Intraspecific Variation in Genome Size in Angiosperms: Identifying its Existence

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INTRODUCTION

The existence and amount of genome size variation within species of higher plants have been topical since the early days of genome size research, when commercial cytophotometers became available. The problem is multifaceted, with instrumental-technical, preparative, material-dependent and taxonomical aspects. Importantly, the scientific approach of the investigator can also be significant. Demonstration of stability of genome size in different samples of an organism can be taken as a proof for actual stability, because any fault in methods would cause variation rather than constancy of the measurement results. The finding of variation should instantly provoke the question whether it is real variation in DNA amount or simply an artefact. Repeatability of variation is not necessarily a proof, because it could be due to an interfering factor that varies. This has been clearly shown in studies on endogenous staining inhibitors (Greilhuber, 1986, 1988; Noirot et al., 2000, 2005; Price et al., 2000). Then there is the variation or discrepancy between results on the same species by different authors (see, for instance, the treatments by Greilhuber and Obermayer, 1998a, b; Baranyi and Greilhuber, 1999; Bennett et al., 2003). Was the material the same? If not, does it matter for the conclusions? Who is using the better laboratory) are reviewed below. They deal with (a) the special problem of the ‘plastic’ or ‘fluid’ genome and (b) the existence or otherwise of genome size variation in two cultivated plant species and in a wild progenitor of one of them. Furthermore, the apparent or real higher error-susceptibility of slide-based Feulgen densitometry compared with flow-cytometry has suggested an investigation of the critical steps of the quantitative Feulgen procedure. Despite the huge amount of methodological literature, some of its basic aspects, such as the decay in quality of fixed plant material over time while being stored at various temperatures, have obviously never been investigated before.

DASYPYRUM VILLOSUM, PLASTIC GENOME OR PLASTIC DATA?

Dasypyrum villosum (syn. Haynaldia villosa; 2n = 14) is a Mediterranean grass species of the tribe Triticeae, which exhibits a remarkable intra-individual fruit colour polymorphism from yellow to almost black. Being based on the colour of maternal tissue, this polymorphism necessarily has a modificatory basis. Accordingly, it has been shown that plants grown from fruits of any colour again produce pale and dark fruits [see references in Cremonini et al. (1994) and Cremonini (2003)]. Innocenti and Bitonti (1983) reported a longer mitotic cycle in seedlings from pale than from dark caryopses. Cremonini et al. (1994) studied heterochromatin banding patterns of metaphase chromosomes and nuclear DNA content in plants grown from pale and dark caryopses. The material originated from a natural population in Italy. In addition to minor chromosome-structural differences correlated with fruit colour, they found 20–24 % more DNA in early prophase...
TABLE 1. Flow cytometric comparison of genome size (given in percentage of *Pisum sativum*) in young plants of *Dasypyrum villosum* grown from yellow and dark brown caryopses.

<table>
<thead>
<tr>
<th>Accession no.</th>
<th>Yellow</th>
<th>Dark brown</th>
<th>Ratio</th>
</tr>
</thead>
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<tr>
<td>GRA 1313/9</td>
<td>111.3</td>
<td>111.8</td>
<td>0.995</td>
</tr>
<tr>
<td>HGRA 960/8</td>
<td>111.9</td>
<td>114.8</td>
<td>0.974</td>
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<td>GRA 1105/9</td>
<td>112.1</td>
<td>114.3</td>
<td>0.980</td>
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<tr>
<td>GRA 1310/9</td>
<td>112.1</td>
<td>111.8</td>
<td>1.002</td>
</tr>
<tr>
<td>GRA 2706/97</td>
<td>113.2</td>
<td>113.3</td>
<td>0.999</td>
</tr>
<tr>
<td>GRA 961/83</td>
<td>113.4</td>
<td>115.0</td>
<td>0.986</td>
</tr>
<tr>
<td>GRA 1170/9</td>
<td>113.4</td>
<td>112.9</td>
<td>1.004</td>
</tr>
<tr>
<td>GRA 2707/98</td>
<td>114.0*</td>
<td>111.7</td>
<td>1.020</td>
</tr>
<tr>
<td>GRA 1022/8</td>
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<td>111.7</td>
<td>1.023</td>
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<td>GRA 2262/9</td>
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<td>112.8</td>
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<td>0.999</td>
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<tr>
<td>GRA 2630/95</td>
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</tr>
<tr>
<td>GRA 992/88</td>
<td>118.7</td>
<td>116.0</td>
<td>1.023</td>
</tr>
<tr>
<td>GRA 2713/9</td>
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<td>0.994</td>
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<tr>
<td>Means</td>
<td>114.8</td>
<td>114.2</td>
<td>1.004</td>
</tr>
<tr>
<td>s.d.</td>
<td>2.522</td>
<td>2.314</td>
<td>0.015</td>
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<tr>
<td>CV</td>
<td>2.198</td>
<td>2.026</td>
<td>0.015</td>
</tr>
<tr>
<td>N</td>
<td>17</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Mean of ratios</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paired t-test</td>
<td></td>
<td></td>
<td>1.004</td>
</tr>
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</table>

The accessions (IPK Gatersleben) were from various Mediterranean countries.

The DNA stain was propidium iodide.

*Pisum sativum* ‘Kleine Rheinländerin’ was co-chopped for internal standardization.

If not stated otherwise, three *D. villosum* plants were studied per accession and fruit colour.

From Oermayer and Greilhuber (unpubl. res.).

* Six plants averaged.

nuclei in seedlings from pale caryopses than from dark ones. Frediani et al. (1994) described a similar but somewhat smaller difference in DNA content, together with an increase of genome size during germination, which was greatest when the initial genome was the smallest. A quantitative modulation of a certain near-telomeric sequence was found in chromosomes of germinating seedlings from the two types of caryopses, indicating the existence of a variable element in the genome of *D. villosum* (Frediani et al., 1994).

This problem was re-investigated with flow cytometry using propidium iodide as the DNA fluorochrome and *Pisum sativum* ‘Kleine Rheinländerin’ as a co-processed standard (R. Oermayer and J. Greilhuber, unpubl. res.). Twenty-nine accessions of *D. villosum* from various countries of the Mediterranean area were obtained from the germplasm collection of the Institut für Pflanzenzogenetik und Kulturpflanzenvorschung (IPK) in Gatersleben. Intergradation in colouring of fruits was found, and caryopses were classified as yellow, mid-brown and dark-brown. From these, plantlets were grown and their genome size was determined. Comparison of yellow versus dark brown was possible in 17 accessions, which should allow any differences to be revealed (Table 1). A paired *t*-test was not significant (*P* = 0.212), the mean ratio of genome sizes in yellow versus dark brown was 1.0048. No other possible within-accession comparison of the three colour groups was significant. We conclude therefore, that there is no genome size plasticity associated with fruit colour in *D. villosum*.

However, there were differences in genome size up to 1.07-fold between our accessions, with a maximum difference between individuals of 1.11-fold. Within accessions the genome sizes of seedlings from pale and dark caryopses were significantly correlated (*y* = 0.995; *r* = 0.654). Individuals with extreme values and the standard were co-processed for flow cytometry and a separation of the *Dasypyrum* peaks was found (Fig. 1). These are strong arguments that the differences observed are more than just technical ‘noise’, but they are not a complete proof of real variation, because differences in content of endogenous staining inhibitors can mimic DNA content differences (Price et al., 2000), although co-processing should minimize this risk.

In an extensive study on genome size variation in Italian *D. villosum*, Caceres et al. (1998) mention nothing of fruit colour-related differences between plants, but they report up to 17.6% difference between means of populations and up to 66.2% difference between individual plants within a population. In the light of the present data such a large variation appears improbable. It is possible that the use by Caceres et al. (1998) of hot hydrolysis (1 N HCl, 60 °C, 8 min) with enzyme-macerated slides could be responsible for such divergences. It is well known that the very short optimum and the fast rise and decay of the hot hydrolysis curve constitute an experimental risk. It is then questionable which value one should ascribe to the various correlations with genome size reported by Caceres et al. (1998).
GENOME SIZE VARIATION IN 
GLYCINE MAX

There are strong indications for an adaptive role of genome size via cell size and cell cycle time (Bennett, 1972; Price and Bachmann, 1976; Cavalier-Smith, 1985, 2005; Gregory, 2003). Plants with short reproductive cycles, such as ephemeral and annual herbs, have genomes that are smaller on average than those with long cycles such as perennial herbs (Bennett, 1972). Aggressive weeds, which are assumed to reproduce vigorously and quickly, have genomes smaller than those of non-weeds (Bennett et al., 1998). The obvious significance of genome size for developmental speed has stimulated studies in cultivated taxa, which have investigated the relationship between genome size and the time needed for seed ripening, which is largely correlated with the geographical latitude of the plants’ cultivation. If developmental speed depends at least in part on genome size, selection for more rapid development could have occurred in parallel with selection for smaller genomes. This may have indeed been the case in maize landraces, in which Laurie and Bennett (1985) and Rayburn et al. (1985) have independently found a decrease in genome size from south to north in North America in connection with a decrease in amount of knob heterochromatin. In contrast, Baranyi and Greilhuber (1995, 1996) found uniform genome sizes in highly selected cultivars, landraces and wild accessions of Pisum sativum worldwide, including samples from equatorial regions and Scandinavia.

Glycine max (soybean) cultivars in North America have been investigated by Graham et al. (1994), using flow cytometry and propidium iodide as the DNA stain, but with no internal standard. They found genome size to vary by about 1-11-fold, the more northern-adapted cultivars having genomes significantly smaller than those of southern-adapted ones. In 95 Chinese accessions of soybean a similar range of variation was recorded, but maturity groups were not available (Rayburn et al., 1997). However, a flow-cytometric and Feulgen densitometric re-investigation of the same North-American soybean cultivars by Greilhuber and Obermayer (1997), who used internal standardization, revealed uniformity of genome size (see also Greilhuber, 1998). A closer inspection of the data of Graham et al. (1994) by Greilhuber and Obermayer (1998b) showed that the significance of their correlation resulted from one single extraordinarily low value and a few deviations from the USDA (United States Department of Agriculture) maturity group list. If these are corrected and the low value is excluded, the significance disappears. In a further study, Obermayer and Greilhuber (1999) presented evidence for genome size stability in Chinese soybeans, showing that there is actually no difference between the five accessions ranking highest and the five ranking lowest in the study by Rayburn et al. (1997) (Fig. 2). Chung et al. (1998) studied genome size in 12 lines of soybean with flow cytometry in relation to a correlation with seed size and weight and leaf size. They found a small variation, up to 1-046-fold, in genome size, that was strongly correlated with seed weight and leaf size, although seed size varied more than five-fold and leaf size varied more than two-fold between extreme lines. It would be interesting to know to what extent leaf cell size and nuclear size, and hence chromatin dispersion, also vary in parallel with organ size in these lines. It would be worthwhile to study the possible influence that chromatin compaction can have on uptake of propidium iodide by the nuclei, and whether this factor could have contributed to this minor variation in genome size estimates.

ARACHIS HYPOGAEA

The groundnut (Arachis hypogaea, 2n = 40, 4x) is another important grain legume in which genome size variation in the range of 1-15-fold has been described recently (Singh et al., 1996). The method of measurement was Feulgen densitometry. As the variation was reported even within varieties of the same subspecies, Temsch and Greilhuber (2000) undertook a re-analysis of the most divergent samples studied by Singh et al. (1996), using flow-cytometry and Feulgen densitometry. Not only was the variation not confirmed, but they even found the mean of the C-values to be very different from that given by Singh et al. (1996) (Fig. 3). In fact, the values reported by Singh et al. (1996) are about twice as high as they should be. It is noteworthy that a value for A. hypogaea similar to that obtained by Temsch and Greilhuber (2000) had already been recorded in the literature (Arumuganathan and Earle, 1991). The uniformity of genome size in A. hypogaea is in line with the low molecular variability of this species (Stalker et al., 1995).

ARACHIS DURANENSIS

Arachis duranensis (2n = 20, 2x) is one of the possible wild ancestors of the groundnut, A. hypogaea. The species is morphologically variable and exhibits more molecular
variation than *A. hypogaea* (Stalker et al., 1995). Singh et al. (1996) reported 1:11-fold variation in genome size in 28 accessions. In 11 of these, in which data on the altitude of the collection sites were given, a strong negative correlation between C-values and altitude was found. In a re-investigation of this case, covering 20 accessions, Temsch and Greilhuber (2001) also found some variation. At 1:07-fold, it was not as high as that reported by Singh et al. (1996), but there was also a negative correlation with altitude of the sampling sites as given by the ICRISAT. Paradoxically, however, the measurement data obtained from the same 11 accessions with known altitude above sea level published by Singh et al. (1996) and by Temsch and Greilhuber (2001) were not significantly correlated (Temsch and Greilhuber, 2001). The explanation is this: Singh et al. (1996), although certainly having all of the altitude data documented by the ICRISAT (one co-author was an authority of the germplasm collection at that time), selected 11 samples which fitted such an arbitrarily assumed regression line. If all altitudes above sea level now available (Stalker et al., 2003) are considered and correlated against the corresponding genome size data by Singh et al. (1996), no significant correlation is observed (Fig. 4).

**IMPORTANCE OF BEST PRACTICE METHODOLOGY**

Flow cytometry and Feulgen densitometry are complementary techniques with advantages and disadvantages of their own, as has been shown in two studies that give highly comparable results (Doležel et al., 1998; Vilhar et al., 2001). While gross errors in DNA content estimates by flow cytometry using intercalating fluorochromes seem to be rare, except when inhibitors are involved (Price et al., 2000; Noirot et al., 2000), or when wrong assumptions for standard species are made (see Doležel et al., 2003; Doležel and Bartoš 2005), it is surprising that two-fold errors occur in recently published studies purportedly using established techniques and instruments on plant material which cannot be regarded as particularly difficult. Work on the legume *Cajanus* is one example, where Bennett and Smith (1976) and Greilhuber and Obermayer (1998a) found C-values only half as large as those recorded by Ohri et al. (1994) while Ohri and Singh (2002) have used new measurements to defend the criticized data against Greilhuber and Obermayer (1998b). The discrepancies in *Arachis* as shown in this paper are another example. In the accumulation of genome size data (Bennett and Leitch, 2003), questions of best practice in Feulgen cytophotometry are obviously more important than ever. Therefore, we have studied some basic aspects of the Feulgen method in plants (Greilhuber and Temsch, 2001; J. Greilhuber, unpubl. res.), some of which have either not been studied at all before, or not in plant material. Our point of view, including recent observations, is summarized below.

Plant material frequently contains endogenous staining inhibitors (presumably often of phenolic nature) that can strongly affect the measurement results (Greilhuber, 1986, 1988). Before fixation, plant parts preferred for study (e.g. ovules) should be separated from other parts, which might contain such inhibitors in higher concentration. The working standard (biological reference material for calculating absolute DNA amounts and making data universally comparable) should be free of inhibitors. Actively growing root
tips of *Pisum sativum* meet this condition. In addition, this species has desirable karyological characteristics for quantitative work (Vilhar et al., 2001): it is apparently stable in genome size world-wide (Baranyi and Greilhuber, 1995, 1996), and it is easy to obtain and to germinate. Any available cultivar can be chosen and calibrated against *P. sativum* ‘Minerva Maple’ as a precaution. ‘Minerva Maple’ is obtainable from Professor M. D. Bennett, Royal Botanic Gardens, Kew, Surrey, UK.

Plant material with unknown characteristics should be fixed both with 1:3 acetic methanol or acetic ethanol and 4 % buffered formaldehyde. Formaldehyde polymerizes condensed tannins within the vacuole (Greilhuber, 1986). Phenolics often turn brown in formaldehyde and are thus recognized. The working standard should both be co-fixed in the same vessel as the test material and in a separate vessel concurrently. Caution should be exercised if the results differ considerably between fixatives, when the test material shows unusual variation in staining, or if the co-fixed standard shows inhibition. With formaldehyde fixation, increased time, temperature and concentration, and lower pH will all reduce Feulgen staining intensity.

In contrast to formaldehyde, acetic alcohols as nucleoprotein-precipitating fixatives do not have this influence. This is the rationale for fixing a working standard in parallel when formaldehyde is used. After about 1-5 h the formaldehyde should be washed out for about 1 h or longer with several changes of acetic methanol. Formaldehyde- and acetic alcohol-fixed material should soon be transferred (say, after 24 h in the refrigerator) to ethanol and stored at −20 °C. It then retains full staining capacity for at least 7 years (J. Greilhuber, unpubl. res.). Quality decay of acetic-alcohol fixations kept in ethanol and acetic methanol at room temperature, at about 6 °C (refrigerator) and at −20 °C was studied by J. Greilhuber (unpubl. res.) (Fig. 5). The results indicate that fixations can be kept for about 2 weeks at ambient temperature, but then decay becomes considerable. It is stronger when the fixative has not been replaced by ethanol. This information may be helpful when the material is collected in the field. Decay in the refrigerator is surprisingly slow, with 3 months of storage seeming possible. Temperature while storing fixations should always be regarded a critical parameter and kept as low as possible. This is especially relevant during field work.

Fixed material should be cut into small pieces and washed before hydrolysis. Prolonged hydration is not a problem, and several days in water in the refrigerator should have no adverse effect.

Hydrolysis is well known to be the most critical step of the Feulgen method. For most routine purposes, in plants the Feulgen procedure should be conducted on small pieces of tissue. In general, there is no advantage in carrying out the procedure on slides, which needs large quantities of reagents and eventually a pre-maceration. The classical hot hydrolysis (1 N HCl, 60 °C, about 10 min) has a very short optimum which is difficult to control and for which different authors give different times. The cold hydrolysis curve (5 N HCl, 20 °C, 60 min) has a very flat region of reasonable duration (55–65 min are optimum for acetic

**FIG. 4.** Diagram showing the available data in *Arachis duranensis* on the altitude above sea level of collection sites (Stalker et al., 2003) correlated against the genome size measurements by Singh et al. (1996). The data points included by Singh et al. (1996) are shown in black ($r = -0.778$, $P \leq 0.005$), those not included are shown in white. The overall correlation is not significant ($r = -0.210$, $P = 0.283$). Original figure by E. M. Temsch.

**FIG. 5.** Quality decay of fixations over time, shown as reduction in Feulgen staining intensity, in *Pisum sativum* root tip meristems, stored in acetic methanol or 96 % ethanol, at room temperature, in the refrigerator (approx. 6 °C), and at −20 °C. At any point of time the telophase DNA content of deep-frozen ethanol stored root tips served as reference (100 %) (20 telophase nuclei were measured in each of two or three slides).
alcohol, 90 min for formaldehyde) (Fig. 6). It is clear that hot hydrolysis is too difficult to control for quantitative work. Although cold hydrolysis has long been known to be best practice (Fox, 1968), it has been ignored by many workers on DNA amounts. It seems that diverse organisms such as *Pisum sativum*, *Homo sapiens* and downy mildews have the same hydrolysis optimum (Voglmayr and Greilhuber, 1998; J. Greilhuber, unpubl. res.). The time that formaldehyde-fixed material is stored in ethanol has apparently little effect on the shape of the hydrolysis curve (Fig. 6), although removal of bound formaldehyde from tissue in ethanol has been reported (Helander, 1994). Thus, a steeper decline of the curve in aged material could be supposed to occur, but this is not observed in at least the first year of storage at \(20^\circ\text{C}\).

The most critical step of the whole procedure after hydrolysis is the post-hydrolysis wash. Hydrolysed material is unstable, and DNA is lost relatively quickly (Fig. 7). Rinsing should be kept as short as necessary, with several changes of cool distilled water for a total of 5–10 min to minimize DNA loss. In Schiff’s reagent (1.5 h, \(20^\circ\text{C}\)), the DNA–Schiff complex becomes surprisingly stable while staining occurs (see below), but prolonged staining leads to a reduction of stain intensity, probably because DNA fragments are lost.

SO\(_2\) water can be applied at room temperature (approx. \(21^\circ\text{C}\)) for about 45 min (but not longer than 2 h) to remove all unbound Schiff’s reagent. The material should then be transferred to water. J. Greilhuber (unpubl. res.) kept stained material for 9 d in distilled water in the refrigerator and observed no loss of stain. Softening in 45 % acetic acid should be optimized (15 min at \(20^\circ\text{C}\)), because loss of stain is again accelerated by longer treatments. Squashes in 45 % acetic acid should be made of test material and standards in parallel. Air-drying the slides after coverslip removal over dry ice, etc. results in flattened nuclei, which is important for optical reasons. A short ethanol wash is usually done, but its duration is not critical (Greilhuber and Temsch, 2001). Squashes are measured without coverslips under an oil-immersion objective. No advantage is seen in using coverslips except for protection after measurement. Slides stored at room temperature in the dark are reasonably stable for at least a few weeks (Greilhuber and Temsch, 2001).

It is not the purpose of this paper to deal with the instrumental side of measurement technology. However, it is noteworthy that some authors continue to use, with reference to Sharma and Sharma (1980), a so called ‘one-wavelength method’ of absorbance cytophotometry, which is suspected to suffer strongly from distributional error. As far as one can see from the poor descriptions, monochromatic light is used in this method with no scanning device. It is also not the one-wavelength, two-area method of Garcia (1965) and not the archaic plug method (e.g. Swift, 1950). It seems that the nuclei are put into a fixed measurement area and the overall absorbance is recorded. Indeed, Sharma and Sharma (1972, p. 273) wrote that with rather homogeneous samples such as interphase nuclei this method ‘can successfully be applied’. Further, Sharma and Sharma (1980, p. 311) wrote ‘This method holds good for homogeneous samples such as interphase.’ This is a misconception and a misunderstanding what ‘homogeneous’ means in this context, namely homogeneity of the whole field of measurement and not only of the object. The distributional error is neither avoided nor corrected with this practice. It is the task of the reviewers of submitted manuscripts to insist on a lucid presentation of the methodological approach to measure DNA, to avoid publication of data which are based on physically unsound techniques.
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