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ENDOPOLYPLOIDY PATTERNS IN ORGANS OF *TRIFOLIUM* SPECIES (FABACEAE)

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The pattern of endopolyploidy in the genus *Trifolium* was studied in mature organs of *T. montanum* and *T. repens* at reproductive stage, with comparative data for *T. pratense*, all from natural populations. Endopolyploidy in root, stem, petiole, leaf, inflorescence stalk, sepal, petal, stamen and carpel was detected by flow cytometry. 2C, 4C and 8C nuclei were found in organs of *T. montanum* and *T. repens*, and additionally 16C nuclei in organs of *T. repens*. The organs of *T. montanum* and *T. repens* differed in degree of endopolyploidy based on cycle values calculated from flow cytometry data; it was lowest in leaf and sepal in *T. montanum* and *T. repens*, and highest in *T. montanum* in petal and carpel and in *T. repens* in petiole and inflorescence stalk. These results are also seen in the two or more peaks of interphase nuclei in the flow cytometry histograms. There were significant correlations between the organs of *T. pratense* and *T. repens* as well as substantial differences between *Trifolium* species in the degree of endopolyploidy. *T. pratense* showed higher absolute endopolyploidy than *T. montanum* and *T. repens*. Principal component analysis showed that individuals of *T. repens* and *T. montanum* are more similar to each other than to individuals of *T. pratense* in degree of endopolyploidy. The observed variation between species might be explained by phylogenetic relationships and genome size differences.

Key words: Cycle value, endopolyploidy degree, endoreduplication, flow cytometry, *Trifolium*.

INTRODUCTION

Endoreduplication is doubling of nuclear DNA material in a single cell without mitosis. Single endoreduplication cycles or series of cycles lead to endopolyploidy (Traas et al., 1998; Joubés and Chevalier, 2000). The co-occurrence of cells with different ploidy levels in organs/tissues of a single individual is called polysomaty (Smulders et al., 1994). Endopolyploidy is essential for the normal development and physiology of polysomatic plants (Barow, 2006; Lee et al., 2009).

Systemic somatic endopolyploidization has been reported in many plant species (de Rocher et al., 1990; Galbraith et al., 1991; Gilissen et al., 1993; Kudo and Kimura, 2001; Barow and Meister, 2003; Lim and Loh, 2003). Those studies suggest very frequent occurrence of endopolyploidy in angiosperms, but it is characteristic for certain families. The species in important and species-rich families such as Brassicaceae, Fabaceae, Poaceae and Solanaceae are predominantly polysomatic (Barow and Meister, 2003). Different species within a single family may express different degrees of endopolyploidy, as in Poaceae and Fabaceae (Barow and Meister, 2003).

Degree of endopolyploidy varies between organs (Barow and Meister, 2003; Barow, 2006). In certain organs, endopolyploidy degree may vary between tissues and even different types of cells such as trichomes and pavement cells (Barow and Jovtchev, 2007). Generally, endopolyploidy is highest in stamens, petioles and cotyledons, lower in flower organs and roots, and lowest in upper leaves (Barow, 2006). The pattern of endopolyploidy is characteristic for the developmental stage (Smulders et al., 1994; Sliwinska and Lukaszewska, 2005; Kolano et al., 2008; Ogawa et al., 2012). These circumstances indicate spatial and temporal regulation of endopolyploidy.

Many factors can influence the occurrence and degree of endopolyploidy in plant species. A broad study by Barow and Meister (2003) confirms that family affiliation is the major factor influencing the degree of endopolyploidy, while life cycle, genome size and organ type have minor but also significant

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effects. Annuals and biennials are more frequently endopolyploid than perennials are (Barow and Meister, 2003). In angiosperms a negative correlation between endopolyploidization and genome size has been observed (Nagl, 1976; de Rocher et al., 1990; Barow and Meister, 2003; Bainard et al., 2012). Jovtchev et al. (2006) demonstrated a highly positive correlation between DNA content and nuclear volume and a significant positive correlation between nuclear volume and cell volume. Proper functioning of cells depends on their size, and the functioning of tissues and organs of course depends on their cells. Cells grow in size through water uptake and vacuolar growth, or through endopolyploidy when nuclear DNA content increases (Maluszynska et al., 2012). Endopolyploidization might speed up the size increase of polysomatic species and compensate for small genomes (Barow, 2006). DNA endoreduplication might be seen as an evolutionary alternative which compensates a deficiency in the phylogenetically determined nuclear DNA amount in some species (Nagl, 1976). Bainard et al. (2012) found a correlation between both genome size and endoreduplication and some phenotypic traits. However, phylogeny and life strategy have a stronger impact on endopolyploidy than genome size (Barow and Meister, 2003; Jovtchev et al., 2006). Environmental factors may also influence endopolyploidy degree (Barow and Jovtchev, 2007). These processes may reflect adaptation to certain habitats during evolution (Barow, 2006).

The relationships between polyploidy and endopolyploidy are not clear. According to some authors, artificial polyploids and the corresponding diploid ancestors show similar polysomaty patterns (Smulders et al., 1994; Ogawa et al., 2012). Other researchers found that artificially generated tetraploids had lower endopolyploidy than their ancestral diploids (Biradar et al., 1993; Mishiba and Mii, 2000; Sliwinska and Lukaszewska, 2005; Chen et al., 2011). In other work, natural polyploids showed lower endopolyploidy than diploids of the same or closely related taxa (Jovtchev et al., 2007). Moreover, differences in degree of endopolyploidy between natural polyploids and their diploid counterparts were higher than between artificially generated polyploids and the corresponding diploids (Jovtchev et al., 2007).

Fabaceae is a family with several polysomatic species (Kondorosi et al., 2000; Barow and Meister, 2003; Kocová and Mártonfi, 2011; Bainard et al., 2012). *Trifolium* L., with more than 250 species, is one of the largest genera in Fabaceae (Zohary and Heller, 1984). *Trifolium* includes diploid and polyploid species with different basic chromosome numbers. The ancestral chromosome number in *Trifolium* is diploid 2n=16 (x= 8) (Ellison et al., 2006). The species analyzed in this study belong to subgenus *Trifolium*. Diploid *T. pratense* L. is in sect. *Trifolium*; diploid *T. montanum* L. and tetraploid *T. repens* L. are in sect. *Trifoliastrum* S.F.Gray (Ellison et al., 2006). These three species are characterized by a perennial habit. Red clover (*T. pratense*) and white clover (*T. repens*) are among the most important pasture legumes and are widely distributed in temperate regions throughout the world. They can adapt to a wide range of edaphic and other environmental conditions. Mountain clover (*T. montanum*) is adapted to life on dry calcareous and sandy soils. Its habitats are dry meadow, forest clearings, steppe, grassy places and mountain slopes.

This study was designed to detect endopolyploidy and determine its degree in organs of *T. montanum* and *T. repens*. Previous work (Barow and Meister, 2003; Kocová and Mártonfi, 2011) showed the occurrence of endopolyploidy and differences of endopolyploidy degrees in organs of *T. pratense*. We assumed that it also occurs in its co-geners *T. montanum* and *T. repens*, and intended to compare the endopolyploidy patterns in *T. pratense* with those in the presently analyzed *T. montanum* and *T. repens*. Possible factors responsible for differences in endopolyploidy between the species – phylogenetic position, genome size and ploidy level – are discussed.

MATERIALS AND METHODS

PLANT MATERIAL

All plant material used for this research was collected in Slovakia from natural populations. Mature plants in reproductive stage were collected: *T. repens* in Košice (48°44'07"N, 21°14'19"E – 48°44'25"N, 21°15'00"E) and *T. montanum* in Kružná (48°38'17"N, 20°27'32"E), Rožňavské Bystré (48°39'27"N, 20°25'23"E) and Lipovce (49°03'29"N, 20°56'33"E). Whole plants were stored at 4°C until used for analyses of endopolyploidy: 31 plants of *T. repens* and 28 of *T. montanum*. Four or 5 plants of *T. pratense* and *T. repens* (from Košice) or a minimum 10 *T. montanum* plants (from Kružná) were potted. Three repotted plants of each species were used to determine genome size and chromosome number. The *T. pratense* and *T. repens* plants were grown in pots outdoors. The *T. montanum* plants were grown in pots in standard laboratory conditions.

The comparative data for *T. pratense* were taken from Kocová and Mártonfi (2011).

DETERMINATION OF CHROMOSOME NUMBER

To determine the chromosome numbers of the species, root tips of cultivated *Trifolium* were immersed in 8-hydroxyquinoline for ~4 h, fixed in a

solution of 97% ethanol and concentrated acetic acid (3:1) for 24 h, washed in distilled water and hydrolyzed in 1 N HCl at 60°C for 5 min. Then the root tips were washed in distilled water again and prepared for microscopy. A drop of 45% acetic acid was added to root tips lying on the microscopic slide and covered with cellophane (Murín, 1960). Meristems of root tips were squashed under cellophane, then the slides were dried and the cellophane removed. Meristems were stained in 10% Giemsa stain solution in Sørensen phosphate buffer. After 24 h the slides were washed in distilled water. Meristems were observed under oil immersion at 100× with a Leica DM 2500 microscope equipped with a DFC 290 HD camera, using the Leica ver. 3 application suite.

DETERMINATION OF GENOME SIZE

Leaves of three different plants of *T. pratense*, *T. montanum* and *T. repens* (n = 9) were used to estimate nuclear DNA content by flow cytometry. The internal reference standards for estimating genome size in *T. repens* and *T. montanum* was *Solanum lycopersicum* cv. Stupicke (2C = 1.96 pg DNA, Doležel et al., 1992; 1C is the DNA content in the unreplicated gametic nucleus of an organism; Bennet and Smith, 1976; see also Greilhuber et al., 2005), and *Raphanus sativus* cv. Saxa (2C = 1.11 pg DNA, Doležel et al., 1992) was used for *T. pratense*. The seeds of the standards were acquired from the Institute of Experimental Botany, Olomouc, Czech Republic, and grown in the laboratory. Leaf pieces (~1 cm^2) of the *Trifolium* species and the standards were chopped together with a razor blade in a Petri dish in 1 ml general purpose buffer (0.5 mM spermine.4HCl, 30 mM sodium citrate, 20 mM MOPS, 80 mM KCl and 20 mM NaCl, 0.5% (v/v) Triton X-100, pH 7.0) according to Loureiro et al. (2007). The resulting nuclear suspension was filtered through 42 μm mesh nylon filter. Then the samples were supplemented with 10 $\mu g.ml^{-1}$ propidium iodide for DNA staining, 10 $\mu g.ml^{-1}$ RNase and 2 μl mercaptoethanol.

Measurements were made with a Partec CyFlow ML (Partec Gmbh, Münster, Germany) flow cytometer at the Institute of Biological and Ecological Sciences, P. J. Šafárik University, Košice, Slovakia. This laser flow cytometer is equipped with an argon-ion laser operating at 532 nm. Three plants of each species were measured 3 times on 3 different days, making 9 measurements for each species. Histograms of the data were displayed on a linear scale (x-axis). At least 10 000 nuclei per measurement were collected and the coefficients of variation (CV) of the G0/G1 peaks of both the samples and the internal standards did not exceed 5%. Data were analyzed with FloMax 2.7 (Partec Gmbh, Münster).

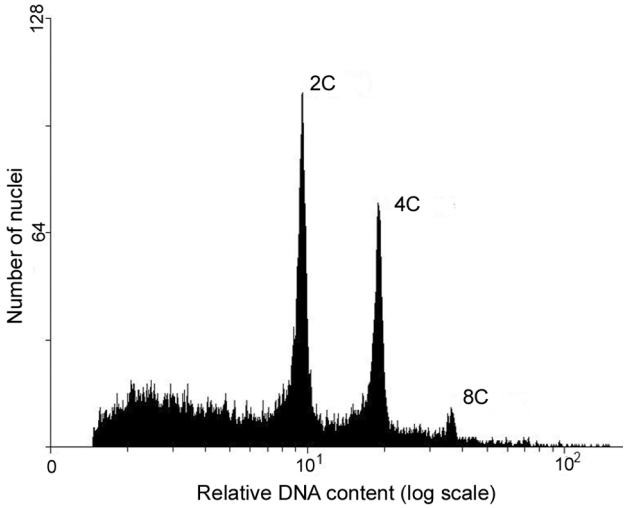

Fig. 1. Typical flow cytometry histogram for suspension of nuclei from *T. repens* inflorescence stalk.

Nuclear genome size in the *Trifolium* species was calculated as follows (Doležel and Bartoš, 2005):

$$\text{Sample 2C DNA content} = \frac{\text{2C sample peak mean}}{\text{2C standard peak mean}} \times \text{standard 2C DNA content (pg)}$$

DETERMINATION OF ENDOPOLYPLOIDY DEGREE

Samples for endopolyploidy analyses were prepared from root, leaf, petiole, stem, inflorescence stalk, sepal, petal, stamen and carpel within one day (*T. repens*, *T. montanum*) or two days (*T. montanum*) after collecting from the field. For preparation of root and stem samples of *T. montanum*, only parts of the organs were excised, mainly the middle part. For preparation of inflorescence stalk samples of *T. montanum*, parts of the upper stem below the inflorescence were used. For preparation of leaf samples, one leaflet in the case of *T. montanum* or all three leaflets in the case of *T. repens* were used. Small flower parts such as sepals, petals, stamens and carpels were removed from the inflorescence with a razor blade, tweezers and dissecting needles and prepared, usually 15 pieces from a single plant per sample. In the next step, samples of organ pieces for flow cytometric determination of endopolyploidy were prepared in the same way as samples for genome size estimation, but without adding an internal standard.

Samples were measured using the same flow cytometer as for genome size estimation. The data are displayed on a logarithmic scale (x-axis) (Fig. 1). Histograms were evaluated using WinMDI 2.9 (Trotter, 2000). To improve the quality of the flow cytometry histograms (peak symmetry, CV values, effect of debris), some measurements were excluded

prior to calculating endopolyploidy. The pattern of endopolyploidy in organ samples is described by cycle value, representing degree of endopolyploidy. Cycle value was defined by Barow and Meister (2003) using the following formula:

$$\text{Cycle value} = \frac{(0 \times n_{2C} + 1 \times n_{4C} + 2 \times n_{8C} + 3 \times n_{16C} \ldots)}{(n_{2C} + n_{4C} + n_{8C} + n_{16C} \ldots)}$$

where n2C, n4C, n8C, n16C represent the numbers of nuclei (identical with peak height) with the corresponding ploidy level (2C, 4C, 8C, 16C). An organ with a cycle value under 0.1 was considered not to be endopolyploid (Barow and Meister, 2003).

STATISTICAL METHODS

Normality of the data distribution was achieved by decimal logarithmic transformation (log10 transformation). ANOVA was used to test differences between *Trifolium* species for particular organs and differences between organs within species. Tukey's HSD test was applied for separation of species and organs into homogeneous groups. Spearman's rank correlation was used to test the associations between *Trifolium* species in organ-specific endopolyploidy expression. All analyses were done in R environment ver. 2.13.1 (R Development Core Team, 2011) using the basic base and stats packages .

Principal component analysis (PCA) was performed to determine the overall variability of endopolyploidy degree among individuals of all three taxa. Each of the 92 studied individuals were considered operational taxonomic units (OTUs) and a data matrix with organs in columns and individuals in rows was constructed. Any missing data in the matrix were replaced by the mean cycle value for the respective organ of that species. Centered and scaled raw data were then subjected to PCA using the dudi.pca function of the ade4 package (Dray and Dufour, 2007) in R environment ver. 2.13.1 (R Development Core Team, 2011).

RESULTS

CHROMOSOME NUMBER AND GENOME SIZE DETERMINATION

Our karyological analyses showed that the somatic chromosome number for diploid *T. pratense* is 2n=2x=14, for diploid *T. montanum* it is 2n=2x=16, and for tetraploid *T. repens* it is 2n=4x=32.

Genome size of 2C DNA is estimated at 0.9 ± 0.015 pg (mean ± SD) for *T. pratense*, 2.48 ± 0.022 pg for *T. montanum* and 2.19 ± 0.010 pg for *T. repens*.

DETERMINATION OF ENDOPOLYPLOIDY DEGREE

We found that *T. montanum* and *T. repens* are polysomatic species (Tabs. 1–4).

All organs of *T. montanum* were endopolyploid and varied in degree of endopolyploidy (Tabs. 1, 2). In organs of *T. montanum* we identified 2C, 4C and 8C nuclei. A 4C peak occurred on the histograms for all organ samples. The frequency of nuclei with 4C DNA content was lowest in leaf (13.0%) and highest in carpel (40.0%) and petal (41.0%). The shares of 8C nuclei were very low; they were identified in root, stem, petiole, inflorescence stalk and carpel. Every organ showed considerable variability of cycle values (Tab. 2). The cycle value was lowest in a leaf sample (0.06, non-endopolyploid) and highest (0.8) in a carpel sample. Degree of endopolyploidy in *T. montanum* was lowest in leaf (0.13) and sepal (0.17) and highest in reproductive organs, petal (0.41) and carpel (0.44).

We calculated the mean cycle value of all measured organs in each species to obtain an absolute cycle value (absolute species degree of endopolyploidy = mean cycle value of organs per species). Absolute degree of endopolyploidy in *T. montanum* was calculated at 0.30 (Tab. 2).

Organs of *T. repens* showed 2C, 4C, 8C, and at very low frequency also 16C nuclei (only petiole, Tab. 3). Two organs, leaf and sepal, had less than 10% shares of 4C nuclei. Nuclei with 8C DNA content were identified in stem, petiole, inflorescence stalk, stamen and carpel. Only petiole showed 16C level (0.2%). *T. repens* organs showed variability of cycle value. It was lowest in leaf and sepal. Some samples of those two organs were endopolyploid, that is, above the cycle value threshold of 0.1, but their mean cycle values for the total sample were 0.08 for leaf and 0.09 for sepal, so these organs should not be considered endopolyploid (Tab. 4). Root, with mean cycle value 0.15, was the organ with the lowest endopolyploidy degree in *T. repens*. Inflorescence stalk and petiole (both 0.39) showed the highest endopolyploidy degree. Petiole sample had the highest cycle value (0.83). Absolute endopolyploidy degree in *T. repens* was 0.23 (Tab. 4).

Differences in the degree of organ-specific endopolyploidy between species were revealed by ANOVA and then Tukey's HSD test (Fig. 2). Generally, *T. repens* expressed the lowest endopolyploidy degrees. Cycle values for petiole, inflorescence stalk, stem and stamen of *T. pratense* were significantly higher than those for the corresponding organs of *T. montanum* and *T. repens*. The cycle value of *T. pratense* stem (0.78) was more than double the values for stem of *T. montanum* (0.31) and *T. repens* (0.28). The cycle value of *T. repens* root (0.15) was less than half the values for root of

TABLE 1. Percentage of nuclei with different DNA content in organs of *Trifolium montanum* (means ± SD)

	Number of samples	2C peak	4C peak	8C peak
Root	18	67.4 ± 8.6	31.0 ± 7.0	1.6 ± 4.2
Stem	21	69.0 ± 5.4	30.7 ± 5.2	0.3 ± 1.5
Petiole	18	67.1 ± 7.5	32.3 ± 7.4	0.5 ± 2.3
Leaf	16	87.0 ± 4.6	13.0 ± 4.6	–
Inflorescence stalk	20	69.5 ± 6.3	30.3 ± 6.4	0.2 ± 1.0
Sepal	18	82.5 ± 5.3	17.5 ± 5.3	–
Petal	13	59.0 ± 6.9	41.0 ± 6.9	–
Stamen	17	75.3 ± 5.7	24.7 ± 5.7	–
Carpel	18	57.0 ± 12.0	40.0 ± 9.5	3.0 ± 5.3

TABLE 2. Exploratory data analysis of cycle values of *Trifolium montanum* organs. Organs with cycle value < 0.1 are nonendopolyploid. Homogeneous groups were separated by Tukey's HSD test. Identical letters designate organs whose mean cycle values do not significantly differ at $P < 0.05$ and which belong to the same group

		Cycle value					
	Number of samples	Minimum	Mean	Maximum	Standard deviation	Variation coeficient	Homogenic groups
Root	18	0.12	0.34	0.7	0.12	34.37	de
Stem	21	0.18	0.31	0.44	0.06	19.16	cd
Petiole	18	0.22	0.33	0.48	0.08	24.54	cde
Leaf	16	0.06	0.13	0.21	0.04	34.29	a
Inflorescence stalk	20	0.15	0.31	0.43	0.06	20.8	cd
Sepal	18	0.1	0.17	0.3	0.05	31.32	ab
Petal	13	0.31	0.41	0.52	0.07	16.57	ef
Stamen	17	0.18	0.25	0.39	0.06	23.48	bc
Carpel	18	0.21	0.44	0.8	0.16	35.42	f
All measured organs	159		0.30				

TABLE 3. Percentage of nuclei with different DNA content in organs of *Trifolium repens* (means ± SD)

	Number of samples	2 C peak	4 C peak	8 C peak	16 C peak
Root	4	84.9 ± 0.6	15.1 ± 0.6	–	–
Stem	27	73.0 ± 6.1	25.8 ± 4.8	1.2 ± 2.6	–
Petiole	29	65.8 ± 10.1	29.8 ± 7.1	4.2 ± 4.8	0.2 ± 1.0
Leaf	29	92.5 ± 2.2	7.5 ± 2.2	–	–
Inflorescence stalk	30	65.5 ± 7.0	30.5 ± 5.4	4.0 ± 4.0	-
Sepal	30	90.6 ± 3.1	9.4 ± 3.1	–	–
Petal	28	78.2 ± 6.6	21.8 ± 6.6	–	–
Stamen	30	82.5 ± 5.8	17.2 ± 5.1	0.4 ± 1.9	–
Carpel	31	68.8 ± 4.9	31.0 ± 5.1	0.3 ± 1.5	–

T. pratense (0.41) and *T. montanum* (0.34). For petal of *T. repens* (0.22) it was about half the value for petal of *T. pratense* (0.43) and *T. montanum* (0.41). *T. repens* petiole and inflorescence stalk showed higher cycle values (both 0.39) than for *T. montanum* (0.33 and 0.31, respectively). The cycle values of organs of *T. pratense* and *T. repens* were significantly correlated (Spearman's rho = 0.89, $P<0.05$), indicating similar organ-specific endopolyploidy degree patterns for the two species. No such correlation was found between *T. montanum* and *T. pratense* or *T. repens*.

TABLE 4. Exploratory data analysis of cycle values of *Trifolium repens organs*. Organs with cycle value < 0.1 are nonendopolyploid. Homogeneous groups were separated by Tukey's HSD test. Identical letters designate organs whose mean cycle values do not significantly differ at P < 0.05 and which belong to the same group

	Cycle value						
	Number of samples	Minimum	Mean	Maximum	Standard deviation	Variation coeficient	Homogenic groups
Root	4	0.14	0.15	0.16	0.01	6.28	abc
Stem	27	0.2	0.28	0.47	0.08	28.5	cd
Petiole	29	0.15	0.39	0.83	0.15	38.86	e
Leaf	29	0.04	0.08	0.13	0.02	29.3	a
Inflorescence stalk	30	0.22	0.39	0.61	0.1	25.94	e
Sepal	30	0.04	0.09	0.21	0.03	33.83	a
Petal	28	0.1	0.22	0.37	0.07	30.18	bc
Stamen	30	0.08	0.18	0.45	0.07	39.36	b
Carpel	31	0.21	0.32	0.42	0.05	16.71	d
All measured organs	238		0.23				

TABLE 5. Eigenvectors of organs with three component axes PC1, PC2 and PC3 given by PCA

	PC1	PC2	PC3
Root	-0.08937	0.082458	-0.06029
Stem	-0.24731	-0.00747	-0.01124
Petiole	-0.1547	-0.0605	-0.01557
Leaf	-0.00333	0.012425	-0.0089
Inflorescence stalk	-0.16825	-0.05067	0.008321
Sepal	-0.03248	0.020692	0.001795
Petal	-0.06366	0.077088	0.006443
Stamen	-0.08998	0.041689	0.098964
Carpel	-0.05241	0.057109	-0.00319

The first three component axes explained 68.46% (PC1), 12.27% (PC2) and 6.98% (PC3) of total variability (Fig. 3). Three groups of individuals corresponding to *T. pratense*, *T. repens* and *T. montanum* were clearly delimited. *T. pratense* is separated from both *T. repens* and *T. montanum* along the first axis, and *T. repens* is separated from *T. montanum* along the second axis. With respect to degree of endopolyploidy, individuals of *T. repens* and *T. montanum* are more similar to each other than to individuals of *T. pratense* (Fig. 3). The separation of *T. pratense* from the rest along the PC1 axis is due mostly to differences in endopolyploidy degree of stem (eigenvector value –0.25, Tab. 5), inflorescence stalk (–0.17) and petiole (–0.15). *T. repens* and *T. montanum* are separated mostly due to differences in endopolyploidy degree of root (0.08), petal (0.08) and carpel (0.06) along the PC2 axis.

DISCUSSION

Flow cytometry is a quick and reliable method for studying endopolyploidy (Biradar et al., 1993; Mishiba and Mii, 2000; Sliwinska and Lukaszewska, 2005; Barow and Meister, 2003). Our flow cytometry results revealed the presence of endopolyploidy in *T. montanum* and *T. repens* (Tabs. 1–4). Endopolyploidy was found earlier in *T. pratense* (Barow and Meister, 2003; Kocová and Mártonfi, 2011), so all three species are polysomatic plants.

Endopolyploidy probably is a very common physiological feature of *Trifolium* species. Our data support the view that Fabaceae is a family with predominantly endopolyploid taxa (Barow and Meister, 2003).

In the present study we found that endopolyploidy degree depended on the type of organ within

species and on the species, in line with many other studies (Smulders et al., 1994; Mishiba and Mii, 2000; Barow and Meister, 2003). In flow cytometry studies the most straightforward evidence of endopolyploidy is the presence of an 8C peak on the histograms. In both *T. montanum* and *T. repens* the frequency of 8C nuclei was low for most of the studied organs, in contrast to the high proportion of nuclei with 8C and 16C DNA content reported in *T. pratense* (Kocová and Mártonfi, 2011). By the criterion of a high proportion of 16C nuclei in organs, endopolyploidy degree was highest in *T. pratense* in petiole, inflorescence stalk and stem. Generally the cells of *Trifolium* species do not have nuclei with very high DNA content such as found in *Portulaca grandiflora* Hook., where Mishiba and Mii (2000) reported 64C for sepal and 32C for petal of di/tetraploid, 64C for mature leaf of diploid, and 32C for mature leaf of tetraploid.

Another recent approach to studying endopolyploidy is endopolyploidy degree estimation via calculation of cycle value (Barow and Meister, 2003). Based on this approach, significant differences in endopolyploidy degree of organs were shown in *T. pratense* (Kocová and Mártonfi, 2011) and here in *T. montanum* and *T. repens* (Tabs. 2, 4). The occurrence of endoreduplication in cells depends on the type and function of cells, and this is connected with cell size (Maluszynska et al., 2012). Our results support the idea that organs with similar functions and types of tissues and cells have similar endopolyploidy degree. Examples of this are organs with a high proportion of vascular tissue: petiole, inflorescence stalk and stem. They have similar proportions of nuclei with the same DNA content and consequently endopolyploidy degree. Generally these organs have higher endopolyploidy degree than others in many species of different families (Barow and Meister, 2003).

A common feature of endopolyploidy degree in the three studied *Trifolium* species is that leaf and sepal gave the lowest cycle values. Low endopolyploidy degree in leaf has been found in other Fabaceae species (Bainard et al., 2012; Barow and Meister, 2003). Fabaceae species show considerably lower cycle values than other polysomatic species, for example those in Cucurbitaceae, Chenopodiaceae (Barow and Meister, 2003) and Brassicaceae (Bainard et al., 2012; Barow and Meister, 2003). Other notable differences, at infrafamilial level, are seen in the endopolyploidy degree of reproductive organs. Carpels of *Pisum sativum* L. (0.24) and *Vicia faba* L. (0.18) have lower endopolyploidy degree than stamens (1.01 and 0.74 respectively; Barow and Meister, 2003), whereas in *Trifolium* species the reverse is the case; cycle value of carpel > cycle value of stamen (but in *T. pratense* the difference in endopolyploidy degree between carpel and stamen is not significant).

There were significant differences in the cycle values of organs between the studied *Trifolium* species (Fig. 2). They could be due to several factors: taxonomic position, genome size, ploidy level of species, and environmental conditions (e.g., temperature, microclimatic conditions, habitat, life cycle, breeding history).

Generally the organs of *T. pratense* showed higher degrees of endopolyploidy than in *T. montanum* and *T. repens*. The absolute endopolyploidy degree was highest (0.46) in *T. pratense* (data from Kocová and Mártonfi, 2011), intermediate in *T. montanum* (0.30) (Tab. 2) and lowest in *T. repens* (0.23) (Tab. 4). This might be explained by the closer phylogenetic relatedness of *T. montanum* and *T. repens*. They both belong to sect. *Trifoliastrum*, while *T. pratense* is a taxon of sect. *Trifolium*. PCA analysis (Fig. 3) also indicated that *T. montanum* and *T. repens* are closer to each other than either of them to *T. pratense*. Barow and Meister (2003) and Bainard et al. (2012) pointed to taxonomic position as the most important factor leading to the occurrence of endopolyploidy, and Bainard et al. (2012) reported that endopolyploidy degree correlates with various morphological and ecological traits, in addition to phylogenetic position.

The ploidy level of a species is another factor affecting degree of endopolyploidy (Biradar et al., 1993; Mishiba and Mii, 2000; Sliwinska and Lukaszewska, 2005; Jovtchev et al., 2007; Chen et al., 2011). Ellison et al. (2006) reported the same chromosome numbers as ours for diploid *T. pratense* (2n=2x=14), diploid *T. montanum* (2n=2x=16) and tetraploid *T. repens* (2n=4x=32). The natural polyploid *T. repens* has lower endopolyploidy than *T. pratense* and *T. montanum*. Natural polyploids generally show lower endopolyploidy than their corresponding diploids (Jovtchev et al., 2007). Since *T. montanum* and *T. pratense* are diploids, we would expect their organ cycle values to be more similar than we found here. Apparently the guideline of ploidy level does not apply to these two taxa.

Our measured genome sizes of *T. pratense* (0.9 pg) and *T. repens* (2.19 pg) lie between the values determined by another authors, for *T. pratense* 0.97 pg (Arumugathan and Earle, 1991) and 0.85 pg (Vižintin et al., 2006) and for *T. repens* 2.07 pg (Arumugathan and Earle, 1991) and 2.24 pg (Vižintin et al., 2006). In this study we estimated the genome size of *T. montanum* (2.48 pg) for the first time. Based on our data, *T. pratense*, *T. montanum* and *T. repens* belong to the category of species of very small genome size (Soltis et al., 2005). Endopolyploidization is typical for plants with small genomes (de Rocher et al., 1990; Galbraith et al., 1991; Gillissen et al., 1993; Smulders et al., 1994;

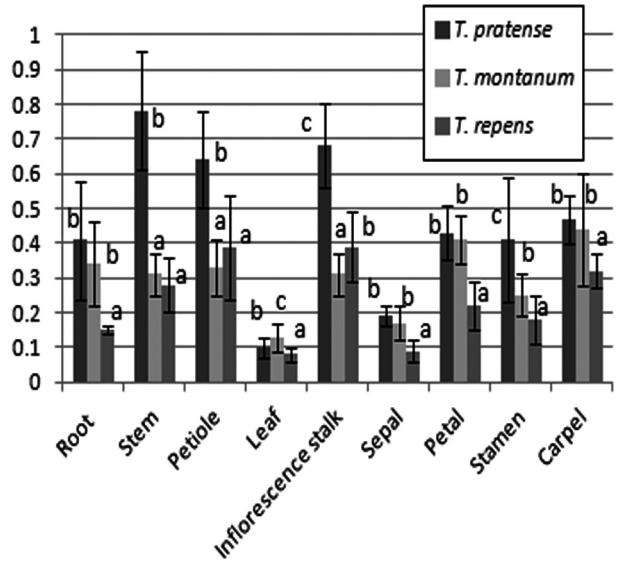

Fig. 2. Cycle values of organs in *T. pratense*, *T. montanum* and *T. repens*. Bars represent mean cycle values. Data are means ± SD. Organs with cycle value < 0.1 are nonendopolyploid. Differences in endopolyploidy level between species for each organ tested by ANOVA (log10-transformed data, $P < 0.05$). Homogeneous groups were separated by Tukey's HSD test. Identical letters designate organs whose mean cycle values do not significantly differ at $P < 0.05$ and which belong to the same group for that organ. Data for *T. pratense* taken from Kocová and Mártonfi (2011).

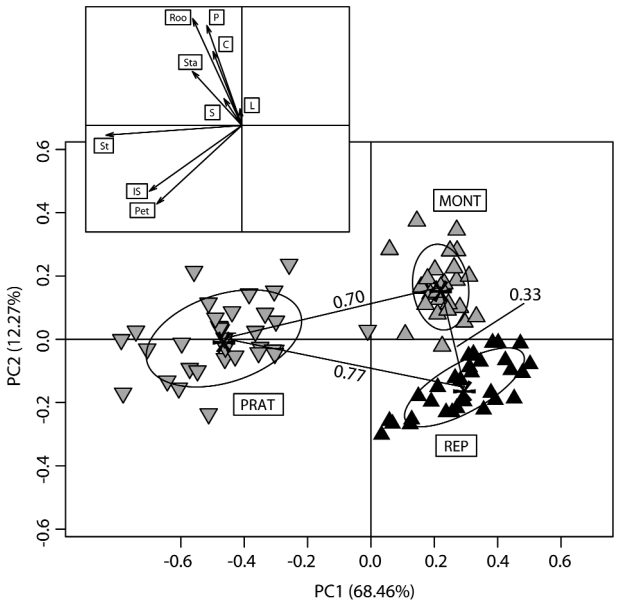

Fig. 3. Plot of eigenvalues of 92 individuals of *T. pratense* (PRAT), *T. repens* (REP) and *T. montanum* (MONT) by principal component analysis. Euclidean distances of group centroids (asterisked) were calculated. Eigenvectors of organs given in inset. Roo – root; St – stem; Pet – petiole; L – leaf; IS – inflorescence stalk; S – sepal; P – petal; Sta – stamen; C – carpel.

Kondorosi et al., 2000; Kudo and Kimura, 2001). Our findings do not entirely confirm this: *T. pratense*, with the lowest genome size of the studied taxa, showed higher endopolyploidy than *T. montanum* and *T. repens*, but *T. repens* showed lower degree of endopolyploidy than *T. montanum* despite its smaller genome size. The ploidy level of the species may be responsible for the divergence from expectations. The higher ploidy of *T. repens* may have been at least partly a factor in its lower endopolyploidy. Diploid *Medicago lupulina* with genome size of 1.41 pg showed higher degree of endopolyploidy than tetraploid *Medicago sativa* L. with genome size of 3.73 pg (Bainard et al., 2012). On the other hand, in two diploid *Plantago* species, *Plantago lanceolata* L. with higher genome size (2.85 pg) showed higher endopolyploidy than *Plantago major* L. (1.46 pg) (Bainard et al., 2012). Whether genome size strongly influences the degree of endopolyploidy of different species within genus remains open to question. Probably it is less important than taxonomic relatedness (Barow and Meister, 2003). The higher degree of endopolyploidy observed in *T. pratense* may be associated with its long history of cultivation. Centuries of breeding, selection and mass production of this fodder plant would no doubt affect its physiology, possibly including endopolyploidy, and might account for the higher degree of endopolyploidy in *T. pratense*.

The distribution pattern of endopolyploidy in organs of *T. repens* was very similar to that of *T. pratense* (supported by correlation analysis), the only difference being endopolyploidy of stem. Stem showed the highest endopolyploidy in *T. pratense*, while in *T. repens* it showed lower endopolyploidy than in petiole or inflorescence stalk. No correlations between *T. montanum* and *T. pratense* or *T. repens* were found. In *T. montanum*, petal and carpel showed the highest degree of endopolyploidy, in strong contrast to the pattern of the other two species. The correlation between *T. repens* and *T. pratense* may be due in part to shared habitat conditions (water availability, nitrogen content, microclimate). The *T. pratense* and *T. repens* plants were collected near paths and a driveway in mown lawns in towns, but the *T. montanum* plants were collected from undisturbed dry meadows.

Based on our results we should expect many more of the 250-plus *Trifolium* species to be polysomatic. Phylogenetic relatedness and genome size

seems to be the most important factors influencing degree of endopolyploidy in the genus *Trifolium*. More detailed studies of them should help identify which factors – the ones studied here and others such as habitat type, climate and breeding history – have the greatest influence on the endopolyploidy pattern in *Trifolium*. A further task is to clarify the relationship between degree of endopolyploidy and phylogeny in those species. For these and other questions regarding endopolyploidy the genus *Trifolium* offers an excellent platform for research.

AUTHORS' CONTRIBUTION

VK collecting plants, preparing samples, measuring by flow cytometry, karyological analysis, determining genome size, treating data for determination of endopolyploidy degree, writing manuscript; VK treating data for determination of endopolyploidy degree, statistical methods, writing manuscript; NS collecting plants, preparing samples, discussions; PM supervisor of endopolyploidy studies in department, discussions, writing manuscript. The authors declare that there are no conflicts of interests.

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