WELLINGTON RONILDO CLARINDO

UTILIZAÇÃO DE TÉCNICAS CITOGENÉTICAS, DE CITOMETRIA DE FLUXO E DE IMAGEM PARA CARACTERIZAÇÃO DO GENOMA DE *Coffea canephora* e *C. arabica*.

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Genética e Melhoramento, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 26 de fevereiro de 2008.

Prof. Wagner Campos Otoni
(Co-Orientador)

Pesq. Eveline Teixeira Caixeta
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Prof. Ney Sussumu Sakiyama

Pesq. Edgard Augusto de Toledo Picoli

Prof. Carlos Roberto de Carvalho
(Orientador)

Aos meus amados pais,

Luiz Carlos Clarindo (*in memoriam*) e Maria das Graças Clarindo.

DEDICO

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BIOGRAFIA

WELLINGTON RONILDO CLARINDO, filho de Luiz Carlos Clarindo e Maria das Graças Clarindo, nasceu em Ponte Nova, Minas Gerais, no dia 19 de agosto de 1979.

Em 1998, ingressou na Universidade Federal de Viçosa, graduando-se Bacharel e Licenciado em Ciências Biológicas em março de 2003.

Durante o período de graduação, foi monitor do Departamento de Bioquímica e Biologia Molecular e bolsista do PIBIC/CNPq do Departamento de Biologia Geral, onde desenvolveu atividades de pesquisa em Citogenética e Citometria Vegetal.

Em março de 2003, ingressou no Programa de Pós-graduação em Genética e Melhoramento, em nível de mestrado, submetendo-se à defesa de dissertação em agosto de 2004.

Em agosto de 2004, ingressou no Programa de Pós-graduação em Genética e Melhoramento, em nível de doutorado, submetendo-se à defesa de tese em fevereiro de 2008.

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RESUMO

CLARINDO, Wellington Ronildo, D. Sc., Universidade Federal de Viçosa, fevereiro de 2008. **Utilização de técnicas citogenéticas, de citometria de fluxo e de imagem para caracterização do genoma de *Coffea canephora* e *C. arabica*.** Orientador: Carlos Roberto de Carvalho. Co-Orientadores: Wagner Campos Otoni e Eveline Teixeira Caixeta.

A aplicação de técnicas citogenéticas, de citometria de fluxo e de imagem tem possibilitado a caracterização do genoma de *Coffea arabica* L. e *Coffea canephora* Pierre ex Froehner. Esses trabalhos vêm provendo relevantes informações a estudos evolutivos e para o melhoramento da cultura. Levando em conta a necessidade de ampliação das informações acerca do genoma de *C. arabica* e *C. canephora*, esse estudo aprimorou estratégias citogenéticas e citométricas para gerar novos dados acerca do genoma dessas espécies. Inicialmente, metodologias citogenéticas aplicadas em suspensões de agregados celulares de *C. canephora* geraram cromossomos com morfologia adequada para caracterização morfométrica e montagem do cariograma da espécie. A aplicação das técnicas de bandeamento Ag-NOR e Hsc-FA possibilitaram a identificação da NOR ativa no braço curto do cromossomo 6. Após empregar os mesmos procedimentos utilizados para *C. canephora* em agregados celulares de *C. arabica*, foram obtidos cromossomos metafásicos e prometafásicos apresentando resolução suficiente para realização das análises citogenéticas e para montagem do cariograma da espécie. As análises morfométricas evidenciaram tanto cromossomos morfologicamente idênticos quanto diferentes, sugerindo que *C. arabica* é um alotetraplóide, não segmental, formado a partir do cruzamento de duas espécies com genoma similar. A citometria de fluxo foi empregada para mensurar o conteúdo de DNA nuclear e determinar a composição de bases de *C. canephora* e *C. arabica*. Análises preliminares mostraram que os procedimentos envolvendo o uso dos tampões OTTO e de compostos antioxidantes resultaram histogramas apresentando picos de núcleos em G_0/G_1 com coeficientes de variação considerados adequados para análises citométricas. Além disso, foi demonstrado que, em comparação com *Pisum sativum* e *Raphanus sativus*, *Solanum lycopersicum* foi a planta utilizada como

padrão de DNA conhecido que gerou as estimativas mais acuradas acerca do conteúdo de DNA nuclear de *C. arabica* e *C. canephora*. Numa segunda etapa foi verificado que o tamanho médio do genoma nuclear de *C. arabica* (2,62 picogramas) e *C. canephora* (1,41 picogramas) obtido por meio do fluorocromo iodeto de propídeo foi idêntico ao mensurado com núcleos corados com SYBR Green I. Entretanto, o corante SYBR Green I proporcionou análises citométricas mais precisas, visto que os picos gerados por núcleos na fase G_0/G_1 apresentaram coeficientes de variação abaixo dos obtidos com iodeto. Além do conteúdo absoluto de DNA, a composição de bases também foi mensurada para as duas espécies utilizando núcleos corados com 4',6'-diamidino-2-fenilindole (DAPI) e cromomicina A_3 (CMA_3). *C. arabica* apresentou um percentual de bases AT equivalente a 63,04% e GC 36,96%, enquanto o percentual de base AT de *C. canephora* foi 65,27% e GC 34,73%. A associação de metodologias citogenéticas e citométricas de fluxo e imagem permitiu estimar o conteúdo de DNA de cada cromossomo de *C. arabica* e *C. canephora*. Os resultados obtidos também mostraram que *C. arabica* é um verdadeiro allotetraplóide e geraram evidências que *C. canephora* é um possível progenitor dessa espécie. Os dados apresentados forneceram novas informações acerca das características, composição e organização do genoma de *C. arabica* e *C. canephora* e representam um avanço na prospecção do genoma do cafeeiro.

ABSTRACT

CLARINDO, Wellington Ronildo, D. Sc., Universidade Federal de Viçosa, February, 2008. **Utilization of cytogenetic, flow and image cytometric techniques for genome characterization of *Coffea canephora* and *C. arabica*.** Adviser: Carlos Roberto de Carvalho. Co-Advisers: Wagner Campos Otoni and Eveline Teixeira Caixeta.

The application of cytogenetic, flow and image cytometric techniques has been enabling the genome characterization of *Coffea arabica* L. and *Coffea canephora* Pierre ex Froehner. These approaches have been providing relevant information for evolutionary studies and breeding programs. Considering the need of increasing the information available on the genomes of *C. arabica* and *C. canephora*, the present study improved cytogenetic and cytometric strategies in order to provide new data about the genomes of these species. Initially, cytogenetic methodologies applied to suspensions of cell aggregates of *C. canephora* yielded chromosomes with adequate structure for morphometric characterization and assembly of the karyogram for this species. Application of the banding techniques Ag-NOR and Hsc-FA allowed the identification of the active NOR in the short arm of chromosome 6. After employing the same procedures used in *C. canephora* to cell aggregates of *C. arabica*, metaphasic and prometaphasic chromosomes were obtained presenting satisfactory resolution for the accomplishment of the cytogenetic analyses and assembly of the karyogram of this species. The morphometric analyses evidenced both morphologically identical and distinct chromosomes, which suggests that *C. arabica* is a allotetraploid, non-segmental, originated from the crossing between two species with similar genome. Flow cytometry was used to quantify the nuclear DNA and determine the base composition of *C. canephora* and *C. arabica*. Preliminary analyses showed that the procedures involving use of the OTTO buffers and antioxidant substances resulted in histograms showing peaks of G_0/G_1 nuclei, with coefficients of variation considered adequate for cytometric analyses. Moreover, it was demonstrated that, in comparison to *Pisum sativum* and *Raphanus sativus*, *Solanum lycopersicum* was the plant used as standard of known DNA that offered the most accurate estimation of the nuclear DNA contents of *C. arabica* and *C. canephora*. In a second stage, it

was verified that the mean size of the nuclear genome of *C. arabica* (2.62 picograms) and *C. canephora* (1.41 picograms), obtained through use of the fluorochrome propidium iodide, was identical to that measured in nuclei stained with the dye SYBR Green I. However, SYBR Green I rendered more precise cytometrical analyses, seeing that the peaks resulting from G_0/G_1 nuclei presented coefficients of variation under those obtained with propidium iodide. Apart from the absolute DNA content, the base composition was also determined for both species by use of nuclei stained with 4',6'-diamidino-2-phenylindole (DAPI) and chromomycin A_3 (CMA_3). *C. arabica* presented a percentage of AT bases equivalent to 63.04%, and 36.96% GC, while *C. canephora* presented 65.27% AT and 34.73% GC. The association of cytogenetic methodologies with flow and image cytometries allowed estimation of the DNA content of each chromosome of *C. arabica* and *C. canephora*. The results obtained also showed that *C. arabica* is a true allotetraploid, and additionally provided evidence that *C. canephora* is a possible progenitor of that species. The presented data supplied new information on the characteristics, composition and genome organization of *C. arabica* and *C. canephora*, and represented an advance in the genome research of the coffee tree.

1 – INTRODUÇÃO GERAL

1.1 – Aspectos gerais do gênero *Coffea*

O gênero *Coffea*; família Rubiaceae, subfamília Cinchonoideae, tribo Coffeeae (Pinto-Maglio 2006); é composto por aproximadamente 100 espécies (Baruah et al. 2003, Pinto-Maglio 2006), nativas das florestas intertropicais da África e Madagascar, que diferem enormemente com relação às características morfológicas e adaptativas (Lashermes et al. 1997).

Esse gênero possui dois subgêneros, *Coffea* e *Paracoffea* (Berthou 1983, Lashermes et al. 1997), sendo que o primeiro compreende as espécies com alto teor de cafeína, inclusive as de maior interesse agronômico: *Coffea arabica* L. e *Coffea canephora* Pierre ex Froehner (Berthou 1983, Lashermes et al. 1993, 1997, Raina et al. 1998, Campa et al. 2005a, b, Mahé et al. 2007).

O café é um dos cinco produtos agrícolas mais comercializados e consumidos no mundo (Mahé et al. 2007, FAO 2007). Cerca de 100 milhões de pessoas trabalham direta ou indiretamente com a produção e o processamento do café (International Coffee Organization 2007). Dentre as espécies do gênero, *C. arabica* e *C. canephora* são as de maior cotação no mercado internacional, sendo que cerca de 70% do café negociado no mundo é do tipo Arábica (*C. arabica*) e 30% do tipo Robusta (*C. canephora*) (Mahé et al. 2007, FAO 2007, International Coffee Organization 2007).

O Brasil lidera o mercado mundial de café em produção e exportação e é o segundo maior consumidor mundial do grão (FAO 2007). O principal tipo de café plantado no Brasil é Arábica, embora existam algumas áreas (Espírito Santo, norte do Rio de Janeiro, Vale do Rio Doce em Minas Gerais, Mato Grosso, Rondônia, sul da Bahia e Acre) plantadas com o café Robusta (Caixeta 2001).

Atualmente o “Projeto Genoma do Café” tem como principal objetivo obter Etiquetas de Seqüências Expressas (ESTs, Projeto Genoma EST-Café) em diferentes condições e tecidos. Contando com a participação de vários pesquisadores e instituições de ensino e pesquisa, inclusive a Universidade Federal de Viçosa (UFV), os trabalhos tiveram início em fevereiro de 2002 com os seguintes objetivos:

a) obter ESTs e anotar no genoma do café;
b) determinar os genes expressos nos diversos tecidos e órgãos, assim como em situações de estresses bióticos e abióticos, e realizar a análise funcional das seqüências geradas;
c) construir mapas genéticos de café visando aumentar a densidade de marcadores moleculares para auxiliar no melhoramento da cultura;
d) fortalecer a capacidade de pesquisa do Consórcio nas áreas de Genômica, Bioinformática e Biologia Estrutural, tanto laboratorial como de recursos humanos.

Em 2004 os pesquisadores declararam que a primeira fase do projeto de seqüenciamento do genoma do café foi completada. Os estudos resultaram na construção de um banco de dados com mais de 200 mil seqüências de DNA, permitindo, dessa forma, a identificação de mais de 30 mil genes responsáveis pelos diversos mecanismos morfo-fisiológicos de crescimento e desenvolvimento do cafeeiro.

De acordo com o contrato de cooperação assinado pela Embrapa, Fapesp e Instituto Uniemp (Fórum Permanente das relações Universidade-Empresa) o banco de dados obtido pode ser acessado gratuitamente por seis instituições públicas de ensino e pesquisa ligadas à Fapesp e 17 órgãos e instituições que integram o Consórcio Brasileiro de Pesquisas e Desenvolvimento do Café (CBP&D/Café).

De posse das seqüências contidas no banco de dados, inúmeros outros estudos podem ser conduzidos e os impactos potenciais desses trabalhos beneficiam a indústria alimentícia, os produtores da cultura do cafeeiro e os consumidores.

Além disso, o programa de melhoramento do cafeeiro também possui como objetivo identificar marcadores moleculares para estabelecimento de mapas genéticos e integração desses a mapas físicos.

Nesse contexto, estudos empregando a citogenética e as citometrias de fluxo e de imagem podem gerar dados relevantes acerca das características do genoma das espécies do gênero *Coffea*. Tais aspectos podem contribuir com o estabelecimento de mapas físicos, com estudos evolutivos e taxonômicos, com o planejamento de projetos de seqüenciamento e em estudos com marcadores moleculares.

1.2 – Citogenética do gênero *Coffea*

Os primeiros estudos citogenéticos realizados no gênero *Coffea* objetivaram estabelecer o número de cromossomos das espécies. Homeyer (1933) (citado por Krug 1934) reportou que o número básico de cromossomos de *Coffea* spp. é x = 11, o mesmo apresentado pela maioria dos gêneros da família Rubiaceae. Além disso, o mesmo autor também relatou a ocorrência de 2n = 44 cromossomos em *C. arabica*, a única espécie tetraplóide e autógama do gênero. Os mesmos resultados foram observados por Krug (1934), Mendes (1938a, b), Conagin e Mendes (1961), Rijo (1974) e Carvalho e Fazuoli (1993). Esse autores também mencionaram que *C. arabica* é uma espécie tetraplóide e as demais são diplóides com 2n = 22 cromossomos.

Numa segunda etapa, os pesquisadores pocuraram caracterizar morfologicamente os cromossomos de *Coffea* spp. Inicialmente, Mendes (1938b) observou que os cromossomos da maioria das espécies são relativamente pequenos (3,5 a 1,5 μm) e morfologicamente similares. Esse autor separou os cromossomos, de acordo com o tamanho total, em três grupos denominados A, B e C.

Bouharmont (1963) realizou um extensivo estudo com o objetivo de caracterizar os cromossomos de 16 espécies de café. Com base nos resultados, resumidos na Tabela 1, o autor reforçou as observações relatadas por Mendes (1938b), ao mencionar que os cromossomos de *Coffea* são relativamente pequenos, similares, sendo possível distinguir apenas 5 cromossomos (1, 2, 3, 4 e 11). Em virtude desses fatores, o autor apresentou um único idiograma médio para o gênero *Coffea*.

Em decorrência das características dos cromossomos mitóticos de *Coffea* spp., alguns pesquisadores realizaram estudos em células meióticas de algumas espécies do gênero. Pinto-Maglio e Cruz (1987), visando ampliar os conhecimentos acerca do genoma de *Coffea* spp., caracterizaram os cromossomos paquitênicos com constrição secundária de nove espécies do gênero. Os resultados obtidos nesse estudo estão resumidos na Tabela 2.

Tabela 1 – Tamanho e porcentagem correspondente no gemona dos cromossomos do gênero *Coffea* (Bouharmont 1963).

Cromossomo	Comprimento médio (μM)	Porcentagem correspondente no genoma
1	2,41 – 1,92	13,6
2	1,98 – 1,61	10,9
3	1,88 – 1,50	10,2
4	1,79 – 1,46	9,8
5, 6, 7, 8, 9 e 10	1,62 – 1,09	9,2 a 7,2
11	1,18 – 0,97	6,4

Tabela 2 – Classificação dos cromossomos com constrição secundária de nove espécies de *Coffea* (Pinto-Maglio e Cruz 1987).

Espécie	Nível de ploidia	Número de cromossomos com constrição secundária	Classificação do cromossomo quanto à posição do centrômero
C. arabica	4x	2 (cromossomos 1 e 2)	S
C. canephora	2x	1	A
C. congensis	2x	1	M
C. dewevrei	2x	1	S – A
C. eugenioides	2x	1	A
C. kapakata	2x	1	A
C. liberica	2x	1	S
C. racemosa	2x	2 (cromossomos 1 e 2)	M – S / S
C. salvatrix	2x	2 (cromossomos 1 e 2)	A / S

M = metacêntrico; S = submetacêntrico e A = acrocêntrico

Em 1998, os mesmos pesquisadores analisaram citogeneticamente todos os cromossomos paquitênicos de *C. arabica* e reportaram que a espécie apresenta 4 cromossomos metacêntricos (1, 8, 11, 18), 14 submetacêntricos (2, 3, 4, 5, 6, 7, 9, 10, 12, 15, 16, 17, 19, 20) e 4 acrocêntricos (13, 14, 21 e 22). Além disso, foram observados três cromossomos com organizador nucleolar (14, 21 e 22).

Considerando que as técnicas convencionais de coloração com orceína acética e reativo de Schiff não permitiram grandes avanços nos estudos citogenéticos em *Coffea*, Pierozzi (1993) aplicou metodologias de bandeamento com o intuito de evidenciar regiões cromossômicas que permitissem diferenciar os cromossomos. Apesar de ter observado regiões heterocromáticas, reveladas pelo bandeamento C, e porções Ag-NOR positivas, a autora enfatizou a dificuldade em aplicar essas técnicas nos cromossomos de *Coffea* e conseguiu caracterizar parcialmente o cariótipo de *C. canephora*, *C. liberica* e *C. racemosa*.

Adequando a metodologia de bandeamento C a plantas com cromossomos relativamente pequenos, Pierozzi et al. (1999) obtiveram um padrão de bandas e descreveram o idiograma de *C. canephora* e *C. dewevrei*. As duas espécies apresentaram 2n = 22 cromossomos, sendo dois com a constrição secundária no braço longo (1, 3). Os autores também mencionaram que *C. canephora* possui 8 cromossomos submetacêntricos (1, 2, 3, 4, 6, 7, 8 e 9) e 4 metacêntricos (5, 10, 11) e que *C. dewevrei* contém 9 cromossomos submetacêntricos (1, 2, 3, 4, 5, 6, 7, 8 e 9) e 2 metacêntricos (10, 11).

Técnicas de bandeamento com fluorocromos, que se ligam preferencialmente a regiões ricas em AT (DAPI – 4'-6'-diamidino-2-fenillindol) e GC (CMA_3 – Cromomicina A_3), e de hibridização *in situ* fluorescente (FISH) também foram empregadas para diferenciar longitudinalmente os cromossomos mitóticos de *C. arabica* (Artero 2006), *C. canephora*, *C. salvatrix*, *C. sessiliflora* (Lombello e Pinto-Maglio 2004b), *C. humulis*, *C. kapakata*, *C.* sp. *Moloundou* e *C. stenophylla* (Lombello e Pinto-Maglio 2004a).

Apesar de todos esses estudos, os primeiros cariogramas de *C. arabica*, *C. canephora* e *C. eugenioides* foram montados por Fontes (2003). A autora utilizou as técnicas de dissociação celular e secagem ao ar (Carvalho e Saraiva 1993, 1997) e sistema de análise de imagens para identificar os pares de cromossomos homólogos dessas espécies. Entretanto, em decorrência do baixo índice metafásico, da morfologia extremamente similar e do tamanho diminuto, apenas os cromossomos de *C. canephora* foram caracterizados em 1 cromossomo metacêntrico (1), 10 submetacêntricos (2, 3, 4, 5, 6, 7, 8, 9, 10, 11) e um (6) que possui, no braço curto, um satélite associado à constrição secundária.

Com base nos resultados obtidos por meio das análises citogenéticas, todos os autores mencionados reportaram que os cromossomos das espécies do gênero

Coffea são relativamente pequenos e morfologicamente similares. Esses aspectos têm prejudicado a identificação dos pares de homólogos, a montagem de cariogramas acurados (Mendes 1938b, Bouharmont 1963, Pinto-Maglio 2006) e a aplicação de técnicas de citogenética molecular (Raina et al. 1998, Lashermes et al. 1999, Pinto-Maglio 2006). Em virtude disso, Pinto-Maglio (2006) mencionou que é necessário o desenvolvimento de metodologias que permitam identificar os cromossomos de *Coffea* spp. Ainda, a montagem de cariogramas mais acurados poderia contribuir para a compreensão das modificações evolutivas que ocorreram no genoma dessas espécies.

1.3 – Evolução do genoma das espécies do gênero *Coffea*

A partir dos resultados obtidos em estudos citogenéticos (Bouharmont 1963) e moleculares (Lashermes et al. 1996, Mahé et al. 2007), concluiu-se que o gênero *Coffea* originou-se de um único ancestral (origem monofilética), que possuía o número cromossômico básico idêntico ao apresentado atualmente pelo gênero, X = 11, cerca de 5-25 milhões de anos atrás (Lashermes et al. 1996). Com base em observações citológicas, Rijo (1974) constatou que a individualização das diferentes espécies de *Coffea* ocorreu por meio de alterações cromossômicas diminutas, citogeneticamente não identificáveis, ou por mutações gênicas.

Por ser a única espécie tetraplóide do gênero, *C. arabica* tem sido a espécie mais estudada evolutivamente com o intuito de identificar os seus genitores (Orozco-Castillo et al. 1996, Raina et al. 1998, Lashermes et al. 1999) e evidenciar as modificações que ocorreram no seu genoma ao longo da evolução (Lashermes et al. 1999).

Essa espécie vem sendo considerada alotetraplóide segmental (Orozco-Castillo et al. 1996, Pinto-Maglio and Cruz 1998, Raina et al. 1998, Baruah et al. 2003, Pinto-Maglio 2006) ou anfidiplóide (Lashermes et al. 1999) formado a partir do cruzamento de duas espécies diplóides (2n = 22), com genomas muito semelhantes, do gênero *Coffea*. Dentre as espécies diplóides, *C. eugenioides* (Höfling and Oliveira 1981, Berthou et al. 1983, Lopes et al. 1984, Orozco-Castillo et al. 1996, Raina et al. 1998, Lashermes et al. 1999, Ruas et al. 2003), *C. canephora* (Lashermes et al. 1997, Lashermes et al. 1999, Ruas et al. 2003), *C. congensis* (Höfling e Oliveira

1981, Lashermes et al. 1997, Raina et al. 1998) e *C. brevipes* (Lashermes et al. 1997) são cogitadas como possíveis progenitores.

As características dos cromossomos mitóticos de *Coffea*, além de dificultar o mensuramento dos dados morfométricos, têm prejudicado o progresso dos estudos evolutivos no gênero, especialmente em *C. arabica*. Em virtude desses aspectos, Lashermes et al. (1999) mencionaram que a identificação dos cromossomos dessa espécie é laboriosa, difícil e que a discriminação dos genomas constituintes de *C. arabica* é impossível.

1.4 – Citometrias de fluxo e imagem em *Coffea* spp.

Além de metodologias citogenéticas clássicas e moleculares, análises citométricas de fluxo e imagem também foram efetuadas em espécies de *Coffea* spp. visando estimar o conteúdo de DNA nuclear. O mensuramento do tamanho do genoma em *Coffea* spp. tem gerado informações que contribuem com os estudos taxonômicos (Cros et al. 1994, 1995) e evolutivos (Noirot et al. 2003b) no gênero. Além disso, o conteúdo 2C de DNA nuclear tem sido correlacionado com características adaptativas das espécies (Cros et al. 1995, Noirot et al. 2003b) e com a fertilidade de híbridos interespecíficos (Cros et al. 1995) e tem sido utilizado para identificar (pseudo) variações intra e interespecíficas no tamanho do genoma (Cros et al. 1994, 1995, Noirot et al. 2003b).

Os primeiros estudos citométricos em *Coffea* spp. utilizaram a citometria de fluxo, uma ferramenta que analisa parâmetros ópticos de partículas coradas em suspensão com auxílio do citômetro de fluxo. Para estimar o conteúdo de DNA nuclear por citometria de fluxo, suspensões de núcleos e/ou células permeáveis são coradas com fluorocromos DNA específicos e a quantidade de luz emitida é quantificada (Price et al. 2000). A posição do pico G_0/G_1 no histograma do espécime analisado deve ser comparada com a posição do pico G_0/G_1 da planta padrão, cujo conteúdo de DNA é conhecido (Doležel e Bartoš 2005). Este procedimento permite mensurar o tamanho do genoma nuclear em picogramas (pg) de DNA e/ou em pares de bases (Mbp); sendo que 1 pg de DNA corresponde a 978 Mpb (Doležel et al. 2003).

Na Tabela 3 estão apresentados os resultados encontrados em três estudos citométricos realizados em espécies de *Coffea*.

Tabela 3 – Conteúdo médio de DNA nuclear de espécies do gênero *Coffea* obtido por meio de suspensões nucleares coradas com iodeto de propídeo e analisadas em citômetro de fluxo.

Espécie	Ploidia	Conteúdo médio de DNA nuclear (pg)		
		Cros et al. (1994)	Cros et al. (1995)	Noirot et al. (2003b)
C. arabica	4x	2,470	2,610	___
C. bertrandii	2x	1,650	___	___
C. brevipes	2x	1,520	1,550	1,523
C. canephora	2x	1,460	1,540	1,440
C. congensis	2x	1,530	1,620	1,478
C. costatifructa	2x	___	___	1,150
C. eugenioides	2x	1,360	1,390	1,364
C. farafaganensis	2x	1,340	___	___
C. heterocalyx	2x	___	___	1,737
C. humilis	2x	1,610	1,780	1,764
C. kapakata	2x	___	___	1,323
C. liberica	2x	1,590	1,680	1,406/1,511
C. millotti	2x	1,710	___	___
C. pocsii	2x	___	___	1,083
C. pseudozanguebariae	2x	1,120	1,090	1,131
C. racemosa	2x	0,930	0,950	1,035
C. salvatrix	2x	1,460	___	1,221
C. sessiliflora	2x	1,000	1,040	1,109
C. sp. F. Bridson	2x	1,260	1,330	___
C. sp. Moloundou	2x	1,600	1,690	___
C. stenophylla	2x	1,280	1,350	1,286

Cros et al. (1994, 1995) e Noirot et al. (2003b) utilizaram em seus trabalhos diferentes genótipos de todas as espécies estudadas. Os autores observaram que ocorre variação intra-específica do tamanho do genoma nas espécies de *Coffea*. Esse fenômeno vem sendo justificado pela ocorrência de uma série de compostos provenientes do metabolismo secundário de *Coffea* spp. Essas substâncias são

liberadas durante a preparação das suspensões nucleares afetando a acessibilidade dos fluorocromos, em especial do iodeto de propídeo, à molécula de DNA (Noirot et al. 2000, 2002, 2003a, b, Price et al. 2000, Loureiro et al. 2006b, Alan et al. 2007).

Como os metabólitos secundários influenciam na coloração estequiométrica da molécula de DNA causando erros no mensuramento do tamanho do genoma nuclear (Noirot et al. 2000, 2002, 2003a, b, Price et al. 2000, Loureiro et al. 2006b, Alan et al. 2007), a variação intra e interespecífica do conteúdo de DNA pode ser induzida pelas diferentes concentrações desses compostos nas variedades (Noirot et al. 2000).

No caso do gênero *Coffea*, a cafeína e os ácidos clorogênicos, dois compostos abundantes nas folhas e frutos, podem promover pseudo-variações intra e interespecíficas no tamanho do genoma mensurado pela citometria de fluxo (Noirot et al. 2003a, b), pois o conteúdo desses compostos varia entre as plantas de café.

A cafeína (1,3,7-trimetilxantina) e outras oxi-purinas são conhecidas como compostos que se ligam ao DNA modificando o superenovelamento da molécula, além de serem capazes de formar um complexo com o fluorocromo intercalante (Noirot et al. 2003a). O conteúdo desses compostos nas células depende do estádio de desenvolvimento e do órgão, e também difere nas folhas e sementes entre as espécies diplóides do gênero *Coffea* (Campa et al. 2005a).

Em contraste com a cafeína, a síntese de ácidos clorogênicos (ACG) é fortemente influenciada pelos fatores ambientais (Noirot et al. 2003a, Doležel e Bartös 2005). Os ACGs são metabólitos secundários precursores de polifenóis e compartilham propriedades químicas com taninos (Noirot et al. 2003a, Doležel e Bartös 2005). De acordo com Greilhuber (1998), os taninos atuam na condensação da cromatina, limitando, assim, o acesso do iodeto de propídeo ao DNA. Além disso, os taninos afetam as proteínas da membrana nuclear, impedindo a coloração dos núcleos (Noirot et al. 2003a). Os ACGs podem se ligar à cafeína e a outras purinas alcalóides tais como: a paraxantina, a teofilina, a teobromina ou a teacrina (Campa et al. 2005b). Portanto, a cafeína aumenta a acessibilidade do fluorocromo à molécula de DNA, enquanto os ácidos clorogênicos diminuem (Noirot et al. 2003a).

Os erros citométricos induzidos pela cafeína e ACGs demandam o desenvolvimento de métodos de citometria de fluxo que permitam a determinação acurada do conteúdo de DNA nuclear (Noirot et al. 2003a).

Além da citometria de fluxo, a citometria de imagem também foi utilizada para mensurar o tamanho do genoma nuclear de algumas espécies de *Coffea* (Fontes 2003). A citometria de imagem é a alternativa digital à microespectrofotometria (Doležel e Bartös 2005), pois essa ferramenta associa um microscópio a um sistema de análise de imagens (Vilhar et al. 2001). A citometria de imagem envolve a captura de núcleos ou cromossomos, corados estequiometricamente pela reação de Feulgen, por meio de uma vídeo câmera CCD conectada a um microscópio (Vilhar et al. 2001). As imagens são convertidas em pixels, que estão relacionados a uma cor e uma intensidade específica de cinza. Dessa forma, a imagem é processada automaticamente pelo algoritimo do programa do sistema de análise de imagens, gerando valores de absorbância relacionados com a área. Tais valores são denominados densidade óptica integrada (DOI) (Hardie et al. 2002).

Fontes (2003) foi a primeira autora a utilizar a citometria de imagem para mensurar o tamanho do genoma nuclear de três variedades de *Coffea*. Segundo a autora, *C. canephora* 'Conillon' apresentou 2C = 1,57 pg, *C. arabica* 'Catuaí Vermelho', 2C = 2,62 pg e 'Mundo Novo' 2C = 2,89 pg.

2 – OBJETIVOS GERAIS

Considerando a necessidade de emprego de metodologias que permitam ampliar as informações acerca das características do genoma de *Coffea arabica* e *Coffea canephora*, o presente estudo teve como objetivos gerais:

- Citogenéticos:
    1- Adaptar um protocolo citogenético, em agreagados celulares de *C. arabica* e *C. canephora*, que forneça cromossomos metafásicos e prometafásicos com resolução suficiente para análise.
    2- Montar os cariogramas de *C. arabica* e *C. canephora*.
    3- Ampliar o conhecimento acerca do tipo de poliploidia atribuída ao genoma de *C. arabica*.
- Citométricos:
    4- Mensurar o conteúdo de DNA nuclear de *C. arabica* e *C. canephora* utilizando diferentes plantas como padrões de DNA conhecido, empregando distintos fluorocromos, e testando diferentes protocolos de estabelecimento das suspensões nucleares.
    5- Estimar o conteúdo de DNA de cada cromossomo de *C. arabica* e *C. canephora*.
    6- Utilizar dados qualitativos e quantitativos para incorporar a base do conhecimento da origem e da organização do genoma de *C. arabica*.

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Title: A high quality chromosome preparation from embryogenic cell suspension aggregates culture of *Coffea canephora*

Running title: Chromosome preparation from cells of *Coffea*

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Abstract

The morphological similarity, relatively small size of the chromosomes and low metaphasic indexes obtained in root meristems have hindered karyotypic characterization and the application of banding techniques in *Coffea* spp. We have developed a method based on the use of embryogenic cell suspension aggregates treated with amiprophos-methyl and macerated in enzymatic solution. This method generated cytogenetic preparations in which the chromosomes showed well-defined primary and secondary constrictions, facilitating the pairing of homologues and assembly of the karyogram, as well as the identification of active NOR and heterochromatin associated with the secondary constriction. This alternative technique could help on the analysis of other species with similar karyotypic characterization problems.

Key words: Ag-NOR, Cell aggregates, Chromosome, *Coffea canephora*, Hsc-FA, Tissue culture.

Introduction

Coffea canephora Pierre ex Froehner has 2n = 2x = 22 chromosomes (Krug 1937). Pierozzi *et al.* (1999) reported that *C. canephora* has small chromosomes (less than 3 µm), three of them metacentric (5, 10 and 11) and the remaining submetacentric. The authors observed that chromosomes 1 and 3 had a secondary constriction (SC) and that chromosome 3 seemed to possess a nucleolus organizer region (NOR).

Karyotypic characterization in *C. canephora* has been difficult, due to morphological similarity, relatively small size of the chromosomes and low metaphasic indexes obtained in root meristems (BD, Fontes 2003. Thesis, Univ. of Viçosa, MG, Brazil). In order to obtain high metaphasic chromosome indexes of *Apium graveolens* and *Brassica carinata*, Murata (1983) developed a method replacing the root meristem by cell suspension aggregates as source of material, exploring the advantage of the high multiplication level of cells in liquid culture. The low metaphasic indexes obtained in root meristems of *Coffea* species have also hindered the application of banding techniques (BD, Fontes 2003. Thesis, Univ. of Viçosa, MG, Brazil).

Chromosome banding has been an important tool for the identification of homologue pairs, mainly in karyotypes with morphologically homogeneous chromosomes (Andras *et al.* 2000). In plants, Ag-NOR banding has been the choice technique for cytogenetic comparison of different species (Dagne and Heneen 1992, Panzera *et al.* 1996).

Active NORs are identified by Ag-NOR banding (Panzera *et al.* 1996, Roussel *et al.* 1996, Morais-Cecílio *et al.* 2000, Neves *et al.* 2005) most likely because the NORs engaged in nucleolus formation retain during the metaphase some of the proteins related to rDNA transcription, such as RNA polymerase I subunits, UBF and SL1 transcription factors (Roussel *et al.* 1996, Scheer and Hock 1999). The ability of these proteins to reduce silver enables a differential staining of mitotic NORs (Goodspasture and Bloom 1975, Morais-Cecílio *et al.* 2000, Neves *et al.* 2005) and designates the potential of ribosomal genes to be transcribed during interphase (Roussel *et al.*1996).

NORs have also been identified by techniques such as the detection of NOR-associated heterochromatic segments (NOR H-segments). NOR H-segments were

identified by Sato (1988) using the acridine orange (AO) fluorochrome after C-banding, which corresponded to telomeric bands in chromosomes of *Vicia faba*, *Allium fistulosum*, *Chrysanthemum coronarium*, *Lycoris aurea* and *Nothoscordum fragans*. AO is a fluorescent dye that stains single-stranded DNA red-orange and double-stranded DNA yellowish-green (Sharma and Sharma 1999, Exbrayat 2000). The fluorescent banding of heterochromatin associated to the SC with the use of acridine orange (Hsc-FA banding), applied to the chromosomes of *Zea mays* and *Capsicum annuum* by Almeida and Carvalho (2004), also detected the NOR, by making evident the NOR H-segments.

We are interested in standardizing the protocol for obtaining metaphasic chromosomes from embryogenic cell suspension aggregates of *C. canephora*, as well as to apply the Ag-NOR and Hsc-FA banding techniques for NOR localization on these chromosomes.

Materials and methods

Plant material

The embryogenic cell suspension aggregates cultures of *C. canephora* 'Robusta' were kindly provided by Mr. Marcelo Antoniol Fontes (Laboratory of Coffee Biotechnology, Universidade Federal de Viçosa-UFV). The embryogenic cell suspension aggregates culture were maintained by regular subcultures every 15 days according to the methodology described by Cordeiro, A. T. (1999, Thesis, Univ. of Viçosa, MG, Brazil). The mitotic analysis was carried out at the Laboratório de Citogenética e Citometria, Departamento de Biologia Geral, UFV.

Cytogenetic preparations

The microtubule inhibiting agent APM (amiprophos-methyl) was added to the culture media to a final concentration of 3 µM, for a period of 2, 3, 4, 5, 6, 12 and 24 h. Next, cell aggregates were fixated in methanol:acetic acid (Merck®) solution (3:1), with three fixative changes at 10 min intervals. Cell aggregates were stored at -20^{0}C, from one to several days.

The cell aggregates were washed and digested with the enzymatic solution flaxzyme (Novo Ferment™) and distilled water, in a ratio of 1:30 (enzyme: distilled water), for 30 min at 34 ^{0}C. Next, the aggregates were washed for 10 min in distilled water, fixed again and stored at -20 ^{0}C. The slides were prepared by the technique of cell dissociation of the enzymatic macerated aggregates, and subsequently air-dried on a hot plate at 50 ^{0}C (Carvalho and Saraiva 1993, 1997). Some slides were immediately stained with a 5% Giemsa solution (Merck) in phosphate buffer (pH 6.8) for 5 min, washed twice in distilled water and air-dried.

Ag-NOR Banding

The Ag-NOR-banding technique was applied as described by Goodpasture and Bloom (1975) with minor time and temperature modifications for plants. In short, the 50% $AgNO_3$ solution was dropped on the slides, which were then incubated at 34 ^{0}C for 18 h. The coverslips were removed and the slides were washed in distilled water for 2 min.

Hsc-FA Banding

The Giemsa-stained slides were distained with methanol:acetic acid (3:1) solutions for application of the Hsc-FA technique, adapted from Almeida and Carvalho (2004). Slides aged for 7 days were incubated in phosphate buffer (pH 6.5) at 85 ^{0}C for 20 min and stained with 0.01% (w/v) AO solution for 15 min. The slides were washed in distilled water for 2 min and in phosphate buffer for 3 min. Three drops of buffer were added to the slides and these were covered by coverslips.

Image Analysis

Images of chromosomes were captured with a CoolSNAP-Pro *cf* (Roper Scientific) video camera on an Olympus™ BX-60 fluorescence microscope with a 100x objective lens and a WB filter. The frame was digitized using an Image Pro-Plus® analysis system (Media Cybernetics®). Image analysis was performed on a Power Macintosh G4 computer, using the freely available (http://reg.ssci.liv.ac.uk)

Image SXM software (Barrett 2002). This is a spin-off of the public domain image analysis application NIH Image, which was developed by Rasband (1998).

Results

Cell aggregates treated with 3 μM APM for 3 or 4 h generated adequate cytogenetic preparations with few overlapped chromosomes, showing well-defined primary and SC which facilitated the pairing of homologues and assembly of the karyogram (Fig. 1a, b). Blocking times inferior to 3 h resulted mostly in prophase images and above 4 h in metaphase images with highly compacted chromosomes.

The cytogenetic analysis of *C. canephora* showed 2n = 22 chromosomes and the presence of SC on the distal short arm of chromosome 6 (Fig. 1a, b, c). This constriction was confirmed as an active NOR by Ag-NOR banding (Fig. 1d).

Using Hsc-FA banding, the preparations showed a uniformly red-orange fluorescence for all chromosomes and a strong yellowish-green fluorescence band on the SC of chromosome 6 in metaphase (Fig. 2a) and, with more resolution, in the flanking NOR in prophase (Fig. 2b).

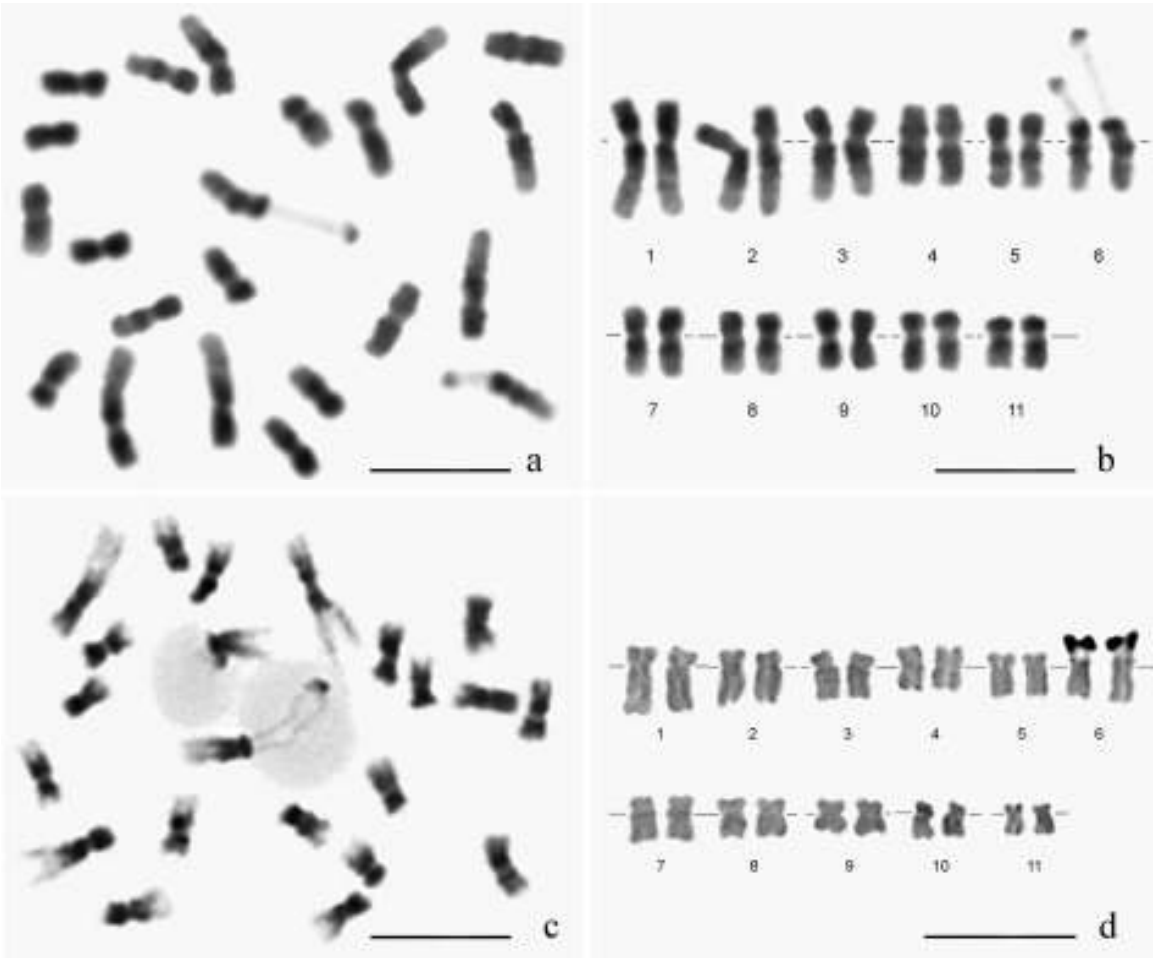

Fig. 1. Chromosomes obtained from cell suspension aggregates culture of *C. canephora* treated with 3 μM APM for 3 h. a) Dispersed metaphasic chromosomes, stained with 5% Giemsa, displayed well-defined constrictions emphasizing heterochromatin pattern distribution and the secondary constriction. b) Karyogram of *C. canephora* (2n = 22) evidencing the distinct morphologies of 11 pairs of chromosomes and the stalk stirred on chromosome 6 secondary constriction. c) Prometaphasic chromosomes stained with Giemsa emphasizing the nucleoli-associated NACs. Note the two chromatids NOR-associated inside of the remaining nucleoli. d) The karyogram assembly by Ag-NOR staining confirmed the active NOR evidenced as on band at the subterminal position of chromosomes 6. Barr = 5 μm.

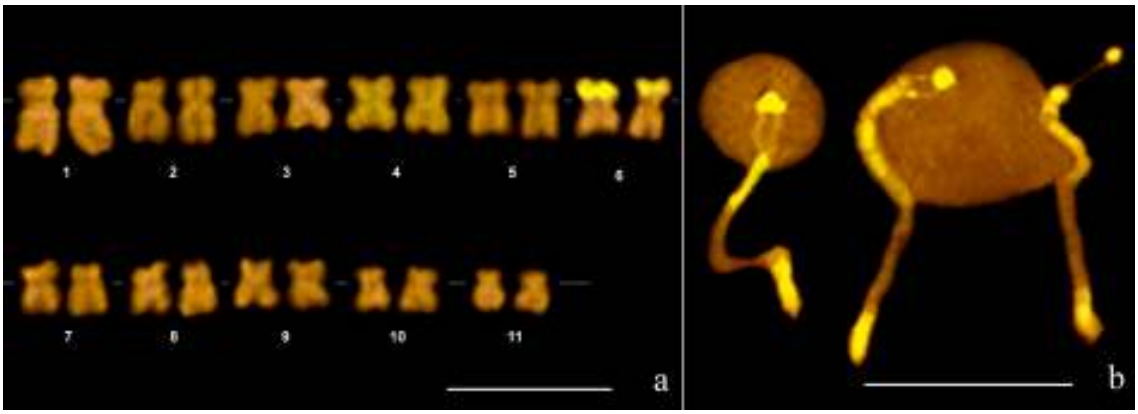

Fig. 2. a) Metaphasic chromosomes obtained from cell suspension aggregates culture of *C. canephora* stained by Hsc-FA, emphasizing one band emitting yellowish-green fluorescence at the subterminal position of chromosome 6. b) Chromosomes 6 associated the nucleoli from distinct cells and stained by Hsc-FA. Note the yellowish-green band flanking the secondary constriction. Barr = 5 µm.

Discussion

The cell suspension aggregates cultures protocol established in the present study allowed the preparation of excellent chromosomes sets for karyogram assembly and banding procedures. Although root meristems of *C. canephora* have been successfully used as source of material in other cytogenetics studies (Pierozzi *et al.* 1999, BD, Fontes 2003. Thesis, Univ. of Viçosa, MG, Brazil), Conagin and Mendes (1961) point out that the low seed germination rate and low metaphasic index represent difficulties for obtaining adequate preparations.

Murata (1983) developed a method that substituted the root meristem by cell suspension aggregates to obtain metaphasic chromosomes of *Apium graveolens* and *Brassica carinata*. The method included: (1) pretreatment to accumulate metaphasic cells, (2) cell wall digestion and protoplast isolation, and (3) use of an air-drying technique. In the present study, the protoplast isolation step was not used, making the process faster, less expensive and suitable for slide preparation.

In order to characterize *C. canephora* chromosomes, some authors used tissue sections for slide preparation (Bouharmont 1959, mentioned by Sybenga 1960), while others used a squashing technique (Louarn 1976, Selvaraj 1987,

Pierozzi *et al.* 1999). In general, these methods are unsuitable to obtain chromosomes with adequate morphology at the same focal plane without debris or overlaps (Carvalho and Saraiva 1993), and the overlying cytoplasm, characteristic of such preparations, obscures the fluorescence to such an extent that banding patterns can not be generated (Andras *et al.* 2000). The cell dissociation of macerated aggregates associated with air-dried slide preparation allows the obtention of individual chromosomes with clear edges showing the primary and secondary constrictions, adequate to characterize homologue pairs and to assemble the karyogram of *C. canephora* (Fig. 1a, b).

Bouharmont (1959), cited by Sybenga (1960), mentioned that *C. canephora* has a satellite on chromosome 4. Louarn (1976) affirmed that this species presents 2 chromosomes (1 and 4) with secondary constrictions. Pierozzi *et al.* (1999) reported the presence of this constriction on chromosomes 1 and 3. In the present study, it was determined that only the chromosome 6 of *C. canephora* has a SC (Fig. 1a, b, c).

Considering that, during mitosis, active NORs are those in which the rDNAs are associated with proteins that associate with silver (Roussel *et al.* 1996, Morais-Cecílio *et al.* 2000, Besendorfer *et al.* 2002, Brasileiro-Vidal *et al.* 2003, Neves *et al.* 2005), the presence of a single active NOR was confirmed at the distal short arm of chromosome 6 (Fig. 1d). This result indicates that chromosome 6 possesses the chromosomal domain around which nucleoli are organized at the end of mitosis, when rDNA transcription is initiated.

Additional results obtained for SC after Hsc-FA staining showed intense fluorescence at the NOR-adjacent heterochromatic region (Fig. 2a, b). This banding pattern showed that the SC in *C. canephora* corresponds to the same pattern described in *Zea mays* and *Capsicum annuum* by Almeida and Carvalho (2004).

Ultrastructural analysis by three-dimensional electron microscopy of the nucleolus-associated chromatin (NAC) of *Zea mays* radicle cells evidenced large heterochromatin masses adjacent to the nucleolar periphery. The NAC was composed of one clear zone, the SC of the NOR, and one electron-opaque zone, the heterochromatic segments (Motte *et al.* 1988). In this study, the NAC of *C. canephora* was distinctly stained by Hsc-FA. The SC, associated to the nucleolus, exhibited red-orange fluorescence, and the heterochromatic segments flanking the SC displayed an intense yellowish-green fluorescence (Fig. 2b).

Early studies on the staining of higher eukaryotic chromosomes indicated many different degrees of heterochromatic condensation, sequence compositions and size of heterochromatic blocks (Bennetzen 2000). Almeida and Carvalho (2004) suggested that β-heterochromatin would be responsible for the positive Hsc-FA band in the SC in maize and pepper chromosomes. The authors mentioned that this type of heterochromatin has been differentiated by the denaturing conditions during Hsc-FA staining.

The main feature that enables the inclusion of NORs as heterochromatic loci is the repetitive nature of the rDNA units. In many species, inactivation of the majority of rDNA genes seems to involve a high level of rDNA chromatin condensation. This concerted action was found to be responsible for establishing the structural characteristics of heterochromatin, such as DNA methylation and H3 histone hypoacetylation and methylation of the lysine 9 residue (Neves *et al.* 2005). Methylation of lysine-9 in histone H3 by Suv39h methyltransferases is a characteristic mark for heterochromatin and provides a binding site for HP1, one of the major heterochromatin proteins (Grewal and Elgin 2002). HP1 binding correlates with global chromatin organization, consistent with a structural role of HP1 in heterochromatin formation (Cheutin *et al.* 2003).

Hsc-FA staining allowed the identification of the NOR, as well as Ag-NOR staining used in this study. Additionally, its accuracy makes it suitable for locating NORs without the need for ribosomal gene FISH mapping. However, it is important to point out that this type of banding is not conclusive about the activity of the rDNA genes (Almeida and Carvalho 2004), for which Ag-NOR banding is essential.

Besides AO, other fluorescent dyes have been used to identify NORs. NOR H-segments have been identified when plant chromosomes undergo banding with the fluorochrome chromomycin A3 (Sato 1988), since heterochromatin is a GC-rich chromosomic portion. Using 4'6-diaminodino-2-phenylindol (DAPI) and propidium iodide (Andras *et al.* 2000), the 2 pairs of NORs in a tetraploid line of *Lycopersicon peruvianum* were identified. In some regions the chromatin was bound to both dyes, yielding a purple color. In the other regions, of which the most obvious were the NORs and telomeres, the chromatin bound PI most exclusively. The chromosome pattern revealed by staining consisted of purple chromosome bodies with distinct red NORs and telomeres.

This study represents an advance on the cytogenetics of coffee, given that it reports an alternative technique which makes possible the obtainance of new cytogenetic data on *C. canephora*, which could help on analyses of other species with similar karyotypic characterization problems.

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Title: First *Coffea arabica* karyogram showing that this species is a true allotetraploid.

Running title: Allotetraploid genome of *C. arabica*.

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Abstract

Evolutive studies have verified that *Coffea arabica* (2n = 44) is a natural segmental allopolyploid originated from a cross between two diploid (2n = 22) *Coffea* species. Data obtained by classical cytogenetic analyses showed that *C. arabica* chromosomes are small and morphologically similar, which hampers the karyogram assembly with well-identified homologue pairs. In the present study, the *C. arabica* complement was reanalyzed using an improved cytogenetic protocol that allowed the obtention of high quality prometaphasic and metaphasic chromosomes. The results showed chromosomes that are cytogenetically distinct (1, 2, 19, 20, 21 and 22) or identical (3 – 4, 5 – 6, 7 – 8, 9 – 10, 11 – 12, 13 – 14, 15 – 16 and 17 – 18), with regard to total length, short and long arm sizes, and chromosome class. Our work suggests that *C. arabica* is a true allotetraploid, non-segmental, originated from different species exhibiting similar and distinct chromosomes.

Key words: *Coffea arabica*, coffee, chromosomes, allotetraploid genome, genome evolution, segmental allopolyploid, amphidiploid.

Introduction

The genus *Coffea* (family Rubiaceae, subfamily Cinchonoideae, tribe Coffeeae) comprises over 100 native species distributed in tropical and subtropical regions (Campa et al. 2005a, b, Pinto-Maglio 2006). Besides its agricultural and economical relevance (Anthony et al. 2001, Aggarwal et al. 2007, Mahé et al. 2007), *Coffea arabica* L. has been the main focus of some evolutive researches. These studies aim to characterize and identify the diploid progenitors (Orozco-Castillo et al. 1996, Raina et al. 1998, Lashermes et al. 1999), as well as to elucidate the genomic organization of this species (Lashermes et al. 1999).

Coffea species originated from a single coffee-ancestor (monophyletic origin), which possessed a basic set of 11 chromosomes (Mahé et al. 2007), about 5-25 million years ago (Lashermes et al. 1996). The origin of each *Coffea* species occurred as a result of genetic mutations or tiny morphologically undetectable chromosomal alterations (Rijo 1974). Hence, the basic chromosome number in *Coffea* spp. remained x = 11 (Krug 1934, Mendes 1938a, b, Charrier and Berthaud 1985), which is the most common number in the genus Rubiaceae (Pinto-Maglio 2006).

Based on molecular (Orozco-Castillo et al. 1996) and cytogenetic (Pinto-Maglio and Cruz 1998, Raina et al. 1998, Pinto-Maglio 2006) approaches, *C. arabica*, the only *Coffea* species with 2n = 44 chromosomes, has been considered a natural segmental allotetraploid that contains two subgenomes, originated from a cross between two diploid species, 2n = 22 (Lashermes et al. 1993, Orozco-Castillo et al. 1996, Lashermes et al. 1997, 1999, Baruah et al. 2003). Some wild diploid *Coffea* species have been considered the potential progenitors, as *C. eugenioides* (Höfling and Oliveira 1981, Lopes et al. 1984, Orozco-Castillo et al. 1996, Raina et al. 1998, Lashermes et al. 1999, Ruas et al. 2003), *C. canephora* (Lashermes et al. 1997, Lashermes et al. 1999, Ruas et al. 2003), *C. congensis* (Höfling and Oliveira 1981, Lashermes et al. 1997, Raina et al. 1998) and *C. brevipes* (Lashermes et al. 1997). Notwithstanding these approaches, Orozco-Castillo et al. (1996) and Lashemers et al. (1999) mentioned that more works are required to elucidate the genomic origin and reorganization of *C. arabica*.

Considering that an accurate karyological characterization is important for evolution studies (Galasso et al. 2001), Clarindo and Carvalho (2007) and Freitas et

al. (2007) performed improved cytogenetic procedures in *Glycine max* (2n = 40) and *Paullinia cupana* (2n = 210) respectively, species that also possess small and morphologically similar chromosomes. From improved methods, these authors assembled accurate karyotypes and verifed the polyploid nature of the genome of these species. In contrast, the cytogenetic protocols applied to *Coffea* spp. have not provided slides with suitable metaphases and prometaphases, thus hindering the characterization, identification and pairing of the chromosomes, and assembly of the karyogram (Mendes 1938a, Bouharmont 1963, Raina et al. 1998, Lashermes et al. 1999, Pinto-Maglio 2006).

Therefore, the procedures adopted for the cytogenetic characterization of *G. max* and *P. cupana* were adapted for *C. arabica*. The aims of this approach were: (1) to conform a cytogenetic protocol that supplies high quality metaphasic chromosomes in *C. arabica*; (2) to identify the homologue pairs; (3) to assemble the first clear karyogram; and (4) to verify if the generated data support the suggested segmental allotetraploid nature of the *C. arabica* genome.

Material and methods

Biological material

Embryogenic cell aggregate suspension cultures of *C. arabica* 'Catuaí Vermelho' were used as source for mitotic chromosomes (Clarindo and Carvalho 2006). The suspensions were established from foliar explants of *C. arabica* according to the tissue culture methodology described by Berthouly and Michaux-Ferriere (1996) and van Boxtel and Berthouly (1996). The mitotic analysis was carried out at the Laboratótio de Citogenética e Citometria, Departamento de Biologia Geral, Universidade Federal de Viçosa – UFV (Brazil).

Cytogenetic preparations

The microtubule inhibiting agents amiprophos-methyl (Nihon Bayer Agrochem K. K.®) or oryzalin (Sigma®) were added to the culture media to a final concentration of 1.0 – 5.0 μM for a period of 3 – 8 h, at 30 ^{0}C (Planchais et al. 2000, Clarindo and

Carvalho 2006). The embryogenic aggregates were subsequently washed with distilled water for 20 min, and then fixed in fresh methanol:acetic acid (Merck®) solution (3:1). The fixative was changed three times and the samples were stored at -20 ^{0}C (Carvalho and Saraiva 1993, 1997). Embryogenic cell aggregates were washed and incubated for 30 min to 2 h, at 34 ºC, in pectinase solution (Sigma®) in the proportions of 1:10 – 1:40 (enzyme:water). The aggregates were washed for 10 min in distilled water, fixed again and stored at –20 °C (Clarindo and Carvalho 2006).

C. arabica slides were prepared with the technique of cell dissociation of enzymatic macerated aggregates, and subsequently air-dried on a hot plate at 50 °C (Carvalho and Saraiva 1993, 1997). Some slides were immediately stained with a 5% Giemsa solution (Merck®) in phosphate buffer (pH 6.8) for 5 min, washed twice in distilled water and air-dried. The other slides were immediately placed in a fixative solution of 4% formaldehyde (Merck®), for 4 h at 25 ºC, and hydrolyzed in 5 M HCl (Merck®) for 18 min at 25 ºC, then stained with Schiff's reagent (Merck®) for 12 h at 4 ºC. The slides were washed three times (for 3 min each time) in 0.5% SO_2 water (Merck®).

Image Analysis

Images of the chromosomes were captured with a CoolSNAP-Pro cf (Roper Scientific®) monochromatic CCD video camera of 12 bits gray, assembled on an Olympus™ BX-60 microscope with a source of stabilized light, PlanApo objective magnification of 100x with 1.4 numeric aperture, aplanat achromat condenser with aperture 1.4, one neutral density filter (ND6) and another of interference green color (IF550). Image analysis of the digitalized frame was performed using the Image Pro-Plus® 6.1 software (Media Cybernetics®). The morphometry of the *C. arabica* chromosomes was measured according to the arm length (in micrometers) criteria described by Levan et al. (1964) and revised by Guerra (1986). Chromosomes of 15 mitotic cells (metaphases or prometaphases) were used in the cytogenetic characterization.

Results and discussion

As in the cytogenetic approach performed in *C. canephora* by Clarindo and Carvalho (2006), embryogenic cell aggregate suspensions were favored, owing to the low seed germination rate and metaphasic indexes obtained from *Coffea* spp. root meristems (Conagin and Mendes 1961). According to Murata (1983) and Clarindo and Carvalho (2006), embryogenic cell aggregate suspensions represent an alternative source of mitotic cells, because of their high proliferation rate in adequate tissue culture conditions.

An adequate protocol for mitotic arrest is a key step for supplying adequate plant metaphasic chromosomes for cytogenetic analyses (Singh 1993, Sharma and Sharma 1999, Planchais et al. 2000); therefore, we strictly tested and monitored the effective time and concentration of microtubule inhibition agents. As a result, high-quality cytogenetic preparations of *C. arabica* were generated. Slides prepared with cell aggregates treated with 3 μM amiprophos-methyl for 3 – 4 h presented the best metaphasic indexes (10%) and mitotic chromosomes at prometaphase and metaphase with subtle range of chromatin condensation (Fig. 1). Clarindo and Carvalho (2006) considered this treatment equally suitable for accumulation of excellent *C. canephora* chromosomes.

Dissociation of the aggregates treated for 30 min with 1:30 pectinase solution, associated with the air-drying procedure (Carvalho and Saraiva 1993, 1997), provided suitable *C. arabica* slides. These preparations showed well-spread chromosomes without cytoplasm background, chromatin damages or overlaps. These chromosomic aspects were imperative for observation of primary constrictions, pairing of homologues and assembly of the first *C. arabica* mitotic karyograms (Fig. 1). Through application of the air-drying technique, karyotypic characterization was also performed in other species with homomorphic small chromosomes, such as *Glycine max* (Clarindo et al. 2007) and *Paullinia cupana* (Freitas et al. 2007).

The CCD video camera was a fundamental tool to capture good images of metaphases and prometaphases. The programs of the digital image analysis system contributed to discriminate between subtle measurements of *C. arabica* chromosomes, and were used for carrying out detailed analyses of each pair of

homologous, characterized by similar chromosomic morphology, and posterior assembly of a karyogram (Fig. 1).

In this study, mitotic cells with chromosomes exhibiting distinct chromatin condensation levels were obtained, this aspect being very important for screening differences between chromosomes of the same class and with seemingly similar lengths. The *C. arabica* karyograms displayed 2n = 44 chromosomes, of which 5 chromosomes are metacentric (7, 8, 13, 14, 20), 16 submetacentric (1 – 6, 9 – 12, 15 – 19, 21) and 1 acrocentric (22) (Fig. 1). The 44 chromosomes of *C. arabica* present lengths varying from 5.30 µM to 2.06 µM in the larger prometaphasic chromosomes (Fig. 1 above). Analyzing metaphasic cells, Raina et al. (1998) reported that *C. arabica* exhibits metacentric and submetacentric chromosomes.

Bouharmont (1963), Raina et al. (1998) and Lashermes et al. (1999) mentioned that the chromosomic discrimination in *C. arabica* is laborious and very difficult because of their very small size and rather uniform morphology. In spite of these cytogenetic adversities, our results demonstrated that suitable prometaphasic and metaphasic chromosomes can be obtained from *C. arabica* cell aggregates by adapting and monitoring the cytogenetic procedures. Furthermore, by screening minor morphological differences, it is also possible to distinguish between individual chromosomes in the *C. arabica* genome at mitosis.

The morphometric analyses also evidenced the allotetraploid nature of the *C. arabica* genome, since cytogenetically distinct chromosomes (1, 2, 19, 20, 21 and 22) could be observed with regard to total length, short and long arm sizes or chromosome class. In contrast, the other chromosomes (3 – 4, 5 – 6, 7 – 8, 9 – 10, 11 – 12, 13 – 14, 15 – 16 and 17 – 18) showed identical cytogenetic characteristics, indicating that *C. arabica* originated from progenitors with similar mitotic chromosomes.

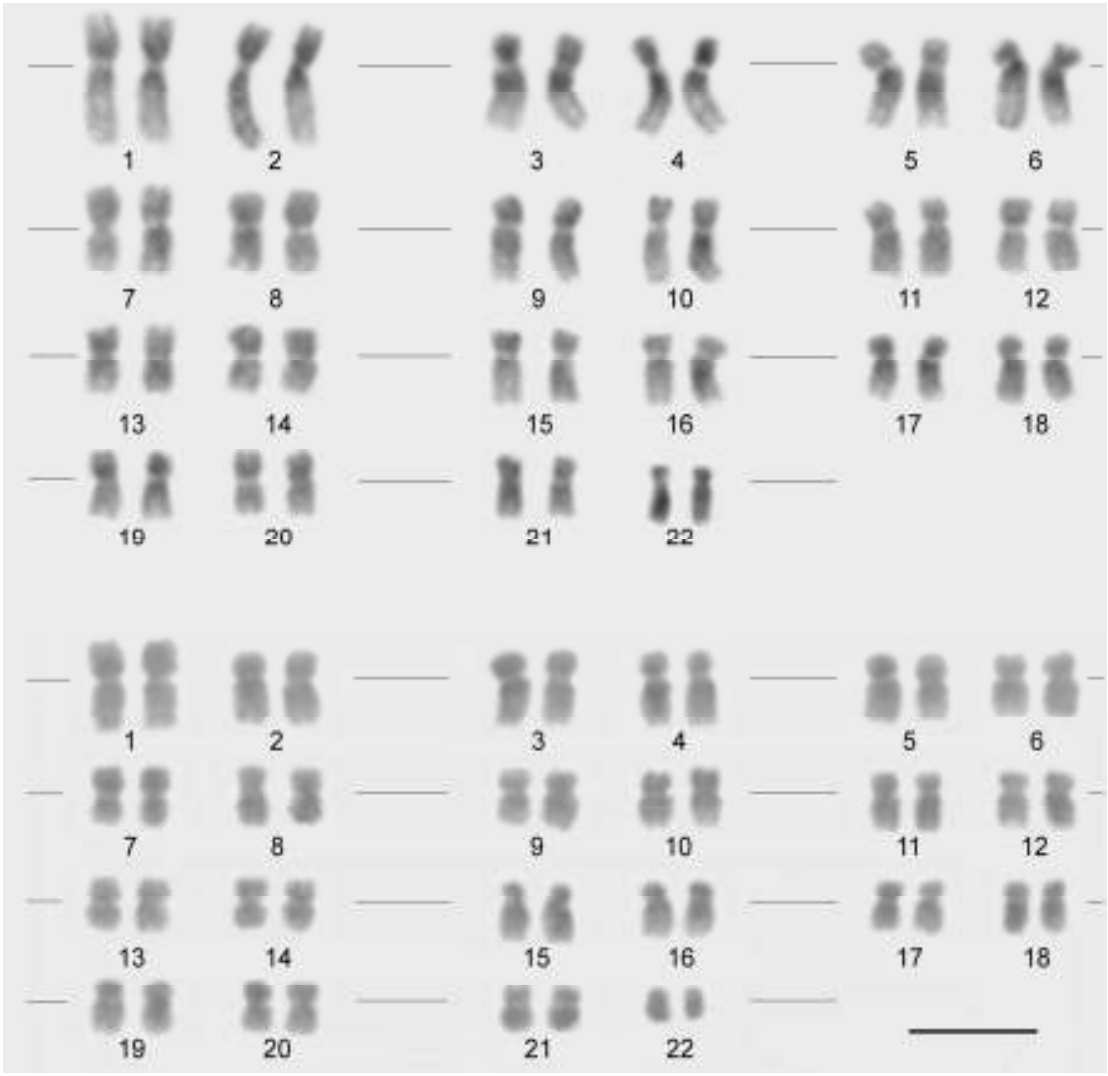

Fig. 1 – *C. arabica* karyograms assembled from chromosomes, stained with 5% Giemsa (above) or Schiff's reagent (below), obtained from embryogenic cell aggregates treated with 3 μM amiprophos-methyl for 3 h (above) and 4 h (below), and showing well-defined centromeric constrictions. The *C. arabica* karyograms displayed 2n = 44, consisting of 5 metacentric (7, 8, 13, 14, 20), 16 submetacentric (1 – 6, 9 – 12, 15 – 19, 21) and 1 acrocentric (22) chromosomes. The morphometric analyses evidenced distinct (1, 2, 19, 20, 21 and 22) and identical (3 – 4, 5 – 6, 7 – 8, 9 – 10, 11 – 12, 13 – 14, 15 – 16 and 17 – 18) chromosomes, with regard to total length, short and long arm sizes and chromosome class. (above) Prometaphasic chromosomes displaying lower chromatin condensation level than in Fig. 1 below, with lengths varying from 5.30 to 2.06 μm. (below) Metaphasic chromosomes exhibiting lengths varying from 3.53 to 1.26 μm. Barr = 5 μm.

Based on classical or molecular cytogenetic analyses, several researchers reported that *C. arabica* is a segmental allotetraploid plant (Orozco-Castillo et al. 1996, Pinto-Maglio and Cruz 1998, Raina et al. 1998, Baruah et al. 2003) generated from a cross between two wild diploid species of *Coffea* with similar genomes (Orozco-Castillo et al. 1996, Raina et al. 1998, Lashermes et al. 1999). The segmental allopolyploid term was initially coined by Stebbins (1947). This author reported that segmental allopolyploids contain two partially differentiated genomes, characterized by a considerable number of homologous chromosomal segments, or even whole chromosomes, as observed in the *C. arabica* karyogram (identical chromosomes 3 – 4, 5 – 6, 7 – 8, 9 – 10, 11 – 12, 13 – 14, 15 – 16 and 17 – 18). Sybenga (1992) complemented Stebbins's previous considerations by affirming that a segmental allopolyploid is a type of intermediate between allopolyploids and autopolyploids, inasmuch as the differentiation of the progenitor genomes is insufficient for effective allopolyploidy.

C. arabica was also considered an amphidiploid plant by Lashermes et al. (1999). Based in molecular cytogenetic studies, they concluded that this species arose from hybridization between *C. eugenioides* and *C. canephora*, or related ecotypes, followed by polyploidization. Moreover, these authors mentioned that the emergence of *C. arabica* involved an unreduced gamete. In spite of distinct denominations, Stebbins (1949) reported that amphidiploids may be segmental allopolyploids originated after hybridization between two or more diploid species separated by barriers of hybrid sterility.

Stebbins (1949) and Sybenga (1992) questioned the idea that segmental allopolyploids persist in nature. These authors reported that segmental allopolyploidy is an unstable condition, because the occurrence of chromosomal alteration and recombination between homoeologous chromosomes would eventually turn them into true auto- or allopolyploids. Stebbins (1949) complemented that allopolyploids originated from segmental allopolyploids rarely or never form multivalents in meiosis. In this study, the occurrence of distinct chromosomes (1, 2, 19, 20, 21 and 22) suggests that *C. arabica* is a true allotetraploid. Besides, *C. arabica* exhibits a diploid-like meiotic behavior (Orozco-Castillo et al. 1996, Lashermes et al. 2000) probably owing to the expression of pair-controlling genes, such as *Ph*1 (*Pairing homoeologues*) in wheat, which inhibit the pairing between these chromosomes (Sybenga 1992, Lashermes et al. 2000).

The very small size of homomorphic chromosomes has often been considered a great obstacle for accurate cytogenetical characterization of *C. arabica*. However the cytogenetic methodologies here described, routinely used in our laboratory, allowed the assembly of the first karyograms of this species, showing well-defined chromosomes. Our results demonstrate that, despite the challenging working material, the characterization of *C. arabica* chromosomes was feasible, and contributed for resolving that *C. arabica* is not a segmental type, but a true allotetraploid species.

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Title: Revisiting the nuclear genome size and base composition in *C. arabica* and *C. canephora*.

Running title: Coffee DNA content and base composition.

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Abstract

The previous flow cytometry (FCM) analyses in *C. arabica* and *C. canephora* displayed distinct mean 2C nuclear DNA content values. The occurrence of these differences have been attributed to the stoichiometric errors in the DNA staining promoted by interference of secondary metabolites, caffeine and chlorogenic acids, in the PI accessibility to the DNA. This current study was conducted in order to obtain adequate *C. arabica* and *C. canephora* nuclei suspensions, using OTTO buffers, and to revisited the nuclear genome size, testing different standards, and the base composition of these species. The statistical analyses performed by *F* test evidenced that the nuclear DNA content values measured for *C. arabica* and *C. canephora* in distinct days and replications were statistically identical. Comparing the mean *C. arabica* and *C. canephora* genome size values by Duncan's test was evidenced that, in comparison with *Pisum sativum* and *Raphanus sativus*, *Solanum lycopersicum* was the most adequate standard for coffee genome size estimation. The mean nuclear DNA content, using *S. lycopersicum* as internal standard, of *C. canephora* was 2C = 1.41 ± 0.004 pg (1.38 X10^9 bp) and of *C. arabica* 2C = 2.62 pg (2.56 x 10^9 bp). *C. canephora* showed 65.27% AT and 34.73% GC, while *C. arabica* exhibited 63.04% AT and 36.96% GC. Our results demonstrated that each standard plant is affected differently by secondary metabolites of coffee and, therefore, it is important to test distinct plants as internal and external standard and to compare the results obtained from these standard types.

Key words: *C. arabica*, *C. canephora*, genome size, base composition, flow cytometry.

Introduction

Coffee is an important commodity and plantation crop of the Rubiaceae family. Although the *Coffea* genus shows more than 100 species, only *Coffea arabica* L. (2n = 44 chromosomes) and *Coffea canephora* Pierre ex Froehner (2n = 22 chromosomes) are commercially cultivated (Lashermes et al. 1993, Baruah et al. 2003, Aggarwal et al. 2007, Mahé et al. 2007) contributing with about 70 and 30% of coffee production worldwide, respectively (International Coffee Organization 2007, http://www.ico.org).

Nuclear DNA content has been considered a relevant parameter for systematic, evolutive (Hardie et al. 2002, Noirot et al. 2003b, Lee et al. 2004) and ecology studies (Cros et al. 1994, 1995), plant breeding programs (Cros et al. 1995, Lee et al. 2002) and genome sequencing projects (Hardie et al. 2002, Doležel and Bartoš 2005). Regarding to coffee, genome size of *C. arabica* (Cros et al. 1994, 1995) and *C. canephora* (Cros et al. 1994, 1995, Noirot et al. 2003b) have been measured, in picograms (pg), mainly by flow cytometry (FCM). This tool quickly and reliably quantifies the fluorescence emitted by individual microscopic particles (cells, nuclei or chromosomes) in suspension, stained with DNA-specific fluorochromes that flow, in single file, within of the equipment (Doležel 1997, Yanpaisan et al. 1999, Doležel and Bartoš 2005).

The previous FCM analyses in *C. arabica* and *C. canephora* displayed distinct 2C nuclear DNA content mean values. However those works differed with regard to the standards, buffers and/or plant cultivar used. The first FCM evaluation of the genome size of 5 *C. arabica* and 11 *C. canephora* genotypes was performed by Cros et al. (1994). These authors observed nuclear DNA content values varying between 2.30 to 2.72 pg for *C. arabica* and 1.18 to 1.61 pg for *C. canephora*. Subsequently, Cros et al. (1995) measured the 2C DNA content of 6 *C. arabica* and 11 *C. canephora* genotypes. The values obtained were 2.38 to 2.84 pg for *C. arabica* and 1.32 to 1.76 pg for *C. canephora*. Working only with coffee diploid species, Noirot et al. (2003b) reported that the mean nuclear DNA content of 4 *C. canephora* populations oscillated from 1.33 to 1.52 pg.

In all *Coffea* FCM investigations, intraspecific genome size variations were outlined (Cros et al. 1994, 1995, Noirot et al. 2003b). Greilhuber (1998, 2005), Price et al. (2000) and Noirot et al. (2000, 2002, 2003a, 2005) mentioned that some

nuclear DNA content differences occur owing to interference of cytosolic compounds in the accessibility of the dye to the DNA. Noirot et al. (2003a) highlighted that caffeine and chlorogenic acids (CGAs), two abundant compounds of the leaves and liberated during chopping procedure (Walker et al. 2006), are involved with stoichiometric errors (pseudo-variations) in *Coffea* genome size estimates. The amount of these secondary metabolites oscillates intra- and interspecifically (Noirot et al. 2000, Ky et al. 2001, Campa et al. 2005a, b). Therefore, the *Coffea* nuclear DNA content should vary between and within species and over time (Noirot et al. 2003a).

Due to the occurrence of pseudo-variations and, consequently, inaccurate genome size estimations caused by caffeine and CGAs, procedures for minimize the stoichiometric errors have been suggested by some authors, as: (1) to test distinct standard plants (Doležel e Bartös 2005, Noirot et al. 2005); (2) to compare the results obtained with internal and external standards (Noirot et al. 2005); (3) to centrifuge the nuclei suspensions and to discard the supernatant (Cros et al. 1994, 1995, Noirot et al. 2000, Doležel e Bartös 2005); (4) to add antioxidant compounds to the FCM buffers (Price et al. 2000, Doležel e Bartös 2005); (5) to maintain target and standard plants in the same environmental conditions in greenhouse (Noirot et al. 2002, 2003a, b, 2005); and (6) to realize replicate measurements (Doležel e Bartös 2005) in distinct periods (Bennett et al. 2007).

In accordance with the suggestion of these authors, this approach was conducted in order to obtain adequate *C. arabica* and *C. canephora* nuclei suspensions using OTTO buffers, quantify the genome size of *C. arabica* and *C. canephora* using distinct standard plants, compare the results obtained with internal and external standards, and determine the base composition of these species.

Materials and methods

Plant material

C. arabica ‘Catuaí Vermelho’ (target), *C. canephora* ‘Apoatã’ (target), *Pisum sativum* L. 'Ctirad' (standard, 2C = 9.09 pg, Doležel et al. 1998), *Raphanus sativus* ‘Saxa’ (standard, 2C = 1.11 pg, Doležel et al. 1992) and *Solanum lycopersicum* L. ‘Stupické’ (standard, 2C = 1.96 pg, Doležel et al. 1992) were cultivated in glasshouse

under the same environmental conditions. Seeds of the three standards were kindly supplied by Dr. Jaroslav Doležel (Experimental Institute of Botany, Czech Republic). The cytometric analyses were carried out at the Laboratório de Citogenética e Citometria, Departamento de Biologia Geral, Universidade Federal de Viçosa (UFV).

Experimental design

In order to minimize the stoichiometric staining errors and determine a suitable internal standard for *C. arabica* and *C. canephora* genome size estimation, the FCM measurements were carried out with *P. sativum*, *R. sativus* and *S. lycopersicum*, which were used as internal and external standards. Besides these species, *C. canephora* and *C. arabica* also were used as internal and external standards for genome size estimation of the other coffee species.

Nuclei isolation

Fragments (2 cm^2) of young leaves of the target and standard plants were simultaneously and separately chopped (Galbraith et al. 1983), for 30 s (Noirot et al. 2005), with a razor blade in a 60 x 15 mm Petri dish with 0.5 mL of OTTO-I (Otto 1990, Doležel and Göhde 1995) lysis buffer supplemented with 2.0 mM dithiothreitol (Sigma®) and 50 µg mL^{-1} RNAse (Sigma®). Subsequently, 0.5 mL of the same buffer was added and the homogenate was filtered in a 30 µm nylon filter (Partec®) into a 2.0 microcentrifuge tube (Eppendorf®), and then centrifuged (ALC® microCentrifugette®4214) at 100 g for 5 min. The supernatant was poured out and the pellet was resuspended and incubated for 5 – 20 min in a 100 µL OTTO-I lysis buffer. The nuclei suspension was stained with 1.5 mL OTTO-I:OTTO-II solution (Otto 1990, Doležel and Göhde 1995) in the 1:2 proportion (Loureiro et al. 2006a, b) and, finally, filtered through a 20 µm nylon mesh (Partec®).

Genome size determination

The nuclei suspension staining was performed in the dark during 10 – 40 min. The OTTO-I:OTTO-II solution was supplemented with 75 µM propidium iodide (PI, Sigma®, excitation/emission wavelengths: 480-575/550-740 nm, Shapiro 2003), 2.0

mM dithiothreitol (Sigma®) and 50 µg mL^{-1} RNAse (Doležel et al. 1992, Meister 2005). The genome size of the target was measured using peaks corresponding to the average of the G_0/G_1 relative 2C nuclei DNA content of the target and standard plants. The more appropriate genome size mean values (pg) was converted to base pairs (bp) by considering that 1 pg of DNA corresponds to 0.978×10^9 bp (Doležel et al. 2003).

Base composition

The average levels of AT% and GC% of the *C. arabica* and *C. canephora* genome were calculated separately according to Schwencke et al. (1998). We used 4',6'-diamidino-2-phenylindole (DAPI, Sigma®, excitation/emission wavelengths: 320-385/400-580 nm, Shapiro 2003) and chromomycin A_3 (CMA_3, Sigma®, excitation/emission wavelengths: 365-460/475-680 nm, Jensen 1977 and Shapiro 2003) to specifically stain AT and GC rich portions, respectively. The DAPI staining was performed from nuclei incubating with 1.5 µM DAPI (Doležel et al. 1992) and 2.0 mM dithiothreitol (Sigma®) in OTTO-I:OTTO-II (1:2) buffer (Otto 1990, Doležel and Göhde 1995, Loureiro et al. 2006a, b) during 20 min. The CMA_3 staining was carried out in OTTO-I:OTTO-II (1:2) buffer (Otto 1990, Doležel and Göhde 1995, Loureiro et al. 2006a, b) supplemented with 2.0 mM dithiothreitol (Sigma®), 50 mM $MgCl_2$ (Merck®) and 42.5 µM CMA_3, during 1 h (Jensen 1977, Shapiro 2003). Both staining procedures were performed in the dark.

C. arabica and *C. canephora* base composition were measured using the most adequate internal standard evidenced by total genome measurement. The target and standard G_0/G_1 fluorescence peaks with DAPI, CMA_3 and PI were compared and the base composition was determined applying the following equations (Godelle et al. 1993):

$$AT_{sample} = AT_{reference} \times (R_{DAPI}/R_{PI})^{1/r}$$

and

$$GC_{sample} = GC_{reference} \times (R_{CMA3}/R_{PI})^{1/r}$$

where *R* is the ratio of fluorescent intensity of target to that of standard, *r* (binding length) = 3 or 4 for DAPI and CMA_3 (Meister and Barow 2007). Finally, the relative levels of AT and GC in the genome was obtained by calculating (Meister and Barow 2007):

$$AT\ (\%) = 100 \times [AT_{sample}/(GC_{sample} + AT_{sample})]$$

and

$$GC\ (\%) = 100 \times [GC_{sample}/(GC_{sample} + AT_{sample})]$$

Equipment handling

The suspensions were analyzed in a Partec PAS® flow cytometer (Partec® Gmbh, Munster, Germany), equipped with a Laser source (488 nm) and an UV lamp (388 nm). PI fluorescence emitted by nuclei was collected through a RG 610 nm band-pass filter while DAPI and CMA_3 fluorescence were collected through a GG 435 nm to 500 nm band-pass filter. The equipment was calibrated and aligned using microbeads and standard solutions according to the manufacturer's recommendations (Partec®). FlowMax® software (Partec®) was used for data analyses.

Three independent repetitions were performed in three distinct days, being that more than 10,000 nuclei were analyzed in each. The coffee mean genome size values were statistically analyzed by the *F*-test and Duncan's method. This analysis was performed on statistical Genes Program (Cruz 1997).

Results and discussion

For the first time, the coffee genome size was calculated using distinct standards. Doležel and Bartös (2005) and Noirot et al. (2005) recommended testing different standard plants and comparing the results provided with internal and external standards. Seeing that each plant nuclei is affected differently by secondary metabolites (Loureiro et al. 2006a, b, Bennett et al. 2007), this procedure allowed the stoichiometric error detection in the DNA content measurement and selection of the most adequate standard.

The Duncan's test ($P < 0.05$), applied for the independent *C. arabica* and *C. canephora* measurements, evidenced that the mean 2C DNA content values obtained from *S. lycopersicum* internal and external standards were identical. On the order hand, the *C. arabica* and *C. canephora* nuclear genome size values quantified with *P. sativum* or *R. sativus* external standard were lower than the values obtained with internal standard (Fig 1, 2, Table 1). The mean PI fluorescence of the *P. sativum*

or *R. sativus* G_0/G_1 nuclei, of the homogenate containing co-chopped *C. arabica* or *C. canephora* and standard, was different with regard to mean PI fluorescence of the standard nuclei that was processed and analyzed separately (Fig. 1, 2). This fact indicates that the secondary metabolites (Price et al. 2000), caffeine and chlorogenic acids (Noirot et al. 2003a, b), interfered in the DNA stoichiometric staining of *P. sativum* and *R. sativus* and provided variations in the coffee genome size estimation.

Besides of *S. lycopersicum*, *P. sativum* and *R. sativus*, either *C. arabica* or *C. canephora* was also used as an internal and external standard to measure the 2C DNA content of the other species. Doležel and Bartoš (2005) mentioned that the FCM estimative is more accurate when the chromatin structure of the standard and the sampled nuclei are similar, and reacts in a common way to compounds that interfere in the fluorochrome accessibility to the DNA. The Duncan's test showed that the mean *C. arabica* or *C. canephora* 2C DNA content values, measured using coffee internal and external standards, do not differed. In addition, the same statistical test evidenced that the mean *C. arabica* or *C. canephora* nuclear DNA content, quantified from *S. lycopersicum* and coffee-trees internal and external standard, was statistically identical (Table 1).

Based on statistical comparison of the *C. arabica* and *C. canephora* genome size estimations, obtained from distinct plant and type standards, *S. lycopersicum* proved to be the most appropriate standard-plant for coffee FCM measurements. Due to the occurrence of errors provide by flow cytometer instability and by variation in sample preparation and staining, the internal standard is commonly regarded to be more adequate for genome size estimation (Doležel 1991, Greilhuber 1998, Noirot et al. 2000, 2003a, 2005, Price et al. 2000, Doležel e Bartös 2005, Bennett et al. 2007). Particularly in species that possess great amount of secondary metabolites, as coffee tree, FCM quantifications applying the internal standard minimize the negative effect of cytosolic compounds in the nuclei staining (Noirot et al. 2000, 2003a, 2005, Price et al. 2000). Therefore the sample and standard nuclei are influenced to the same extent (Bennett et al. 2007).

In spite of the advantage of the internal standard, Noirot et al. (2000) and Bennett et al. (2007) observed in their approaches that the use of an internal calibration standard does not completely avoid inaccurate genome size estimations. Therefore, other procedures were adopted in this work in order to reduce the interference of the coffee secondary metabolites in the nuclei stoichiometric staining.

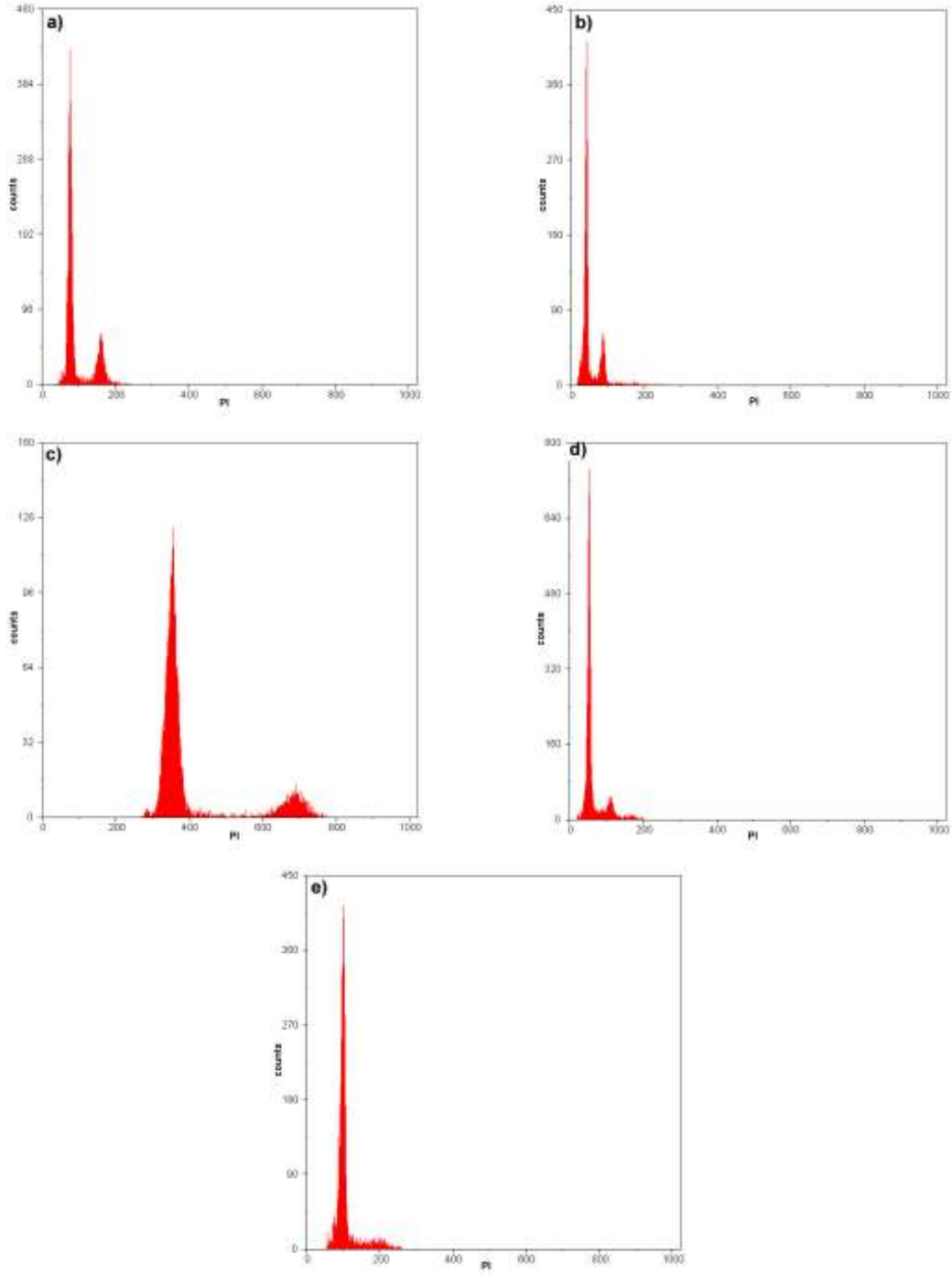

Fig 1 – FCM histograms showing G_0/G_1 peaks obtained from individual processing and analysis of each nuclear suspension stained with PI. a) *S. lycopersicum* G_0/G_1 peak (external standard, 2C = 1.96 pg, channel 75). b) *R. sativus* G_0/G_1 peak (external standard, 2C = 1.11 pg, channel 42). c) *P. sativum* G_0/G_1 peak (external standard, 2C = 9.09 pg, channel 355). d) *C. canephora* G_0/G_1 peak (2C = 1.37 to 1.45 pg, channel 54). e) *C. arabica* G_0/G_1 peak (2C = 2.56 to 2.67 pg, channel 100).

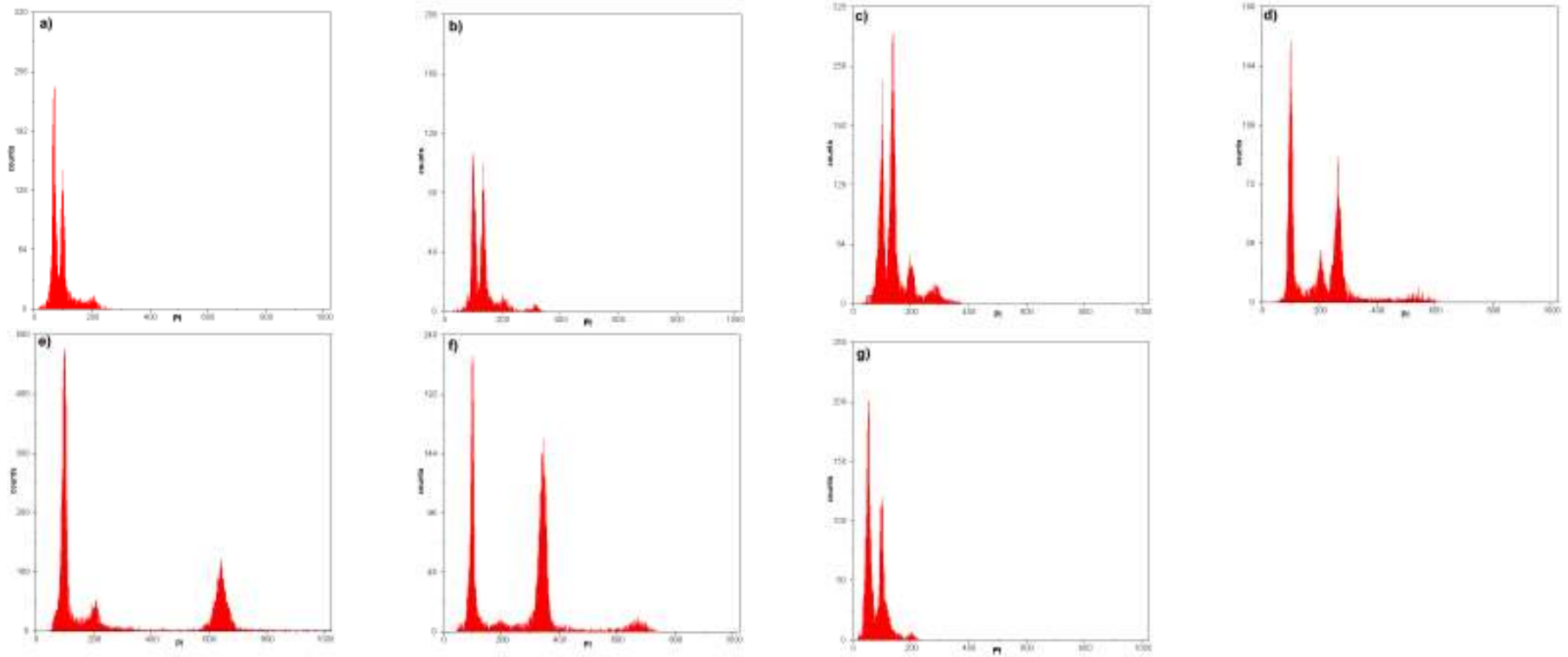

Fig 2 – FCM histograms showing G_0/G_1 peaks obtained from simultaneous processing and analysis of nuclear suspensions stained with PI. a) *S. lycopersicum* (standard, 2C = 1.96 pg, channel 100) and *C. canephora* (2C = 1.41 pg, channel 72). b) *S. lycopersicum* (standard, channel 100) and *C. arabica* (2C = 2.62 pg, channel 134). c) *R. sativus* (standard, 2C = 1.11 pg, channel 100) and *C. canephora* (2C = 1.57 pg, channel 141). d) *R. sativus* (standard, channel 100) and *C. arabica* (2C = 2.92 pg, channel 263). e) *P. sativum* (standard, 2C = 9.09 pg, channel 637) and *C. canephora* (2C = 1.43 pg, channel 100). f) *P. sativum* (standard, channel 345) and *C. arabica* (2C = 2.63 pg, channel 100). g) *C. canephora* (2C = 1.41 pg, channel 54) and *C. arabica* (2C = 2.62 pg, channel 100).

Table 1 – Average nuclear genome size (pg) of *C. canephora* and *C. arabica* measured from distinct standard (Fig 1, 2).

C. canephora			*C. arabica*		
Species	Internal standard	External standard	Species	Internal standard	External standard
S. lycopersicum	1.408$^{A, c}$ (1.400 – 1.410)	1.410$^{A, b}$ (1.400 – 1.410)	*S. lycopersicum*	2.620$^{A, c}$ (2.620)	2.620$^{A, b}$ (2.620)
R. sativus	1.571$^{A, a}$ (1.570 – 1.580)	1.450$^{B, a}$ (1.440 – 1.460)	*R. sativus*	2.916$^{A, a}$ (2.910 – 2.930)	2.666$^{B, a}$ (2.660 – 2.670)
P. sativus	1.426$^{A, b}$ (1.420 – 1.440)	1.372$^{B, c}$ (1.370 – 1.380)	*P. sativus*	2.638$^{A, b}$ (2.630 – 2.640)	2.559$^{B, c}$ (2.540 – 2.560)
C. arabica	1.407$^{A, c}$ (1.390 – 1.410)	1.406$^{A, b}$ (1.390 – 1.410)	*C. canephora*	2.620$^{A, c}$ (2.620)	2.620$^{A, b}$ (2.620)

Mean DNA content values (above) and between-nine range, obtained from three independent replications realized in three days, in parenthesis (below).

The average genome size values obtained from analyses using the internal or external standard were statistically compared for each standard species (horizontal line). The mean 2C DNA contents followed by the same letter (A or B) are not significantly different according to the Duncan's test ($P < 0.05$).

The same statistical test was used for comparison of the mean nuclear DNA content values obtained separately in each type standard (internal and external, vertical line). The mean 2C DNA content followed by the same letter (a, b or c) are not significantly different.

For the nuclei extraction, we used healthy, vigorous target and standard leaves of plants grown in the same controlled environments, as suggested by Noirot et al. (2000, 2003a, b, 2005) and Bennett et al. (2007). Pinto et al. (2004) mentioned that, in general, the amount, the type and complexity of the secondary compounds change along the age of the plant tissue.

The nuclei suspensions were submitted to the distinct times in the OTTO buffers. This procedure supplied histograms with G_0/G_1 peaks exhibiting distinct resolutions and CVs. The most adequate G_0/G_1 peaks presented CVs ranging between 2.75 to 5.86%, being that only the *P. sativum* G_0/G_1 peaks showed CVs larger than 5%. These CV values were obtained from nuclei suspension maintained in OTTO-I buffer-extraction for 10 min and OTTO-I:OTTO-II buffer-staining for 30 min.

In agreement with Doležel and Bartoš (2005), CVs lower that 5% are considered suitable for FCM measurements. Due to the occurrence of cytosolic compounds that interfere in the fluorescent staining of the nuclear DNA, Pinto et al. (2004) reported that for the majority woody species, considered recalcitrant for DNA FCM (Noirot et al. 2000, 2005, Loureiro et al. 2006b, 2007), is very difficult, and in some cases impossible, to obtain CV values at the level suggested as suitable by Doležel and Bartoš (2005). CV superiors to the 5% in species rich in secondary metabolites, as coffee tree, were observed in FCM estimations of *Eucalyptus* spp. – 6.3 to 12.8% (Grattapaglia and Bradshaw 1994), *Eucalyptus globulus* – 4.6 to 6.8% (Pinto et al. 2004), *Quercus* spp. – 3.3 to 6.9% (Zoldos et al. 1998) and *Scutellaria baicalensis* – 3.48 to 6.77% (Alan et al. 2007).

The G_0/G_1 peaks of coffee and *S. lycopersicum* (most adequate internal standard) nuclei suspension exhibited CVs oscillating between 2.75 to 4.80%. This result suggests that the FCM procedures supply peaks with resolution appropriate for analyses, now that the CV is a fundamental statistical parameter, which indicates the precision of the individual FCM measurements (Doležel and Bartoš 2005, Castro et al. 2007).

Based on CVs obtained in this approach, we concluded that the FCM protocol facilitates the release of enough quantity of intact and isolated nuclei of coffee and standards. Differently of the other works that also aim the coffee DNA content quantification (Cros et al. 1994, 1995, Noirot et al. 2003b), this

approach used the Otto's buffers (Otto 1990, Doležel and Göhde 1995) for preparation of the nuclei suspension. Doležel and Bartoš (2005) mentioned that these buffers generate histograms with unsurpassed resolution. This aspect was observed by Loureiro et al. (2006a), especially in species with small genome size, and has also been verified in our laboratory routine. Loureiro et al. (2006b) demonstrated that nuclear suspensions made in LB01 (Doležel et al. 1992) and Galbraith's (Galbraith et al. 1983) buffers were more susceptible to tannic acid, a secondary metabolite that possess similar properties with regard to CGAs (Noirot et al. 2003a), than in Otto's (Otto 1990, Doležel and Göhde 1995) and Tris.$MgCl_2$ (Pfosser et al. 1995) buffers.

The OTTO-I contains Tween 20, a non-ionic detergent that facilitates the release, in association with chopping procedure (Galbraith et al. 1983), of nuclei without cytoplasmic debris and avoids the nuclei aggregation with cellular fragments (Loureiro et al. 2007). The citric acid presents in OTTO-I fixes the nuclei, improving the chromatin accessibility to the fluorochrome, due to maintaining of this structure (Doležel and Bartoš 2005, Loureiro et al. 2006a). As a result of the citric acid property, the most nuclei in suspension are stoichiometricly stained resulting histograms with high resolution and low CVs (Doležel and Bartoš 2005), as obtained in this study.

Other important factor that contributed for obtention of histograms with CVs oscillating 2.75 to 5.86% was the incorporation of an antioxidant in the buffers. In order to minimize the interference of secondary metabolities, caffeine and CGAs, in the accessibility of the PI to the DNA, the dithiothreitol was added in the Otto's buffers. As well as the β-mercaptoethanol, polyvinylpyrrodine, ascorbic acid, potassium metabisulphite and ascobarte (Noirot et al. 2000, Price et al. 2000, Alan et al. 2007, Greilhuber et al. 2007); the antioxidant dithiothreitol is an reducing agent that avoids the polyphenol, formed manly by CGAs in coffee (Noirot et al. 2003a), oxidation. Due to polyphenol reduction, the chromatin proteins are preserved and the chromosome structural integrity is maintained (Doležel and Bartoš 2005, Loureiro et al. 2006b, Greilhuber et al. 2007).

Our results suggest that the chemical and physical characteristics of the Otto's buffers were important to reduce the negative effect of coffee compounds in the DNA staining. These buffers avoid the nuclear DNA degradation and

provide an appropriate environment for specific and stoichiometric staining of the nuclear DNA.

In this approach the nuclei homogenate centrifugation and the supernatant poured outing procedures (Cros et al. 1995, Noirot et al. 2000) were adopted. This step was fundamental to decrease the amount of undesirable products of the chopping procedure; as nuclei fragments, nonnuclear debris, secondary metabolites and autofluorescent organelles (chloroplasts) (Galbraith et al. 1983, Pinto et al. 2004); corroborating also with obtention of histograms with low CVs.

The *F* test ($P < 0.05$) evidenced that the nuclear DNA content values measured for *C. arabica* and *C. canephora* in distinct days and replications were statistically identical (Table 1). This result suggests that the flow cytometer equipment was carefully calibrated in each FCM measurement. Besides, the procedures for obtention of the nuclei suspensions are reproducible, seeing that none intersample variations resulting from chopping time and intensity and material quantify was evidenced by statistical test. Doležel and Bartoš (2005) and Suda (2004) recommended that each FCM analyses should be repeat at least three times on three different days to avoid errors.

Considering the G_0/G_1 nuclei peak of *S. lycopersicum* (most appropriate standard) in channel 100, the average genome size was 2C = 1.41 ± 0.004 pg (1.38 $X10^9$ bp) for *C. canephora* and 2C = 2.62 pg (2.56 x 10^9 bp) for *C. arabica* (Fig 2). Other genome size values were reported by distinct authors, such as 2.47 (Cros et al. 1994) and 2.61 pg (Cros et al. 1995) for *C. arabica* and 1.67 (Marie and Brown 1993), 1.46 (Cros et al. 1994), 1.54 (Cros et al. 1995) and 1.44 pg (Noirot 2003) for *C. canephora*. Doležel et al. (1998), Bogunic et al. (2003) and Castro et al. (2007) reported that these differences can be provided to the distinct methods, internal standards, buffers or fluorochromes used, as well as to the plant cultivar analyzed.

However, Noirot et al. (2003a) demonstrated that two compounds present in *C. canephora* and *C. arabica*, the inhibitors caffeine and CGAs (Ky et al. 2001, Campa et al. 2005a, b), modified the PI accessibility to the DNA of *Petunia hybrida* (standard). These authors suggested that these compounds can be the main factor for the occurrence of pseudo-intraspecific genome size variation in *Coffea* species. The action mode of these compounds appears to

involve either an intercalation into the DNA or an interaction with histones hindering the access of PI to the DNA. It is also possible that these inhibitors act directly on the PI molecule altering its fluorescence intensity (Greilhuber 1998, Price et al. 2000, Noirot et al. 2003a).

Based on absolute DNA content results, *S. lycopersicum* (64.5 AT% and 35.5% GC, Doležel et al. 1992, Meister and Brown 2007) was used as internal standard to determine the *C. arabica* and *C. canephora* base composition. Seeing that the DAPI and CMA_3 fluorochromes possess two binding lengths, 3 and 4 (Meister and Barow 2007), the two values were used and the mean base composition was measured (Fig 3, Table 2). *C. canephora* showed 65.27% AT and 34.73% GC, and *C. arabica* 63.04% AT and 36.96% GC (Table 2).

The results showed in this study suggest that secondary metabolities interfered in the coffee genome size estimation using *P. sativum* and *R. sativus* as standard and that *S. lycopersicum* is the most adequate standard for coffee nuclear DNA content determination. Besides, the FCM procedures adopted in this study provide histograms with CVs considered appropriated. Accordingly, the present data demonstrated that is relevante to test distinct standard plants, to compare the results obtained with internal and external standards, to centrifuge the nuclei suspensions and to discard the supernatant and to realize replicate measurements for genome size determination in species rich in secondary metabolites.

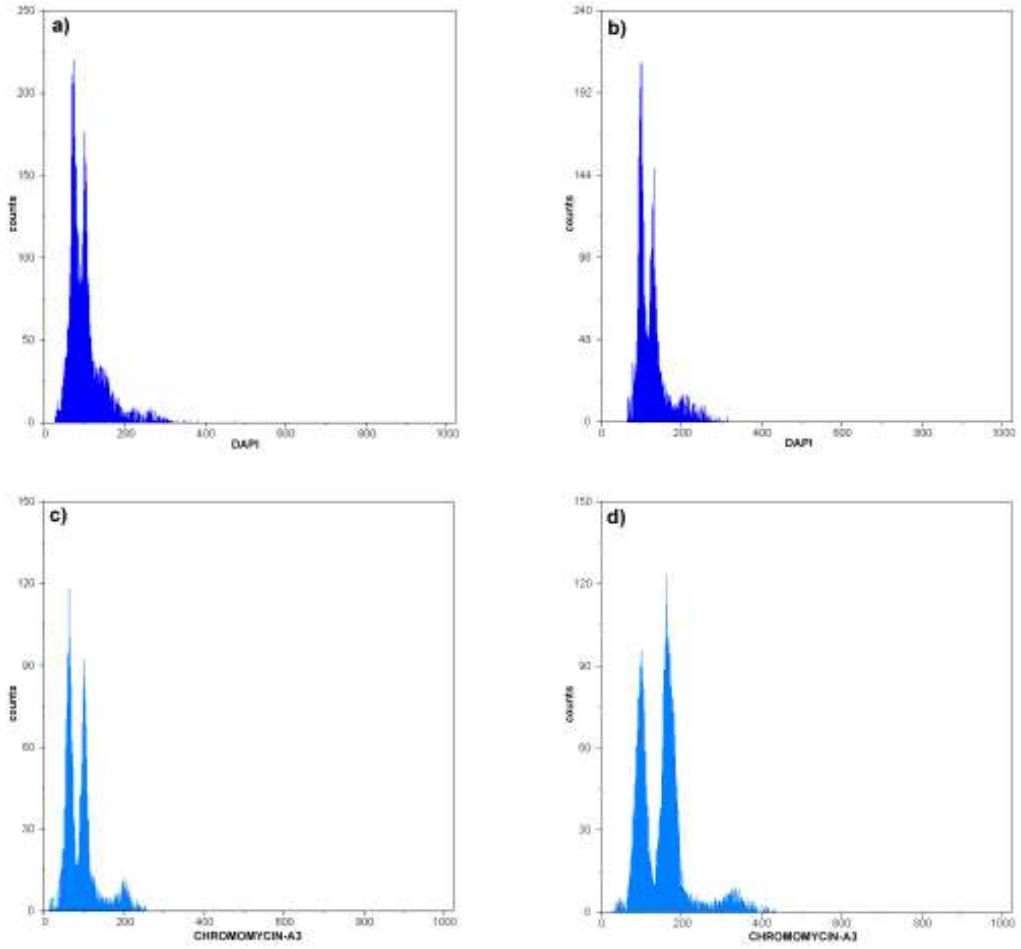

Fig 3 – FCM histograms of fluorescent intensity of G_0/G_1 nuclei stained with DAPI (a, b) and CMA_3 (c, d). a) G_0/G_1 peaks of *C. canephora* (channel 72, 65.27% AT) and *S. lycopersicum* (internal standard, channel 100, 64.5% AT). b) G_0/G_1 peaks of *C. arabica* (channel 133, 63.04% AT) and *S. lycopersicum* (internal standard, channel 100). c) G_0/G_1 peaks of *C. canephora* (channel 64, 34.73% GC) and *S. lycopersicum* (internal standard, channel 100, 35.5% GC). d) G_0/G_1 peaks of *C. arabica* (channel 165, 36.96% GC) and *S. lycopersicum* (internal standard, channel 100).

Table 2 – *C. canephora* and *C. arabica* base composition measured by FCM (Fig. 3), in three independent repeats, using *S. lycopersicum* (64.5% AT and 35.5% GC) as internal standard. The original base composition was obtained quantifying independently AT% and GC% using Godelle et al. (1993) procedure.

Coffee species	Original base composition[a]				Mean base composition[b]			Adjusted base composition[c]		
	r = 3		r = 4							
	AT%	GC%	AT%	GC%	AT%	GC%	AT% + GC%	AT%	GC%	AT% + GC%
C. canephora	64.50	34.50	64.50	34.13	64.50	34.32	98.82	65.27	34.73	100.00
C. arabica	64.34	38.05	64.38	37.40	64.36	37.73	102.10	63.04	36.96	100.00

(a) As the fluorochromes DAPI and CMA_3 show distinct binding lengths (3 or 4) the AT% and GC% was measured for each value. (b) The mean base composition calculated from of the original base composition values. (c) These values were recalculated to add up 100%, as described by Meister and Barow (2007).

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Title: Flow cytometric analysis using SYBR Green I for genome size estimation in coffee.

Running title: Genome size estimation using SYBR Green I.

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Abstract

Plant genome size has been measured by flow cytometry (FCM) using propidium iodide (PI) as a dye for nuclei DNA staining. However, some authors have reported the occurrence of genome size estimation errors, especially in plants rich in secondary metabolites, such as the coffee tree. In this context, we tested an alternative cytometric protocol using SYBR Green I as a fluorochrome for stoichiometrically staining nuclear double-stranded DNA (dsDNA) in *Coffea canephora* (2x) and *C. arabica* (4x). The results showed that the respective mean genome size measured from nuclei stained with SYBR Green I and PI was statistically identical. However, the G_0/G_1 peaks of nuclei stained with SYBR Green I exhibited lower coefficient of variations (CVs) (1.57 to 2.85%) compared to those stained with PI (2.75 to 4.80%). CV statistical data suggests that SYBR Green I was more adequate for stoichiometric nuclei staining in this methodology. Our results evidence that SYBR Green I can be used in FCM measurements of plants, with the advantages of minimizing errors in nuclear DNA content quantification, staining relatively quicker, with more affinity, and being less mutagenic than PI.

Key words: *C. arabica*, *C. canephora*, Flow cytometry, genome size, SYBR Green I, propidium iodide.

Introduction

Flow cytometry (FCM) measurements in *Coffea* species have used 4',6'-diamidino-2-phenylindole – DAPI (Cros et al. 1995, Noirot et al. 2002) or propidium iodide – PI (Cros et al. 1994, 1995, Noirot et al. 2002, 2003b) for staining nuclei suspensions. The base specific-fluorochrome DAPI (Galbraith et al. 1983, Leitch and Bennett 2007) has been used in FCM assessments in coffee and others organisms presumably because it binds specifically (Otto 2000) to double-stranded DNA (dsDNA) independently of chromatin structure, resulting in peaks with a low coefficient of variation (CV) (Doležel and Göhde 1995, Doležel and Bartoš 2005, Meister and Barow 2007, Suda et al. 2007). Besides, some researchers consider this dye to be particularly easy to excite and measure (Doležel and Bartoš 2005). As DAPI provides high resolution G_0/G_1 peaks with low CVs (Shapiro 2005), this stain has been widely used for ploidy screening (Roux et al. 2003, Meister and Barow 2007, Parc et al. 2007, Suda et al. 2007, Clarindo et al. 2008), base ratio determination (Shapiro 2003, Meister and Barow 2007, Suda et al. 2007) and chromosome sorting (Neumann et al. 2002, Kubaláková et al. 2003).

Despite all DAPI applications, this fluorochrome is not considered adequate for genome size estimation (Doležel et al. 1992, Doležel et al. 1998, Doležel and Bartoš 2005), as observed in coffee trees (Noirot et al. 2002), since this dye binds preferentially to AT-rich portions of the dsDNA (Galbraith et al. 1983, Shapiro 2003, Traganos 2004, Leitch and Bennett 2007) and that errors of 100% in FCM measurements have been observed (Doležel et al. 1992, Doležel et al. 1998).

DNA intercalators (ethidium bromide – EB or PI) have been used for absolute DNA estimation in FCM protocols (Watson 2004, Shapiro 2003, Doležel and Bartoš 2005). Despite these fluorochromes showing similar DNA binding properties, most FCM works have used PI as dye because EB does not rapidly cross the nuclear membrane and PI possesses a higher binding affinity for dsDNA than EB (Shapiro 2003, 2005). In the first and second Plant Genome Size Meetings (1997 and 2003), PI was recommended as the fluorochrome of choice for genome size studies (Leitch and Bennett 2007, http://www.kew,org/genomesize/pgsm/index), mainly because genome size

quantified by PI-FCM has been considered perfectly correlated with that measured by Feulgen microspectrophotometry (Doležel et al. 1998).

Although PI has been considered the most adequate fluorochrome for FCM measurements, some works have reported the occurrence of pseudo-intraspecific genome size variations in samples stained with this dye (Cros et al. 1994, 1995, Noirot et al. 2000, 2002, 2003a, b, Price et al. 2000). This variation occurs by non-stoichiometric binding of PI to the DNA (Noirot et al. 2000, 2002, 2003a, b, Price et al. 2000, Loureiro et al. 2006b, Alan et al. 2007, Bennett et al. 2007).

Noirot et al. (2003a) showed that secondary metabolites (caffeine and chlorogenic acids), present in *Coffea* spp., interfere in the PI intercalation to the DNA. Chlorogenic acids (CGAs), which are polyphenol precursors, insert within dsDNA limiting the access of PI. Besides, these compounds can act on nuclear membrane proteins hindering stain uptake by the nucleus (Greilhuber 1998). In contrast, caffeine (1,3,7-trimethylxanthine) forms complexes with CGAs reducing their negative effects on PI accessibility (Noirot et al. 2003a).

Recently, another nucleic acid stain, the intercalating dye SYBR Green I (Marie et al. 1999, Demontis et al. 2007, Sakurai et al. 2008), has been used for the rapid and accurate quantitative and qualitative enumeration of viral particles by FCM (Marie et al. 1999). This fluorochrome quickly stains dsDNA (Marie et al. 1999), exhibits an exceptional affinity to this molecule (Demontis et al. 2007) and displays fluorescence enhancement upon dsDNA binding (Invitrogen 2006, http://probes.invitrogen.com/servlets/publications?id=146).

We tested an alternative cytometric protocol using SYBR Green I as a fluorochrome for stoichiometric staining of nuclear dsDNA in *C. canephora* (2x) and *C. arabica* (4x). Additionally, results obtained with PI and SYBR Green I were compared.

Materials and methods

Plant material

C. canephora ‘Apoatã’ (sample), *C. arabica* ‘Catuaí Vermelho’ (sample), and *Solanum lycopersicum* L. ‘Stupické’ (standard, 2C = 1.96 pg, Doležel et al.

1992) were cultivated in a glasshouse under the same environmental conditions. Seeds of the standard were kindly supplied by Dr. Jaroslav Doležel (Experimental Institute of Botany, Czech Republic). Cytometric analyses were carried out at the Laboratório de Citogenética e Citometria, Departamento de Biologia Geral, Universidade Federal de Viçosa (UFV, Brazil).

Sample preparation

Young leaves of the sample and standard were simultaneously placed in distilled water at 4 ºC and cut into 2 cm^2 fragments. Nuclei was extracted by chopping (Galbraith et al. 1983) in 0.5 mL of OTTO-I lysis buffer (Otto 1990) containing 0.1 M citric acid (Merck®), 0.5% Tween 20 (Merck®), 2.0 mM dithiothreitol (Sigma®) and 50 µg mL^{-1} RNAse (Sigma®), pH 2.3. Subsequently, 0.5 mL of the same buffer was added and the suspension was filtered through a nylon filter (Partec®) with a 30 µm pore diameter, transferred to microfuge tubes (Eppendorf®) and centrifuged (ALC® microCentrifugette®4214) at 100 g for 5 min. The supernatant was discarded and the pellet was resuspended and incubated for 10 min in 100 µL OTTO-I lysis buffer.

Staining of the nuclei suspension

The nuclei suspension was stained with 1.5 mL OTTO-I:OTTO-II solution (400 mM $Na_2PO_4.12H_2O$, Merck®) (Otto 1990, Doležel and Göhde 1995), pH 7.5, in a 1:2 ratio (Loureiro et al. 2006a, b) and finally filtered through a 20 µm nylon filter (Partec®). Some suspensions were stained in OTTO-I:OTTO-II solution supplemented with 2.0 mM dithiothreitol, 75 µM PI (Sigma®, excitation/emission wavelengths: 480-575/550-740 nm, Shapiro 2003) and 50 µg mL^{-1} RNAse (Doležel et al. 1992, Meister 2005). Other suspensions were stained in OTTO-I:OTTO-II solution supplemented with 2.0 mM dithiothreitol, SBYR Green I (excitation/emission wavelengths: 497 nm/520 nm, Invitrogen 2006 – http://probes.invitrogen.com/servlets/publications?id=146) diluted 1,000-fold (Marie et al. 1999, recommendations of the Invitrogen 2006 – http://probes.invitrogen.com/servlets/publications?id=146) and 50 µg mL^{-1} RNAse. The SBYR Green I stock solution (10,000x concentrate in DMSO, 500

µL) was subdivided into 50 µL aliquots, and to each aliquot 4.95 mL of DMSO were added. 100 µL of this last solution was mixed with 0.90 mL of OTTO-I:OTTO-II buffer. The nuclei suspension staining was performed in the dark during 10, 20, 30 and 40 min.

Equipment handling

The suspensions were analyzed with a Partec PAS® flow cytometer (Partec® Gmbh, Munster, Germany), equipped with a laser source (488 nm). PI fluorescence emitted by nuclei was collected through a RG 610 nm band-pass filter, while SYBR Green I fluorescence was collected through an EM 520 nm band-pass filter. The equipment was calibrated and aligned using microbeads and standard solutions according to the manufacturer's recommendations (Partec®). FlowMax® software (Partec®) was used for data analyses.

In the order to minimize possible intersample variations resulting from chopping time and intensity and material quantification, the same nuclei suspension was FCM analyzed after staining for specific times (10, 20 30 and 40 min). The peak of the standard nuclei was set to channel 150. Three independent replications were performed over a period of three days, with more than 10,000 nuclei being analyzed in each replication.

Genome size estimation

C. canephora and *C. arabica* genome size were measured using peaks corresponding to the average of the G_0/G_1 relative 2C nuclei DNA content of the sample and standard plants. The average 2C value (pg) was converted to base pairs (bp) assuming that 1 pg of DNA corresponds to 0.978×10^9 bp (Doležel et al. 2003). The data were statistically analyzed by the *F*-test to compare the average coffee DNA content values. The statistical tests were performed using the Genes Program (Cruz 1997).

C. arabica and *C. canephora* 2C DNA content was calculated from the internal standard *S. lycopersicum*. Subsequently, either *C. arabica* or *C. canephora* was used as an internal standard to measure the 2C DNA content of the other species, because the FCM estimate is more accurate when the

chromatin structure of the standard and the sampled nuclei are similar and reacts in a common way to compounds that interfere with the accessibility of fluorochromes to the DNA (Doležel and Bartoš 2005).

Results and discussion

Incubation of nuclei suspensions in OTTO-I:OTTO-II buffer for specific times was very important for obtaining histograms with different resolutions and CVs oscillating from 1.57 to 4.80%. The chopping procedure, associated with nuclei isolation using OTTO-I (10 min) and OTTO-I:OTTO-II (30 min for PI or 10 min for SYBR Green I), allowed the release of enough quantity of intact and isolated nuclei and, consequently, histograms showed the best resolution levels and CVs (Fig 1a – f).

In plants, the most used FCM buffers are Galbraith's (Galbraith et al. 1983), LB01 (Doležel et al. 1992), Otto's (Otto 1990, Doležel and Göhde 1995) and Tris.$MgCl_2$ (Pfosser et al. 1995). In this study we're chosen Otto's because it contains citric acid in OTTO-I, a compound that improves chromatin accessibility to the fluorochrome and makes the chromatin structure of the sample and the standard uniform. Therefore, it minimizes differences in staining intensity, resulting in histograms with high resolution and low CVs (Doležel and Bartoš 2005, Loureiro et al. 2006a). This fact has been observed in our laboratory routine, and was confirmed by these analyses.

Furthermore, Otto's procedure is carried out in two steps, with the nuclei suspensions being centrifuged and the supernatant discarded between the two steps. This procedure decreases nonnuclear debris (Cros et al. 1995) and the amount of secondary metabolites (Cros et al. 1995, Noirot et al. 2000), contributing also to the obtention of histograms with low CVs. Even then, Doležel and Bartoš (2005) reported that the precision of FCM measurements, as verified by the CV (Ormerod 2000, Shapiro 2003, Doležel and Bartoš 2005, Castro et al. 2007), depends also on stoichiometric nuclei staining.

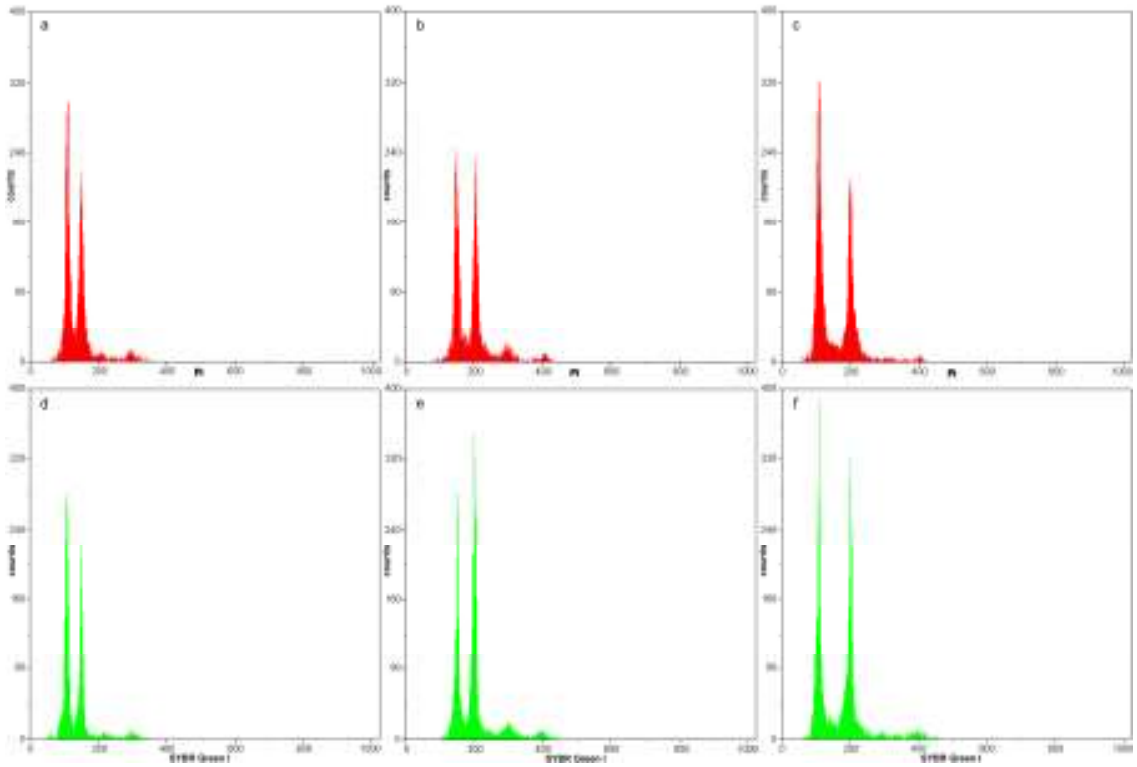

Figure 1 – Absolute genome size estimation in *C. canephora* (2x) and *C. arabica* (4x) from nuclei suspensions stained with PI and SYBR Green I. (a – c) Histograms showing G_0/G_1 peaks with CVs ranging between 2.75 to 4.80%, obtained from PI-stained nuclear suspensions prepared from leaves of: a) *C. canephora* (channel 108) and *S. lycopersicum* (internal standard, channel 150), b) *C. arabica* (channel 200) and *S. lycopersicum* (internal standard, channel 150), c) *C. canephora* (channel 108) and *C. arabica* (channel 201). (d – f) Histograms exhibiting G_0/G_1 peaks with CVs oscillating between 1.57 to 2.85%, obtained from SYBR Green I-stained nuclear suspensions prepared from leaves of: d) *C. canephora* (channel 108) and *S. lycopersicum* (internal standard, channel 150), e) *C. arabica* (channel 200) and *S. lycopersicum* (internal standard, channel 150), f) *C. canephora* (channel 108) and *C. arabica* (channel 201).

Coffea species produce large quantities of secondary metabolites, specially caffeine and CGAs (Noirot et al. 2003a, b), which may promote non-stoichiometric staining of the nuclei suspension, generating histograms with high CVs (Cros et al. 1994, 1995) and causing errors in assessments of nuclear DNA content by FCM (Noirot et al. 2000, 2003a, b, Price et al. 2000, Loureiro et al. 2006b, Alan et al. 2007). CGAs alter the chromatin and membrane proteins structure, interfering with the intercalation of PI to the DNA (Greilhuber 1998). Caffeine is also an intercalating substance, but this compound can form complexes with CGAs reducing the negative effects on PI accessibility to the DNA (Noirot et al. 2003a).

In this study, histograms of nuclei suspensions stained with SYBR Green I exhibited CVs varying between 1.57 to 2.85%, while the suspensions incubated with PI yielded histograms with CVs oscillating from 2.75 to 4.80%. This indicates that the FCM procedures with SYBR Green I were more suitable, since a lower variance from the mean fluorescence value of the nuclei population was obtained. Furthermore, this result suggests that the interaction between SYBR Green I and DNA was less affected by caffeine and chlorogenic acids.

SYBR Green I is a fluorescent molecule that binds strongly to dsDNA (Demontis et al. 2007). SYBR Green I exhibits exceptional affinity to DNA and a large fluorescence enhancement upon DNA binding. In comparison with EB, a fluorochrome with similar properties to PI, SYBR Green I has a much larger fluorescence enhancement when bound to dsDNA (Rengarajan et al. 2002, Leggate et al. 2006). The quantum yield of the DNA/SYBR Green I complex (~0.8) is several times greater than that of the DNA/EB complex (~0.15). Another additional advantage of SYBR Green I is the fact that this stain is significantly less mutagenic than EB and PI (Invitrogen 2006 – http://probes.invitrogen.com/servlets/publications?id=146).

SYBR Green I quickly stains DNA (10 – 15 min) (Marie et al. 1999), since it has a negligible background in the absence of DNA, allowing a rapid staining procedure. In our work, this dye stained the nuclei suspension in 10 min, while the nuclei suspension was stained by PI after 30 min. Even though, Loureiro et al. (2006b) reported that the interaction between secondary metabolites and

DNA is very rapid, this SYBR Green I property contributed to the obtention of low CVs.

As the nuclei suspensions were submitted to identical staining procedures and flow cytometer analyses, the results obtained in this study suggest that the rapid and strong binding of SYBR Green I to DNA, in comparison with PI, contributed to the stoichiometric staining of the sample and standard nuclei, generating histograms with CVs lower than 3%, which is considered perfectly acceptable for FCM measurements (Marie and Brown 1993).

When the DNA was stained with SYBR Green I, RNAse was added to the OTTO buffer solution because this dye also presents affinity for RNA, just like EB and PI. Furthermore, SYBR Green I is pH sensitive, with the pH of the staining solution for optimal sensitivity of the dye being in the range of 7.5 to 8.3 (Invitrogen 2006 – http://probes.invitrogen.com/servlets/publications?id=146). Therefore the pH of the OTTO-I:OTTO-II buffer was adjusted to 7.5.

The *F* test applied to the mean values of nuclear DNA content of *C. arabica* and *C. canephora*, obtained from the histograms with the best CVs (1.57 to 2.85% for SYBR Green I and 2.75 to 4.80% for PI) showed that the values obtained with nuclei suspensions stained with PI or SYBR Green I are identical (Table 1). This statistic test also indicated that the *C. arabica* and *C. canephora* genome size obtained when one of the coffee species was used as an internal standard was the same obtained when with *S. lycopersicum*, irrespectively of the dye (Table 1). These results further suggest that SYBR Green I is really adequate for staining of nuclei suspensions used in FCM and that *S. lycopersicum* is an adequate standard for FCM measurements in coffee trees.

The *F* test also indicated no statistical difference in the 2C DNA content of *C. arabica* and *C. canephora* measured over the three day period. This demonstrates that the FCM equipment was carefully calibrated, that the FCM procedures adopted in this work supplied suspensions containing enough isolated and intact nuclei, and that these analyses are reproducible. Furthermore, this result also provides evidence that the use of SYBR Green I yields FCM histograms with high resolution and low CVs, indicating that the nuclei of the sample and standard were stoichiometrically stained.

Table 1 – *C. arabica* and *C. canephora* genome size measured from FCM analyses with different standards (*S. lycopersicum, C. canephora* or *C. arabica*) and fluorochromes (PI or SYBER Green I).

Sample/standard	Stain	Mean 2C DNA content (pg)	Mean 1C DNA content (pg)	Base pairs (x 10^9)*
C. canephora/*S. lycopersicum*	PI	1.408[a]	0.704	0.688
C. canephora/*S. lycopersicum*	SYBR	1.411[a]	0.706	0.690
C. canephora/*C. arabica*	PI	1.409[a]	0.704	0.688
C. canephora/*C. arabica*	SYBR	1.413[a]	0.708	0.692
C. arabica/*S. lycopersicum*	PI	2.620[b]	1.310	1.281
C. arabica/*S. lycopersicum*	SYBR	2.622[b]	1.312	1.283
C. arabica/*C. canephora*	PI	2.620[b]	1.310	1.281
C. arabica/*C. canephora*	SYBR	2.623[b]	1.312	1.283

*1C mean values converted to bp (base pairs), assuming that 1 pg of DNA corresponds to 0.978 x 10^9 bp (Doležel et al. 2003). Mean genome sizes followed by the same letter (a or b) are not significantly different according to the *F* test ($P < 0.05$).

Our results indicate that SYBR Green I is adequate for *C. arabica* (2.622 pg ± 0.002) and *C. canephora* (1.410 pg ± 0.004) genome size estimation, since it yielded histograms with higher resolution and, consequently, lower CVs compared to PI. This fluorochrome can be used in FCM measurements of plants with the advantages of minimizing errors in nuclear DNA content quantification, staining relatively quicker, with more affinity, and being less mutagenic than PI.

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Title: Chromosomal DNA content of *Coffea arabica* and *Coffea canephora* measured by image cytometry.

Running title: *Coffea* chromosomal DNA content

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Abstract

Genome size has been measured in various plants, including coffee species, seeing that knowledge of the 2C DNA content provides useful information for taxonomic, ecological and evolutive studies, comparative analyses of the genomic structure, plant breeding programs and genome sequencing projects. Besides the nuclear DNA content, some authors have also quantified the chromosomal DNA content, this way expanding the data about genomic structure. Accordingly, this study was conducted in order to estimate the DNA content of each *C. canephora* and *C. arabica* chromosome, associating cytogenetic methodologies with flow and image cytometry tools. Firstly, the genome sizes of *C. canephora* (1.41 pg) and *C. arabica* (2C = 2.62 pg) were quantified by flow cytometry. The nuclear DNA content was proportionally distributed based on integrated optical density (IOD) mean values calculated by image cytometry. Then, the DNA content of each *C. canephora* and *C. arabica* chromosome was calculated in picograms and base pairs. The ten most adequate prometaphases or metaphases of *C. arabica* and *C. canephora* were used for accomplishment of the cytometric measurements and assembly of karyograms. The qualitative (cytogenetic characterization) and quantitative (chromosomal DNA content) analyses evidenced that *C. arabica* is a true allotetraploid, non-segmental, originated from a cross between *Coffea* diploid species exhibiting similar genomes. Besides, the same analyses also reinforce that *C. canephora* is a possible progenitor of *C. arabica*, as observed by other authors.

Key words: *C. arabica*, *C. canephora*, Chromosomal DNA content, Cytogenetics, Flow cytometry, Image cytometry

Introduction

The nuclear DNA content of the two most cultivated coffee species, *Coffea arabica* L. (2n = 44 chromosomes) and *Coffea canephora* Pierre ex Froehner (2n = 22 chromosomes) (Anthony et al. 2001; Baruah et al. 2003; Aggarwal et al. 2007; Mahé et al. 2007), has been measured, in picograms (pg), by flow (FCM) and image (ICM) cytometries. Knowledge of the 2C DNA content in *Coffea* spp. has contributed to identify intra and interespecific variations (Cros et al. 1994; 1995; Fontes 2003), to correlate with fertility of interespecific hybrids (Cros et al. 1995) and with adaptive traits (Cros et al. 1995; Noirot et al. 2003b), and to evolutionary studies (Noirot et al. 2003b).

In other plants, such as *Zea mays* (Lee et al. 2002; Rosado et al. 2005), *Pinus* spp. (Bogunic et al. 2003), *Triticum* spp. and *Secale cereale* (Lee et al. 2004), besides the genome size, the chromosomal DNA content has also been quantified. Measurement of the chromosomal DNA content extends the data about the genome (Lee et al. 2004), generating important information for taxonomy (Lee et al. 2002; Rosado et al. 2005; Carvalho et al. 2006), evolutionary studies (Lee et al. 2004; Rosado et al. 2005), comparative analyses of the genomic structure and plant breeding programs (Lee et al. 2002). Moreover, the nuclear and chromosomal DNA contents, reported in base pairs (bp), have been considered useful for genome sequencing projects (Hardie et al. 2002; Doležel and Bartoš 2005) and for genetic mapping.

Lee et al. (2002) quantified the *Zea mays* chromosomal DNA content by analyzing isolated chromosomes with the flow karyotyping tool. This technique generates flow karyotypes based on the classification of the chromosomes according to their relative DNA, base and protein contents, and morphological parameters (Laat and Blaas 1984; Lucretti et al. 1993; Kovářová 2007). Nevertheless, flow karyotyping requires suspensions with large amounts of intact chromosomes, as well as methodologies that provide sufficient resolution to distinguish all of them (Lucretti et al. 1993; Doležel and Lucretti 1995; Neumann et al. 1998; Doležel et al. 2001; 2004; 2007).

Bogunic et al. (2003) measured the chromosomal DNA amount of five *Pinus* species, dividing the total genome size by the mean chromosome length.

However, this procedure does not consider the differences of DNA content among the chromosomes.

Rosado et al. (2005) developed a quantitative methodology for measuring chromosomal DNA content, associating cytogenetic and cytometric tools. In this approach, the DNA content of each *Zea mays* A and B chromosomes was quantified from distribution of the nuclear genome size values of G_0/G_1 nuclei, obtained by FCM, proportionally to the integrated optical density (IOD) of the chromosomes, generated by ICM.

This study was conducted in order to: (1) quantify the nuclear genome size of *C. canephora* and *C. arabica* by FCM, (2) adapt a cytogenetic protocol that supplies adequate prometaphasic and metaphasic chromosomes for accomplishment of the ICM procedures, (3) adjust a protocol for stoichiometric staining of the chromosomes by Schiff's reaction, and (4) assess the DNA content of each *C. canephora* and *C. arabica* chromosome by associating cytogenetic, FCM and ICM methods.

Materials and methods

Biological material

Leaves of *C. canephora* 'Apoatã' and *C. arabica* 'Catuaí Vermelho' were used for establishment of embryogenic cell suspension aggregates culture, according to protocols described by Berthouly and Michaux-Ferriere (1996) and van Boxtel and Berthouly (1996). Leaves of the same plants were also used in FCM assessments. *Solanum lycopersicum* L. 'Stupické' seeds (2C = 1.96 pg, Doležel et al. 1992), kindly supplied by Dr. Jaroslav Doležel (Experimental Institute of Botany, Czech Republic), were used as standard for the FCM analysis. All plants were cultivated in a greenhouse, under equal environmental conditions. The cytogenetic and cytometric analyses were carried out at the Laboratório de Citogenética e Citometria, Departamento de Biologia Geral, Universidade Federal de Viçosa – UFV (Brazil).

Flow cytometry analysis

Fragments (2 cm^2) of young leaves of sample (*C. canephora* or *C. arabica*) and standard (*S. lycopersicum*) were briefly and simultaneously chopped with a razor blade (Galbraith et al. 1983), in a 60 x 15 mm Petri dish with 0.5 mL of OTTO-I (Otto 1990, Doležel and Göhde 1995) lysis buffer, supplemented with 2.0 mM dithiothreitol (Sigma®) and 50 µg mL^{-1} RNAse (Sigma®), pH 2.3. Subsequently, 0.5 mL of the same buffer was added, the homogenate was filtered through a 30 µm nylon sieve (Partec®) into a 2.0 mL microcentrifuge tube (Eppendorf®), and then centrifuged at 100 g for 5 min. The supernatant was poured out and the pellet was resuspended and incubated for 10 min in 100 µL OTTO I lysis buffer. The nuclei suspension was stained with 1.5 mL of 1:2 (Loureiro et al. 2006a, b) OTTO-I:OTTO-II solution (Otto 1990, Doležel and Göhde 1995), supplemented with 75 µM propidium iodide (PI, Sigma®, – excitation/emission wavelengths: 480-575/550-740 nm, Shapiro 2003), 50 µg mL^{-1} RNAse (Sigma®) and 2.0 mM dithiothreitol (Sigma®), pH 7.5 (Doležel et al. 1992).

After 30 min in the dark, the suspensions were analyzed in a Partec PAS® flow cytometer (Partec® Gmbh, Munster, Germany) equipped with a Laser source (488 nm). PI fluorescence emitted from nuclei was detected by a RG 610 nm band-pass filter and converted to 1024 channels. The equipment was calibrated for linearity and aligned with microbeads and standard solutions, according to recommendations by the manufacturer. FlowMax® software (Partec®) was used for data analysis. The nuclei peak of the internal standard was set to channel 150. Over 10,000 nuclei were analyzed, and three independent repetitions and average values of 2C DNA content are reported in pg and bp. The histograms with CV (coefficient of variation) above 5% were rejected.

Image cytometry analysis

The microtubule inhibiting agent amiprophos-methyl (Nihon Bayer Agrochem K. K.®) or oryzalin (Sigma®) was added to the culture media to a final concentration of 3.0 µM for a period of 3 – 4 h, at 30 ^{0}C (Planchais et al. 2000,

Clarindo and Carvalho 2006). The embryogenic aggregates were subsequently washed with distilled water for 20 min, and then fixed in a 3:1 (v/v) methanol:acetic acid solution (Merck®) for 24 h. This solution was replaced by 95% alcohol, as suggested by Greilhuber and Ebert (1994) and Baranyi and Greilhuber (1999). The fixative solution was changed three times and the samples were stored at -20 ^{0}C (Carvalho and Saraiva 1993, 1997).

Embryogenic cell aggregates were washed and incubated for 30 min, at 34 ºC, in pectinase solution (Sigma®) in the proportion of 1:30 (enzyme:water). Next, the aggregates were washed for 10 minutes in distilled water, fixed again and stored at –20 °C (Clarindo and Carvalho 2006). *C. canephora* and *C. arabica* slides were prepared by dissociation of the aggregates, air-dried (Carvalho and Saraiva 1993, 1997), and immediately placed in a fixative solution of 17:5:1 methanol:37% formaldehyde:acetic acid (Merck®), for 12 h, at 25 ºC.

The Feulgen reaction procedures were performed according to Greilhuber and Ebert (1994) and Vilhar et al. (2001), with few modifications. After fixation, the slides were washed in distilled water, air-dried, and hydrolyzed in 5 M HCl (Merck®) for 10 – 40 min at 25 ºC, then stained with Schiff's reagent (Merck®) for 12 h at 4 ºC. After that, the slides were washed three times (for 3 min each time) in 0.5% SO_2 water (Merck®).

Image Analysis

Images of the chromosomes were captured with a Photometrics CoolSNAP Pro® (Roper Scientific®) monochromatic CCD video camera of 12 bits gray, assembled on an Olympus™ BX-60 microscope with a source of stabilized light, PlanApo objective magnification of 100x with a 1.4 numeric aperture, aplanat achromat condenser with aperture of 1.4, one neutral density filter (ND6) and another of interference green color (IF550). The Köhler method was applied prior to each capture session to adjust the optimal light path and, consequently, to reduce stray light.

The frame was digitized using the Kit Cool-SNAP Pro®, and image cytometric analysis was performed using the Image Pro-Plus® 6.1 analysis system (Media Cybernetics®). Calibration and evaluation of the image analysis

system was performed and consisted of three tests: stability (Vilhar and Dermastia 2002); linearity (Vilhar et al. 2001; Hardie et al. 2002), carried out with a set of certified neutral density filters: 0.15, 0.30, 0.40, 0.60, 0.90 and 2.50 (Edmund Industrial Optics®); and uniformity (Puech and Giroud 1999), performed with 11 stepped density filters (Edmund Industrial Optics®). The image cytometry tools stabilized after 12 min, and the software of the image analysis system automatically calculated a R^2 = 0.9978 for linearity test and a CV = 0.68% for uniformity test.

Chromosomic DNA content

The average DNA content of each chromosome of *C. canephora* and *C. arabica* was measured, in pg, by distributing the nuclear 4C value obtained by FCM proportionally to the mean IOD values of each metaphasic chromosome pair, calculated by ICM (Rosado et al. 2005). The values were estimated using the formula:

$$2C_c = \frac{(IOD_c \times 4C_n)}{IOD_m}$$

being $2C_c$ = mean 2C chromosomal DNA content; IOD_c = mean IOD for each chromosome pair in all metaphases; $4C_n$ = 4C nuclear DNA content; IOD_m = mean IOD of all metaphases. The mean values were converted to bp (base pairs), considering that 1 pg of DNA corresponds to 0.978 x 10^9 bp (Doležel et al. 2003).

Results and discussion

Flow cytometry analysis

The G_0/G_1 nuclei stained with PI, from the three independent repetitions, corresponded to the peaks at channels 107, 108, 109 for *C. canephora* and 200, 200, 201 for *C. arabica*. The peaks of G_0/G_1 nuclei showed CV ranging from 2.75 to 4.80% (Fig. 1a, b). These results demonstrate that the FCM equipment had been carefully calibrated and that the suspensions contained

sufficient isolated and intact PI-stained nuclei. Doležel and Bartoš (2005) noted that CVs below 5% are acceptable for FCM assessments. However, distinct authors have reported CV values above 5% in species that are, as coffee trees, rich in secondary metabolites, such as *Eucalyptus* spp. – 6.3 to 12.8% (Grattapaglia and Bradshaw 1994), *Eucalyptus globulus* – 4.6 to 6.8% (Pinto et al. 2004), *Quercus* spp. – 3.3 to 6.9% (Zoldos et al. 1998) and *Scutellaria baicalensis* – 3.48 to 6.77% (Alan et al. 2007). Considering this aspect, Pinto et al. (2004) reported that, for most woody species, the CV values recommended by Doležel and Bartoš (2005) are very difficult, or in some cases even impossible to obtain.

Considering the G_0/G_1 nuclei peak of *S. lycopersicum* in channel 150, the mean value for DNA content was 2C = 1.41 ± 0.013 pg (1.38 $X10^9$ bp) for *C. canephora* and 2C = 2.62 ± 0.008 pg (2.56 x 10^9 bp) for *C. arabica*. These values were identical to those observed by Fontes (2003), using ICM. Differently, other genome size values were reported by various authors, for instance 2.47 (Cros et al. 1994) and 2.61 pg (Cros et al. 1995) for *C. arabica* and 1.67 (Marie and Brown 1993), 1.46 (Cros et al. 1994), 1.54 (Cros et al. 1995) and 1.44 pg (Noirot 2003b) for *C. canephora*. The difference in these results can be attributed to the particular methodologies, standards, buffers or fluorochromes used, as well as to the plant cultivar analyzed. However, the interference of secondary metabolites (caffeine and chlorogenic acids) with the dye (PI) accessibility to the DNA has been considered the main cause of intraspecific variations in *Coffea* species (Noirot et al. 2002; 2003a, b).

Coffea species produce secondary metabolites, especially caffeine and chlorogenic acids (Noirot et al. 2003a, b), which cause stoichiometric errors in the estimation of nuclear DNA content by FCM (Noirot et al. 2000; Price et al. 2000; Noirot et al. 2003a, b; Loureiro et al. 2006b; Alan et al. 2007). Therefore, FCM procedures were adapted in order to minimize stoichiometric errors.

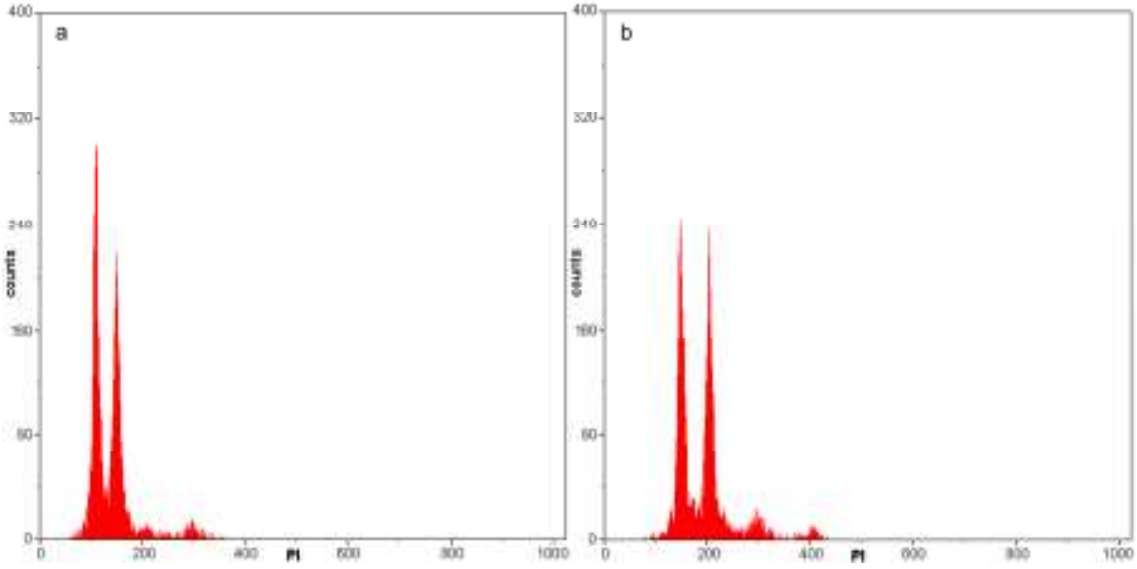

Figure 1 – Estimation of the nuclear genome size in *C. canephora* and *C. arabica* from nuclei suspensions stained with PI. (a and b) Histograms showing G_0/G_1 peaks with CVs ranging from 2.75 to 4.80%, obtained from PI-stained nuclear suspensions prepared from leaves of: a) *C. canephora* (channel 108, 2C = 1.41 pg) and *S. lycopersicum* (internal standard, channel 150, 2C = 1.96 pg) and b) *C. arabica* (channel 200, 2C = 2.62 pg) and *S. lycopersicum* (internal standard, channel 150, 2C = 1.96 pg).

In this study we used Otto's buffers because Doležel and Bartoš (2005) related that these buffers provide FCM histograms exhibiting G_0/G_1 peaks with unequalled resolution for most plant species, especially those species with lower genome size (Loureiro et al. 2006a), like coffee trees. OTTO-I contains citric acid, a compound that improves chromatin accessibility to the fluorochrome and makes the chromatin structure of the sample and the standard uniform. This way, it avoids differences in staining intensity, resulting in histograms with high resolution and low CVs (Doležel and Bartoš 2005; Loureiro et al. 2006a), as confirmed by our analyses. Besides citric acid, we also added the antioxidant dithiothreitol, a substance that preserves chromatin integrity and minimizes stoichiometric errors in the DNA staining (Doležel and Bartoš 2005).

Discarding the supernatant after centrifugation of the nuclear homogenate drastically reduces FCM interferences (Otto 1990; Cros et al.

1995; Doležel and Göhde 1995; Noirot et al. 2000), since the supernatant contains secondary metabolites and autofluorescent organelles (chloroplasts, for instance). The above-mentioned procedures supplied suspensions with few artifacts, such as fluorescent nonnuclear debris and nuclear aggregates, thus generating histograms with low CVs (Cros et al. 1995).

In this study the nuclear genome size was measured by FCM, since this tool is a more practical and rapid methodology for absolute DNA measurements than ICM (Doležel and Bartoš 2005). FCM and ICM have been employed, combined or separately, for determination of the 2C DNA content (Doležel et al. 1998; Doležel and Bartoš 2005; Greilhuber 2005), and the results of both techniques have been considered in perfect accordance (Doležel et al. 1998),

Image cytometry analysis

The slides prepared with *C. canephora* or *C. arabica* embryogenic cell aggregates, treated with 3 µM amiprophos-methyl for 3 – 4 h and macerated with 1:30 pectinase solution for 30 min, exhibited the most adequate metaphasic and prometaphasic chromosomes, with different levels of chromatin condensation. The fixation of these slides with a combination of three substances (methanol, formaldehyde and acetic acid) contributed to maintain the chromosome integrity and, consequently, avoided any bias by providing high chromatin stability (Greilhuber and Temsch 2001; Vilhar et al. 2001; Greilhuber 2005).

Air-drying the preparations provided chromosomes flattened on the slide, without cytoplasmic debris and chromatin deformations. This methodology was fundamental for ICM application in *Coffea* chromosomes, since the squashing technique, usually applied in nuclear ICM (Greilhuber and Ebert 1994; Vilhar et al. 2001; Greilhuber 2005) and cytogenetic studies in *Coffea* (Raina et al. 1998; Pierozzi et al. 1999), is unsuitable to provide chromosome preparations with those required characteristics (Carvalho and Saraiva 1993, 1997).

Only the preparations hydrolyzed with 5 M HCl solution for 18 min at 25 ^{0}C and maintained in Schiff's reagent for 12 h at 4 ^{0}C presented stoichiometrically

stained chromosomes (Fig 2 a, b). Hydrolysis is the most critical step in ICM (Greilhuber and Temsch 2001; Greilhuber 2005), requiring meticulous determination of the appropriate HCl concentration, time and optimal temperature (Greilhuber and Temsch 2001) to ensure the stoichiometric DNA staining with Schiff´s reagent.

The CCD video camera was an appropriate tool for scattering the mitotic images showing well-individualized chromosomes, which were used for assembling the karyograms of *C. canephora* and *C. arabica*, and for the IOD estimation of each chromosome by programs of the image analysis system. The ten karyograms of *C. arabica* showed 2n = 44 chromosomes, being five metacentric (7, 8, 13, 14 and 20), 16 submetacentric (1 – 6, 9 – 12, 15 – 19, 21) and one acrocentric (22) pairs. The ten karyograms of *C. canephora* displayed 2n = 22 chromosomes, of which two pairs are metacentric (4, 9) and nine are submetacentric (1 – 3, 5 – 8, 11) (Fig 2 a, b, Table 1).

These results demonstrate that, even though the very small homomorphic chromosomes of coffee have often been considered a great hindrance for cytogenetic characterization (Raina et al. 1998; Lashermes et al. 1999), the cytogenetic methodologies used in this work supplied prometaphasic and metaphasic chromosomes adequate for the karyogram assembly of *C. arabica* and *C. canephora* (Fig 2 a, b).

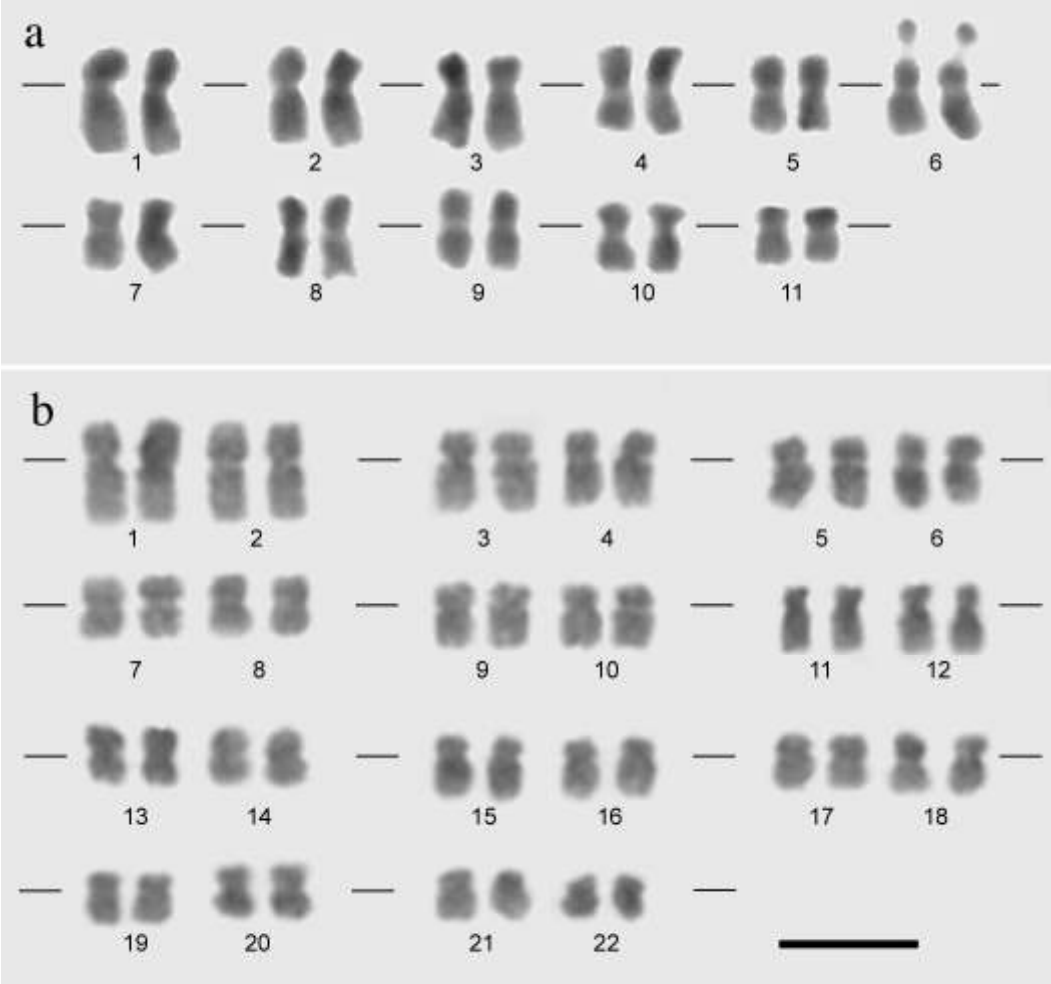

Figure 2 – (a) *C. canephora* and (b) *C. arabica* karyograms assembled from chromosomes stained with Schiff's reagent, obtained from embryogenic cell aggregates treated with 3 μM amiprophos-methyl for 3 h, and showing well-defined centromeric constrictions. a) *C. canephora* karyogram displaying 2n = 22 chromosomes, of which two are metacentric and nine submetacentric. The qualitative and quantitative analyses showed chromosomes exhibiting different cytogenetic characteristics and chromosomal DNA content (1, 2, 3, 4 and 5), and chromosome groups presenting the same chromosomal DNA content and distinct cytogenetic characteristics (6-7, 8-9 and 10-11). b) *C. arabica* karyogram displaying 2n = 44, consisting of five metacentric, 16 submetacentric and one acrocentric pairs. The cytogenetic and ICM analyses evidenced distinct (1, 2, 19, 20, 21 and 22) and identical (3 – 4, 5 – 6, 7 – 8, 9 – 10, 11 – 12, 13 – 14, 15 – 16 and 17 – 18) chromosomes. Bar = 5 μm.

Table 1 – Chromosomal DNA content measured from of the nuclear DNA content distribution, previously established by FCM, proportionally to the average IOD values, quantified by ICM.

C. canephora					*C. arabica*				
Chrom[a]	Class[b]	2C DNA	1C DNA	bp (x 10^9)[c]	Chrom[a]	Class[b]	2C DNA	1C DNA	bp (x 10^9)[c]
1*	S	0.180	0.090	0.088	1*	S	0.180	0.090	0.088
2	S	0.160	0.080	0.078	2	S	0.170	0.085	0.084
3	S	0.150	0.075	0.073	3-4	S	0.150	0.075	0.073
4	M	0.130	0.065	0.063	5-6	S	0.130	0.065	0.063
5	S	0.130	0.065	0.063	7-8	M	0.120	0.060	0.059
6	S	0.120	0.060	0.059	9-10	S	0.120	0.060	0.059
7	S	0.120	0.060	0.059	11-12	S	0.110	0.055	0.054
8	S	0.110	0.055	0.054	13-14	M	0.110	0.055	0.054
9	M	0.110	0.055	0.054	15-16	S	0.110	0.055	0.054
10**	S	0.100	0.050	0.049	17-18	S	0.100	0.050	0.049
11***	S	0.100	0.050	0.049	19**	S	0.100	0.050	0.049
					20	M	0.100	0.050	0.049
					21***	S	0.100	0.050	0.049
					22	A	0.060	0.030	0.029

[a]Chrom – chromosome; [b]Class: M – metacentric, S – submetacentric and A – acrocentric (determined by arm ratio – long/short); [c]1C mean values converted to bp (base pairs), considering that 1 pg of DNA corresponds to 0.978 x 10^9 bp (Doležel et al. 2003); *, ** and ***, chromosomes with identical cytogenetic characteristics (class, total length, short and long arm sizes) and chromosomal DNA content.

Using the absolute nuclear DNA content, previously established by FCM, and the average IOD values, quantified by ICM, the mean DNA content was measured for all chromosomes of *C. canephora* and *C. arabica* (Table 1). It is important to emphasize that the protocol established in this study was appropriate for determining the DNA amount of each chromosome of *C. canephora* and *C. arabica*.

Using the flow karyotyping technique, Lee et al. (2002) quantified the chromosomal DNA content of *Zea mays* lines. In spite of this species shows ten morphologically distinct chromosomes (Carvalho et al. 1993; 1997; Caixeta and Carvalho 2000; 2001; Lee et al. 2002), Lee et al. (2002) only measured the chromosomal DNA content of chromosomic groups, since it was not possible to individualize each chromosome. Unfortunately, the application of flow karyotyping in plants is cumbersome inasmuch as, in comparison to human cells, the root meristem shows low mitotic index and a cell wall (Veuskens et al. 1999). Chromosome instability, monochromatidic chromosome formation (Veuskens et al. 1999) and absence of significant differences in DNA content among chromosomes (Doležel et al. 2001, 2004) also constitute factors that have hindered flow karyotyping analyses. Besides, Lee et al. (2002) mentioned that the chromosomal DNA content measured by flow karyotyping is inferior to the nuclear genome size, a fact also observed by Lee et al. (2004).

Bogunic et al. (2003) reported the chromosomal DNA amount of five *Pinus* species by dividing the total genome size by the mean size of the chromosomes. However this procedure is not considered adequate for accomplishment of quantitative analyses, since the specific differences of each chromosome are not available. Comparing the 2C DNA content of *Triticum* spp. and *Secale cereale* with other parameters measured with the image analysis system, Lee et al. (2004) observed that the chromosome density volume, calculated by multiplying the chromosomal area and the mean gray value, is more reliable for genome size estimation in comparison with total chromosome length and chromosome area.

In this study, the qualitative and quantitative analyses evidenced structurally identical chromosomes and with the same chromosomal DNA content in the karyotype of *C. arabica* (3 – 4, 5 – 6, 7 – 8, 9 – 10, 11 – 12, 13 – 14, 15 – 16 and 17 – 18). As well as related by Orozco-Castillo et al. (1996),

Pinto-Maglio and Cruz (1998), Raina et al. (1998) and Lashermes et al. (1999), this result suggests that *C. arabica* originated from a cross between two diploid *Coffea* species with similar genomes. Furthermore, the cytogenetically distinct chromosome pairs of *C. arabica* (1, 2, 21 and 22) also exhibited different chromosomal DNA content. Conversely, the chromosome pairs 19 and 20, which present distinct morphology, exhibited the same chromosomal DNA content (Fig. 1b, Table 1). These data confirm the allotetraploid nature of the genome of *C. arabica*.

C. canephora chromosomes 1, 2, 3, 4 and 5 exhibited different cytogenetic characteristics and chromosomal DNA content. In contrast, the chromosome groups 6 – 7, 8 – 9 and 10 – 11 showed the same chromosomal DNA content, but distinct total length, arm ratio or chromosome class (Fig. 1a, Table 1).

In this approach, it was also observed that the morphologically distinct chromosomes of *C. arabica* (1, 19 and 21 – submetacentric with 2C = 0.18, 0.10 and 0.10 pg, respectively) possess cytogenetic characteristics (class, total length, short and long arm sizes) and chromosomal DNA content identical to those of *C. canephora* chromosomes 1, 10 and 11, respectively. This fact indicates that *C. canephora* is a possible progenitor of *C. arabica*, as also observed by Lashermes et al. (1997, 1999) and Ruas et al. (2003) in their molecular researches.

The quantitative and reproducible technique presented in this study, associating cytogenetic and cytometric tools, was suitable for measuring the DNA content of each chromosome of *C. canephora* and *C. arabica*. The values of chromosomal DNA content shown in this work, reported in pg and bp, increase de cytometric information available about the genome of both, economically most important coffee species. The new cytometric and cytogenetic data on *Coffea* provided evidence that *C. arabica* is a true allotetraploid, and that *C. canephora* is a possible diploid progenitor of that species. Besides, these results can contribute with molecular biology, genome sequencing projects and breeding programs in coffee.

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