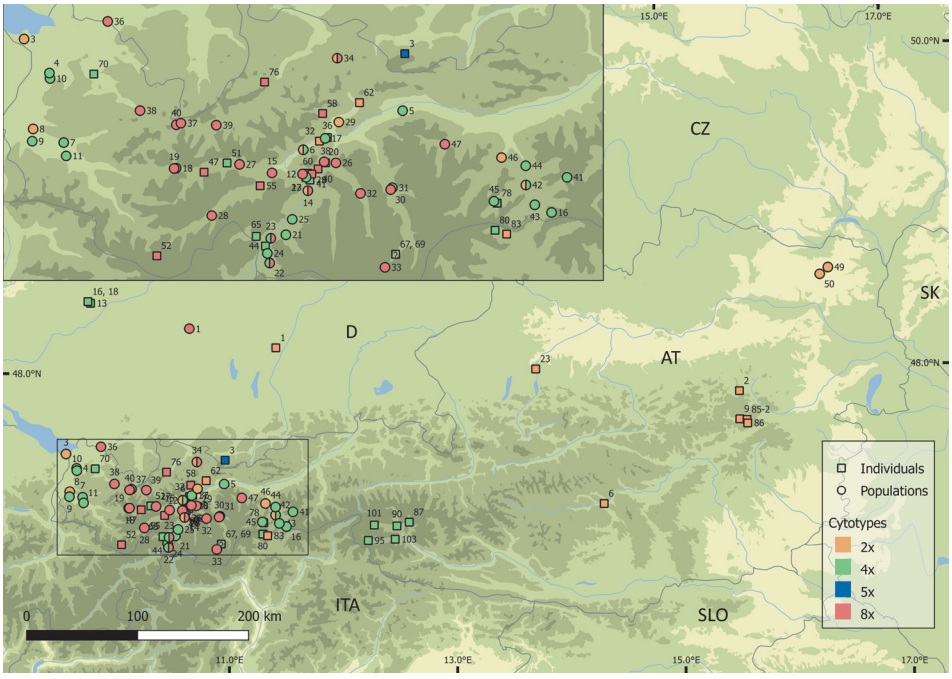

Fig. 1. – Map showing the geographic origin of three majority and one minority (pentaploid) ploidy cytotypes recorded for *Valeriana officinalis* in Austria and southern Germany. Forty-seven populations and single individuals from 38 additional sites were collected. 2x, 4x, 5x, and 8x refer to di-, tetra-, penta- and octoploidy, respectively.

A one-way ANOVA as implemented in Statistica was performed to infer the amount of variance in relative genome size within (as a clue for intraspecific variation in genome size) and among populations of identical ploidy. We further tested for relationships between geographic distance among sampling sites and relative genome size of individuals using a Mantel test. Pairwise Euclidean distances of relative genome size among individuals were calculated using the dist-function in the statistic package R (R Development Core Team 2011) and pairwise spatial distances from the geographic coordinates of sampling sites using the GeographicDistanceMatrixGenerator_v1.2.3 (Ersts 2016). Mantel tests were performed using the function mantel.rtest implemented in the R package ade4. Correlation between matrices was tested in a permutation one matrix 9999-times.

Estimates of the absolute genome sizes

The absolute genome size (2C-value) was determined for individuals of each of the three major DNA ploidy levels. Individuals were selected in order to include the recorded intra-ploidy variation in relative genome size. Individuals with low and high ratios were chosen from each of four di-, tetra- and octoploid populations, 24 individuals in total. An additional octoploid individual of known chromosome number was also analysed. Measurements were made using a Partec CyFlow ML flow cytometer equipped with a diode-pumped solid-state green laser (100 mW, 532 nm, Cobolt Samba; Cobolt AB, Stockholm,

Sweden). In order to avoid fluorescence signal overlap we used *Solanum pseudocapsicum* L. (2C-value = 2.59 pg: Temsch et al. 2010) as an internal standard. Fresh leaves of the sample and the standard were cochopped with a razor blade in 1 ml Otto I buffer and filtered through 29 µm nylon mesh (Sefar AG, Rüschlikon, Switzerland). RNA was digested with RNase A (final concentration 0.15 mg ml^{-1}) at 37 °C for 30min. Nuclei were stained with propidium iodide (PI) dissolved in Otto II buffer (2 ml, final concentration PI 50 µg ml^{-1}) for 1.5 h at 4 °C. Each sample was measured three times. Coefficients of variation < 4% for the G0/G1 peaks only were considered. The 2C-value (Greilhuber 2005b) was calculated by multiplying the 2C-value of the standard with the sample/standard fluorescence ratio. The range in variation in genome size was calculated as the (highest value – lowest value) × 100/lowest value. Monoploid genome size (1Cx-value) was calculated for euploid chromosome-counted individuals. A one-way ANOVA was used to test for differences in monoploid genome size among individuals of different ploidy. The Bonferroni test was applied as a posthoc test.

Counting of chromosomes

Chromosomes of 47 individuals of the three major ploidy levels were counted. We aimed at obtaining precise chromosome numbers as they are required for identifying aneuploids and for the determination of the sizes of monoploid genomes. Root tips were pretreated in 0.002 M 8-hydroxyquinoline for 2 hours at room temperature and subsequently for 2 hours at 4 °C, fixed in Carnoy (95% ethanol: acetic acid = 3: 1) and stored at –20 °C until preparation. Root tips were hydrolyzed in 5 N HCl for 20 minutes at room temperature, the meristems subsequently isolated on a slide in a drop of 45% acetic acid and carefully squashed under a cover slip. The slides were immediately frozen in liquid nitrogen (–196 °C) or on a cold-plate (–80 °C), the cover slip was removed using a razor blade and the slide was air-dried. The preparations were stained with Giemsa (Carl Roth, Karlsruhe, Germany) solution (diluted 1: 10) for 5 minutes, air-dried and embedded in Euparal (Carl Roth). Preparations were analysed using a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) at 1000-fold magnification. Late prophase and metaphase plates were hand sketched.

AFLP analyses

Seventy-nine individuals analysed for relative genome size were randomly selected from each of three major ploidy levels with approximately equal frequencies and phenotyped by means of AFLP. Seventeen populations and 3–5 individuals per population were analysed. Seven individuals were analysed twice to test the reproducibility of results. Total DNA was extracted from silica dried leaf material using the NucleoSpin Plant II Kit (Macherey-Nagel, Düren, Germany). The principal steps of the AFLP analysis followed Vos et al. (1995) with several minor modifications. Restriction and ligation were performed in 12 µl reaction mixes containing BSA (0.046 mg ml^{-1}), T4 ligase buffer (0.92×), NaCl (0.045 M), 300 ng of genomic DNA, MseI (1 unit) and EcoRI (5 units) enzymes (Promega, Madison, USA), MseI- (4.160 pmol $µl^{-1}$) and EcoRI-adapters (0.416 pmol $µl^{-1}$) (Sigma-Aldrich, St. Louis, USA) and T4 DNA ligase (0.6 units, Promega). The reaction mix was incubated for 3 h at 37 °C, followed by an inactivation step for 12 hours at 17°C. The restriction-ligation product was subsequently diluted 10-fold. The primer-pairs

EcoRI-A/MseI-C and EcoRI-G/MseI-C (Applied Biosystems, Foster City, USA) were chosen for preselective amplification of fragments. The preselective PCR was done in 10 µl reaction mixes containing 5.86 µl ultrapure water, 1.14 µl 10× RedTaq buffer (10 mM $MgCl_2$), 0.22 µl 10 mM dNTP mix (Ambion, Austin, USA), 0.58 µl primer (5 µM MseI: Sigma-Aldrich + 5 µM EcoRI: Applied Biosystems), 0.2 µl RedTaq polymerase (Sigma-Aldrich) and 2 µl diluted restriction/ligation product. The reactions were held at 72 °C for 2 min followed by 26 cycles of 94 °C for 5 s, 56 °C for 30 s and 72 °C for 2 min, with a final 30 min extension at 60 °C. The pre-selective PCR product was visualized on a 1.0% agarose gel and diluted ten-fold. The primer pairs EcoRI-ACA (FAM)/MseI-CAC, EcoRI-ACT (NED)/MseI-CTC, EcoRI-ACG (VIC)/MseI-CAA, EcoRI-GTC (FAM)/MseI-CAG, EcoRI-GTC (NED)/MseI-CTA, EcoRI-GAC (VIC)/MseI-CAG (Applied Biosystems) were chosen for the selective amplification of fragments. The selective PCR was done in 10 µl reaction mixes containing 5.5 µl ultrapure water, 1.0 µl 10× RedTaq buffer (10 mM $MgCl_2$), 0.22 µl 10 mM dNTP mix (Ambion), 0.54 µl primer MseI (5µM: Sigma-Aldrich), 0.54 µl primer EcoRI (5µM: Applied Biosystems), 0.2 µl RedTaq polymerase (Sigma-Aldrich) and 2 µl diluted (1: 10) preselective PCR product. Thermocycling started at 94 °C for 2 min, 65 °C for 30 sec, and 72 °C for 2 min. Denaturation was reduced to 1 sec in the following cycles. The annealing temperature progressively decreased by 1 °C per cycle (from 65 °C to 57 °C) and was then held constant for 23 cycles. The program finished with a final 30 min extension at 60 °C. The differentially labelled fragments were multiplexed and purified using Sephadex G-50 superfine (Merck Millipore, Billerica, USA). One µl of the purified product was mixed with 10 µl mastermix containing 980 µl HiDi-formamide and 10 µl of the size standard GeneScan™ 500 ROX™ (Applied Biosystems) and separated on a capillary sequencer (Applied Biosystems). Raw data were visualized and manually scored using Genographer v1.6.0. (Montana State University 1998, http://hordeum.oscs.montana.edu/genographer), and the data exported as a presence/absence matrix. Only bands from 100 bp to 550 bp were considered to avoid homoplasy in shorter fragments.

Genetic admixture

Even though not fully appropriate due to violation of some population genetics-based assumptions, genetic admixture analysis in the program STRUCTURE v2.3.3 (Pritchard et al. 2000) was used to identify genetic clusters. Separate analyses were run for the entire data and each ploidy level. The admixture model with correlated allele frequencies and a first row containing 0 for the recessive allele, as implemented in Falush et al. (2007) was used. Polyploids were coded as proposed by Stöck et al. (2010). Each individual was coded as a formal octoploid (highest ploidy present in the dataset), assigning the missing chromosome sets in other ploidies as "missing data" (–9). The data were tested with K ranging from 1–10, with 10 replicate runs for each K and a burn-in period of 20×10^4 and 50×10^4 iterations. In order to find the most probable value of K, the STRUCTURE output files were analysed using the Structure Harvester v0.6.94 (Earl et al. 2012) and the Evanno's delta K (Evanno et al. 2005) and the outputs of the repeated runs of each K were averaged using CLUMPP v1.1.1. (Jakobsson & Rosenberg 2007). Similarity coefficients between each pair of STRUCTURE runs were calculated using the R-script STRUCTURE-sum v2011 (Ehrich et al. 2007).

In order to test for effects of genetic admixture (i.e. hybridization) on the genome size of individuals, we correlated relative genome size and the degree of genetic admixture recorded in the analysis of the entire data using the Spearman's correlation coefficient implemented in Statistica v6 (StatSoft 2002). Correlations were performed for each identified genetic cluster and separately for the (potentially introgressed) di-, tetra- and octoploid individuals. When a correlation was significant whether geographic distance between cytotypes is associated with the genome size of the introgressed cytotype was tested using linear regression (function lm of R). Relative genome size was the dependent variable, the shortest geographic inter-cytotype distance between sampled sites was the independent variable.

Results

DNA ploidy level and relative genome size

Coefficients of variation for the G0/G1 peak of the samples analysed for DNA ploidy ranged from 1.03 to 3.96 (mean = 2.19±0.58 SD). Three major distinct classes of sample/standard fluorescence ratios were identified. The classes corresponded to the di- (116 individuals), tetra- (292) and octoploid (261) DNA ploidy levels. One individual was inferred to be of intermediate DNA ploidy (about DNA pentaploid) (Fig. 2). Sample histograms of the majority of the ploidy cytotypes are provided in Electronic Appendix 3. DNA ploidies were assigned using 47 individuals for which the chromosome number was determined (Electronic Appendix 4). Six populations were of mixed ploidy (populations 6, 14, 23, 24, 34 and 42) and the others were uniformly di- (7 populations), tetra- (17) and octoploid (18). Measurements are provided in Electronic Appendix 5.

Variation in relative genome size was highest within tetraploids (up to 27.75% difference among individuals) and lowest in diploids (Table 1). Coprocessing of selected tetra- (population 6 - individuals 4 and 18; 17-11 and 17-17) and octoploid individuals (12-2 and 12-19; 20-5 and 20-14; 26-4 and 26-11; 28-1 and 28-3) with extreme values resulted in two distinct fluorescence peaks (Fig. 3) indicating real intra-ploidy variation in genome size within populations. Only a single peak was recorded for the two pooled diploid samples. Di-, tetra- and octoploid populations differed from each other in their mean relative genome size up to 6.85%, 13.88% and 8.63%, respectively (Fig. 4) with the more diverged populations statistically significantly different from each other (Electronic Appendix 6). Overall, 63.2%, 48.6% and 38.0% of the total variance occurred within populations (Electronic Appendix 6). Furthermore, there was a correlation between the relative genome size of individuals and the geographic distance separating them, which was significantly positive at $P = 0.05$ for only the tetraploids (Mantel test, $P < 0.001$; diploids: $P = 0.996$; octoploids: $P = 0.383$).

The greatest inaccuracy in measuring the relative genome size was ±2.55% (mean 0.85±0.59% SD, N individuals = 20). The DAPI fluorescence signal of the *Pisum* standard decreased by –1.43% when coprocessed with a valerian sample relative to the value obtained when analysed alone, but the difference was not statistically significant ($t = 2.030$, $df = 11$, $P = 0.067$).

Table 1. – Maximum difference in relative genome size (Δ) and absolute genome size (2C) among di-, tetra- and octoploid individuals of *Valeriana officinalis*. 'Δ selected' provides the range in relative genome size covered by individuals measured in addition to absolute genome size.

Ploidy	Relative genome size				2C
	$N_{individuals}$	N_{sites}	Δ	Δ selected	Δ
Diploids	116	20	13.64%	8.97%	3.03%
Tetraploids	292	40	27.75%	23.62%	21.11%
Octoploids	261	30	19.12%	19.12%	15.23%

Absolute genome size

Absolute holoploid genome size (2C-value) ranged from 2.95 pg to 3.04 pg (mean 2.98±0.03 pg) for the diploid individuals, 4.56–5.53 pg (5.10±0.38 pg) for the tetraploids and 8.16–9.40 pg (8.77±0.49 pg) for the octoploids (Table 2). When comparing the variation in the intra-ploidy level for individuals for which both the absolute and relative genome sizes were measured, we generally recorded less variation in absolute genome size. This decrease was strongest in diploids (i.e. the variation between individuals was reduced from 8.97% to 3.03%: Table 1).

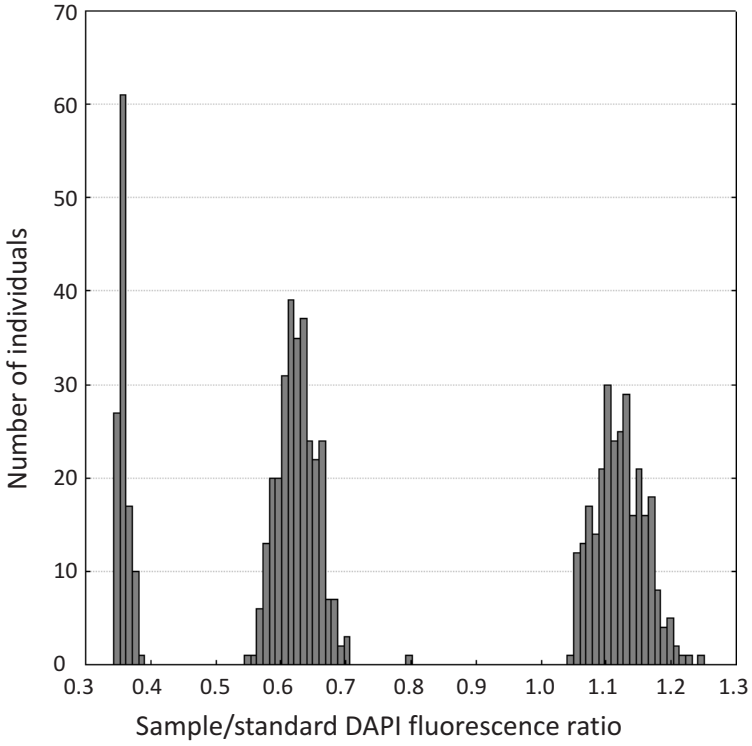

Fig. 2. – Relative genome size (sample: standard DAPI fluorescence ratio) inferred for 670 individuals of *Valeriana officinalis* using DAPI staining. The three major size classes correspond to DNA of di-, tetra- and octoploids, respectively.

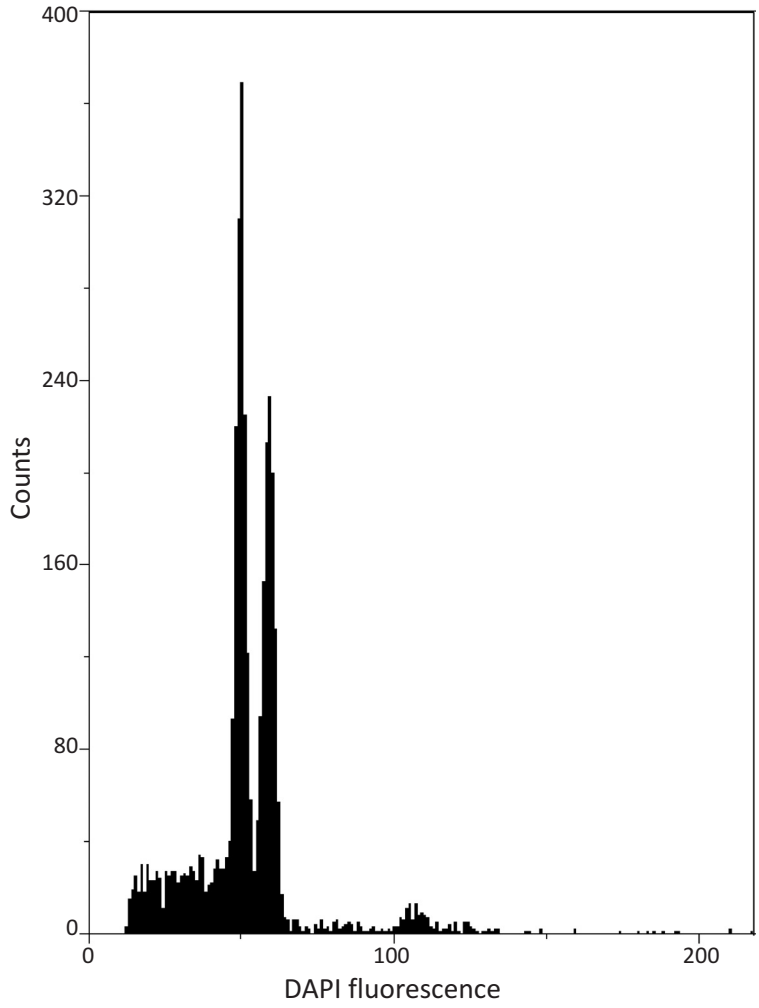

Fig. 3. – Flow cytometric histograms of tetraploid individuals no. 4 and 18 from population 6. The co-processed individuals differed in their mean DAPI-fluorescence by 18.20%. The value closely fitted the estimate based on separate measurements of these individuals (Δ 18.06%). The comparison is an example of the variation in intraploidy genome size frequently recorded in polyploid populations of *Valeriana officinalis*.

Monoploid genome size (1Cx-value) was calculated for five di-, seven tetra- and four octoploid individuals for which there was an exact euploid chromosome number (Electronic Appendix 4). Cytotypes differed significantly in monoploid genome size in all pairwise comparisons (one-way ANOVA: $F = 32.83$, $df = 2$, $P < 0.001$, Bonferroni's test $P \leq 0.003$) with monoploid genome size tending to decrease with ploidy: the mean for the diploids was 1.48 pg, 1.29 pg for the tetraploids, and 1.10 pg for the octoploids (Table 2). We recorded up to 21.11% and 12.89% difference in monoploid genome size among euploid tetra- and octoploids, respectively, compared to 0.92% among the euploid diploids. The greatest inaccuracy in measuring the absolute genome size was ±1.25% (mean 0.30±0.24%).

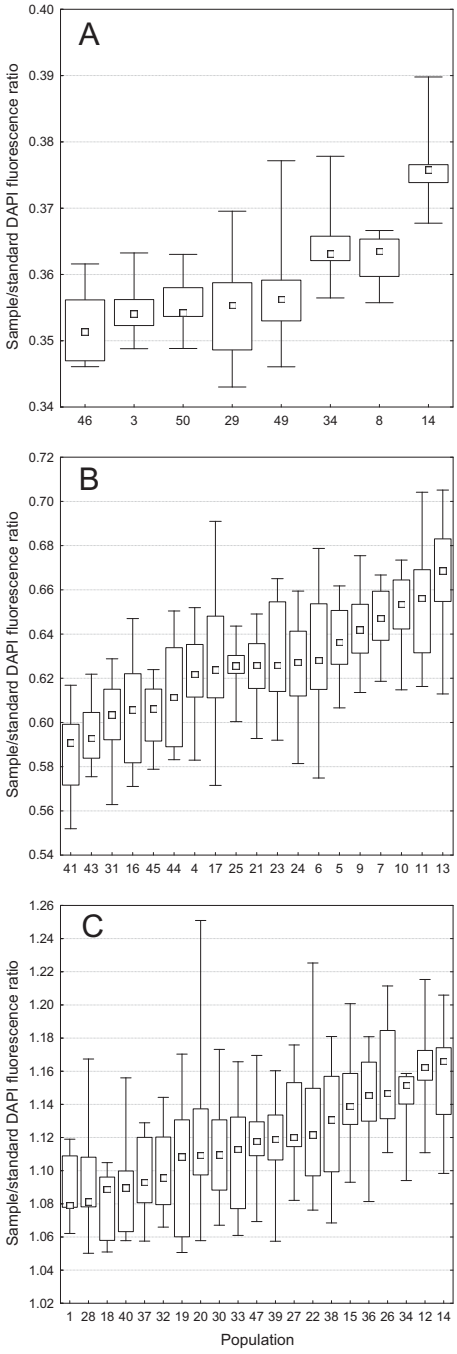

Fig. 4. – Variation in relative genome size within (A) di-, (B) tetra- and (C) octoploid populations of *Valeriana officinalis*. The box plots show the median, the 25%/75% percentiles and the maximum / minimum values of the sample/standard fluorescence ratio. Populations are arranged in ascending order of their medians.

Table 2. – Absolute genome sizes (2C and 1Cx) of 25 individuals of *Valeriana officinalis* of three ploidy levels. Index PI is the mean sample/standard PI fluorescence (calculated from three repeat measurements). Δ 2C is the difference in genome size compared to the lowest value recorded within each ploidy level. Individuals are arranged in ascending order based on their 2C-values. 2x, 4x and 8x refer to di-, tetra- and octoploids, respectively.

Population	Individual	Ploidy	Index PI	Chromosome number	2C [pg]	Δ 2C [%]	1Cx [pg]
3	10	2x	1.139	14	2.949	0.00	1.475
3	14	2x	1.143	14+1	2.960	0.36	–
46	13	2x	1.144	14	2.964	0.49	1.482
50	7	2x	1.145	14	2.964	0.52	1.482
50	18	2x	1.148	14	2.973	0.82	1.487
46	10	2x	1.149	14	2.976	0.92	1.488
34	15	2x	1.152	14+1	2.983	1.15	–
34	19	2x	1.173	14+1	3.038	3.03	–
						mean	**1.483**
6	4	4x	1.762	28	4.563	0.00	1.141
13	19	4x	1.841	27	4.768	4.50	–
23	14	4x	1.857	28	4.809	5.40	1.202
17	11	4x	1.885	28	4.883	7.00	1.221
13	11	4x	2.054	28	5.323	16.65	1.330
23	18	4x	2.102	28	5.444	19.30	1.361
17	17	4x	2.109	28	5.461	19.67	1.365
6	18	4x	2.134	28	5.527	21.11	1.382
						mean	**1.286**
14	15	8x	3.151	–	8.161	0.00	–
26	11	8x	3.216	56	8.330	2.06	1.041
28	3	8x	3.219	–	8.336	2.14	–
20	14	8x	3.285	56	8.507	4.24	1.063
34	12	8x	3.420	56	8.858	8.54	1.107
14	17	8x	3.489	–	9.036	10.72	–
28	1	8x	3.515	–	9.103	11.54	–
20	5	8x	3.585	–	9.285	13.77	–
26	4	8x	3.631	56	9.404	15.23	1.176
						mean	**1.097**

Aneuploidy was recorded for three diploid individuals, which had one extra chromosome and in one tetraploid individual with 2n = 27. Two of the aneuploid diploids had the largest absolute genome sizes (2C-value) compared with their euploid counterparts. The third hyper-aneuploid diploid was next-to-last as was the hypo-aneuploid tetraploid in terms of this value among the eight individuals studied (Table 2).

AFLPs and the association of genetic admixture with relative genome size

The six primer combinations resulted in 311 AFLP fragments ranging in size from 102 bp to 534 bp. The repeatability (technical difference rate; Bonin et al. 2004) was 95.8% to 99.7%. For diploid individuals 58–115 fragments (all studied diploids together 197), tetraploids 57–122 (232) and octoploids 42–92 (248) were recorded. In addition, di-, tetra- and octoploids had 22, 19 and 48 unique (private) fragments, respectively. In the admixture analyses the highest ΔK was assigned to K = 4, but the ΔK for K = 2 was only slightly lower

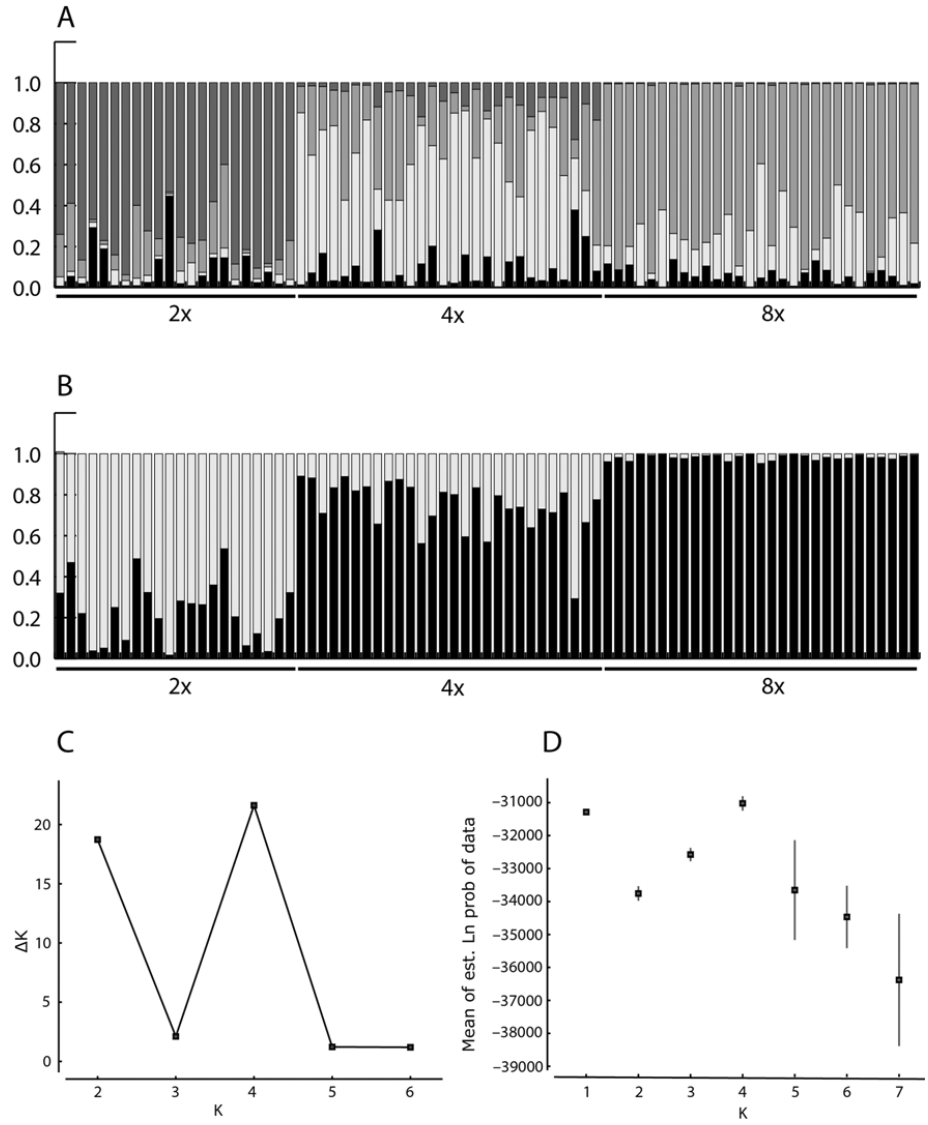

Fig. 5. – Results of the AFLP-based admixture analyses of 79 individuals of *Valeriana officinalis* showing the cluster membership of di- (2x), tetra- (4x) and octoploids (8x) for (A) K = 4 and (B) K = 2, (C) ΔK values, and (D) mean likelihood of K values for Ks ranging from 1 to 7. Individuals are sorted in ascending order based on their relative genome size.

(Fig. 5). Ten STRUCTURE runs for both K = 4 and K = 2 indicated nearly identical individual membership coefficients with pairwise similarity coefficients of 0.972±0.015 SD and 0.989±0.006 SD, respectively. For K = 2, the genetic clusters approximately corresponded to di- and octoploids. At least some individuals of each ploidy level exhibited genetic admixture, which was greatest for the tetraploids (Fig. 5). The relative genome size was significantly positively correlated with the percentage of the diploid genetic partition

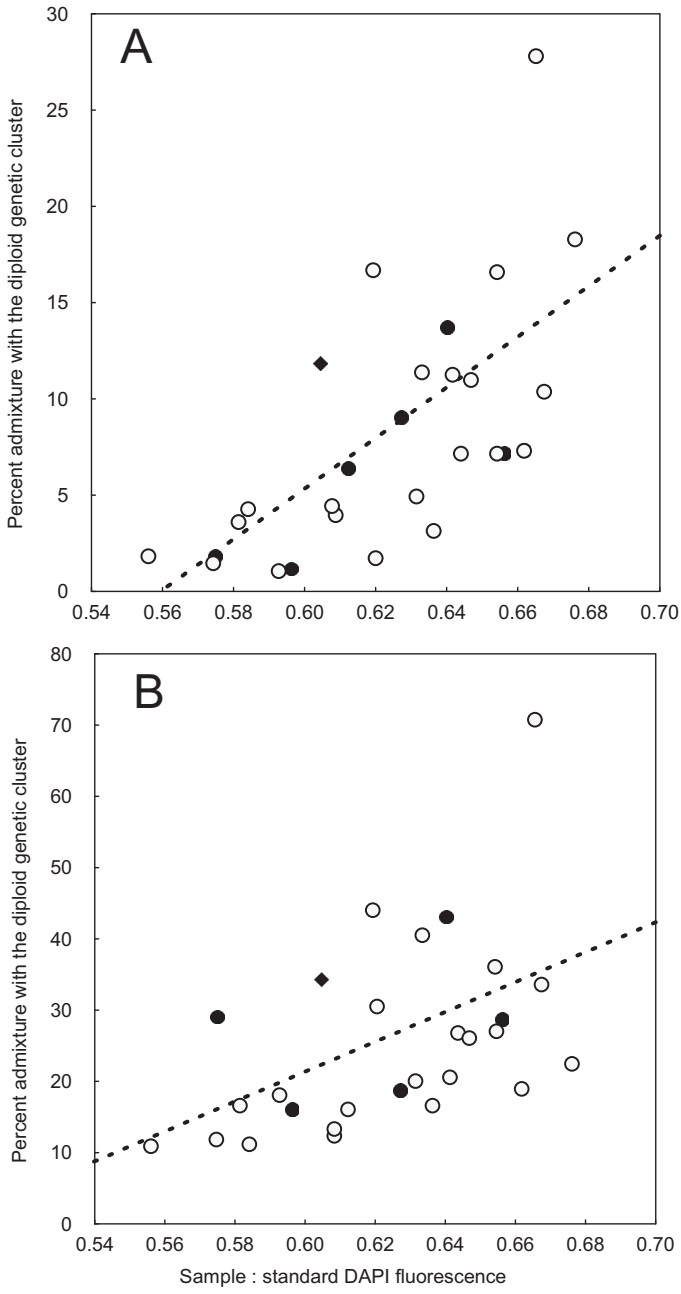

Fig. 6. – Regression of percentage admixture from diploids against relative genome size (sample: standard DAPI fluorescence) of tetraploid individuals of *Valeriana officinalis*. Genetic clusters were identified using a STRUCTURE analysis. a) and b) correspond to alternative partitions of genetic variation into four and two clusters, respectively. Filled symbols distinguish individuals for which the chromosome numbers were determined (dots: 2n = 28, diamond: 2n = 27–28).

(and accordingly negatively with the octoploid genetic cluster) in tetraploid individuals ($P < 0.05$; Fig. 6A), whereas the relation was insignificant in the di- and octoploids (data not shown). Although additional genetic partitions were assigned for $K = 4$ (Fig. 5), two clusters corresponded to di- and octoploids and two to tetraploids and an unspecific (or unidentified) group. Again, the relative genome size of tetraploid individuals was significantly positively correlated with the percentage of the diploid genetic partition ($P < 0.05$; Fig. 6B). In contrast, correlations between relative genome size and the degree of genetic admixture were statistically not significant in tetraploids in the other genetic clusters as well as generally for the di- and octoploid individuals. The parallel increase in the genome size of the tetraploids and the diploid genetic partition is also seen in Fig. 5, while such a trend was not obvious for the di- and octoploids. Relative genome size of tetraploids was furthermore slightly but significantly negatively related to geographic distance between the di- and tetraploid individuals (linear regression: genome size = $0.6323 - 0.0006 \times$ geographic distance in km, $P = 0.02$).

Discussion

Assessment of the variation in genome size

In agreement with earlier reports, we found individuals of the three major ploidy levels (di-, tetra- and octoploid), whereas the high intra-ploidy variation in relative genome size was unexpected. Our data indicated that the intra-ploidy variation in genome size is real, i.e. was not caused by systematic or random errors. We recorded two distinct fluorescence signals (peaks) for coprocessed individuals (Fig. 3) that differed in relative genome size when measured separately, which is a strong indicator that the differences are due to real variation in genome size and not "technical noise" (Noirot et al. 2000, Greilhuber 2005a, Greilhuber et al. 2007). To evaluate measurement error as a possible cause, measurement inaccuracy was determined by making replicate measurements of single individuals and found to be at most ±2.55%, much smaller than the variation recorded within any of the DNA ploidy levels. We finally considered the possibility that variation among individuals may be due to a stoichiometric error caused by staining inhibition. Although, we coprocessed the sample and the standard, which should minimize the risk of endogenous staining inhibition mimicking DNA differences, secondary compounds of plants can affect these measurements (Greilhuber 1998, Price et al. 2000). However, we did not record a statistically significant decrease in nuclei fluorescence of the internal standard when coprocessed together with the valerian sample compared to the standard measured alone. The insignificant role of fluorescence inhibition may be explained by the chemical properties of valepotriates, which are unstable, thermolabile compounds, decomposing rapidly in acidic or alkaline conditions (Bos et al. 2002). As the extraction buffer Otto I used is strongly acidic (pH 2–3) and considering the fact that valepotriates occur mainly in roots and rhizomes, we suppose that the content and effect of valepotriates in leaf extracts should be negligible.

Variation in relative genome size among the individuals analysed using both flow cytometric methods exceeded their corresponding absolute variation in genome size for all three ploidy levels (Table 1). The maximum difference in relative genome size among individuals thus increased the corresponding values in absolute genome size by 5.94%

for the diploids, 3.89% for the octoploids and 2.51% for the tetraploids (Table 1). The higher variation recorded for the DAPI-stained samples could be explained by the higher error involved in measuring relative genome size (0.85±0.59% SD) compared to absolute genome size (0.30±0.24% SD) and the use of single values for the establishment of relative genome size but averaged values for absolute genome size.

Processes causing changes in genome size within ploidy levels

Intra-ploidy variation in genome size has two primary sources: mechanisms leading to a change in the DNA content of monoploid genomes (or of chromosomes; e.g. Bennetzen et al. 2005, Grover & Wendel 2010) and change in chromosome number (e.g. Zoldoš et al. 1998). Our data indicate that both sources played a role in the evolution of *Valeriana officinalis*, but evidence differed for the ploidy levels studied.

Monoploid genome size was almost constant in diploids (1Cx = 1.48–1.49 pg, corresponding to a maximum difference among individuals of 0.92%) but varied widely in tetraploids (1 Cx = 1.14–1.38 pg; 21.11%). Variation in octoploids was intermediate to the former (1 Cx = 1.04–1.18 pg or 12.89%). Hence, our data support variation in genome size due to variability in the DNA content of monoploid genomes in polyploids but not in diploids or only marginally so. Monoploid genome size tentatively fitted the results of Hidalgo et al. (2010), who report 1Cx = 1.49 pg for diploid and 1Cx = 1.02 pg for octoploid *V. officinalis*. As with our data monoploid genome size was negatively correlated with ploidy, a relation which is in accordance with the prevailing downward direction in genome size evolution (i.e. genome downsizing) in the angiosperms (Leitch & Bennett 2004). However, although decrease in genome size with ploidy in valerian followed the overall trend, it was less (on average –26.0% in octoploid compared to diploid valerian) than the decrease reported for both eudicots (–56.0%) and Asterids (–40.9%) (Leitch & Bennett 2004), which belong to the *Caprifoliaceae*.

Change in monoploid genome size may either be gradual, through DNA loss or gain, or instantaneous via hybridization. We suppose that the latter process has contributed to the variation in relative genome size inferred for tetraploids at least. This interpretation is based on the positive correlation of relative genome size with the proportion of the diploid genetic cluster shared by tetraploids (Fig. 6) and the larger average monoploid genome size of diploids compared to tetraploids (Table 2). Interestingly, in two of the six genetically studied populations (6 and 42) we found diploids co-occurring with a tetraploid individual that had the highest percentage of admixture (55%) recorded in our data. An effect of introgression in increasing genome size is supported by the regression of the relative genome size of tetraploids against their geographic distance to the most proximate diploids, which was significantly negative. Genome size additivity in hybrids among taxa differing in nuclear DNA content is not exceptional and has been detected in several plant genera (e.g. *Elytrigia*: Mahelka et al. 2005; *Dryopteris*: Ekrt et al. 2010; *Amaranthus*: Jeschke et al. 2003; *Cerastium*: Vít et al. 2014). The increase in relative genome size could not be explained in this study by chromosome addition, because genetically admixed and thus supposedly hybrid individuals analysed for chromosome number were eutetraploid (with one individual possibly hypo-tetraploid). Furthermore, the higher variability in monoploid genome size in tetraploids (21.11%) indicates that it is more likely that variation in the DNA content of chromosomes accounts for the increase.

The recording of aneuploidy in diploid *V. officinalis* is a novel finding of this study. Two (out of three) of the hyper-diploid individuals had holoploid genomes, which were larger (by up to 3.03%) than those of the euploids, suggesting a significant contribution of the extra chromosomes to the variation in genome size in diploids. Suitability of FCM for detecting variation in genome size due to extra chromosomes has been demonstrated for various plant groups. For example, Zoldoš et al. (1998) report an up to 1.06-fold difference in genome size due to extra chromosomes within a population of diploid *Quercus petraea* (*Fagaceae*) and Sharbel et al. (2005) statistically significant differences in relative fluorescence between eudiploids and diploids carrying a B chromosome in *Boechera holboellii* (*Brassicaceae*). In contrast, in tetraploid *V. officinalis* variation due to aneuploidy did not exceed the variation recorded in euploids, indicating that the extent of variation in increments of DNA per monoploid genome precluded the identification of aneuploidy in tetraploids. Inclusion of variability in extra chromosomes (i.e. B chromosomes) in the regular variability in genome size is also assumed for tetra- and diploid *Festuca pallens* (*Poaceae*) (Šmarda & Bureš 2006, Šmarda et al. 2007) and species of the *Anthoxanthum aristatum/ovatum* complex (*Poaceae*) (Chumová et al. 2016).

Potential evolutionary causes of population differentiation in genome size

Though stability of monoploid genome size in species is considered the norm (Bennett & Leitch 1995), intraspecific variation is recorded in several species (e.g. Schmuths et al. 2004, Šmarda & Bureš 2006, Suda et al. 2007, Slovák et al. 2009). In *V. officinalis*, 38.0–63.2% of the total intra-ploidy variation in relative genome size occurred within populations, which argues for the existence of variation in genome size within even narrowly defined species (i.e. "types"). In accordance with this interpretation is the recently recorded 1.12-fold variation in genome size in two subspecies of tetraploid *Valeriana pratensis* in southern Germany (Gregor et al. 2016).

Population demographic processes may promote differentiation in genome size in space through isolation by distance. We did not find, however, a significant correlation between spatial distance and difference in genome size between individuals for both di- and octoploids at a geographic scale. The geographic scale in our study corresponds to (part of) the distributions of the diploid type *V. exaltata* and octoploid *V. procurrens*, indicating that genome size is not geographically structured in these proposed taxa in the area studied. A comparable pattern of intra-ploidy differentiation is recorded in *Picris hieracioides* (*Asteraceae*), a species with a 1.37-fold range in genome size. The variation persists after splitting the data into two altitudinal morphotypes (Slovák et al. 2009). The significant negative correlation between genome size and geography found for tetraploid valerians, in contrast, might be due to hybridogenous introgression in areas where they are sympatric with diploids.

Frequencies of cytotypes of deviant ploidy and of aneuploids

There was one DNA pentaploid individual in our sample (Fig. 2). In addition, there are two reports of hexaploid *V. officinalis* (Heuberger et al. 2012, Hidalgo & Vallès 2012), confirming that individuals of intermediate ploidy level occur sporadically in natural populations. These deviant ploidy levels are expected from occasional interploidy crosses or non-reduction of gametes. Hypothetically the origin of the pentaploid can be

explained by the fusion of reduced gametes from di- and octoploid individuals. We further provide the first account of aneuploidy in diploid *V. officinalis*. So far, there is only one report of extra chromosomes in this taxon by Corsi et al. (1984), who describe a B chromosome in tetraploid *V. officinalis* subsp. *collina* in Italy. The extra chromosomes recorded in our study may be B chromosomes as they varied in number (0–1) between and within populations, which is a characteristic of this type of chromosome. B chromosomes are not essential for the organism, are often heterochromatic and rich in repetitive sequences (Sharbel et al. 2004), features, however, which we have not yet established. In the numerous chromosome counts for *V. officinalis* aneuploidy is never mentioned (Titz 1964, 1969, Titz & Titz 1979, 1981), and one could suppose that the occurrence of aneuploidy is relatively rare. However, contrary to this, we recorded this condition in two out of six diploid populations.

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Souhrn

Polyploidie, aneuploidie a změny v obsahu DNA monoploidního genomu nebo chromozomů jsou nejdůležitějšími příčinami variability ve velikosti jaderného genomu. S cílem odhalit mechanismy podílející se na evoluci velikosti genomu jsme studovali tyto jevy ve středoevropských populacích okruhu *Valeriana officinalis*, který zahrnuje diploidy, tetraploidy a oktoploidy tvořící morfologicky definované typy, jakož i typy přechodné, jejichž existence komplikuje tradiční taxonomické hodnocení. K odhalení genetické struktury studovaného materiálu jsme použili metodu AFLP. Potvrdili jsme převahu diploidů, tetraploidů a oktoploidů, kteří zhruba odpovídali geneticky definovaným skupinám a lišili se mezi sebou i v monoploidní velikosti genomu. Některé studované rostliny, zvláště na tetraploidní úrovni, byly ovlivněny hybridizací, která způsobila zvýšení variability ve velikosti genomu. Ta byla u tetraploidů negativně korelována s geografickou vzdáleností k nejbližším diploidním populacím. Výrazná variabilita ve velikosti genomu byla však zjištěna i v rámci ostatních ploidních úrovní, u kterých byla způsobena aneuploidií. Variabilita v monoploidní velikosti genomu byla zjištěna u polyploidů, ale nikoliv u diploidů, což naznačuje, že polyploidi více podléhají změnám v evoluci genomu. 38,0–63,2 % variability v relativní velikosti genomu uvnitř ploidní úrovne se nacházelo na vnitropopulační úrovni. Závěrem lze konstatovat, že okruh *Valeriana officinalis* se vyznačuje variabilitou ve velikosti genomu v důsledku čtyř hlavních evolučních mechanismů: polyploidizace, aneuploidie, změn v obsahu DNA chromozomů a hybridizace, a že jejich význam se liší mezi ploidními úrovnemi. Přestože stabilita v monoploidní velikosti genomu je považována za stabilní znak druhů, v naší studii jsme odhalili velkou variabilitu uvnitř populací i úzce definovaných druhů.

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