Nuclear DNA content in the subgenus *Coffea* (Rubiaceae): inter- and intra-specific variation in African species

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Abstract: Flow cytometry has been used to estimate nuclear DNA content of 13 *Coffea* species (Rubiaceae) native to Africa. Twelve diploid ($2n = 22$) and one tetraploid ($C.$ *arabica*, $2n = 44$) species were investigated. Isolated nuclei from 77 genotypes were stained with propidium iodide (PI: not base specific). Thirty-nine genotypes were stained with 4',6-diamidino-2-phenylindole (DAPI; AT specific). Nuclear DNA content (2C values), estimated with PI, ranged from 0.95 to 1.78 pg. By aggregative clustering, three groups of accessions with increasing DNA content were identified. Three species, namely *C. sessiliflora*, *C. racemosa*, and *C. pseudozanguebariae*, had a low DNA content (0.90–1.30 pg). Three species, namely *C. eugenioides*, *C. stenophylla*, and *C.* sp. F, were exclusively found in the intermediate group (1.31–1.60 pg). The remaining species were distributed between the intermediate group and the last group (1.61–1.80 pg). The values determined for the *Coffea* species are compared, inter- and intra-specifically, to those of other angiosperm species. The observed differences are discussed according to the ecogeographic origin of the species, their phenological characteristics, and the fertility of the F_1 interspecific hybrids.

Key words: Africa, *Coffea*, flow cytometry, nuclear DNA content, genome evolution.

Résumé : La cytométrie en flux a été utilisée pour estimer la quantité d'ADN nucléaire chez 13 espèces de *Coffea* (Rubiaceae) originaires d'Afrique. Douze espèces diploïdes ($2n = 22$) et l'espèce tétraploïde *C. arabica* ($2n = 44$) ont été analysées. Pour 77 génotypes, des populations de noyaux isolés ont été colorées par l'iodure de propidium (IP: non spécifique des bases). Pour trente neuf génotypes, le 4',6-diamidino-2-phenylindole (DAPI; AT spécifique) a été également utilisé. Les quantités 2C d'ADN nucléaire, estimées avec l'IP, oscillent entre 0.95 et 1,78 pg. Trois groupes correspondant à des quantités croissantes d'ADN ont été mis en évidence. Les trois espèces *C. sessiliflora*, *C. racemosa* et *C. pseudozanguebariae* se classent dans le groupe des plus petites valeurs (groupe 1 : 0,90 à 1,30 pg). Les trois espèces *C. eugenioides*, *C. stenophylla* et *C.* sp. F. se rangent uniquement dans le groupe des valeurs intermédiaires (groupe 2 : 1,31 à 1,60 pg). Les autres espèces se répartissent entre le groupe 2 et le groupe des plus hautes valeurs (groupe 3 : 1,61 à 1,80 pg). Les valeurs déterminées pour les espèces de *Coffea*, sont comparées au niveau intra- et inter-spécifique à celles d'autres angiospermes. Les différences observées sont discutées en fonction de l'origine éco-géographique des espèces, leurs caractéristiques phénologiques et la fertilité de leurs hybrides F_1 interspécifiques.

Mots clés : Afrique, *Coffea*, cytométrie en flux, quantité d'ADN par noyau, évolution des génomes.

Introduction

Large variation in nuclear DNA content, from 1 to more than 125 pg per diploid nucleus, has been reported among angio-sperms (Price 1988). Until recently, most DNA contents were determined by Feulgen microspectrophotometry of root tip mitoses (Bennet and Smith 1991). Since the introduction of flow cytometry, DNA content can be determined more easily using fluorochromes after leaf chopping, protoplast lysis, or nuclei isolation (Arumuganathan and Earle 1991*a*; Dolezel 1991; Ulrich and Ulrich 1991; Dolezel et al. 1992). In the *Coffea* genus, the only reported value, namely −1.67 pg of DNA per nucleus, is that of one genotype of *C. canephora* (Marie and Brown 1993).

Current commercial green coffee production relies on two species, *C. arabica* ($2n = 44$, autogamous, cultivated at altitude mainly in central and south America) and *C. canephora* ($2n = 22$, allogamous, cultivated in lowlands mainly in Africa and Asia). Wild species present great genetic diversity structured in three biogeographic areas: Madagascan floristic

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region (Charrier 1978), east Africa (Bridson and Verdcourt 1988), and the Guinea−Congo region (Chevalier 1947). Some species have agronomic characteristics that could be of value to coffee breeders (Berthaud and Charrier 1988). A large number could be intercrossed (Louarn 1993).

In the study reported here, we evaluated the total nuclear DNA content, using isolated nuclei stained with propidium iodide (PI; not base pair specific), of a representative panel of African *Coffea* species. Nuclei of several genotypes within each species were also stained with 4',6-diamidino-2-phenyl-indole (DAPI; AT specific). Results are discussed according to the ecogeographic origin of the species, their phenological characteristics, and the fertility of their F_1 interspecific hybrids.

Materials and methods

Plant cultivation
Coffee plants were grown from seeds in a greenhouse with a tropical climate (24°C during the day, 18°C at night, relative humidity of 70%). Leaves were collected at the same time from the second node from the apex of the main stem or branches. Leaves were used fresh or frozen in liquid nitrogen and stored at −80°C before processing.

Species and genotypes
Seventy-seven genotypes belonging to 13 African species were analyzed. It was possible to stain with both fluorochromes in only a few genotypes because of the small size of the plants, the availability of leaves, and the need to isolate nuclei for PI staining. In the following list, the number of genotypes per species is given in parentheses: the first number corresponds to genotypes stained with PI, the second to those stained with DAPI. Seven species were native to the Guinea−Congo region: *C. brevipes* (8, 3), *C. canephora* (11, 3), *C. congensis* (9, 3), *C. humilis* (5, 2), *C. liberica* (8, 8), *C.* sp. *Moloundou* (3, 2), and *C. stenophylla* (6, 3). Six species were native to eastern Africa: *C. eugenoïdes* (3, 2), *C. pseudozanguebariae* (5, 2), *C. sessiliflora* (6, 2), *C. racemosa* (4, 2), *C.* sp. F Bridson (3, 3), *C. arabica* (6, 4). Except for *C. arabica* ($2n = 4x = 44$), all other species were diploids ($2n = 2x = 22$).

Nuclei preparation
Preliminary experiments were performed using thinly sliced leaves in different lysis buffers. Unpurified nuclei suspensions were stained with PI according to Aramuganathan and Earle (1991a). Results were heterogeneous in quality, with high CV peaks. Nuclei purification protocols have been tried in an attempt to avoid the problems associated with low penetration of DNA-specific fluorochrome (Dolezel 1991) and improve the quality of the results. On the contrary, preliminary results with DAPI showed that the use of flow cytometry on unpurified nuclei suspensions gave peaks of high resolution and low CV. As already noted by Marie and Brown (1993), the analysis of unpurified nuclei suspensions often seems more convenient with UV-excited dyes such as Hoechst bisbenzimidine or DAPI. Therefore, in this study the experiments using PI were performed on purified nuclei suspensions, and the experiments using DAPI intercalent were on unpurified nuclei suspensions.

The preparation of unpurified nuclei suspension was adapted from Galbraith et al. (1983). Crude samples containing nuclei were prepared from leaf material (approx. 500 mg) by chopping with a sharp razor blade in 1 mL of Chemunex commercial buffer R09-220-500 (Chemunex, Maison Alfort, France). The suspended cell constituents were stained for 20 min using DAPI at a final concentration of 10 μg/mL. Before analysis, the suspension was filtered through a nylon membrane of 17-μm mesh size to remove large debris.

Partially purified nuclei suspension was prepared using three different sucrose buffers. Fresh leaves were ground to a fine powder using liquid nitrogen, mixed with buffer A (0.4 M sucrose, 0.05 M Tris, 2 mM $CaCl_2$, 0.4% β-mercaptoethanol) and filtered through 50-μm Blutex membrane (Scrynel, Rüschlikon, Switzerland). The solution was centrifuged at $3000 \times g$ and 4°C for 15 min. The pellets were resuspended in buffer B (0.25 M sucrose, 0.05 M Tris, 2 mM $CaCl_2$) and centrifuged again at $3000 \times g$ and 4°C for 15 min. The second pellet was carefully mixed with 5 mL of buffer B and 20 mL of buffer C (2 M sucrose, 0.05 M Tris, 2 mM $CaCl_2$) and centrifuged at $16\,000 \times g$ and 4°C for 45 min. The last nuclei pellet could be stored at −80°C for 1 week. Before analysis, 500 μL of phosphate-buffered saline (7.74×10^{-2} M Na_2HPO_4, 2.26×10^{-2} M NaH_2PO_4, pH 7.4) was added to isolated nuclei and warmed to room temperature. The nuclei suspension was incubated for 5 min in PI at a final concentration of 160 μg/mL.

Apparatus
Resuspended PI-stained nuclei were analyzed using a FAC-Scan flow cytometer (Becton Dickinson European, Erembodegem-Aalst, Belgium) equipped with an argon laser (15 mW) emitting at 488 nm. The voltage of the photomultipliers was set at 550 V. Five thousand nuclei were measured in each run.

DAPI-stained nuclei were analyzed using a CA-II flow cytometer (Partec Gmbh, Munster, Germany) equipped with a 100-W high-pressure mercury lamp for UV 360 nm excitation. Emission light was collected using a 420-nm long-pass filter.

Reference samples
With PI, under our experimental conditions, the *C. arabica* peak occurred near the 600-W channel. Measurements were made on a linear scale. To estimate total nuclear content, rice nuclei (*Oryza sativa* type *japonica*, 2C = 1.2 pg; Bennett and Smith 1991) were used as calibration standards between each sample of *Coffea*. To estimate the total DNA content, we compared the mean position of the tested sample with the mean position of the calibration standard. According to Galbraith et al. (1983), the amount of nuclear DNA = (mean position of the tested sample / mean position of the rice nuclei) × 1.2 pg. With DAPI, our standard was internal (*Petunia hybrida*, 2C = 2.85 pg; Marie and Brown 1993). Its peak was arbitrarily fixed on channel 100 on a logarithmic scale. The values measured were translated on a linear scale using a formula demonstrated by Chemunex (unpublished data). For 39 genotypes stained by both dyes it was possible to compare nuclear DNA content and fluorescence intensity observed after staining with DAPI (dependent on the nuclear DNA content and the genome base composition).

Fig. 1. Peaks obtained for three samples of *Coffea* using two different stains. (*a*—*c*) PI on isolated nuclei (see text) (*d*—*f*) DAPI on unpurified nuclei and *Petunia hybrida* as internal standard.
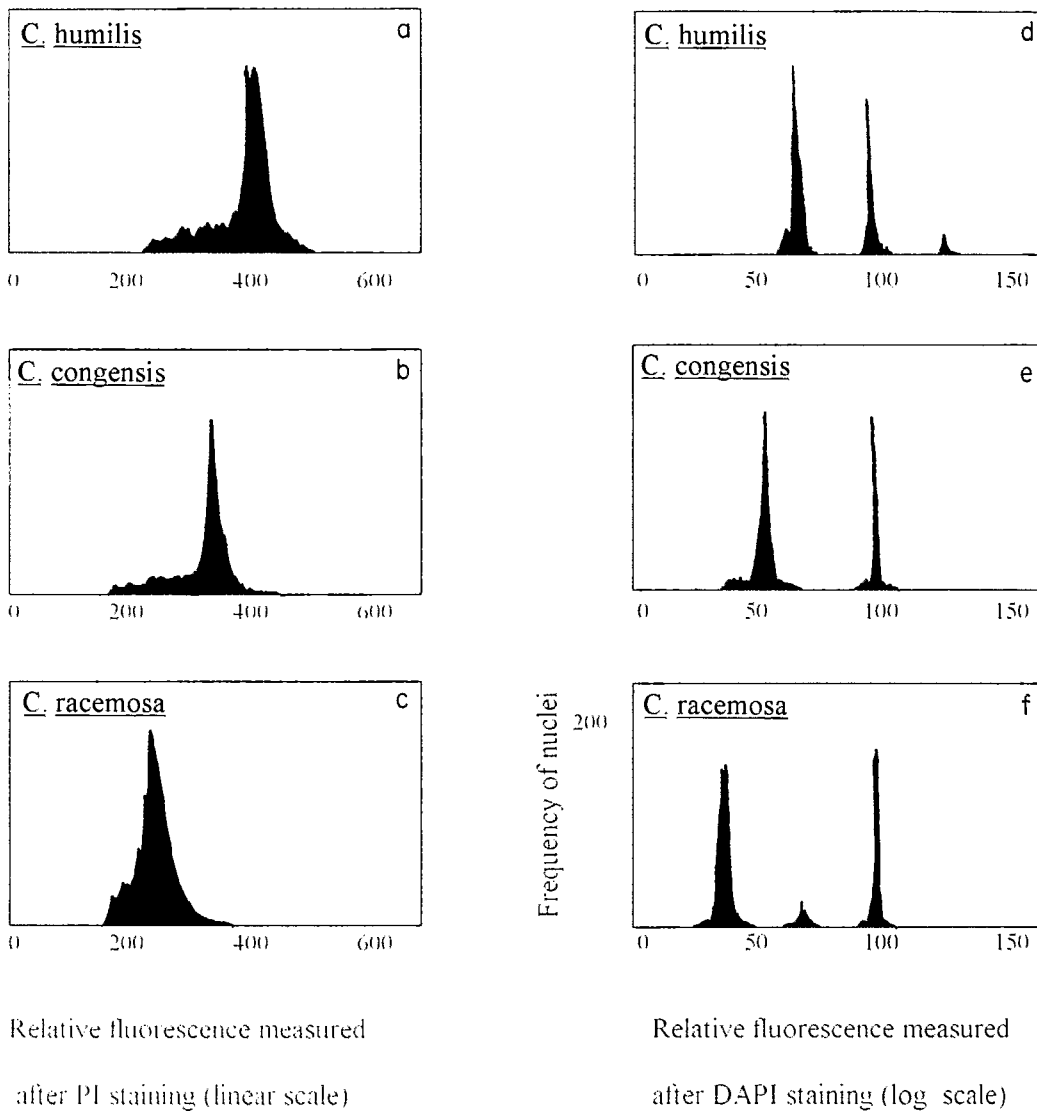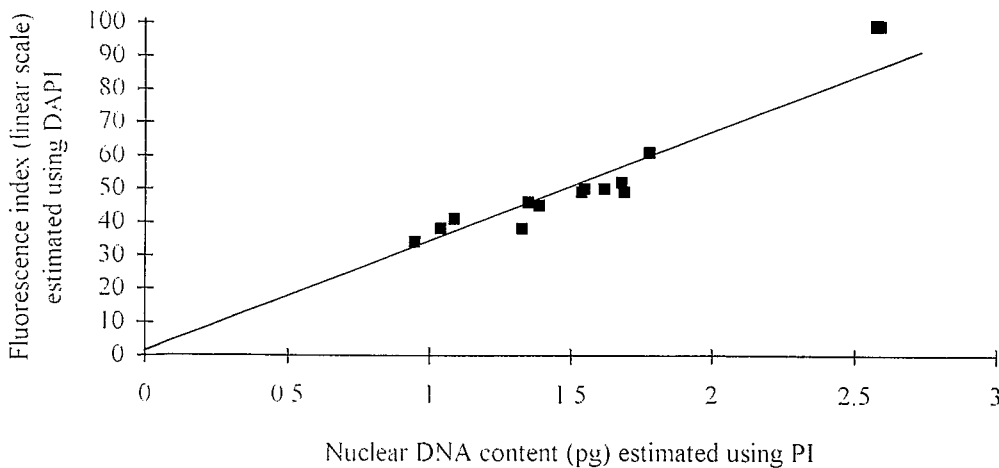

Relative fluorescence measured after PI staining (linear scale)

Relative fluorescence measured after DAPI staining (log scale)

Table 1. Nuclear DNA content of 13 *Coffea* species.

Species	Ploidy	Biogeographic area	DNA content (pg per nucleus)	Aggregative group*
C. arabica	4*x*	East Africa	2.61±0.23	na
C. humilis	2*x*	Guinea—Congo region	1.78±0.33	A
C. sp. *moloundou*	2*x*	Guinea—Congo region	1.69±0.25	A-B
C. liberica	2*x*	Guinea—Congo region	1.68±0.29	A-B
C. congensis	2*x*	Guinea—Congo region	1.62±0.19	B
C. brevipes	2*x*	Guinea—Congo region	1.55±0.18	B
C. canephora	2*x*	Guinea—Congo region	1.54±0.22	B
C. eugenioides	2*x*	East Africa	1.39±0.12	B
C. stenophylla	2*x*	Guinea—Congo region	1.35±0.12	B
C. sp. F	2*x*	East Africa	1.33±0.02	B
C. pseudozanguebariae	2*x*	East Africa	1.09±0.13	C
C. sessiliflora	2*x*	East Africa	1.04±0.16	C
C. racemosa	2*x*	East Africa	0.95±0.13	C

*A, B, and C indicate groups of values not significantly different at $P = 0.05$ according to Newman and Keul's multiple range test. na, not included in the analysis.

Fig. 2. Least squares regression analysis between PI and DAPI of mean values per species. The regression line ($r = 0.95$) passes through (0, 0); the slope $= 33.5 \pm 2.1$ for 95% confidence.

Nuclear DNA content (pg) estimated using PI

Results

Figures 1a−1c show that high resolution of nuclear DNA content was achieved using laser flow cytometry and PI. Addition of sarkosyl (1.5% final) to the nuclei suspension, before addition of PI, improved the peak resolution. The sharp peak and low CV value indicate that the nuclei were well isolated and did not suffer significant damage during staining and analysis. Typically, CV values of 2.0 to 7.0 are obtained by this flow cytometric procedure. Using DAPI on unpurified nuclei suspensions, the simple chopping also provided reliable results (Figs. 1d−1f).

The mean nuclear DNA contents of 13 *Coffea* species are listed in Table 1. Species names and biogeographic area are ordered according to the decreasing value of their mean nuclear DNA content. As expected, *C. arabica* (tetraploid) had the highest nuclear DNA content (2.61 pg per nucleus). For diploid *Coffea* species, nuclear DNA content ranged from 0.95 pg (*C. racemosa*) to 1.78 pg (*C. humilis*). Mean values for each species were compared to test significance using a Newman and Keul's multiple range test (Table 1). Three groups were distinguished: medium−high (groups A and A-B), medium (group B), and low nuclear DNA content (group C). To test accessions grouping, a clustering analysis, using the minimum intragroup variance criteria, was performed on the individual values of the DNA content. As in Newman and Keul's test based on the mean values for the species, three groups with increasing DNA content were identified (Table 2): group 1 (DNA content 0.90−1.30 pg) comprised 14 genotypes of the three species *C. sessiliflora*, *C. racemosa*, and *C. pseudozanguebariae*; group 2 (DNA content 1.31−1.60 pg) included all genotypes of *C. eugenioides*, *C. stenophylla*, and *C. sp. F*, one genotype of *C. pseudozanguebariae*, and several genotypes of the six remaining species; and group 3 (DNA content 1.61−1.80 pg) comprised genotypes of *C. humilis*, *C. sp. Moloundou*, *C. liberica*, *C. congensis*, *C. brevipes*, and *C. canephora*. These species were not found in this group with equal frequency. Most genotypes of *C. humilis* and *C. sp. Moloundou* (0.75−0.80) were found in group 3, compared with one-third for *C. canephora* and *C. brevipes* (0.37−0.36). These

Table 2. Frequency of distribution in aggregative groups obtained on the basis of the nuclear DNA content of 77 *Coffea* accessions.

Species	Group 1	Group 2	Group 3
C. humilis		0.20	0.80
C. sp. moloundou		0.25	0.75
C. liberica		0.34	0.66
C. congensis		0.38	0.62
C. brevipes		0.63	0.37
C. canephora		0.64	0.36
C. eugenioides		1.00	
C. stenophylla		1.00	
C. sp. F		1.00	
C. pseudozanguebariae	0.80	0.20	
C. sessiliflora	1.00		
C. racemosa	1.00		
Limits of groups (in pg of DNA)	0.90−1.30	1.31−1.60	1.61−1.80

NOTE: The aggregative analysis of individual values used the minimum intragroup variance criteria.

results suggest a structured level of difference between diploid *Coffea* species.

Calculated values (linear scale) of the fluorescence index using DAPI for diploid species ranged from 34 for *C. racemosa* to 61 for *C. humilis*. Figure 2 shows a least squares regression analysis performed on mean values for the species obtained with DAPI (AT specific) and mean nuclear DNA content per species estimated using PI. Estimations using these two dyes were well correlated ($r = 0.95$). This means that the percent AT for these species was not significantly different. The least squares regression line passes through a point not significantly different from zero. These results indicate that the nuclear DNA content, for a sample belong-

ing to this representative set of *Coffea* species, could be estimated indirectly using DAPI staining of unpurified nuclei.

Discussion

Coffea nuclear DNA content compared with other angiosperm species

Marie and Brown (1993) reported a DNA content of 1.67 pg per nucleus for one genotype of *C. canephora*, which was the only available value in the literature. Our data are consistent with this estimation (*C. canephora* mean nuclear DNA content is 1.54 pg and can reach 1.70 pg depending on the genotype), but the number of species and genotypes studied allows a general overview of interspecific variation in nuclear DNA content within the *Coffea* genus. The extreme values are 0.95 and 1.78 pg. Arumuganathan and Earle (1991*b*) and Bennett and Smith (1991) have reviewed the nuclear DNA content of a large number of species. *Coffea* species could be considered to have a low nuclear DNA content compared with *Viscum album* (152 pg), *Alium cepa* (32.7 pg), or *Pinus carabeae* (45.6 pg). *Coffea* DNA content is similar to species like *Acacia heterophylla* (1.60 pg), *Beta vulgaris* (1.75 pg), *Daucus carota* (1.19 pg), *Dioscorea alata* (1.47 pg), and *Vigna unguiculata* (1.20 pg) (all data from Marie and Brown 1993). Assuming that the mean base pair weight is 660 Da (Sambrook et al. 1989), the mean coffee haploid genome is estimated as 700 million base pairs.

Intra- and inter-specific variation at the same ploidy level

A twofold variation in DNA content was observed at the diploid level for *Coffea* species and up to 10−15% intraspecific variation. Price (1988) has clearly shown that differences in excess of twofold to threefold are common among congeneric diploid species. In the genus *Helianthus*, total DNA content varies more than fourfold among diploid species (Sim and Price 1985; Cavallini et al. 1989; Michaelson et al. 1991). Bennett (1985) reported that the extent of variation of the nuclear genome within some species may be considerable, reaching 54% for *Glycine max* and 228% for *Collinsia verna*. Variations were also observed in some crop species like *Zea mays* (30%) and *Capsicum annuum* (35%). In contrast, species such as *Hordeum vulgare*, *Vicia faba*, *Sorghum bicolor*, and *Festuca pratensis* do not exhibit such polymorphism (Laurie and Bennett 1985; Bennett and Smith 1991). Interspecific variations are similar to those observed in other genera, and intraspecific variations found for *Coffea* species may be considered small.

Interspecific variation at the polyploid level

The calculated 1C value of *C. arabica* is 1.3 pg. De Laat et al. (1987) showed that in autopolyploid series, as in the genera *Malus* and *Prunus*, the DNA content is exactly doubled. *Coffea arabica* (2*n* = 44) is supposed to be an allotetraploid of amphiploid origin, so it could be the sum of two species with different genome sizes. Berthou et al. (1983) compared total cpDNA of different *Coffea* species and suggested that *C. eugenioides* may be a good putative progenitor. Cros et al. (1993) used chloroplastic probes to show that *C. eugenioides*, *C.* sp. *Moloundou* and *C.* sp. F chloroplast cannot be distinguished from *C. arabica*. In the present study, because of the observed intraspecific variation, any hypothesis concerning *C. arabica* lineage can be excluded.

Coffea diploid variation according to genetic and ecogeographic origin of species

Five of six diploid species with less than 1.5 pg of DNA per nucleus are native to the East African biogeographic area. According to Furuta and Nishikawa (1991), evolution at a given ploidy level may be associated with a gradual increase in DNA content. Kenton (1984) noted that the more specialized species of the *Gibasis linearis* group contain more DNA. This author suggested that the DNA content increases during evolution. In terms of *Coffea* species, Leroy (1982) suggested than the center of origin of the genus is the Kenyan region, which was close to Madagascar until the Gondwanian separation during the Cretaceous period. From this area, species radiation has occurred twice: in Madagascar and in central and western Africa (Guinea−Congo region). Our data clearly indicate that species endemic in East Africa contain less DNA than species endemic in the Guinea−Congo region. Moreover, the higher DNA content is found for *C. humilis*, which is endemic in the west margin of the distribution area at the boundary between Côte d'Ivoire and Guinea (Berthaud 1986). If the radiation route described by Leroy is correct, our results for African species are consistent with an increase in genome size during evolution.

In a large number of species of British flora, Grime and Mowforth (1982) noted a correlation between large genome size and capacity for growth at low temperature. Price (1988) reported that the decrease in DNA content might be a adaptative process of a plant group from mesic to more xeric conditions. *Coffea* species of high nuclear DNA content are native to the evergreen forest (Guinea−Congo region); species of low content are native to dry forest (East Africa).

Price (1988) also report that total DNA content may be positively correlated with cell cycle duration. Wakamiya et al. (1993) observed positive correlations between nuclear DNA contents and growth indices, minimum seed-bearing age, and seed dimensions for 19 North American *Pinus* species. In contrast, strong negative correlations were observed with the lowest mean annual precipitation. *Coffea sessiliflora* and *C. pseudozanguebariae*, which flower frequently, have very small seeds, a small number of flowers per node, and a shorter period of seed maturation, i.e., 2 months versus 7−15 months for species native to western Africa (*C. canephora*, *C. liberica*, *C. congensis*, *C. humilis*, and *C. stenophylla*) (Hamon et al. 1984; Anthony 1992). If we assume that the process of seed maturation implies a constant number of cell divisions from flowering to ripening, a smaller genome size and consequently lowered DNA synthesis requirement could be good adaptative features.

An apparent contradiction is noted with *C. stenophylla*, a Guineo−Gongolian species that have a long period of seed maturation (around 10 months; Berthaud 1986) but only contain 1.35 pg of DNA per nucleus. In fact this low DNA content could be an adaptation to the relatively dry conditions found in the margin area of the west African evergreen forest in Guinea and northern Côte d'Ivoire, where this species was found (Portères 1962; Berthaud 1986).

Relationship between DNA content and fertility of interspecific hybrids

Interspecific hybrids have been made (Louarn 1993) between *C. canephora*, *C. congensis*, and *C. liberica* (Guinea−

Congo region) and *C. racemosa*, *C. sessiliflora*, and *C. pseudozanguebariae* (East Africa). The authour analyzed the male and female fertility of a large number of interspecific hybrids and noted that species originating from the same area are more or less interfertile (pollen viability higher than 55%). The pollen viability of F_1 hybrids between the two groups (species from the Guinea−Congo region and species from East Africa) is below 30%. These results can be explained by differences in genome size, which could be the result of different numbers of copies of highly repetitive sequences and deletion−duplication or polyteny of large chromosomal coding regions (Vedel and Delseny 1987).

In conclusion, the total DNA content of *Coffea* species is low compared with that of other angiosperms and there is an intrageneric twofold variation in genome size. These differences may correspond to genomic evolution correlated with an ecological adaptative process. The three species with a lower DNA content originated from the supposed center of origin of the genus (Kenya). This result could be an argument in favor of the increase of the DNA content during evolution in the *Coffea* subgenus. In addition, species adapted to xeric conditions have a lower DNA content than other species. Species that differ by more than 0.5 pg in nuclear DNA content produce interspecific F_1 hybrids of reduced fertility.

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