Reliable Flow Cytometric Estimation of Nuclear DNA Content in Coffee Trees

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Flow cytometry gave high resolution of genome size in two coffee taxa (*Coffea liberica dewevrei* and *C. pseudozanguebariae*). Propidium iodide (PI) and *Petunia hybrida* were used as dye and internal standard, respectively. Proportionality between the DNA content and the digitized fluorescence signal was checked. Five main results were evident. First, optimal experimental conditions were established for peak location estimation (mean or mode), staining time (at least 2 minutes), high voltage (557 V) for the photomultiplier tube (PMT), and PI concentration (333 µg/ml). Second, a parameterization of the effects of high voltage and PI concentration were deduced from curve fitting. Third, two biases in DNA content estimation were recorded for high voltage and PI concentration, and were minimized. Fourth, the genome sizes of *C. liberica dewevrei* and *C. pseudozanguebariae* were estimated with accuracy 2C = 1.421 ± 0.005 pg and 2C = 1.129 ± 0.005 pg, respectively. Fifth, between-genotype variance was emphasized within each taxon. © 1996 Wiley-Liss, Inc.

Key terms: Propidium iodide, *Coffea liberica dewevrei*, *Coffea pseudozanguebariae*, Rubiaceae

By the late 1970s, flow cytometry (FCM) was established as a powerful technique for DNA content analysis in human cells. The major advantages of FCM are convenience, precision, and rapidity (12). This technique has also been developed for use with plant material. The presence of inclusions such as starch grains, the constitution of organelle genomes, and the occasionally very high content of natural chromophores, e.g., chlorophyll, led to the isolation and analysis of cell nuclei (6). Plant cell nuclei may be isolated by osmotic lysis of protoplasts (3) or by mechanical isolation (15).

Reliable genome size estimations depend greatly on the assumption that DNA content is linearly related to the digitized fluorescence signal. The validity of this assumption depends on such factors as stoichiometry of dye binding, accessibility of DNA to fluorochrome, fluorescence absorption, and a linear amplification system (2). Determination of nuclear DNA content (qDNA) by FCM requires comparison with a reference standard. The use of an internal standard is recommended to avoid any effects due to instrumental drift or differences in staining (12). Such cytometric analysis produces a histogram of fluorescence intensity per object with, normally, two major populations of nuclei in G1 phase forming two main peaks. An estimation of the DNA content of a sample (qDNA$_{smp}$) is given by the formula:

$$qDNA_{smp} = qDNA_{std} \times \frac{\text{peak means of the sample } (y_{smp})}{\text{peak means of the standard } (y_{std})} \quad (Eq. 1)$$

use of which assumes that the y_{smp}/y_{std} ratio is independent of y_{std} for fixed qDNA$_{smp}$ and qDNA$_{std}$. In this case, y_{smp} is related to y_{std} by a straight line through the origin: $y_{smp} = b \cdot y_{std}$. All deviation from the origin constitutes a bias in DNA content estimation.

Our aim was to obtain a high-resolution flow cytometric estimation of genome size in two coffee taxa (the sub-species *Coffea liberica dewevrei* and the species *C. pseudozanguebariae*) using Galbraith's nuclei isolation procedure (15) and propidium iodide (PI) staining. High resolution is necessary for further studies on inheritance of qDNA in an interspecific cross including these two taxa, which are known to be different for this trait (7). Different factors that could affect DNA content measurements were studied: the method of estimating G1 peak locations, staining time, dye concentration, and the photomultiplier (PMT) voltage. For the last two effects, a deviation from the origin (cf. above) was emphasized. An analytical approach allowed a parameterization of these effects, which is discussed. An estimation and a comparison of nuclear DNA content of the two taxa are presented with the within- and between-genotype variances.

MATERIALS AND METHODS
Plant Material

Two coffee taxa (the sub-species from Centrafrique *C. liberica dewevrei* and the species from Tanzania *C. pseudozanguebariae*) and the standard *Petunia hybrida* were used in this study and were grown in a greenhouse with a tropical climate (24°C during the day, 18°C at night, relative humidity of 70%). *Petunia hybrida* was chosen as an internal standard because its nuclear DNA content is 2C = 2.85 pg (19), which is close to those of coffee species (between 0.98 and 1.78 pg) (7).

Sample Preparation

Nuclei were extracted by leaf chopping (15). A slightly modified version (0.5% Triton X-100 and pH = 9.2) of the lysis buffer of Dolezel et al. (11) was prepared just before use. The leaf weight per volume unit of buffer was about 400 mg/ml for coffee tree and 250 mg/ml for petunia (400,000–2,000,000 nuclei/ml). Petunia and coffee tree leaves were chopped simultaneously in a Petri dish with lysis buffer. The solution was filtered through nylon cloth (50 μm mesh size). RNase A (5 U/ml of nuclear suspension, boiled Boehringer Mannheim #109 169 DNase free) was added to the filtrate for an incubation period of at least 2 hours.

Staining of Nuclei

The nuclei were stained with PI (95–98% by thin-layer chromatography, Sigma #P 4170). Except in experiments on the effect of staining time and dye concentration, staining conditions were 333 μg/ml of PI for at least 3 minutes.

Cytometric Measurements

A FACScan cytometer (Becton Dickinson) was used with an argon laser (15 mW) at 488 nm, taking a pulse area of emissions > 590 nm. Histograms were collected over 1,024 channels. Each histogram contained 3,000–10,000 nuclei depending on the width of the histogram. The zero offset of the analog-to-digital converter was checked with nuclei from *Petunia hybrida* so that the ratio between the modal position of the G2 and the G0—G1 nuclei was 1.98, not significantly different from 2 (t = 2.368; df = 5). Nevertheless, PI-stained chicken erythrocyte nuclei (DNA QC, Becton Dickinson) were used to esimate the zero offset. Several measurements were done at different amplification gains with a constant high-voltage setting (400 V). All the data were corrected as advised by Givan et al. (16) by substracting the zero offset (5 channels) from peak locations. During experiments, the gain of the amplifier system was never changed. Except in experiments on the effect of high-voltage settings for the PMT, the high voltage was set to 557 V.

Experimental Designs

For each peak, the user defined two boundaries that delimit a zone in which the average of nuclear fluores-cence is calculated. In a first step, two types of estimations, namely, "mode" and "mean," were compared. The "mode" type took into account only nuclei near the maximum frequency, whereas the "mean" type took into account about 95% of the distribution.

The effect of staining time was analyzed from two experiments. For each experiment, a large sample was obtained from one coffee leaf and several petunia leaves. In the first experiment, the sample was split before staining into 11 sub-samples (0.6 ml) corresponding to 11 staining times (0, 20, 40, 60, 80, 100, 120, 140, 160, 180, and 238 minutes). In the second experiment, the large sample was split into ten sub-samples (0.6 ml) corresponding to staining times from 0 to 18 with 2-minute increments.

The effect of PI concentration was analyzed from a large sample (see above). The filtrate was divided into ten equal aliquots (0.6 ml). PI concentrations from 42 to 420 μg/ml, with 42 μg/ml increments, were used. The effect of high-voltage settings for the PMT was studied using ten different voltages (485, 500, 515, 530, 540, 545, 550, 555, 560, and 565 V) on the same sample. qDNA of nine genotypes of *C. liberica dewevrei* (EB 51, 55, 56, 57, 58, 62, 64, 65, and 69) and of *C. pseudozanguebariae* (H51, 54, 55, 56, 58, 59, 60, 61, and 63) was estimated. Two leaves were collected for each tree. Each leaf was split into two parts. Each part was an independent sample. The whole constituted a nested design with three factors: taxa, genotype nested in taxa, and leaf nested in genotype.

Statistical Methods

Linear regression: estimation and comparison of parameters. Parameters of linear regressions between sample (y_{smp}) and standard (y_{std}) observations were estimated using the least squares method. The slopes of the different linear regressions were compared using the generalized model of variance analysis. A common estimation of the slope was computed when the non-parallelism test was not significant at the 5% level.

Non-linear regression: estimation of parameters. The simplex method (22) was used to estimate parameters in curvilinear fitting. Constraints were applied to some coefficients. For example, the curvilinear relationship between peak location and PI concentration was fitted by Mitscherlich's (8) function ($y = M - k \cdot n^{0.01\,x}$) with three parameters for the coffee tree (M_1, k_1, and n_1) and for petunia (M_2, k_2, and n_2), setting $n_1 = n_2$. The same method was applied to the functions ($y = ax^b + c$) on high voltage.

Nested model of ANOVA. A three-way nested model of ANOVA was used to test variations in qDNA estimation due to taxa (fixed effects), genotype nested in taxa (random effects), and leaf nested in genotype (random effects) (Table 1). When a source of variation was significant (at the 5% level), the corresponding variance was estimated. These estimations led to the intraclass correlation coefficient of Snedecor.

Table 1
Three-Way ANOVA Results[a]

Sources of variations	Sample size	Degrees of freedom	Effect	Mean square	Tests (F)	Observed F
Taxa	2	1	Fixed	MS_T	MS_T/MS_G	1,723 ($\alpha = 0.0000$)
Genotypes (plants)/taxa	9	16	Random	MS_G	MS_G/MS_L	6.23 ($\alpha = 0.0002$)
Leaves/genotype	2	18	Random	MS_L	MS_L/MS_R	0.823 ($\alpha = 0.66$)
Samples/leaf	2	36	Random	MS_R		
Total	72	71				

[a]The tested effects on qDNA estimation were taxa (fixed), genotype nested in taxa (random), and leaf nested in genotype (random).

RESULTS

Effect of the Type of Estimation of Peak Location

Whatever the type of estimation—mode or mean—the location of the coffee peak was linearly related to that of the petunia peak, and slopes did not differ ($F_{obs} = 2.35$; $df_1 = 1$, $df_2 = 15$; $\alpha = 0.15$). The differences between y_{smp} means covaried by y_{std} were also not significant ($F_{obs} = 0.12$; $df_1 = 1$, $df_2 = 16$; $\alpha = 0.73$). Thus, the DNA content estimation was not affected by the type of parameter as long as the two peak locations (Fig. 1) were estimated in the same way. The choice between these parameters for each sample can be determined by the shape of the distribution and the presence of debris.

Effect of the PI Staining Time

Peak locations were stable after 8 minutes. Nevertheless, Figure 2 shows that the y_{smp}/y_{std} ratio was stable from as early as 2 minutes. Only the value obtained immediately after staining seemed overestimated. Similar results were observed with a staining time of 0–238 minutes. In conclusion, a 3-minute staining time was sufficient to reach a level with no detectable variation over at least 4 hours.

Effect of the High Voltage for the PMT

Bagwell et al. (2) advised checking the degree of amplification system linearity by plotting the between-peak distance (y_2—y_1) versus the first peak position (y_1) and the ratio y_2/y_1 versus y_1. In the present study, the regression between (y_{std}—y_{smp}) and y_{smp} was linear (y_{std}—y_{smp} = 1.01 y_{smp}— 7.98; $R^2 = 0.999$). The observed relationship between y_{smp} and y_{std} was also estimated:

$$y_{smp} = 0.495 y_{std} + 4.96 (R^2 = 0.999). \quad (Eq. 2)$$

The intercept of this equation was not significantly different from zero. This could be due to a lack of repetitions. Indeed, a similar experiment was done with PI-stained CEN, which allowed us to obtain several peaks p1, p2, p3, p4, p5 corresponding respectively to 1, 2, 3, 4, 5 nucleus clumps. We calculated the different linear regressions [p2 = f(p1), p3 = f(p1); ...; p3 = f(p2); ... ;p5 = f(p4)]. In all cases, the intercept was significantly different from zero.
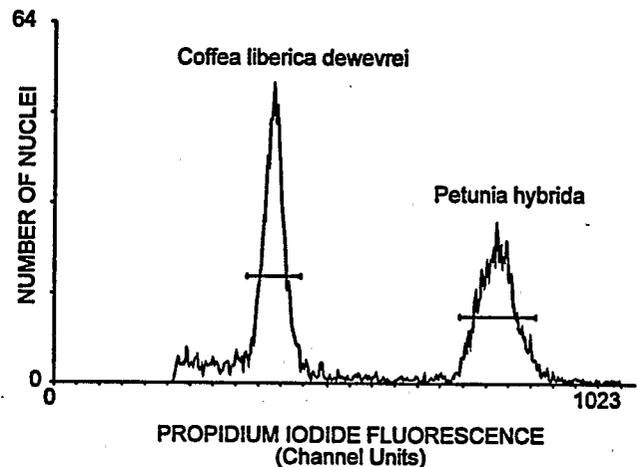

FIG. 1. Histogram of fluorescence intensities in a mixture of *Coffea* (sample) and *Petunia* (standard) nuclei, stained with propidium iodide. The fluorescence pulse area was collected on a 1,024 channel analyzer. The ranges used to define mean estimation of each peak are shown.

For theoretical reasons, we fit the peak locations as a function of voltage by the function $y = a x^b$. Coefficients b_1 and b_2 obtained for y_{smp} and y_{std}, respectively, were very similar (7.3367 vs. 7.498). A second simultaneous fitting of the two curves with three coefficients (a_1, a_2, and b) gave $a_1 = 2.46.10^{-18}$ (coffee), $a_2 = 1.24.10^{-18}$ (petunia), and $b = 7.467$ (Fig. 3).

The expected relationship between y_{smp} and y_{std} was analytically determined from the two functions $y_{smp} = a_1 x^b$ and $y_{std} = a_2 x^b$:

$$y_{smp} = (a1/a2) y_{std}.$$

Substitution of coefficients yielded $y_{smp} = 0.504 y_{std}$.

In Figure 3, the theoretical choice of the function $y = a \cdot x^b$ seemed correct. Nevertheless, the function did not explain the observed intercept of Equation 2, which led to a bias in qDNA estimation. In addition, further statistical studies showed that residuals were not independent of y_{std}. This led to add a third parameter c: $y = a x^b + c$.
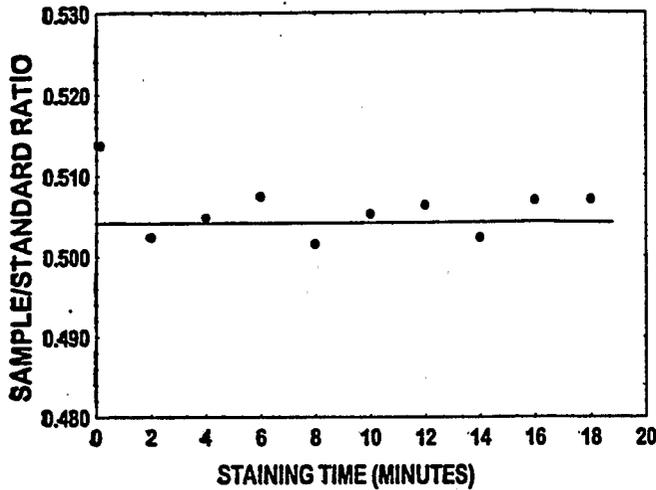
Fig. 2. Effect of the staining time on the y_{smp}/y_{std} ratio. Except for T = 0, the ratio was approximately constant as early as 2 minutes.
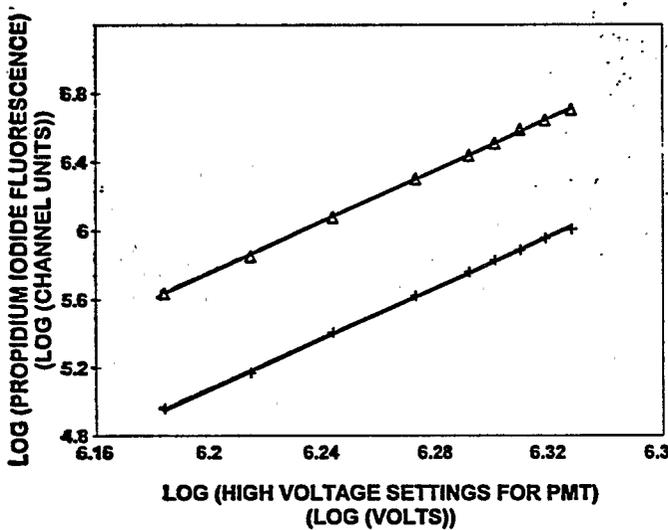

Fig. 3. Effect of the high voltage for the photomultiplier tube (PMT) on the fluorescence (channel units) of PI-stained *Coffea* (+) and *Petunia* (Δ) nuclei. The fitted functions shown are log(y) = −41.23 + 7.467 log(x) and log(y) = −40.54 + 7.467 log(x) with x the PMT voltage and y the peak locations for coffee tree and petunia, respectively.

The simultaneous fitting of the two curves with five co-efficients gave $a_1 = 2.72.10^{-16}$, $c_1 = 28.38$ (coffee), $a_2 = 5.48.10^{-16}$, $c_2 = 66.68$ (petunia), and b = 6.625. The expected relationship between y_{smp} and y_{std} was $y_{smp} = (a_1/a_2)y_{std} + c_1 - a_1c_2/a_2$. The substitution of coefficients yielded: $y_{smp} = 0.496\ y_{std} + 4.68$, which was very similar to the observed relationship (Eq. 2).

In conclusion, variations in voltage led to a bias in DNA content estimation. This bias was equal to $[k/y_{std}\cdot qDNA_{std}]$, where k = $c_1 - a_1c_2/a_2$ and can be minimized by maximizing the location of the internal standard distribution on the channel axis.
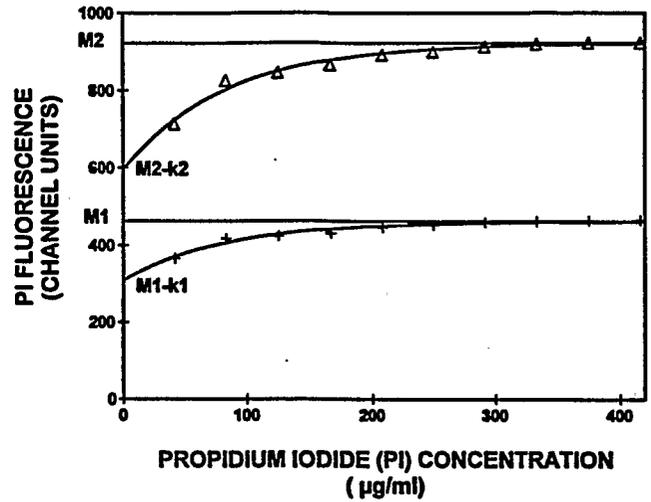

Fig. 4. Effect of propidium iodide (PI) concentration on the fluorescence (channel units) of PI-stained *Coffea* (+) and *Petunia* (Δ) nuclei. Mitscherlich's functions (y = M—kn$^{0.01\ x}$ with x the PI concentration and n = 0.3037) gave the best fit. Asymptotes for coffee tree (M1 = 458) and petunia (M2 = 917.1) are shown also as intercepts (M1—k1 = 304 and M2—k2 = 590.1).

Effect of the Concentration of Propidium Iodide

For theoretical reasons, and from curve shapes (Fig. 4), Mitscherlich's function:

$$y = M - k \cdot n^{0.01\ x}$$

where x is the PI concentration in μg/ml, was selected for the fitting of y_{smp} and y_{std} as a function of PI concentration. Coefficients M_1, n_1, k_1 (coffee) and M_2, n_2, k_2 (petunia) were simultaneously estimated by setting n = n_1 = n_2: M_1 = 458, k_1 = 154, M_2 = 917.1, k_2 = 327, and n = 0.304.

The relationship between y_{smp} and y_{std} was analytically deduced:

$$y_{smp} = \frac{k_1}{k_2}y_{std} - \frac{k_1 M_2}{k_2} + M_1$$

which leads to y_{smp} = 0.471 y_{std} + 26.6. The linear fitting between observed y_{smp} and y_{std} leads to the equation y_{smp} = 0.473 y_{std} + 24.4 (R^2 = 0.995). The analytically derived linear relationship coincided well with the observed one. The intercept was significantly different from zero (α = 0.05). Note that regression would be theoretically through the origin if M_1/M_2 = k_1/k_2, i.e., if the slope is equal to the ratio of the asymptotes.

The relationship between y_{smp}/y_{std} ratio and PI concentration is presented (Fig. 5) with the analytic curve:

$$Y_{smp}/Y_{std} = \frac{M_1 - k_1 \cdot n^{0.01x}}{M_2 - k_2 \cdot n^{0.01x}}$$

This function shows a decreasing hyperbolic shape with an asymptote reached at approximately 333 μg/ml.
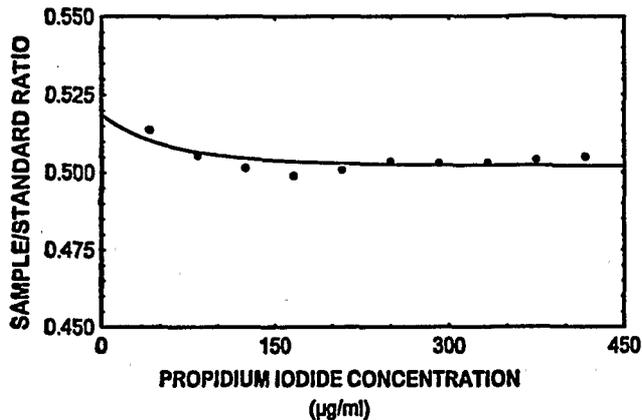
The study of the effect of PI concentration highlighted

FIG. 5. Effect of the PI concentration on the "coffee peak location/petunia peak location" ratio. The curve represents the expected function $y_{smp}/y_{std} = (M_1—k_1 n^{0.01x})/(M_2— k_2 n^{0.01x})$.

two results: 1) the theoretical choice of Mitscherlich's function for fitting was correct; and 2) variations in PI concentration led to bias in qDNA estimation. For an infinite concentration, qDNA can be estimated by $M_1/M_2 \cdot 2.85 = 1.423$ pg. When PI concentration was 333 μg/ml, then qDNA was 1.431 pg, showing no bias. Note that in this case, we considered M_1/M_2 as the qDNA estimator. If, by contrast, the slope k_1/k_2 (k equals the difference between asymptote and intercept) is taken into account, qDNA would be 1.343 pg.

DNA content of the two coffee taxa

The previous results led us to work with a 3-minute staining time, a PI concentration of 333 μg/ml, and a high voltage setting of 557 V. Levene's test showed that within-genotype variances were homogeneous ($F_{obs} = 1.11$; $df_1 = 17$, $df_2 = 54$; $\alpha = 0.372$). ANOVA showed no between-leaf effect, but highly significant between-taxa and between-genotype effects for DNA content (Table 1). Within-genotype and between-genotype variances were 0.000187 and 0.000174, respectively, showing that between-genotype differences represented 52% of the diversity (heritability sensus lato).

The different estimations of DNA content by formula (Eq. 1) of the nine trees of each taxa are shown in Table 2, with the results of the tests of Newman and Keuls. Confidence interval for the means was 1.425 ± 0.005 pg and 1.133 ± 0.005 pg for C. liberica dewevrei and C. pseudozanguebariae, respectively.

Linear regresssions between y_{smp} and y_{std} were computed:

$$y_{smp} = 0.492\, y_{std} + 5.64 (R^2 = 0.972)$$

and

$$y_{smp} = 0.371\, y_{std} + 20.23 (R^2 = 0.796)$$

for the 36 estimates for C. liberica dewevrei and C. pseudozanguebariae, respectively (Fig. 6). Slopes were different between taxa ($F_{obs} = 12.76$; $df_1 = 1$; $df_2 = 68$;

Table 2
Estimation of Nuclear DNA Content in Two Coffee Taxa*

Genotype label	qDNA
C. liberica dewevrei	
EB 65	1.399[a]
EB 56	1.402[a]
EB 51	1.412[ab]
EB 55	1.423[ab]
EB 69	1.425[ab]
EB 58	1.427[ab]
EB 64	1.431[b]
EB 62	1.431[b]
EB 57	1.438[b]
Mean	1.421
C. pseudozanguebariae	
H59	1.108[a]
H51	1.114[a]
H58	1.124[a]
H56	1.125[a]
H55	1.127[a]
H60	1.129[a]
H61	1.131[a]
H54	1.139[a]
H63	1.166[b]
Mean	1.129

*Each qDNA estimation is the mean of four within-genotype samples. Results of Newman and Keuls test for the two taxa are indexed. (For each taxa two genotypes with the same letter are not significantly different at the 5% level.)

$\alpha = 0.0007$). Note that intercepts of these among-genotype regressions (within taxa) were not significantly different from zero at the 5% level.

DISCUSSION
Some Sources of Bias in Nuclear DNA Content Estimation

Different sources of bias in nuclear DNA content estimation have already been emphasized in the literature. They may be sorted according to their origin, which can be related to the instruments (for example, amplification gain (16) and voltage for the PMT (2)), the staining procedure (for example, staining time and dye concentration) (15), the sex of standard (26), the dye type (12,13,17,23), and DNA accessibility (9,14,27).

An increase in the accuracy of qDNA estimation calls for mathematical corrections and well-defined methodological conditions. Checking and recommendations should be routine but are relatively rarely reported, especially in plant studies. In our study, different sources of bias were studied, including those related to instrumentation and staining.

Instrumental sources of bias. The first instrumental source of bias concerns the non-linearity of the amplification system for two parameters: the gain amplification and the high voltage for the PMT.

Givan et al. (16) checked linearity of gain amplification at constant high voltage (550 V) with fluorescein-conjugated calibration beads, PI-stained chicken cells,
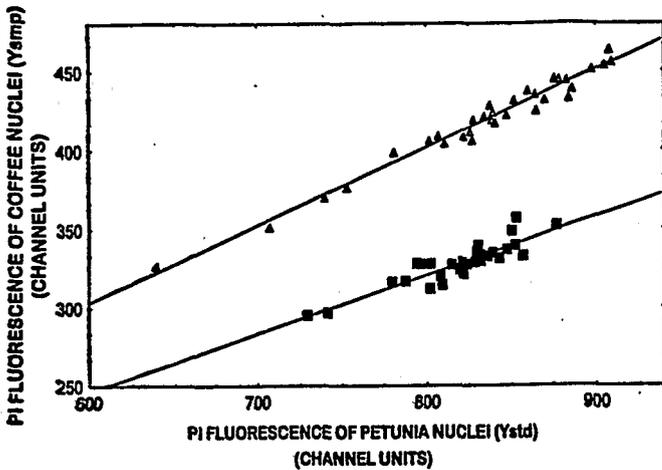
Fig. 6. Relationships between peak locations of PI-stained nuclei of coffee trees and petunia. Each linear regression correspond to a taxon [*C. liberica dewevrei* (Δ) and *C. pseudozanguebariae* (■)]. Observed regressions were y = 5.64 + 0.492 x (R² = 0.972) for *C. liberica dewevrei* and y = 20.23 + 0.371 x (R² = 0.796) for *C. pseudozanguebariae*.

and human cells. Linear relationships between expected and observed fluorescences converged at a positive x-axis intercept of about the fifth channel. In our study we estimated the zero offset at 5 channels, and all the data were corrected accordingly. Moreover, for the genotype series, we always worked at the same amplification gain setting.

Bagwell et al. (2) showed that linearity of voltage amplification should be checked with a constant amplification gain. When linearity is respected, the between-peak difference $(y_2 - y_1)$ plotted against the first peak position (y_1) should be a straight line through the origin. In our study, the linear relationship was indeed obtained, but the intercept was different from zero, leading to an overestimation of DNA content. We first chose the function y = $a \cdot x^b$ to fit y_{smp} and y_{std} as a function of voltage because it agrees with the electronic principle of the voltage effect in the PMT system (25) and because we would expect $y_{smp} = y_{std} = 0$ when the voltage was set to zero. Nevertheless, the intercept of the linear regression $y_{smp} = f(y_{std})$ can only be explained by a third parameter c (y = $a \cdot x^b$ + c), for which unfortunately we have no explanation.

Three methods have been proposed in the literature to eliminate or reduce this type of bias. The first includes two steps: 1) estimation of x-axis intercept (cf. above); and 2) subtraction of this value from peak locations before computation of qDNA (16). The second method of correction uses two standards (26) and theoretically gives an unbiased estimation. Nevertheless, standards and sample should be adequately separated, and the absence of interaction between standards should be verified. The third method minimizes the bias by maximizing the location of the internal standard on DNA histograms by adjustment of the high-voltage settings for PMT (1). To

avoid peak truncation, the right peak must be the less variable and should be the standard. The qDNA estimation is still biased, but this is negligible as long as the high-voltage setting does not change. The first and the last methods were used here with efficiency.

Staining sources of bias. To avoid variation in qDNA estimation due to staining time, optimal incubation periods should be determined (9, 15, 20, 26). The usual staining time with PI ranges from 10 minutes (1, 9) to more than 24 hours (20), but an accumulation of debris with time was recorded. In coffee trees, we found no variation in qDNA from 2 minutes to four hours. To avoid the accumulation of debris, we used a staining time of 2–20 minutes.

The effect of fluorochrome concentration on qDNA estimation has already been investigated. With isolated nuclei from leaves and root tips of *Nicotiana tabacum*, Galbraith et al. (15) found a hyperbolic relationship between the y_{smp}/y_{std} (labeled microspheres) ratio and the mithramycin (Mi) concentration. They assumed a ratio equal to zero when Mi was not added. Similar results were obtained with isolated nuclei from fresh or fixed tissues of *Pisum sativum* stained by 4',6-diamino-2-phenylindol (DAPI) (24). In both cases stained microspheres were used as the internal standard, and increasing fluorochrome concentration led to increasing relative fluorescence. In our study, the ratio decreased when the PI concentration increased. This could be explained either by the difference in standard (microspheres vs. petunia), or by the difference in dye type (base-specific dye vs. intercalating dye).

Mitscherlich's function gave the best fit of y_{smp} and y_{std} as a function of PI concentration. Fitted equations showed different asymptotes (M₁ and M₂) and intercepts (M₁—k₁ and M₂—k₂) for coffee trees and petunia. Intercepts were significantly different from zero, suggesting fluorescence of nuclei without PI. Nevertheless, no peaks were observed in this case, but only debris. Additional experiments at limiting concentrations should be performed to interpret these intercept values. Another consequence of the Mitscherlich fit was the problem of the choice between the k_1/k_2 ratio (the slope of the linear relationship between y_{smp} and y_{std}) and the M₁/M₂ ratio (the asymptote ratio) for qDNA content estimation. The ratio of the asymptotes is easier to handle statistically.

Accurate estimations of DNA were obtained with a saturating PI concentration of at least 330 μg/mL. The value was much higher than that usually used (50 μg/ml) (13,17). This could be explained by the larger quantity of leaves that we chopped.

Nuclear DNA Content of Coffee Taxa

The qDNA of nine coffee trees of two coffee taxa were estimated using the saturating value of M₁/M₂. Previous results (1.68 ± 0.29 pg and 1.09 ± 0.13 pg for *C. liberica dewevrei* and *C. pseudozanguebariae*, respectively) were obtained by Cros et al. (7). The present work improved accuracy (within-genotype coefficient of variation = 1.14%). With four leaves, the confidence interval of the

mean of a genotype was 1.07%, whereas nine genotypes gave an estimation of 1.421 ± 0.005 pg (*C. liberica dewevrei*) and 1.129 ± 0.005 pg (*C. pseudozanguebariae*). The present accuracy should allow further studies on interspecific qDNA inheritance, especially in the segregating G2 generation. In addition, we have shown that flow cytometry is an adequate tool for comparison of genome sizes of *Coffea* species when the main sources of variation affecting DNA content estimation are controlled.

Between-genotype variations were observed in the two taxa. This fact was reported in species belonging to genera as various as *Medicago* (5), *Zea* and *Sorghum* (18), and *Helianthus* (20,21). No between-leaf variations, which were observed in *Helianthus* (20), were recorded here. Nevertheless, it would be interesting to verify if between-taxa and among-genotype variations represent true qDNA variations or differences in accessibility of DNA to PI (4,9,10,14).

ACKNOWLEDGMENTS

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