Plant Syst. Evol. 234: 1–13 (2002)
DOI 10.1007/s00606-002-0186-0

Plant Systematics and Evolution
Printed in Austria

Genome size and the nucleolar number as estimators of ploidy level in *Dactylis glomerata* in the Slovenian Alps

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Received May 31, 2001; accepted March 5, 2002
Published online: November 14, 2002

Abstract. We studied five natural populations of *Dactylis glomerata* L. (Poaceae) growing at different altitudes in the south-eastern fringe of the Alps in northern Slovenia to determine the subspecies. The stomatal length, the pollen diameter and chromosome counts were consistent with the tetraploid taxon *D. glomerata* subsp. *glomerata* (2n = 4x = 28). Genome size was measured in 55 individuals. The mean 2C value was 8.6 pg DNA. The mean 2C values of populations growing at different altitudes showed only 2.1% variation, and no correlation was observed between altitude and genome size. In *D. glomerata* subsp. *glomerata* eight nucleoli were observed in late telophase, indicating that the nucleolus-organising regions inherited from both diploid parent species are functional. We demonstrate that both genome size and the number of nucleoli may be used to determine the ploidy level as an alternative to chromosome counting.

Key words: *Dactylis glomerata*, morphology, ploidy, genome size, DNA image cytometry, Ag-NOR staining, altitude, nucleolus.

The genus *Dactylis* (Poaceae) is one of the most studied examples of a natural polyploid complex (Mizianty 1990, and references therein). It comprises over twenty mainly diploid (2n = 2x = 14) and tetraploid (2n = 4x = 28) taxa treated as species or subspecies and several more taxa described at the lower taxonomic levels. Genetic barriers between the taxa are not always complete, and morphological differentiation does not always correspond to cytological differentiation and vice versa (Mizianty 1991a). The taxonomy of the genus is difficult, as there are no distinct morphological borders between recognised taxonomic units (Mizianty 1986, 1991b), and the precise distribution ranges of individual taxa are not known. The current knowledge on the *Dactylis* complex in the Middle Europe was reviewed by Conert (1994).

In the territory of Slovenia, this genus has not been studied well. The *Dactylis* material has mostly been determined on the basis of morphology alone as belonging to two tetraploid taxa *D. glomerata* L. s. str. (= *D. glomerata* subsp. *glomerata*) and *D. hispanica* Roth (= *D. glomerata* subsp. *hispanica* (Roth) Nyman), and only recently has also the diploid subsp. *aschersoniana* (Graebn.) Thell. been reported (Conert 1994, Trpin and Vreš 1995), but has not yet been karyologically confirmed as diploid material. Apart from these two taxa, two other taxa from the genus *Dactylis* are likely to occur in Slovenia, the tetraploid *D. glomerata* subsp. *slovenica* Domin and the diploid subsp. *reichenbachii* (Hausm.) Stebbins & Zohary.

In the present study, we investigated five populations of *D. glomerata* growing at different altitudes in the south-eastern fringe of the Alps in northern Slovenia, close to the border with Austria. In Carinthia (Austria), in the region adjacent to our study area, Wetschnig (1984) recorded two *D. glomerata* taxa, subsp. *glomerata* and subsp. *aschersoniana*. While subsp. *glomerata* is widespread in Carinthia, subsp. *aschersoniana* shows a more limited distribution, with most localities situated within a 30 km radius from the site of our investigation (Wetschnig 1984). In Carinthia, the two taxa are morphologically very similar and can only be distinguished on the basis of the number and morphology of the metaphase chromosomes, and features associated therewith, such as the length of the stomata and the diameter of the pollen grains (Wetschnig 1984).

D. glomerata subsp. *aschersoniana* and subsp. *glomerata* are phylogenetically closely related. Cytological evidence suggests that subsp. *glomerata* is either a heterozygous autopolyploid of different genomes of subsp. *aschersoniana* (Mizianty 1991a), or an allotetraploid whose one ancestor is subsp. *aschersoniana*, while the other is presently not known (Wetschnig 1983, Mizianty 1991a).

The tetraploid complement of *D. glomerata* subsp. *glomerata* has four chromosome pairs with secondary constrictions, two of which have been inherited from the known diploid parent subsp. *aschersoniana* (Wetschnig 1983; Mizianty 1991a). The nucleolus organising regions (NORs) with tandem repeats of 5.8/18/25S rRNA genes are usually located at the secondary constrictions. Since only transcriptionally active rRNA genes give rise to a nucleolus, the maximal number of nucleoli per nucleus correlates with the number of active NORs (McClintock 1934). *D. glomerata* subsp. *glomerata* features eight secondary constrictions, thus there are presumably eight NORs in the tetraploid chromosome complement. Nevertheless, the highest number of nucleoli that has been observed in interphase nuclei of *D. glomerata* subsp. *glomerata* is five (Wetschnig 1983).

Several studies addressed the nuclear DNA content in tetraploid *D. glomerata*. However, considerable disagreement exists among the published 2C values: 6.4 pg DNA (Horjales et al. 1995), 8.3 pg (Greilhuber and Baranyi 1999), 8.7 pg (Grime et al. 1988), 8.7 pg to 11.2 pg in different populations (Creber et al. 1994; Reeves et al. 1998), 9.8 pg (Grime et al. 1985) and 12.4 pg (Schifino and Winge 1983, cited in Bennett and Smith 1991). The highest published 2C value is thus almost twice as high as the lowest one.

Adaptive value of genome size has also been studied in *D. glomerata*. A negative correlation has been observed between altitude and genome size in tetraploid populations at altitudinal transects in Spain, Italy and France (Creber et al. 1994, Reeves et al. 1998), suggesting that strong nucleotypic selection is acting upon populations with increasing altitude. The smaller genome size at high altitudes may be a reflection of a harsher environment which plants at these sites experience (Creber et al. 1994). In such environments, the vegetation season is short, and a smaller genome size may allow for some favourable phenotypic characters such as a shorter mitotic and meiotic cell cycle duration (Bennett 1987), and consequently faster vegetative development and faster seed ripening.

We studied morphological and karyological parameters in five natural populations of *D. glomerata* to determine the plants at the subspecies level. We first analysed the stomatal length and the pollen diameter as morphological characters reported to correlate with ploidy. We examined specimens of *D. glomerata* collected at different sites in Slovenia and the neighbouring regions to establish whether these characters are adequate for discrimination between the two expected groups of taxa, namely the diploids and the tetraploids. In addition, we determined the ploidy level on the basis of chromosome number and investigated inter- and intrapopulation variation of genome size in the five studied populations. We examined whether a correlation exists between altitude and genome size which may be due to a gradient

of selection pressure along the altitudinal transect. Furthermore, we investigated the suitability of the number of nucleoli as a criterion to determine the ploidy level in *D. glomerata* subsp. *glomerata*, with a special consideration to whether eight NORs are active, as expected from the situation in diploid relatives, in which four NORs are present.

Materials and methods

The sampling sites on the mountain Krvavec. We investigated populations of *D. glomerata* on the slope of the mountain Krvavec (46°16′N, 14°33′E, 'quadrant' – mapping unit measuring 3′ latitude × 5′ longitude – 9753/1) in the Kamniške Alpe in northern Slovenia, in the south-eastern fringe of the Alps. Five sampling sites were selected for the study at an altitudinal transect from 450 m to 1500 m a.s.l.: site 1 near the village Grad (450 m a.s.l.), site 2 near the village Ravne (650 m a.s.l.), site 3 near the village Davovec (850 m a.s.l.), site 4 near the footpath Krvavec – Davovec (1230 m a.s.l.), site 5 on Gospinca (1470 m a.s.l.). The distance between site 1 and site 5 was approx. 4 km. The sites were similar in terms of vegetation (non-intensively cultivated semi-natural grassland), orientation (south-west), steepness of the slope, geology (limestone) and absence of intensive cultivation through which seeds of commercial cultivars could be introduced into the natural populations.

Plant material. At the five Krvavec sites, plant material was sampled which showed some macromorphological characteristics of the diploid *D. glomerata* subsp. *aschersoniana* (i.e. almost glabrous lemma with only tiny prickles on the keel, tip of the lemma more or less gradually tapering to a fine point; Tutin 1980; Pignatti 1983; Oberdorfer 1990; Rothmaler 1990; Conert 1994; Adler 1994). This plant material is referred to as 'the Krvavec populations' in the following text. We collected fully developed plants for morphometrical analysis and ripe seeds for karyological studies.

We also examined 59 herbarium specimens collected at different regions in Slovenia and 9 specimens from the neighbouring regions: 7 from northern Croatia and 2 from north-eastern Italy. This material is referred to as 'the Slovenian reference plants'. Among the Slovenian reference plants, the chromosome number was determined previously for one specimen from Slovenia (tetraploid; B. Druškovič, pers. comm.). One specimen from Poland with a known chromosome number (diploid; *D. glomerata* subsp. *aschersoniana*; M. Mizianty, pers. comm.) was included in the study as a reference for diploids.

All herbarium vouchers are deposited at LJU (plants from the Krvavec populations – numbers 133251, 133252, 133253, 133254).

Morphometrical analysis. Herbarium material was used to analyse two characters associated with the ploidy level: the pollen diameter and the stomatal length. All Slovenian reference plants and three specimens from each of the Krvavec populations were analysed. For each plant, the maximal diameter of 20 pollen grains and the maximal length of the guard cells in 20 stomata (10 stomata from abaxial and 10 from adaxial epidermis in the mid-portion of the second leaf from the top) were measured, and the mean values were calculated to represent the stomatal length and the pollen diameter for each plant. For the measurement of the stomatal length, the dried material was briefly boiled in water and stored in ethanol at 4° C. The leaf segment was placed in a drop of water on a microscope slide, covered with a coverslip and measured under a light microscope with an image analysis system.

Karyological analysis of the Krvavec populations. The seeds were germinated on plastic germinating plates. Primary roots of the seedlings were harvested for both karyological analysis and genome size measurement. The karyological study comprised analysis of ploidy level, measurement of nuclear DNA content and evaluation of nucleolar activity.

For measurement of nuclear DNA content and analysis of nucleolar activity, the root tips were harvested, fixed in ethanol – acetic acid (3:1 v/v) for 24 h at 4°C and stored in 96% ethanol at −20 °C. To determine the ploidy level, the seedlings were incubated in 0.1% colchicine for 4 h at room temperature (appox. 21 °C), and then fixed and stored as described above.

The root tips were stained with the Feulgen reaction. In preliminary experiments, the optimal temperature and time of hydrolysis in HCl during the Feulgen staining procedure were determined for both *D. glomerata* and the standard species *Hordeum vulgare* L. The intensity of staining was measured after incubation of plant material in 5 M HCl for 10 to 100 min at 20 °C and 25 °C (results not shown).

The obtained hydrolysis curves were essentially the same as shown in Greilhuber and Baranyi (1999). Optimal hydrolysis conditions for both species were 30 min at 25 °C and 60 min at 20 °C. The latter treatment was used in subsequent genome size measurements due to a broader hydrolysis plateau at 20 °C compared to the 25 °C treatment.

The Feulgen-stained slides were prepared following in essence the protocol of Greilhuber and Ebert (1994). The root tips were washed in distilled water, hydrolysed in 5 M HCl for 60 min at 20 ± 0.1 °C in an ultrathermostated waterbath, washed in ice-cold distilled water for 5 min, stained with the Feulgen reagent for 120 min at 20 °C, washed in SO_2-water for the total of 45 min with several changes of the SO_2-water, and washed in distilled water. The apical root meristem region was dissected and the meristems were squashed in a drop of 45% acetic acid. The coverslip was removed with the dry-ice method, the slides were immersed in ethanol, air-dried, mounted in DPX (Fisons, UK) and dried in the dark.

For evaluation of the ploidy level, the number of chromosomes was counted in several metaphase plates per slide. The nuclear DNA content (2C and 4C value) was measured with the interphase-peak method based on DNA image cytometry as previously described (Vilhar et al. 2001, Vilhar and Dermastia 2002), with 100 to 200 interphase nuclei measured per slide. The slides were coded during the experiment, so the researcher measuring genome size did not know which population the measured slide belonged to and thus unintentional bias was excluded. The roots of *Hordeum vulgare* L. cv. Ditta (2C = 10.04 pg DNA; Doležel et al. 1998) were used as the standard and were strictly processed in the same vials as the investigated material. The seeds of *H. vulgare* were a gift of Prof. J. Greilhuber, Vienna.

For evaluation of nucleolar activity, image analysis tools were developed to enable simultaneous recording of the nuclear DNA amount and the number of nucleoli in individual interphase nuclei under a light microscope. The nucleolar number was counted in nuclei with clearly visible nucleoli. The nuclei on each slide were classified in two groups according to DNA content: the nuclei in the 2C peak (G1 phase of the cell cycle) and the nuclei in the 4C peak (G2 phase). In each group, the percentage distribution of nuclei with different numbers of nucleoli was calculated. In total, 14 individuals from different Krvavec populations were analysed, and the mean percentage distribution polygon was plotted from data obtained for individual plants.

The nucleolar number was also determined with silver (Ag-NOR) staining. Seedling root tips were fixed in ethanol – acetic acid (3:1 v/v) for 24 h at 4 °C and stored in 96% ethanol at −20 °C. The root tips of 10 individuals were softened in an enzyme solution (cellulase, pectinase) and silver-stained according to the procedure of Bloom and Goodpasture (1976) modified after Kodama et al. (1980). The data were analysed as described above.

Statistical analysis. Standard procedures were applied for statistical analysis as indicated in the Results, using the software package GraphPad Prism 3.02 (GraphPad Software Inc., USA).

Results

Macromorphological characters. At the five Krvavec sites, two *D. glomerata* taxa could be expected to occur, the diploid *D. glomerata* subsp. *aschersoniana* and the tetraploid subsp. *glomerata*, since both taxa were recorded close to our studied area by Wetschnig (1984). In the determination keys, two of the most consistently used and well defined macromorphological characters for discrimination between the two mentioned subspecies are the hairiness of the lemma keel and the shape of the lemma tip (Tutin 1980, Pignatti 1983, Oberdorfer 1990, Rothmaler 1990, Conert 1994, Adler 1994). In the collected material, the lemma was almost glabrous with only tiny prickles on the keel, with the tip of the lemma more or less gradually tapering to a fine point. These characters indicated that the plants might belong to subsp. *aschersoniana*, but no definite determination of the material could be made.

The stomatal length and the pollen diameter as indirect estimators of ploidy level. Two morphological characters known to be often associated with ploidy level, the stomatal length and the pollen diameter, were measured in the Slovenian reference plants. When a graph of the stomatal length against the pollen diameter was plotted, the data point pattern indicated a separation of specimens into two groups (Fig. 1A). According to the literature (Rurka

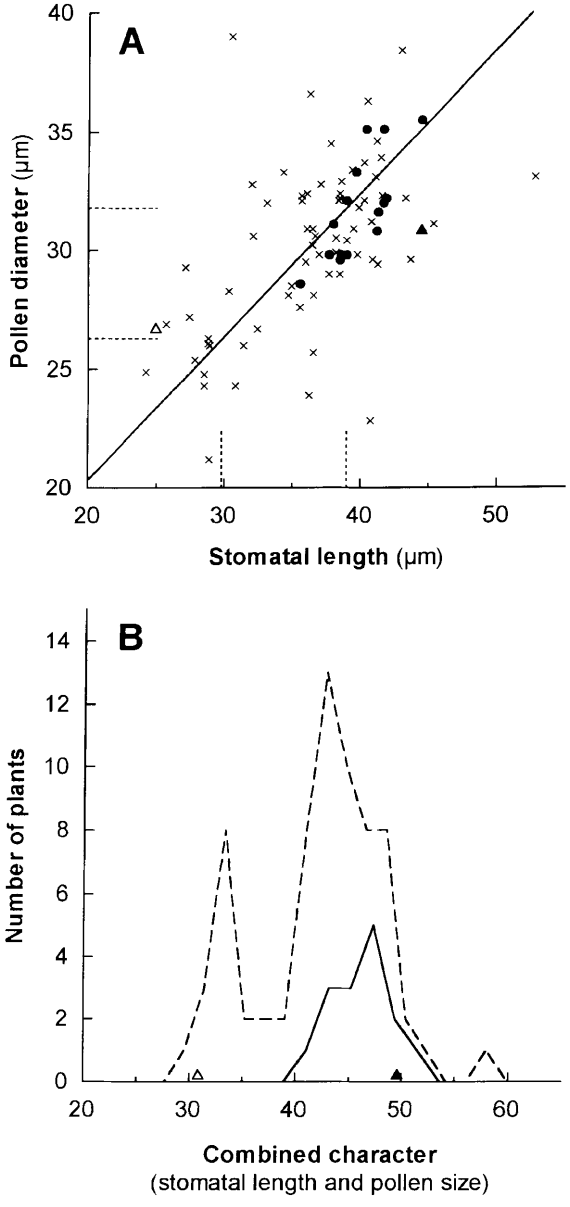

Fig. 1. Variation of the stomatal length and the pollen diameter in *D. glomerata*. **A** Distribution of the stomatal length vs. the pollen diameter. Crosses – the Slovenian reference plants (69 plants from Slovenia and the neighbouring regions); open triangle – the reference diploid plant ($2n = 2x = 14$); closed triangle – the reference tetraploid plant ($2n = 4x = 28$); closed circles – 15 plants from the five Krvavec populations (each population 3 plants). The dashed lines indicate the position of the mode values for the stomatal length and the pollen diameter in the putative diploid and tetraploid groups. **B** Distribution (frequency polygon) of the combined character (stomatal length and pollen diameter) along the line connecting the modes of the diploid and the tetraploid group (line shown in A). Dashed line – the Slovenian reference plants; full line – 15 plants from the Krvavec populations; empty triangle – position of the reference diploid plant; full triangle – position of the reference tetraploid plant

1974, Lindner and Garcia 1997), the group with small stomata and small pollen probably corresponded to diploids, while the one with large stomata and large pollen represented the tetraploid plants. This was also indicated with the position of the data points for the two plants with known chromosome numbers, one diploid and one tetraploid (Fig.1A).

The stomatal length ranged from 24 μm to 42 μm (the conspicuous outlier 53 μm). The frequency distribution for the stomatal length showed bimodality, with the mode for the putative diploids at 30 μm and for the putative tetraploids at 39 μm. Bimodality was less pronounced in the frequency distribution of the pollen diameter, with the mode for the diploids at 26 μm and for the tetraploids at 32 μm. Some overlapping occurred between the putative diploid and tetraploid group in both characters (Fig. 1A). The correlation between the stomatal length and the pollen diameter was statistically significant (Spearman correlation coefficient $r_s = 0.54$, $p < 0.001$).

To analyse the separation of the data into two groups, a 'modal line' was plotted through the point representing the mode values for the stomatal length and the pollen diameter for the putative diploid group, and the point representing the same respective parameters for the putative tetraploid group ('modal line' shown in Fig. 1A). We transformed the data by rotating the coordinate system, so that the new x-axis corresponded to the 'modal line'. The frequency distribution of the data points along this new x-axis was bimodal (Fig. 1B), with one mode representing the putative diploids and the other the putative tetraploids.

The pollen diameter and the stomatal length was also measured in plants from the

Krvavec populations (Fig. 1A, 1B). The data points for these plants were nested within the presumed tetraploid group, at the high range of distribution of both characters (Fig. 1A, 1B). The stomatal length was 39.8 ± 0.6 μm (mean ± standard error, N = 15) and the pollen diameter 31.8 ± 0.6 μm. The mean values of the two characters roughly corresponded to the modes of the respective characters for putative tetraploids as measured in the Slovenian reference plants (the stomatal length 39 μm, the pollen diameter 32 μm).

For the two studied morphological parameters, no statistically significant differences among the Krvavec populations were observed when either the parametric one-way ANOVA with Tukey's multiple comparison test or the nonparametric Kruskal-Wallis test with Dunn's multiple comparison test was applied. Furthermore, no statistically significant correlation between altitude and the two described morphological characters was detected when either parametric (Pearson correlation coefficient) or nonparametric (Spearman correlation coefficient) tests were used.

Genome size as a direct estimator of ploidy level. The number of chromosomes was counted in 19 individuals from the Krvavec populations. In all individuals, the chromosome number was 28, showing that all examined individuals were tetraploid and therefore belonged to the tetraploid taxon *D. glomerata* subsp. *glomerata*, and not to the diploid subsp. *aschersoniana*.

Genome size (2C nuclear DNA content) was measured in 55 individuals from the Krvavec populations (Fig. 2A). The mean 2C value was 8.58 ± 0.05 pg DNA (Table 1, all individuals). The 2C value showed 23% variation and ranged from 7.64 pg to 9.61 pg. (Fig. 2A, 2B). The frequency polygon of 2C values for the 55 individuals revealed a clearly unimodal peak (Fig. 2B) with the coefficient of variation 4.6% (Table 1).

The mean 2C values of the Krvavec populations showed only 2.1% variation and ranged from 8.52 pg to 8.70 pg (Table 1, populations; Fig. 2C). The highest range of intrapopulation variation (23%) was observed in the population growing at 1230 m, while at the other four altitudes the range of variation within the populations was between 8% and 13% (Table 1). In all five Krvavec populations, the difference between the smallest and the largest measured genome size was statistically significant as estimated from the position and the width of the 2C peak with Student's t-test ($p < 0.001$).

When the 2C values measured in the Krvavec populations were compared, no significant differences among populations were detected using either parametric one-way ANOVA with Tukey's multiple comparison test, or nonparametric Kruskal-Wallis test with Dunn's multiple comparison test. To test whether a correlation exists between altitude and genome size, a regression line was plotted over values for individuals (Fig. 2A). The correlation was not significant (Pearson correlation coefficient $r = -0.11$, $p > 0.05$). The correlation between altitude and genome size was also not significant when the mean genome sizes of the populations were used instead of values for individuals ($r = -0.49$, $p > 0.05$), or when nonparametric analysis was applied to values for individual plants (Spearman correlation coefficient $r_s = -0.16$, $p > 0.05$).

The nucleolar number as an estimator of ploidy level. The nucleolar number was counted in the root meristem region of 10 individuals from the Krvavec populations using the silver staining technique. Per slide, 330 to 750 nuclei were analysed (total number 5657). One to eight nucleoli were observed per nucleus (Fig. 3). The highest number of nucleoli was seven in 2 individuals and eight in 8 individuals (Fig. 4). When the frequency distributions obtained for individuals were averaged (Fig. 3), the nucleolar number was one, two or three in approx. 65% of the nuclei. Four nucleoli were observed in approx. 20% of the nuclei. In 15% of the nuclei, the nucleolar number was five or higher.

In addition, the number of nucleoli per interphase nucleus was counted in the root meristem region of 14 individuals from the Krvavec populations using the Feulgen stain-

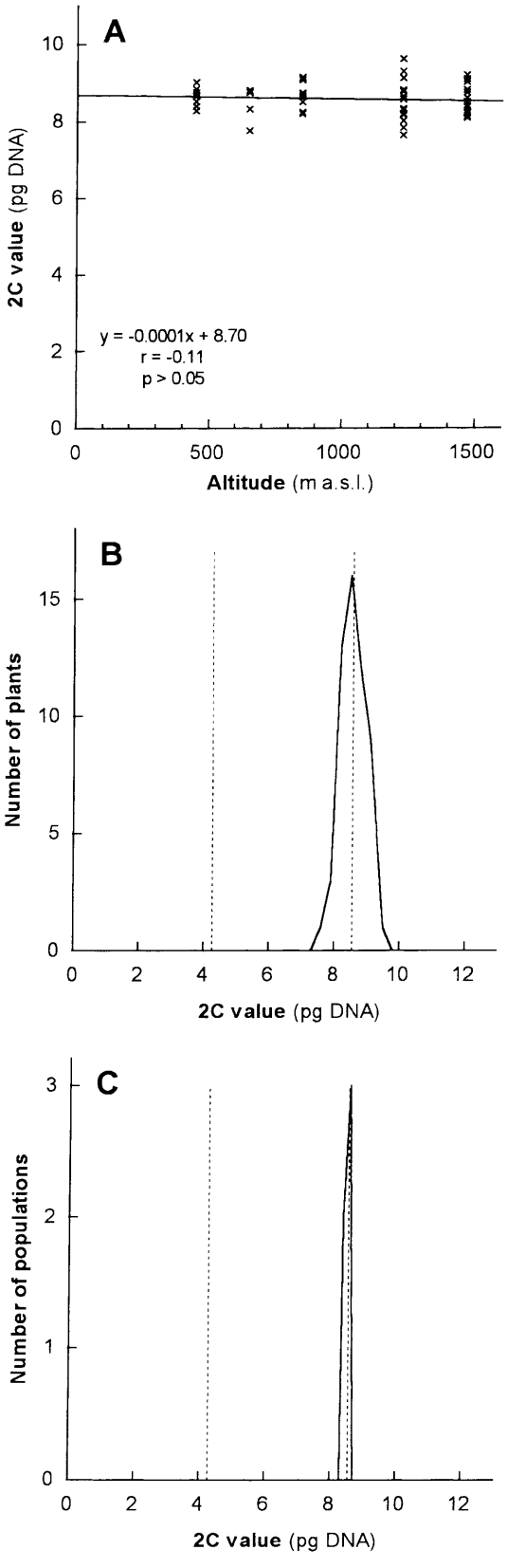

Fig. 2. Variation in genome size in *D. glomerata* subsp. *glomerata*. **A** 2C values of 55 individuals from the five Krvavec populations growing at different altitudes. Absence of correlation between altitude and genome size is shown with the regression line (regression statistics is indicated on the graph). **B** Distribution (frequency polygon) of 2C values recorded in 55 individuals from the five tetraploid Krvavec populations. Gridlines – the position of the mean tetraploid 2C value and the calculated diploid 2C value. **C** Distribution (frequency polygon) of the mean 2C values of the five Krvavec populations. Gridlines as in **B**

ing technique. Per slide, 40 to 170 nuclei with clearly visible nucleoli were analysed (total number 1209). One to seven nucleoli were observed per individual nucleus (Fig. 3). In 11 individuals, the highest number of nucleoli was five or more. The maximal number of nucleoli was four in 3 individuals, five in 2 individuals, six in 4 individuals and seven in 5 individuals. When compared to the silver staining technique, the Feulgen staining gave on average an underestimation of the nucleolus number by one nucleolus per nucleus (Fig. 3).

The Feulgen staining method enabled us to plot the frequency distribution polygons for the nuclei in the G1 and in the G2 phase of the cell cycle (Fig. 3). In both phases of the interphase, the distribution of nuclei with different numbers of nucleoli was very similar. On average, in approx. 80% of both the G1 and G2 nuclei the number of nucleoli was one, two or three. Four nucleoli were observed in approx. 10% of the nuclei. In 7% of the nuclei, the number of nucleoli was five or higher. A similar distribution emerged when nonparametric statistical analysis was used and the medians were plotted instead of the mean values (data not shown).

Discussion

In the taxon *D. glomerata*, morphologically similar individuals with different ploidy levels are often encountered (Lumaret 1988, Miziantly 1990, Wetschnig 1983), and they can only be

Table 1. Genome size in the Krvavec populations of *D. glomerata* subsp. *glomerata* growing at different altitudes. 2C – the mean 2C value of the populations calculated using *Hordeum vulgare* (2C = 10.04 pg DNA) as the standard; SEM – standard error of the mean (N corresponds to the number of individuals), CV – coefficient of variation. The range of variation was calculated as the difference between the highest and the lowest 2C value divided by the mean value. 2C (arbitrary units) – the mean 2C values of the populations were normalised with the mean 2C value of all individuals set at 100

Altitude (m a.s.l.)	N	2C (pg)	SEM	CV (%)	Range of variation (%)	2C (a.u.)
450	10	8.64	0.07	2.4	8.4	100.7
650	6	8.53	0.17	4.9	12.3	99.4
850	10	8.70	0.11	3.9	10.8	101.4
1230	15	8.52	0.14	6.4	23.1	99.3
1470	14	8.54	0.10	4.3	13.0	99.5
Populations	5[a]	8.59	0.04	0.9	2.1	100.1
All individuals	55	8.58	0.05	4.6	23.0	100.0

[a] Number of populations

distinguished with karyological analysis or on the basis of morphological features associated with the ploidy level, such as the stomatal length and the pollen diameter.

Previous reports demonstrated that the diploids of the genus *Dactylis* have smaller stomata and smaller pollen than the tetraploids, although some overlapping occurs between the two ploidy groups in both morphological characters (Müntzing 1937, Rurka 1974, Lumaret et al. 1987, Lindner and Garcia 1997). The herbarium specimens of the Slovenian reference plants very likely included some diploid as well as the more frequently occurring tetraploid *D. glomerata* plants, and a weak separation of the specimens into two groups was observed on the basis of the stomatal length and the pollen diameter (Fig. 1A).

When the stomatal length and the pollen diameter data were transformed as shown in Fig. 1B, the bimodal distribution became clearer than with either of the two characters alone. The data transformation thus improved discrimination between the two groups. However, some overlapping of the two groups remained, and there were some outliers not clearly nested in either of the groups (Fig. 1A, 1B). Although the plants from the Krvavec populations appeared to be nested in the putative tetraploid group (Fig. 1A, 1B), the separation between the two groups was too ambiguous to reliably deduce the ploidy level of individual plants.

Since the morphological characters proved not to be accurate enough to determine the Krvavec populations of *D. glomerata* at the subspecies level, we investigated the number of chromosomes as the ultimate criterion for the ploidy level. In the Krvavec populations, all individuals in which the chromosome numbers were counted had 28 chromosomes, which is consistent with the tetraploid taxon *D. glomerata* subsp. *glomerata* (Wetschnig 1983, Mizianty 1991b).

However, chromosome counting is methodologically laborious, and there is a growing interest to find simpler alternatives for a reliable determination of the ploidy level. We examined two karyological characters directly linked to the ploidy level in *D. glomerata*, genome size and the nucleolar number.

The mean 2C values of the Krvavec populations ranged from 8.5 to 8.7 pg DNA (Table 1, Fig. 2C). The 2C values of the Krvavec populations are in general agreement with the published 2C values for tetraploid *D. glomerata* of 8.7 pg (Grime et al. 1988) and 8.3 pg DNA (Greilhuber and Baranyi 1999). Our data also agree well with 2C values of 8.7 pg to 11.2 pg observed in populations growing at

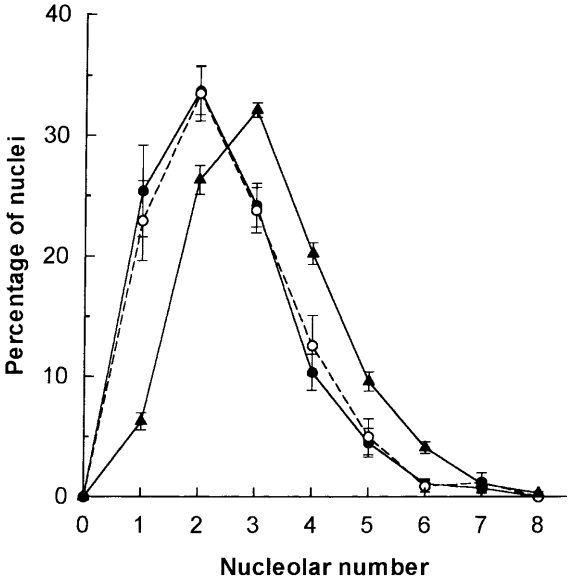

Fig. 3. Distribution (percentage frequency polygons) of nuclei with different numbers of nucleoli in the root meristem of *D. glomerata* subsp. *glomerata*. Triangles, full line – silver-stained slides (330 to 750 nuclei per slide, 10 individuals); circles – Feulgen-stained slides (40 to 170 nuclei per slide, 14 individuals). The values for nuclei in G1 (closed circles, full line) and G2 (open circles, dashed line) phase of the cell cycle are shown separately. For both staining methods, the percentage distribution of nuclei with different numbers of nucleoli was calculated for each individual. The mean percentage distribution polygon calculated from data for individual plants is shown (the mean value ± standard error, N = number of individuals, see above)

different altitudes (Creber et al. 1994, Reeves et al. 1998). Namely, in these two studies the same standard species (*H. vulgare*) was used as in our measurements, but the 2C value of the standard was set at 11.2 pg DNA, while we used the value 10.0 pg (Doležel et al. 1998). When the data are recalculated using 10.0 pg DNA as the standard, the range of 2C value variation observed in *D. glomerata* by Creber et al. (1994) and Reeves et al. (1998) is from 7.8 to 10.0 pg DNA for 28 tetraploid population from different parts of Europe.

Although some variation in the measured genome sizes was found within the Krvavec populations (Table 1, Fig. 2A, 2B), the range of this variation indicates that the populations are strictly tetraploid. Based on our results, a calculated estimate of the 2C value for a diploid individual is 4.3 pg DNA, as indicated in Fig. 2B and 2C. The lowest 2C value measured for individuals from the investigated populations was 7.6 pg and the highest 9.6 pg. While these two individuals were located at the extremes of the recorded 2C values, they were not conspicuous outliers (Fig. 2A, 2B).

Variation of measured genome sizes within and among populations comprises two possible sources of variation which are difficult to discriminate: true biological variation, such as karyotypical and genetic differences among individuals, and methodological noise. The coefficient of variation for the 2C value measured in 55 individuals was 4.6%, for the mean values of the five populations 0.9% (range of variation 2.1%) and within populations from 2.4% to 6.4% (Table 1, Fig. 2B, 2C). A similar level of variation was observed when genome size was measured with flow cytometry in 108 tetraploid individuals of *D. glomerata* (coefficient of variation 5.3%; Horjales et al. 1995). On the other hand, in another grass species, *Sesleria albicans* Kit. ex Schult., the range of variation among ten populations was 1.6%, and the variation within individual populations showed a low coefficient of variation from 0.9% to 1.2% (Lysák et al. 2000). Presently, it is not possible to determine whether the observed differences among individuals of *D. glomerata* point to biological variation, which might indicate a less stable genome than in *S. albicans*, or are rather due to noise.

Difficulties in estimation of ploidy levels with genome size measurement may arise when apart from euploids also aneuploids occur in a studied taxon. Furthermore, the presence of B chromosomes may affect genome size. In *D. glomerata* subsp. *glomerata*, aneuploids have been described by Müntzing (1937), but no other author reported aneuploid chromosome numbers in this taxon (Mizianty 1991a). On the other hand, B chromosomes have been observed in *D. glomerata* subsp. *glomerata* (Mizianty 1990, and references therein; Mizi-

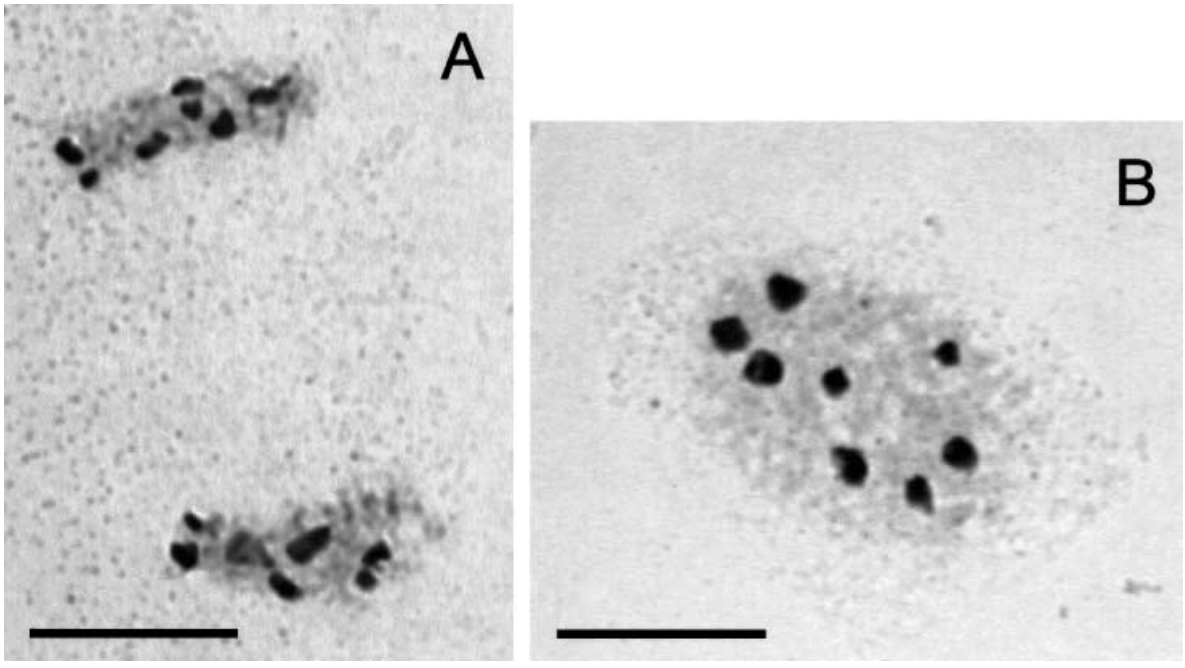

Fig. 4. Silver-stained nuclei of *D. glomerata* subsp. *glomerata* in late telophase (**A**) and in interphase (**B**). Note that eight nucleoli are present in the upper nucleus in A and in B. Bar: 10 μm

anty 1991a). They are usually half the size of the regular chromosome (Mizianty 1990), hence one B chromosome would increase the nuclear DNA content roughly by 2% in a tetraploid and by 4% in a diploid, and only presence of numerous B chromosomes could lead to confusion between the diploid and the tetraploid level in genome size measurements. In the plants from the Krvavec populations, no B chromosomes were observed.

D. glomerata subsp. *glomerata* has four chromosome pairs with secondary constrictions; two pairs were presumably inherited from each of the parent genomes (Wetschnig 1983, Mizianty 1991a). In some allopolyploid plant hybrids, nucleolar dominance has been described (McClintock 1934, Navashin 1934; reviewed in Pikaard 1999), whereby in hybrids one parental set of rRNA genes is transcribed, but the rRNA genes inherited from the other parent are repressed. A cytogenetic manifestation of NOR repression is the loss of the secondary constriction in metaphase chromosomes.

In the diploid *D. glomerata* subsp. *aschersoniana*, two chromosome pairs with secondary constrictions are present and maximally four nucleoli were observed (Wetschnig 1983), showing that there are four active NORs in this putative parent species of subsp. *glomerata*. In *D. glomerata* subsp. *glomerata*, eight secondary constrictions have been phylogenetically preserved (Wetschnig 1983, Mizianty 1991a), indicating that the NORs from both ancestor genomes are co-dominant. We observed maximally eight nucleoli in the silver-stained nuclei of subsp. *glomerata* (Figs. 3, 4). The presence of eight active NORs in this subspecies confirms that the NORs inherited from both parent genomes are functional.

The dynamics of nucleolar coalescence during the cell cycle was studied in Feulgen-stained nuclei (Fig. 3). Notably, the nuclei in the G1 and the G2 phase of the cell cycle showed essentially the same frequency distribution of the nucleolar number (Fig. 3). These results indicate that nucleolar coalescence occurs early in the G1 phase and that the

established number of nucleoli is then preserved during later stages of the interphase.

The silver staining technique proved to be adequate for discrimination between diploids and tetraploids on the basis of the nucleolar number (Figs. 3 and 4). In all ten tetraploid individuals from the Krvavec populations examined with the silver staining method, the maximal nucleolar number was at least seven, which is clearly more than four as reported for diploids (Wetschnig 1983). On average, five or more nucleoli were detected in 15% of the nuclei (Fig. 3), and the lowest proportion of such nuclei in an individual plant was 9% (data not shown). Hence, even a quick screening of the silver-stained slides should be sufficient to determine the ploidy level.

On the other hand, the Feulgen method is not sensitive enough for a reliable discrimination between diploids and tetraploids on the basis of the nucleolar number. Individual nucleoli in the Feulgen-stained nuclei are detected as non-stained regions largely devoid of chromatin. In three out of fourteen plants examined with this technique, the highest observed nucleolar number was four, although these individuals were tetraploids as estimated from genome size.

In total, we determined the chromosome number, genome size or the nucleolar number with the silver staining method in 84 individuals from the Krvavec populations. These karyological characters were consistent with the tetraploid ploidy level in all examined individuals, confirming that the Krvavec populations belong to the taxon *D. glomerata* subsp. *glomerata*.

Apart from determining the ploidy level in the studied populations, we also investigated the adaptive value of genome size as related to altitude. A gradient of climatic conditions along an altitudinal transect results in a shorter vegetation season at higher altitudes, and plants with smaller genomes may be able to complete the annual growth cycle faster than those with larger genomes (Bennett 1987). We detected no correlation between altitude and genome size in the Krvavec populations (Fig. 2A). In contrast, a negative correlation between altitude and genome size has been observed in tetraploid *D. glomerata* at altitudinal transects in Spain, France and Italy (Creber et al. 1994, Reeves et al. 1998). The genetic nature of this variation in genome size is not known. In these studies, the possibility that some variation in genome size among populations might be due to the presence of B chromosomes has not been explored. Notably, in the diploid populations of *D. glomerata* in Israel, populations occupying various habitats differ in the number of B chromosomes (Zohary and Ashkenazi 1958).

Among other grass species, a negative (Rayburn 1990, Poggio et al. 1998) as well as a positive (Rayburn and Auger 1990) correlation between altitude and genome size has been observed in cultivars of *Zea mays* L. from different countries. In *Sesleria albicans*, genome size did not correlate with altitude (Lysák et al. 2000). Recently, a negative correlation between altitude and genome size was detected in wild populations of a dicot species *Arachis duranensis* Krapov. & W. C. Gregory (Temsch and Greilhuber 2001). Hence, while a relationship exists between altitude and nuclear DNA content in some studied cases and genome size may thus have an altitude-related adaptive value, the phenomenon appears to be complex and the present evidence is not sufficient to accept it as a general rule.

In conclusion, morphometrical and karyological methods are presently available to determine the ploidy level in *D. glomerata*. With morphometry, the ploidy level can be estimated from the stomatal length and the pollen diameter. Although this method is not adequate for a definite determination of the ploidy level due to incomplete separation between the diploid and the tetraploid group (Fig. 1A, 1B; Lumaret et al. 1987), it allows for the screening of the herbarium material and for identification of suspect diploid populations of *D. glomerata*, which in general occur in Europe less frequently than the tetraploids (Wetschnig 1984, Mizianty 1990). Following identification of suspect diploid plants

(Fig. 1A, 1B), we will re-investigate the sites at which the putative diploid plants were collected to clarify the distribution range of different subspecies of *D. glomerata* in Slovenia and to karyologically confirm the presence of diploids of *D. glomerata* in Slovenia.

The karyological methods, on the other hand, require living plant material, but are also more accurate than morphometry. While the number of chromosomes is the direct proof of the ploidy level, two other characters can also be used, namely genome size and the nucleolar number. In the present study, we demonstrate that the range of variation in genome size in *D. glomerata* is low enough (Fig. 2B and 2C) to allow for accurate determination of the ploidy level of populations and individuals with Feulgen densitometry of interphase nuclei. This methodological approach is less tedious than chromosome counting, it does not require a metaphase block and can be used also in non-dividing tissues (Price et al. 1980, Hörandl et al. 2000) and in material easily fixed in the field other than root tips, i.e. the leaf bases, vegetative shoot meristems, young flowers and fruits. While flow cytometry may also be used to discriminate among ploidy levels on the basis of genome size in *D. glomerata* (Horjales et al. 1995), this method relies upon fresh plant tissue in a good physiological condition. We believe that measurement of genome size with the Feulgen DNA image cytometry, as demonstrated in our study, is a simple and reliable method for determination of the ploidy level in plants.

This work was supported by a grant from the Ministry of Science and Technology, Slovenia (project J1-1579-0487-99).

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