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HIGH VARIABILITY OF NUCLEAR DNA CONTENT IN CULTIVARS AND NATURAL POPULATIONS OF *POA PRATENSIS* L. IN RELATION TO MORPHOLOGICAL CHARACTERS

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Kentucky bluegrass is a facultative apomict which is propagated from seeds of variable genetic origins, ploidy levels and nuclear DNA contents. This study analyzes the variability of relative nuclear DNA content among cultivars and natural populations, and examines whether this variability is correlated with morphological traits. Relative nuclear DNA content (an indirect measure of chromosomal variability) was determined in 281 plants from 28 accessions (17 cultivars, 11 populations) using flow cytometry of DAPI-stained nuclei. The same plants were also measured for leaf area and stomatal length. Variation of measured relative DNA content between the studied accessions was very high (5.5-fold). Intra-accession variation was very high in six accessions, even though three of these were cultivars. Relative nuclear DNA content was correlated with stomatal length but not with leaf area. The lack of correlation with leaf area might explain why high intra-accession variability of nuclear DNA content was found in released cultivars that had passed uniformity testing during the registration procedure. We suggest that nuclear DNA content measurement should be made part of the cultivar registration process.

Key words: *Poa pratensis* L., Kentucky bluegrass, flow cytometry, intraspecific variation, relative nuclear DNA content, leaf area, stomatal length, apomixis.

INTRODUCTION

Kentucky bluegrass (*Poa pratensis* L.) is one of the most important forage and turf grasses in temperate regions. It has been intensively bred due to its high value in the turf grass and forage industries, and numerous cultivars have been released. Modern cultivated varieties undergo a complex approval process, based mainly on the guidelines of the International Union for the Protection of New Varieties of Plants (Anonymous, 2002). The cultivar release evaluation procedure relies largely on morphological testing of the distinctness, uniformity and stability (DUS) of examined breeding lines.

Poa pratensis is a facultative apomictic species, reproducing by a type of gametophytic apomixis called pseudogamous apospory, characterized by the formation of an unreduced female gametophyte from a somatic cell; the megaspore mother cell either is arrested in its development or else does not enter or complete meiosis (Ozias-Akins, 2006).

Facultative apomicts produce apomictic progeny which are genetically identical to the maternal genotype, and sexual progeny which are genetically different from the maternal genotype. Sexual progeny (often termed aberrant or off-type) can have at least nine different genetic origins. They can originate from self- or cross-pollination and subsequent fertilization of reduced or unreduced eggs with reduced or unreduced pollen. Multiple genetic origins may even occur within the same ovary, resulting in a polyembryonic seed (Huff and Bara, 1993). In apomictic progeny, mitotic and meiotic chromosome number aberrations are frequent and may result in additional variation of nuclear DNA content (Eaton et al., 2004).

The apomictic or sexual nature of *P. pratensis* has been previously studied using chromosome counting, RAPD profiling and flow cytometric analyses of nuclear DNA content (Huff and Bara, 1993; Barcaccia et al., 1997; Eaton et al., 2004; Stephens et al., 2006; Wieners et al., 2006). These investigations have focused mainly on determination of the

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genetic origin of embryos and endosperms based on ploidy level and RAPD profiles. Using this approach, the reproductive pathway could be identified. For example, Wieners et al. (2006) detected four reproductive pathways in 38 accessions of *P. pratensis*.

The most extensive evaluation of ploidy level variation in *P. pratensis* cultivars was an analysis of nuclear DNA content and chromosome number in 22 cultivars, each represented by one individual (Eaton et al., 2004). That study found 3.3-fold variation of nuclear DNA content (2C ranged from 5.39 to 17.69 pg), demonstrating considerable variation among those cultivars, but did not investigate variation of nuclear DNA content within cultivars.

Nuclear DNA influences the phenotype by expression of its genic content and by the physical effects of its mass and volume (the nucleotypic effect). The nucleotype can affect agriculturally important traits, such as minimum maturation time, biomass and yield, via effects on cell size, cell division rate and cell number (Bennett and Smith, 1991).

To our knowledge there is no report of nuclear DNA content variation within cultivars or natural populations of *P. pratensis* in the available literature. Nor has the relationship between nuclear DNA content variation and the morphological characteristics of individual plants been investigated. To assess intra- and inter-accession variability of nuclear DNA content in *P. pratensis*, we measured relative nuclear DNA content in 17 cultivars and 11 natural populations, each represented by several individual plants. To find out whether nuclear DNA content variability was reflected in the phenotype, we analyzed the correlation between nuclear DNA content and two morphological parameters: leaf area and stomatal length.

MATERIALS AND METHODS

PLANT MATERIAL

Caryopses of 17 cultivars and 11 natural populations of *P. pratensis* were sown as a permanent collection in May 2000. Each young seedling was transferred to an individual pot. The plants were grown in the greenhouse of the Biotechnical Faculty of the University of Ljubljana. The plants were morphologically examined during flowering for species confirmation.

NUCLEAR DNA CONTENT MEASUREMENT

Relative nuclear DNA content was measured by flow cytometry according to Bohanec (2003). DNA staining used 4',6'-diamidino-2-phenylindole (DAPI), with *Trifolium repens* cv. Tara (2.07 pg; Arumuganathan and Earle, 1991) as the internal standard. Leaves of *P. pratensis* and the standard species were harvested from living plants immediately prior to measurements. They were chopped together with a razor blade in plastic Petri dishes. Nuclei were released in 0.1 M citric acid containing 0.5% Tween 20. The suspension was filtered through a 30 μm nylon-mesh filter. A three- to four-fold volume of the staining buffer, containing 4 μg/ml DAPI in 0.4 M disodium hydrogen phosphate, was added to the filtered nuclear suspension. Nuclear fluorescence was measured with a Partec PAS flow cytometer (Münster, Germany) using a linear scale, UV excitation (HBO lamp) and a GG 435 long-pass emission filter. At least four nuclear suspensions were prepared separately for each individual plant, and 10,000 nuclei were measured per nuclear suspension. At least ten different individuals (genotypes) were measured for each accession (Tab. 1; total 281 measured individuals). We used Flomax® software (Partec, Münster, Germany) to calculate the positions of the G0/G1 (2C) peaks of the standard species and investigated accessions. Relative nuclear DNA content of *P. pratensis* individuals was calculated from the positions of the 2C peaks of the investigated and standard species, and expressed in arbitrary units. The relative nuclear DNA content of an individual was calculated as the mean value from four independently prepared nuclear suspensions. Analysis of each accession was completed within one day to minimize the seasonal variation effect.

MEASUREMENT OF MORPHOLOGICAL PARAMETERS

Morphological analysis comprised measurements of leaf area and length of stomata. Leaf area was measured for all individuals for which nuclear DNA content had been measured. The youngest fully expanded leaves were harvested in May (five leaves per individual) and in October (ten leaves per individual). The leaf blades were scanned and the blade areas were measured with a Zeiss KS400 3.0 (Jena, Germany) image analysis system. The mean leaf area of an individual was calculated as the mean value of the harvested leaf blades.

Stomatal length was measured for ten accessions: seven cultivars (Haga, Trampas, Barcelona, Conni, Asset, Primo, Menina) and three natural populations (135, 80/94-Pokljuka, 29; Tab. 1). The youngest fully developed leaves were collected, fixed in ethanol-acetic acid (3:1 v/v) and stored in 96% ethanol at 4°C. Leaf blade segments were placed in a drop of water on a microscope slide, covered with a cover slip and measured under a light microscope with a Zeiss KS400 3.0 image analysis system, as described by Vilhar et al. (2002). For each individual the maximum lengths of guard cells in 40 stomata were measured; the means of these lengths are used here to represent stomatal length for an individual plant.

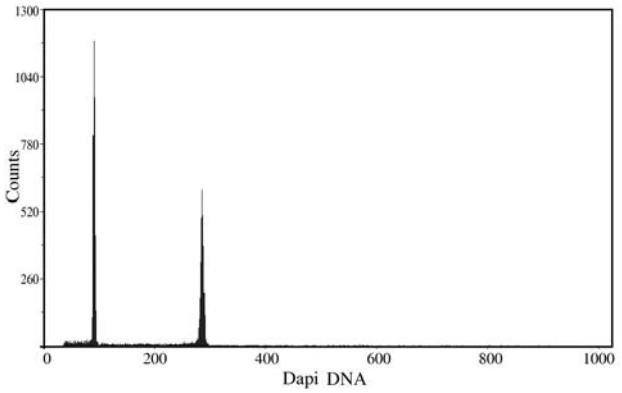

Fig. 1. Flow cytometric histograms of the 2C peaks for the internal standard species *T. repens* cv. Tara (left) and the analyzed species *P. pratensis* cv. Conni (right).

CHROMOSOME COUNTS

One plant of of cv. Leicra with relative nuclear DNA content of 2.043 was chosen for chromosome counting. Root tips were removed, chilled in ice water for one day, fixed in 1:3 acetic ethanol for one day, and finally stored in ethanol at 4°C. Fixed roots were hydrolyzed in 1 N HCl for 15 min and Feulgen-stained with Schiff's reagent. The chromosome spreads were observed with a Nikon Eclipse 80i using an oil immersion lens.

STATISTICAL ANALYSIS

The relative nuclear DNA content of each individual was calculated as the mean of four independently prepared nuclear suspensions. Pearson's correlation coefficient (Microsoft Excel) was used to assess the correlations between the nuclear DNA content and morphological parameters of individual plants. Variance component analysis was performed with Statgraphics Plus 4.0.

RESULTS

NUCLEAR DNA CONTENT

The suspensions of isolated leaf nuclei gave characteristic fluorescence peaks (Fig. 1). The coefficient of variation of the 2C peaks for the investigated and standard species ranged from 0.88% to 4.70%, with the majority around 2%. The relatively low coefficients of variation of the 2C peaks indicate precision of measurements.

Data showing the variability of relative nuclear DNA content among cultivars and populations are given in Figure 2. The relative nuclear DNA content of 281 individual plants from all accessions ranged from 0.862 (natural population *27p*) to 4.702 (cultivar *15c*); this is 5.5-fold variation. Table 1 shows the lowest and highest measured relative nuclear DNA content for each accession. The low standard deviations for the four separately prepared nuclear suspensions from each individual, with corresponding coefficients of variation of up to 1.5% for the four measurements, indicate high repeatability of measurements (Tab. 1; Fig. 3).

The accessions differed in their patterns of relative nuclear DNA content variation. Four representative examples are given in Figure 3. The cultivar Menina (accession *1c*) had uniform relative nuclear DNA content (Fig. 3a). In cv. Conni (accession *15c*) the relative nuclear DNA content of individuals was generally uniform, with a single outlier (Fig. 3b). The relative nuclear DNA content of cv. Parade (accession *4c*) was highly variable (Fig. 3c). In nine cases, the harvested plant material was at first assumed to come from individual plants, but flow cytometric measurement revealed two different values for relative nuclear DNA content (from cultivars *2c, 12c, 14c* and natural populations *20p, 21p, 23p, 24p*, 25p; Fig. 3d). These plants represented two individuals developed from polyembryonic seeds (possessing two distinct embryos) or developed from two separate seeds. Therefore, for further analysis these plants were considered as two individuals.

Among the 28 accessions, 22 possessed fairly uniform relative nuclear DNA content, with its variability caused mainly by one, two or three outliers. The others showed a higher interquartile range without outliers (accessions *2c, 4c, 7c, 21p, 25p*) or with only one outlier (accession *24p*).

RELATIONSHIP BETWEEN RELATIVE NUCLEAR DNA CONTENT AND MORPHOLOGICAL PARAMETERS

The mean leaf area of individuals measured in May ranged from 94 mm^2 (accession *9c*, individual no. 9) to 569 mm^2 (accession *25p*, individual no. 7). For leaves harvested in October, leaf area ranged between 130 mm^2 (accession *3c*, individual no. 9) and 1233 mm^2 (accession *1c*, individual no. 8). Leaf area varied widely within the same individual and between individuals from the same accession (Tab. 1). The variance components of leaf area were calculated for the data obtained in May and October. Of the total variance, 24.6% (May) and 37.5% (Oct.) was ascribed to the inter-accession component, 42.8% and 37.5% to the intra-accession component, and 32.6% and 25.0% to the within-individual component. The correlation between nuclear DNA content and leaf area was not significant (Pearson's correlation coefficient $r_{May} = 0.11$ (n=266), $r_{Oct} = -0.08$ (n=262), $p > 0.05$; Fig. 4a).

Mean stomatal length of individuals ranged from 32 μm (accession *18p*, individual no. 9) to 46 μm (accession *10c*, individual no. 4). Variance component

TABLE 1. Relative nuclear DNA content and morphological parameters in 28 accessions of *P. pratensis*. Cultivars/natural populations are listed by increasing median relative nuclear DNA content

Accession label[a]	Accession name (and origin[b])	Number of individuals[c] (number of outliers)	Inter-quartile range	Minimum relative nuclear DNA content [d]	Maximum relative nuclear DNA content [e]	Mean leaf area in October (mm^2)[f]	Mean stomatal length (μm)[g]
			Cultivars				
1c[h]	Menina (AIS, Slovenia)	10 (2)	0.022	1.393 ± 0.012	1.474 ± 0.034	90 ± 237	37 ± 1
2c	Leikra (Planteforsk, Norway)	11 (0)	1.419	0.847 ± 0.002	3.694 ± 0.028	07 ± 286	
3c	Haga (WPBI, Sweden)	10 (3)	0.027	2.060 ± 0.012	2.587 ± 0.019	81 ± 158	37 ± 3
4c[h]	Parade (VDH, The Netherlands)	10 (0)	1.012	2.022 ± 0.002	3.885 ± 0.014	15 ± 69	
5c	Oxford (DLF-trifolium, Denmark)	10 (1)	0.039	2.545 ± 0.023	2.894 ± 0.021	51 ± 151	
6c	Cocktail (Mommersteeg, The Netherlands)	10 (1)	0.049	2.618 ± 0.010	3.423 ± 0.014	05 ± 69	
7c	Bona (IHAR, Poland)	9 (0)	0.386	2.361 ± 0.002	2.785 ± 0.002	90 ± 120	
8c	Primo (WPBI, Sweden)	10 (1)	0.039	2.704 ± 0.012	2.888 ± 0.006	95 ± 156	42 ± 2
9c	Unna (WPBI, Sweden)	10 (3)	0.091	1.328 ± 0.001	4.132 ± 0.007	60 ± 74	
10c	Asset (VDH, The Netherlands)	10 (1)	0.046	2.742 ± 0.005	2.923 ± 0.021	80 ± 102	44 ± 2
11c	Barcelona (Barenburg, The Netherlands)	10 (1)	0.059	2.864 ± 0.007	4.247 ± 0.007	38 ± 68	42 ± 3
12c	Compact (DLF-trifolium, Denmark)	11 (2)	0.145	1.509 ± 0.009	4.372 ± 0.156	75 ± 132	
13c	Baron (Barenburg, The Netherlands)	10 (1)	0.070	2.947 ± 0.024	3.830 ± 1.461	63 ± 87	
14c	Balin (DP, Denmark)	11 (2)	0.121	2.257 ± 0.010	3.439 ± 0.027	01 ± 170	
15c[h]	Conni (DLF-trifolium, Denmark)	10 (1)	0.031	3.075 ± 0.015	4.702 ± 0.013	12 ± 127	41 ± 3
16c	Trampas (DP, Denmark)	10 (0)	0.068	3.384 ± 0.039	3.554 ± 0.022	13 ± 72	43 ± 3
17c	Delft (Cebeco Zaden, The Netherlands)	10 (2)	0.0633	1.669 ± 0.006	3.525 ± 0.010	92 ± 160	
			Natural populations				
18p	80/94-Pokljuka (AIS, Slovenia)	10 (2)	0.025	1.142 ± 0.004	1.215 ± 0.008	93 ± 53	35 ± 3
19p	1/89 (BF UL, Slovenia)	10 (1)	0.046	1.341 ± 0.002	1.66 ± 0.007	82 ± 221	
20p	31/97-Treblja (AIS, Slovenia)	8 (1)	0.141	1.701 ± 0.017	2.860 ± 0.011	30 ± 159	
21p	0029 (AIS, Slovenia)	11 (0)	0.646	1.485 ± 0.006	2.538 ± 0.008	89 ± 155	
22p	SVG TRG 0013 (AIS, Slovenia)	10 (1)	0.042	1.940 ± 0.021	2.151 ± 0.005	41 ± 202	
23p[h]	135 (AIS, Slovenia)	11 (1)	0.023	2.004 ± 0.012	3.144 ± 0.050	91 ± 88	41 ± 3
24p	362-Dragonja (AIS, Slovenia)	11 (1)	0.602	2.191 ± 0.009	4.284 ± 0.026	35 ± 34	
25p	74/94-Pl. pod Viševnikom (AIS, Slovenia)	12 (0)	1.294	1.156 ± 0.010	3.221 ± 0.018	51 ± 173	
26p	29 (BF UL[e], Slovenia)	10 (0)	0.066	2.609 ± 0.006	2.805 ± 0.010	41 ± 130	41 ± 3
27p	3-Pl. pri jezeru (AIS, Slovenia)	10 (1)	0.065	0.862 ± 0.004	2.957 ± 0.004	26 ± 135	
28p	297-Vršič (AIS, Slovenia)	6 (2)	0.004	2.775 ± 0.013	3.089 ± 0.024	15 ± 121	

[a] Accessions are labeled *c* for cultivars and *p* for natural populations.
[b] Origin of accessions: AIS – Agricultural Institute of Slovenia, Ljubljana, Slovenia; Planteforsk – Norwegian Crop Research Institute, Oslo, Norway; WPBI – Weibullsholm Plant Breeding Institute, Landskrona, Sweden; VDH – D.J. van der Have B.V., Kappele, The Netherlands; DLF-trifolium – DLF-TRIFOLIUM, Roskilde, Denmark; Mommersteeg – Mommersteeg International B.V., Kappele,

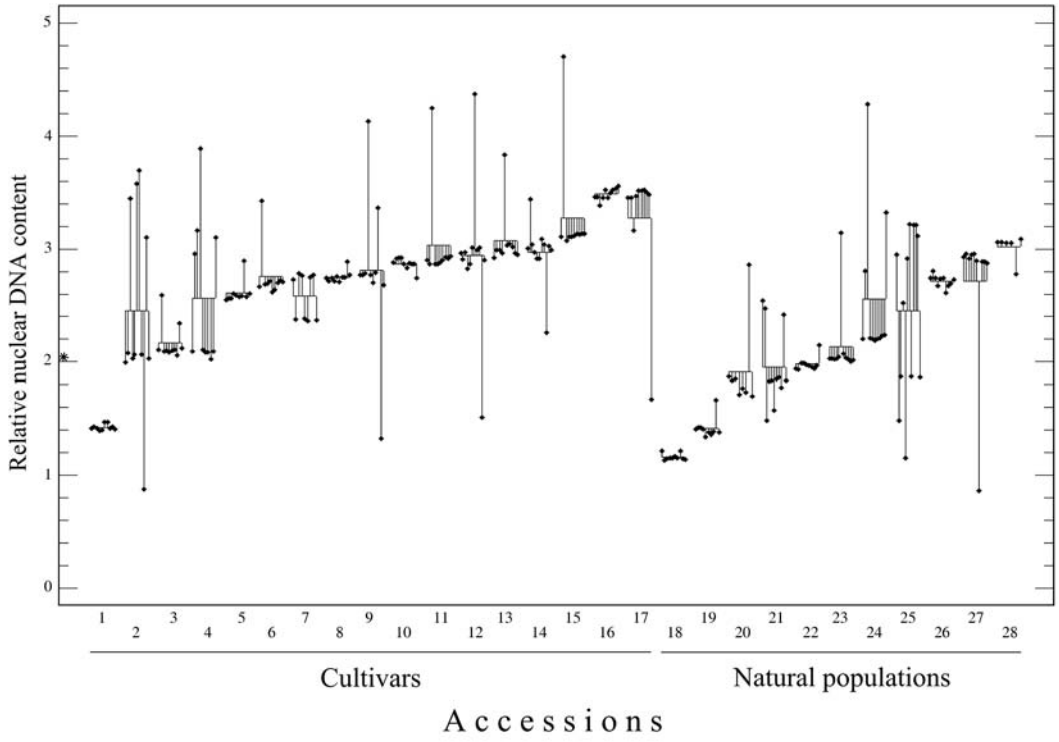

Fig. 2. Relative nuclear DNA content (arbitrary units) for 17 cultivars and 11 natural populations of *P. pratensis*. Points represent the average relative nuclear DNA content (n=4) of individual plants; horizontal lines show average relative nuclear DNA content (n=6–12) of accessions (n=6–12). Asterisk indicates relative nuclear DNA content of hexaploid plants measured in individual plant of cv. Leicra.

analysis explained 45.1% of the variation by the inter-accession component, 39.8% by the intra-accession component, and 15.0% by variation of stomatal lengths in the same individual. The correlation between nuclear DNA content and stomatal length for the examined ten accessions was statistically significant (Pearson's correlation coefficient $r_{Oct} = 0.61$ (n=48), $p < 0.001$; Fig. 4b).

CHROMOSOME COUNTS

Chromosome counts showed that the 2.043 relative nuclear DNA content measured for an individual plant of cv. Leicra corresponds to the hexaploid level (2n=6x=42). This measurement serves to indicate that the ploidy levels of the measured accessions varied approximately from diploid (plant in population 3-Pl. "pri jezeru") to 12-ploid (plant of cv. Conni).

DISCUSSION

Nuclear DNA content is a quantitative character of an organism, which should be more or less constant among individuals of a population so long as there is enough interbreeding to mix up the gene pool. However, there is almost always some degree of chromosomal variation within populations due to duplications and deletions, spontaneous aneuploidy and polyploidy, heterochromatic segments, B-chromosomes and, in special cases, sex chromosomes causing inter-individual DNA content variation (Greilhuber, 1998).

The Netherlands; IHAR – Plant Breeding and Acclimatization Institute, Radzikow, Poland; Barenbrug – Barenbrug Holding BV, Oosterhout Nijmegen, The Netherlands; DP – Dansk Planteforaedling A/S, Store Heddinge, Denmark; Cebeco Zaden – Cebeco Zaden B.V, Vlijmen, The Netherlands; BF UL – University of Ljubljana, Biotechnical Faculty, Ljubljana, Slovenia.

[c] Number of individuals (genotypes) measured per accession depended on seed germinability.

[d,e] Data for the individual with the lowest/highest relative nuclear DNA content measured within accession. Mean ±SD of four separately isolated nuclear suspensions from the same individual.

[f] Area of 10 leaves was measured for each individual. Mean ±SD of accession is for all individuals (see column *Number of individuals*).

[g] Mean length of 40 stomata calculated for each individual. Mean ±SD of accession is for of five individuals. (N = 5).

[h] Distribution of relative nuclear DNA content within accession shown in Figure 3.

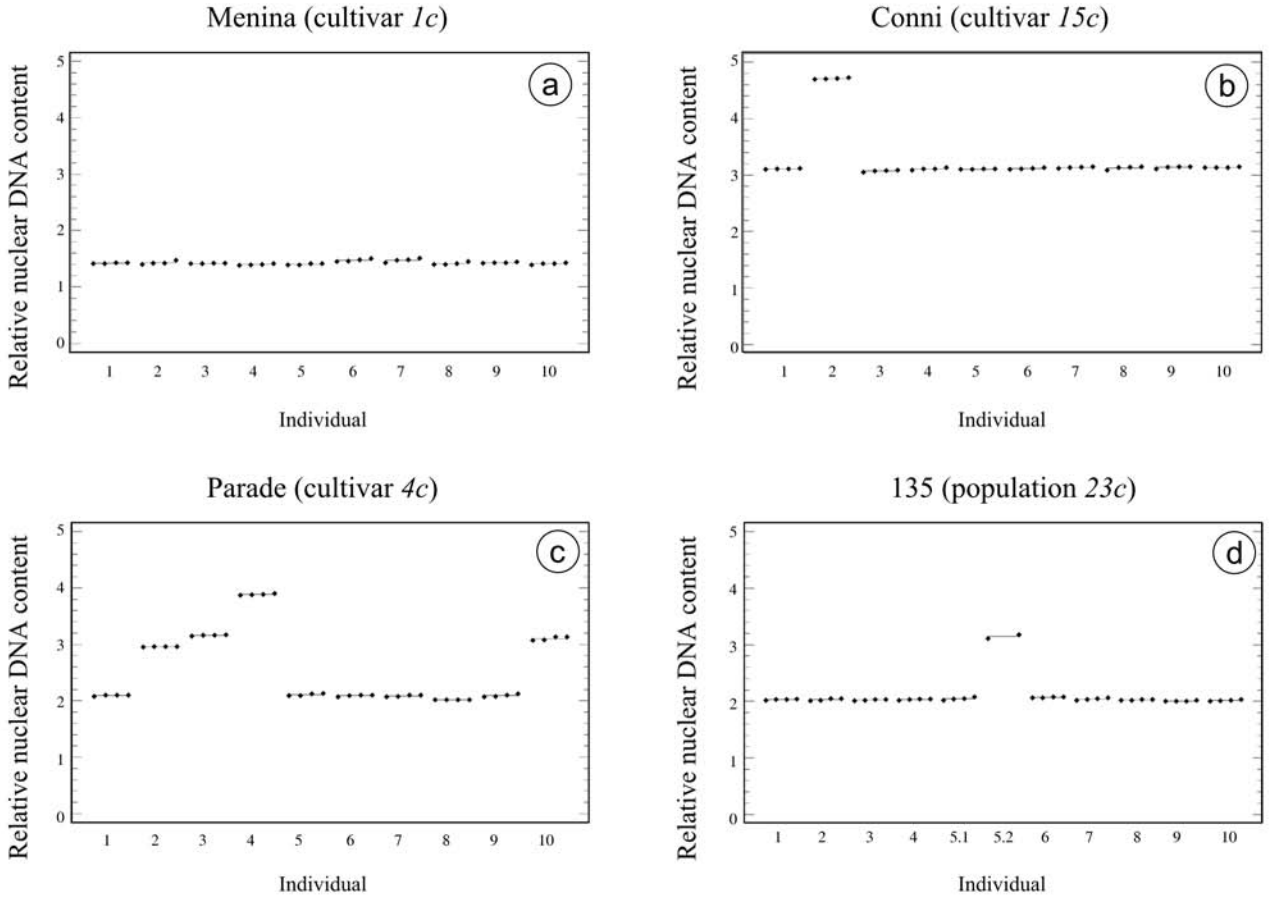

Fig. 3. Detailed data for four accessions exhibiting different patterns of relative nuclear DNA content distribution. Accessions: Menina – *1c* (**a**), Conni – *15c* (**b**), Parade – *4c* (**c**) and 135 – *23p* (**d**). Relative nuclear DNA content was assessed for ten individuals from each accession. Lines indicate the mean relative nuclear DNA content for an individual; dots represent relative nuclear DNA content measured for separately prepared nuclear suspensions of the individual. See Table 1 for detailed description of accessions.

Previously published studies of *P. pratensis* have been limited to nuclear DNA content estimations in cultivars represented by a single individual (Eaton et al., 2004; Wieners et al., 2006), so intra-accession variability was not addressed. Because *P. pratensis* is a facultative apomictic species, we did expect some variation in chromosome number and therefore in nuclear DNA content within accessions, but the measured variation in nuclear DNA content for both cultivars and natural populations was surprisingly high (Fig. 2, Tab. 1). The high nuclear DNA content variation we found between individuals within accessions for some cultivars as well as some natural populations clearly shows that in this regard the cultivars do not differ from natural populations.

Previous studies have shown that variation of nuclear DNA content in different plant species is associated with morphological characters such as cell and plant size. Positive correlations with nuclear DNA content have been reported in various species for stomatal length and seed size, and both negative and positive correlations between genome size and leaf size (Caceres et al., 1998; Chung et al., 1998; Sugiyama, 2002; Tatum et al., 2006). In *P. pratensis* we found a significant positive correlation between nuclear DNA content and length of stomata, but no correlation between nuclear DNA content and leaf blade area. It should be noted, however, that the blade areas of leaves harvested from the same individual were quite variable, as reflected in the high variance component ascribed to variability within leaves. It was not diminished substantially even when the number of measured leaves per individual was increased from five in May to ten in October.

The cultivars and populations in our experiment did not differ in nuclear DNA content variability, although much lower variability might be expected in cultivars, which are subjected to uniformity testing before registration. According to our findings

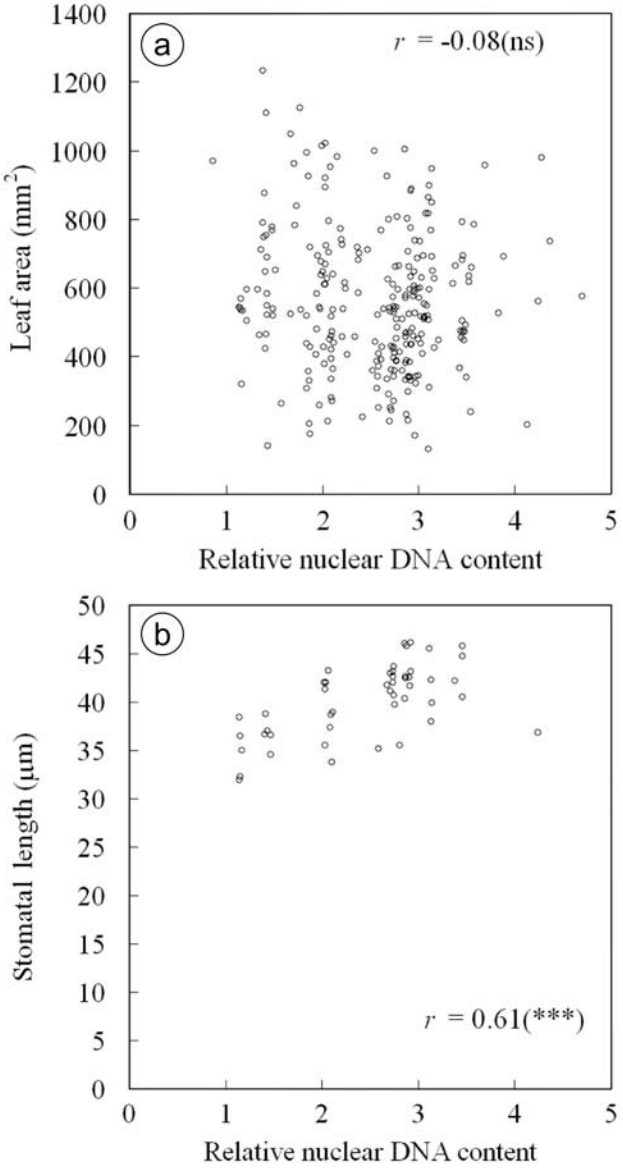

Fig. 4. Relationship between relative nuclear DNA content and mean leaf area measured in October (**a**), and between relative nuclear DNA content and mean length of stomata (**b**). Each point represents an individual. *r* Pearson's correlation coefficient; *** – $p < 0.001$; ns – not significant ($p > 0.05$). See Table 1 for detailed description of accessions used for measurements.

at least some released cultivars do possess high variability of nuclear DNA content, so it seems likely that this character is indeed not highly correlated with gross morphology in *P. pratensis*.

The UPOV guidelines for testing and maintenance (Anonymous, 1990) classify varieties of *P. pratensis* as apomictic or non-apomictic, with fewer plants required for testing in varieties declared to be apomictic. High uniformity of nuclear DNA content would be expected with apomictic cultivars (populations) originating from an individual selected plant. If bulking of several individual plants has been done during the selection process, variation of nuclear DNA content cannot be excluded even in apomictically propagated plants. For sexually propagated cultivars (populations), the expected nuclear DNA content should be uniform within cultivars. The observed variability we found within several cultivars and populations suggests a probable multiple-plant origin of the apomictic cultivars. This suggests that nuclear DNA measurements should be included in the early stages of breeding programs.

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