VARIATIONS IN THE TOTAL NUCLEAR DNA CONTENT IN AFRICAN *COFFEA* SPECIES (*RUBIACEAE*)

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INTRODUCTION

Coffee beans are produced by many species, but current commercial green coffee production relies on two species only: *Coffea arabica* (2n=44, autogamous, cultivated at altitude) and *C. canephora* (2n=22, allogamous, cultivated in lowlands). Coffee trees belong to the botanical genus *Coffea*, sub-genus *Coffea* (*Rubiaceae*), for which many species have been described (Chevalier 1947, Charrier 1978, Leroy 1980, Bridson & Verdcourt 1988, Anthony 1992). *C. arabica*, native to Ethiopia, was introduced into south and central America only three centuries ago. Wild species are only found in inter-tropical Africa and Madagascar (Berthaud & Charrier 1988).

The main breeding objectives are high yielding varieties, improvement of quality, and screening for disease resistance. The hierarchy of breeding criteria changes with time and place, especially when the genetic improvements of *C. canephora* (variety Robusta) and *C. arabica* cultivars are compared. Despite a large number of breeding studies, the genetic structure of the genus remains largely unknown and a genetic map does not currently exist. Genome size varies among angiosperms from 0.15 to 233 pg of DNA per nucleus (Bennet and Smith, 1991; Marie and Spencer Brown, 1993). In addition, for a given genus, major variations are not only recorded between different ploidy levels, but also between species or populations (De Laat et al., 1987).

Until recently, most DNA contents reported in the literature were determined by Feulgen microspectrophotometry of root tip mitoses (Bennet and Smith, 1991). Since the introduction of automated fluorescence, DNA content can be determined more easely using fluorochromes after leaf chopping, protoplast lysis or nuclei isolation (Galbraith et al., 1983, Dolezel 1991, Ulrich and Ulrich 1991, Arumuganathan and Earle, 1991a, Dolezel et al., 1992).

In the present study, a laser flow cytometry was used to investigate the DNA content of a representative panel of *Coffea* species.

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MATERIALS AND METHODS

Plant materials

Coffee plants were grown from seeds in a greenhouse with a tropical climate (24 °C during the day, 18 °C at night, relative humidity 70%). Newly expanded leaves (0.5 g) were collected at the same time, frozen in liquid nitrogen and stored at -80°C before processing. Seventy-five genotypes belonging to 17 species were used. In the following list, the number of genotypes per species is given in parentheses. Seven species (46 genotypes) are native to Central and West Africa: *C. brevipes* (6), *C. canephora* (11), *C. Congensis* (9), *C. humilis* (3), *C. liberica* (8), *C. sp.* Moloundo (3), *C. stenophylla* (6). Seven species (25 genotypes) are native to East Africa: *C. eugenoïdes* (3), *C. salvatrix* (1), *C. pseudozanguebariae* (3), *C. sessiliflora* (6), *C. racemosa* (4), *C. sp F.* Bridson (3),. *C. arabica* (5). Three species are native to Madagascar: *C. bertrandini* (1), *C. farafaganensis* (1), *C. milotti* (1).

Chopping leaves

To release plant nuclei, leaf tissue (approximately 500 mg) was chopped with a razor blade in a glass Petri dish in 1 ml of PBS buffer, and Triton X100 (10%) was added. The suspension of released nuclei was passed through a 50 mm nylon filter and then stained with 80 ml of propidium iodine (1%).

Preparation of nuclei pellets

Leaves were ground to a fine powder using liquid nitrogen, mixed with buffer A (0.4M sucrose, 0.05M Tris, 2mM CaCl2, 0.4% b-mercaptoethanol) and filtered throught 50 µm Blutex. The solution was centrifuged (3,000g, 15min., 4°C) and the pellets were resuspended in 0.25 M sucrose, 0.05M Tris, 2mM CaCl2 and centrifuged again (3,000 g, 15min., 4°C). The second pellet was mixed with 5ml buffer of B and centrifuged (16,000 g, 45min., 4°C) onto 20ml of buffer C (2M sucrose, 0.05M Tris, 2mM CaCl2). The last pellet - rich in nuclei - could be stored at -80°C or below for one week.

Staining and flow cytometry procedure

PBS buffer (500 ml) was added to isolated nuclei and warmed to room temperature. The isolation procedure was modified by the addition of sarkosyl (1.5% final) to the nuclei suspension, before addition of propidium iodine. The mean fluorescence intensity, frequency, and standard deviation of the propidium iodine-stained nuclei at 488nm were recorded with a FACSCAN - argon laser flow cytometer (Becton Dickinson 488 nm, 15 mW). The voltage of the photomultipliers was set at 550V so that the *C. arabica* peak occurred at channel 600. Rice nuclei (*Oryza sativa* 1.2 pg) and chicken erythrocyte red blood cells "CRBC" (2.33 pg) were used as calibration standards.

Estimation of total nuclear content

To estimate total nuclear content, we compared the mean position of the tested sample with the mean value of the calibration standards, according to Galbraith et al. (1983). Amount of nuclear DNA = (mean position of the tested sample / mean position of the CRBC) * 2.33 pg

RESULTS AND DISCUSSION

Comparison of the two nuclear isolation procedures

Figure 1 shows two DNA histograms obtained with the same genotype of *C. arabica,* one by simple chopping (figure 1a) and the other after nuclei isolation (figure 1b). It is clear that the use of nuclei pellets gave better results (lower CV). In addition, for some species like *C. racemosa* from a very dry region, it seems that the nuclei are less accessible to the dye and no signal is detected. In this case, the isolation of nuclei pellets is a real improvement.

Table 1: Total DNA content of Coffea species native in West and Central Africa

Species	Country of origin	Population/Bulk	Genotype	Nuclei DNA amount (pg) Genotype	Mean
C. brevipes	Cameroon	Mont Cameroun	1	1,52	
C. brevipes	Cameroon	Mont Cameroun	4	1,60	
C. brevipes	Cameroon	Mont Cameroun	6	1,70	
C. brevipes	Cameroon	Mont Cameroun	8	1,45	
C. brevipes	Cameroon	Mont Cameroun	12	1,47	
C. brevipes	Cameroon	Mont Cameroun	18	1,36	1,52
C. canephora	Côte-d'Ivoire	Guinean diversity group	3	1,61	
C. canephora	Côte-d'Ivoire	Guinean diversity group	5	1,29	
C. canephora	Côte-d'Ivoire	Guinean diversity group	6	1,36	
C. canephora	Côte-d'Ivoire	Congolese diversity group	8	1,57	
C. canephora	Côte-d'Ivoire	Congolese diversity group	14	1,46	
C. canephora	Côte-d'Ivoire	Congolese diversity group	19	1,61	
C. canephora	Rep. Central Africa	Nana river	4	1,44	
C. canephora	Rep. Central Africa	Nana river	6	1,18	
C. canephora	Cameroon	Cameroonese diversity group	2	1,47	
C. canephora	Cameroon	Cameroonese diversity group	7	1,56	
C. canephora	Cameroon	Cameroonese diversity group	19	1,47	1,46
C. congensis	Rep. Central Africa		4	1,59	
C. congensis	Rep. Central Africa		7	1,57	
C. congensis	Rep. Central Africa		9	1,52	
C. congensis	Cameroon		10	1,54	
C. congensis	Cameroon		13	1,52	
C. congensis	Cameroon		22	1,45	
C. congensis	Congo		14	1,76	
C. congensis	Congo		22	1,46	
C. congensis	Congo		23	1,40	1,53
C. humilis	Côte d'Ivoire		2	1,81	
C. humilis	Côte d'Ivoire		13	1,42	
C. humilis	Côte d'Ivoire		16	1,60	1,61
C. liberica	Côte d'Ivoire	Guinean diversity group	3	1,54	
C. liberica	Côte d'Ivoire	Guinean diversity group	12	1,42	
C. liberica	Côte d'Ivoire	Guinean diversity group	19	1,86	
C. liberica	Rep. Central Africa	Congolese diversity group	3	1,51	
C. liberica	Rep. Central Africa	Congolese diversity group	9	1,43	
C. liberica	Rep. Central Africa	Congolese diversity group	17	1,33	
C. liberica	Cameroon	Koto	4	1,71	
C. liberica	Cameroon	Koto	14	1,88	1,59
C. Sp Moloundou	Cameroon	Moloundou	1	1,70	
C. Sp Moloundou	Cameroon	Moloundou	2	1,32	
C. Sp Moloundou	Cameroon	Moloundou	7	1,79	1,60
C. stenophylla	Côte d'Ivoire	Ira	4	1,32	
C. stenophylla	Côte d'Ivoire	Ira	6	1,22	
C. stenophylla	Côte d'Ivoire	Ira	11	1,46	
C. stenophylla	Côte d'Ivoire	Assabli	3	1,25	
C. stenophylla	Côte d'Ivoire	Assabli	8	1,22	
C. stenophylla	Côte d'Ivoire	Assabli	12	1,23	1,28

Table 2: Total DNA content of Coffea species native in East Africa and Madagascar

Species	Country of origin	Population	Genotype	Nuclei DNA amount (pg)	
				Genotype	Mean
C. eugenioides	Kenya		16.1	1,39	
C. eugenioides	Kenya		16.2	1,27	
C. eugenioides	Kenya			1,43	1,36
C. pseudozanguebariae	Kenya		4	1,25	
C. pseudozanguebariae	Kenya		5	1,01	
C. pseudozanguebariae	Kenya		7	1,09	1,12
C. racemosa	Mozambique		4	0,91	
C. racemosa	Mozambique		9	0,87	
C. racemosa	Mozambique		11	0,87	
C. racemosa	Mozambique		13	1,08	0,93
C. salvatrix			LB1	1,46	
C. sessiliflora	Kenya	Shimba	2	0,87	
C. sessiliflora	Kenya	Shimba	4	0,92	
C. sessiliflora	Kenya	Shimba	7	1,06	
C. sessiliflora	Tanzania	Kitulangalo	7	1,14	
C. sessiliflora	Tanzania	Kitulangalo	10	0,99	
C. sessiliflora	Tanzania	Kitulangalo	13	1,03	1,00
C. Sp F Bridson	Tanzania		10	1,25	
C. Sp F Bridson	Tanzania		17	1,29	
C. Sp F Bridson	Tanzania		20	1,25	1,26
C. bertrandii	Madagascar		HAK 1	1,65	
C. farafaganensis	Madagascar			1,34	
C. millotii	Madagascar		CM1	1,71	
C. arabica			Catimor	2,35	
C. arabica	Ethiopia		1,1	2,60	
C. arabica	Ethiopia		12.1a	2,39	
C. arabica	Ethiopia		12.1b	2,30	
C. arabica	Ethiopia		12.5	2,72	2,47
P. ebracteolatus	Côte d'Ivoire			1,14	

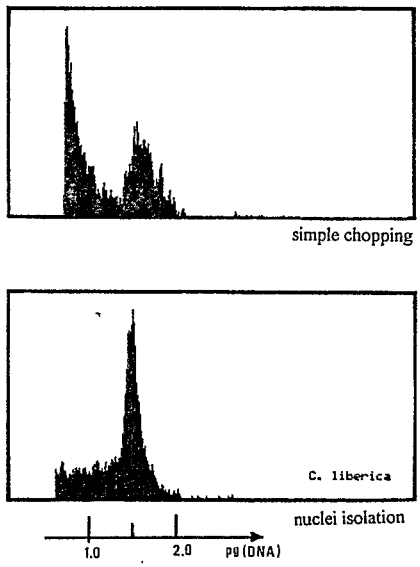

Figure 1 : Comparison of 2 histograms obtained by cytofluorometry for a genotype of *Coffea liberica*

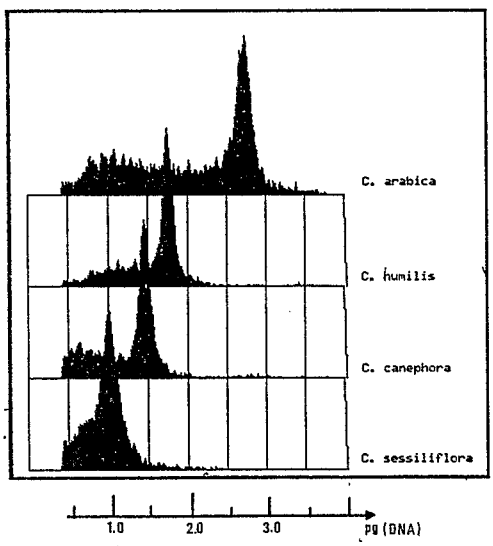

Figure 2 : Comparison of the total DNA amount of 4 *Coffea* species, obtained by cytofluorometry.

Although isolation of nuclei is more time-consuming, the results are more consistent and repeatable. For example, repetition of the complete procedure for 2 leaves of the genotype *C. arabica* 12.1a, harvested at the same time, gave very similar results (2.26 versus 2.39 pg per nucleus - Table 2). In addition, isolated nuclei can be stored in the cold (-80°C) for weeks without loss of resolution. The flow cytometry method could allow the analysis of nuclear DNA content in large populations.

Variation among diploid species

The DNA contents of diploid *Coffea* species and genotypes are reported in Table 1 for West and Central Africa, and in Table 2 for East Africa. Three species (*C. sessiliflora*, *C. pseudozanguebariae* and *C. racemosa*) native to East Africa have the lowest DNA content per nucleus (about 1pg). *C. humilis*, *C. liberica* and *C. sp.* Moloundou exhibit the highest contents (about 1.6 pg)(Table 2). Species from both west and east (*C. stenophylla*, *C. eugenioides* and *C. sp.* F Bridson) have an intermediate content (1.3 pg).

Differences in excess of 2- to 3-fold are common among congeneric diploid species (Price, 1988). For example, in the genera *Malus* and *Prunus*, the total DNA content of diploid species varies from 0.42 to 1.75 pg (Dickson et al., 1992). Walbot and Cullis (1985) described rapid genomic changes in plants. In the genus *Helianthus*, total DNA content varies more than 4-fold among diploid species (Sim and Price 1985, Cavallini et al. 1989). Some authors have tried, without any real possibility of generalisation, to correlate such variations with altitude, longitude or degree of selection (Price 1988). Price also suggests that total DNA content may be positively correlated with cell cycle duration. What is the situation for coffee? 1- If we analyze the results according to the climate of the native region we observe that species native to dry areas have a smaller genome than those from evergreen forest. 2- Let us assume that the process of seed maturation implies a constant number of cell divisions from flowering to ripening. Hamon et al. (1984) and Anthony (1992) report large differences between species. The three species of lower DNA content also have a shorter maturation phase (2 *versus* 11 months). 3-In the literature, no special attention is paid to the potential relation between genome size and genetic distance or possibility of inter-crossing. In extensive studies of crossing possibilities and hybrid fertility between diploid coffee species, Louarn (1993) clearly shows that *C. racemosa, C. sessiliflora* and *C. pseudozanguebariae* are interfertile but genetically isolated from the others by a strong fertility barrier. One constitutive element of this fertility barrier could be related to genome size differences.

Coffee genome size compared to other angiosperm species

Estimated values of total DNA content per nucleus are given in Tables 1 and 2. The 2-C value of *Coffea* species varies from 0.9 to 1.9 pg. Figure 2 shows histograms obtained for four species with different values of nuclear DNA content.

A range from 0.15 pg per nucleus for *Arabidopsis thaliana* to 223 pg for *Trillium rhombifolium* (Liliaceae) is given for angiosperm species by Bennett and Smith (1991). In this review paper, most estimates were made after Feulgen staining. When our results are compared with those obtained by cytofluorometry (Michaelson et al. 1991, Arumuganathan and Earle 1991b, Hamon et al. 1992, Lannaud et al. 1992, Marie and Spencer Brown 1993), the *Coffea* genome is small compared to those of *Allium cepa* (32.7 pg) or *Triticum aestivum* (30.9 pg), and similar to those of *Lycopersicum esculentum* (2.01 pg), *Beta vulgaris* (1.65 pg) and some types of *Dioscorea cayenensis-rotundata* or *Cucumis sativus* (1.77). Translated into mega base pairs (Mbp), with the equivalence: 1pg = 0.960 Mbp, the mean haploid genome size (0.75 pg) of *Coffea* species can be estimated as 0.7 Mpb. The mean chromosome size (x = 11) is therefore about 0.06 Mpb.

De Laat et al. (1987) show in auto-polyploid series, as in the genera *Malus* and *Prunus*, that the DNA content is exactly doubled. *C. arabica* (2n = 4x) is supposed to be a tetraploid plant of amphiploid origin. Two hypotheses could be suggested to explain the value of 2.5 pg for C. *arabica*: the addition of 2 genomes of about 1.3 pg or the sum a genome of 1pg and one of 1.5pg corresponding to the groups previously identified.

Intraspecific DNA polymorphism

Despite clear differences between genetically isolated species, within- and between-species overlap in C-DNA values is seen (Tables 1 and 2). The CV in DNA content was about 30%.

The concept of constancy of the unreplicated haploid nuclear genome (C-value) was introduced by Swift (1950) and was accepted untill the beginning of the 1980s. Bennett (1985) reports that the extent of variation of the nuclear genome in some species may be considerable, reaching 54% for *Glycine max*, 59% for *Gibasis venestula*, 80% for *Poa annua* and 228% for *Collinsia verna*. Variations is also seen in some crop species: *Oryza sativa* (33%), *Zea mays* (30%), *Capsicum annuum* (35%). In contrast, *Hordeum vulgare*, *Vicia faba*, *Triticum aestivum*, *Sorghum bicolor*, and *Festuca pratensis* do not reveal such polymorphism (Laurie and Bennett 1985, Bennett and Smith 1991). Essad (1988) found that for *Medicago* diploids, intra- and interspecific variations were all near multiples of a DNA quantum called a "nucleon", which was estimated to be 0.37 pg at the 2 C level. Michaelson et al. (1991) found that the F1 offspring of two varieties of *Zea mays*, differing in nuclear DNA content, have an intermediate DNA content. Consequently, intraspecies polymorphism of *Coffea* species is consistent with other results.

Significance of variation in DNA C-values

We have observed intra- and interspecific differences in total DNA content in the genus *Coffea*. Furuta and Nishikawa (1991), Lapitan (1992) have reviewed variations in nuclear chromosomal DNA. A large number of explanations could be suggested. The first relates to differences in chromosome length and/or deletion/duplication of some chromosomal segments. No recent findings on this are available for coffee. Bouharmont (1959) published the only work on the subject and referred to interspecific variations in chromosomal length.

Another possible explanation is variation of the repeated sequences. The number of repeated DNA sequences often increases with genome size: 14% for *Arabidopsis*, 15-20% for *Lycopersicum* and 60 to 80% for *Zea* (Ganal et al. 1988). Among the different types of repeated sequences, satellite DNA is often found close to the telomeres and centromeres of plant chromosomes. While satellite DNA is highly homogenous within a species, it often highly divergent across species within a family, as in tomato and barley (Schweizer et al. 1988). Telomeric DNA possesses unique structural features that are important for its function of stabilizing chromosomes by allowing complete replication and preventing progressive loss of terminal nucleotides during replication. The ends of eukaryotic chromosomes consist of tandem copies of a highly conserved repeated DNA sequence with the general form (T/A)nG(1-8) (Ganal et al. 1991). The particular hairpin structure is believed to be very important for recognition of the telomere-synthesizing enzyme, telomerase (Blackburn, 1990). Another repeated sequence family is the gene coding for ribosomal DNA. Corresponding genes are often clustered at one or more sites, most of which are associated with the nuclear region. In tomato, 27 hybridization sites are known. The total length in plants ranges from 7.8 to 18.5 Kb, with copies numbering from 600 to 8500 per haploid genome.

Conclusions and prospects

Analysis of the total DNA content in different species gives important information concerning the total DNA per nucleus for *Coffea* species. The genome size (1 - 1.6 pg), which is similar to that of *Beta vulgaris* (1.77 pg), is small if compared with other angiosperms. Within the genus 2 main groups are revealed. Their relative DNA content per nucleus was correlated both with their ecological origin (dry/humid) and the possibility of crossing them. In addition, species with smaller genomes, which exhibit a shorter flowering to ripening interval, may have a shorter cell duration. Our work gives the overall genetic background, and it is now necessary to identify the genetic basis of observed differences and perhaps use them in coffee breeding programmes. *In situ* hybridization has been used to identify differences in DNA repeated sequences in rye and barley (Jouve et al. 1991, Leith et al. 1991, 1992). Mukai and Gill (1992) have used such techniques with interspecific hybrids of barley and wheat to detect barley chromatids. Another objective could be flow karyotyping and chromosome sorting, as recently reported for *Vicia faba* (Lucretti et al. 1993).

Abstract

Laser flow cytometry has been used to estimate total nuclear DNA content for 75 *Coffea* accessions, corresponding to 16 diploid species (2n=22) and *C. arabica* (tetraploid, 2n=44). Nuclei were isolated and stained by propidium iodine (DNA intercaling dye). 2C values ranging from 0.9 to 1.9 pg per nucleus have been estimated for *Coffea* species. Three species native to East Africa (*C. sessiliflora, C. racemosa* and *C. pseudozanguebariae*) had the smallest genome size (about 1 pg per nucleus). Species native to the African evergreen forest (*C. humilis, C. sp* Moloundou and *C. liberica*) had the highest diploid DNA content (1.6 pg). The genome size of the tetraploid *C. arabica*, native to Ethiopia, was 2.5 pg. In most species, variation in 2C values (up to 25%) was also recorded. Results are compared with data from other angiosperm species and are discussed in terms of their evolutionary significance.

Résumé

La quantité d'ADN par noyau a été estimée pour 75 génotypes de *Coffea* appartenant à 16 espèces diploides (2n=22) et *C. arabica* (tetraploide 2=44) par cytofluorométrie en flux. L'agent fluorochrome utilisé est l'iodure de propidium (intercalent). La valeur 2-C du génome des caféiers oscille de 0,9 pg d'ADN par noyau à 1,9 pg. Trois espèces, originaires d'Afrique de l'Est (*C. sessiliflora, C. racemosa* et *C. pseudozanguebariae)* ont le plus petit génome (environ 1 pg par noyau). A l'opposé 3 espèces de forêt tropicale humide (*C. humilis, C. sp* Moloundou and *C. liberica*) correspondent aux valeurs les plus élevées (1.6). L'espèce tétraploide *C. arabica*, originaire d'Ethiopie, a 2,5 pg par noyau. Au sein des espèces diploides on note une importante variation des valeurs 2-C pouvant atteindre 25% pour une espèce. Les résultats sont comparés aux résultats analogues chez d'autres angiospermes et une tentative d'interprétation des variations observées est donnée.

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