STÉFANIE CRISTINA DE OLIVEIRA

ORIGIN OF THE ALLOTRIPLOID "HÍBRIDO DE TIMOR" THROUGH A KARYOTYPE COMPARISON WITH ITS *COFFEA* ANCESTORS

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

ALEGRE

ESPÍRITO SANTO – BRASIL

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Wellington Ronildo Clarindo

(Prof. Dr. UFES – Orientador)

Aos meus pais José Francisco e Márcia
À minha filha Marjorie

Dedico

"Jamais considere seus estudos como uma obrigação, mas como uma oportunidade invejável para aprender a conhecer a influência libertadora da beleza do reino do espírito, para seu próprio prazer pessoal e para proveito da comunidade à qual seu futuro trabalho pertencer".

Albert Einstein

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BIOGRAFIA

STÉFANIE CRISITNA DE OLIVEIRA, filha de José Francisco de Oliveira e Márcia Cristina de Oliveira, nasceu em Barbacena, Minas Gerais, em 08 de julho de 1986.

Em fevereiro de 2005, iniciou o curso superior na Universidade Presidente Antônio Carlos (UNIPAC), Barbacena, Minas Gerais, colando grau em janeiro de 2009 como Biotecnóloga. No mesmo ano recebeu a Medalha Presidente Antônio Carlos pela dedicação e envolvimento durante todo o curso de Biotecnologia. No período de sua graduação, desenvolveu atividades de pesquisa além de estágios na Embrapa Gado de Leite (Juiz de Fora, MG) e no Laboratório de Associações Micorrízicas, do Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO/Universidade Federal de Viçosa).

Em agosto de 2010, iniciou o curso de Mestrado no Programa de Pós-Graduação em Produção Vegetal da UFES, na área de Biotecnologia e ecofisiologia do desenvolvimento de plantas, defendendo dissertação em julho de 2012. Em agosto de 2013, iniciou o curso de Pós-Graduação em nível de Doutorado, no Programa de Genética e Melhoramento da Universidade Federal do Espírito Santo (UFES), submentendo-se à defesa de tese em dezembro de 2017.

SUMÁRIO

Title: Origin of the allotriploid "Híbrido de Timor" through a karyotype comparison with its *Coffea* ancestors.

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Resumo

Entre as espécies *Coffea*, existe um híbrido natural denominado "Híbrido de Timor" (HT), encontrado na Ilha de Timor em 1927. HT 'CIFC 4106', o qual representa a primeira planta, possui 2n = 3x = 33 cromossomos e valor 1C DNA igual a 1C = 2.10 pg. O número cromossômico, o conteúdo de DNA e evidências geográficas, suportam uma possível origem alotriploide a partir da fusão de uma célula reprodutiva reduzida de *Coffea arabica* (2n = 4x = 44) com outra célula, também reduzida, de *Coffea canephora* (2n = 2x = 22). *C. arabica*, outro alopoliploide pertencente ao gênero, acumula estudos que buscam desvendar seus progenitores. Dados moleculares e cariotípicos sugerem que este alotetraploide verdadeiro seja formado a partir de uma célula reprodutiva reduzida de *C. canephora* (CC) e *C. eugenioides* (EE), seguido por um evento de poliploidização. Neste sentido, acredita-se que o genoma de *C. arabica* seja representado como $C^aC^aE^aE^a$. Com base nas evidências mencionadas, formulamos a seguinte hipótese: o genoma de HT 'CIFC 4106' é CC^aE^a? O presente estudo caracterizou citogenicamente *C. eugenioides*, *C. canephora*, *C. arabica* e HT 'CIFC 4106'. A combinação de dados morfométricos, conteúdo de DNA nuclear e cromossômico e hibridização in situ fluorescente (FISH) com rDNA 5S, expandiu o conhecimento sobre a origem evolutiva e a estrutura do genoma de HT 'CIFC 4106'. O cariograma de HT 'CIFC 4106' evidenciou pares e grupos cromossômicos delimitados de acordo com o tamanho total, classes e conteúdos de DNA cromossômicos. Com base nessas características cariotípicas, foi possível inferir a presença de dois genomas idênticos em HT 'CIFC 4106', possivelmente de *C. canephora* (CC) e um genoma distinto (*C. eugenioides*, E). Os cromossomos de HT 'CIFC 4106' apresentaram classe, conteúdo de DNA idênticos aos cromossomos de *C. eugenioides*, *C. canephora* e *C.*

arabica. Padrões de distribuição de sinais 5S em HT 'CIFC 4106' foram similares aos encontrados nos possíveis progenitores *C. eugenioides* e *C. canephora*. Os dados revelados neste estudo corroboram com a hipótese CC^aE^a do genoma de HT 'CIFC 4106'.

Palavras-chave: Café, alopoliploidia, citogenética, citometria de imagem, FISH.

Abstract

Among the *Coffea*, there is a natural hybrid denominated “Híbrido de Timor” (HT), found on Timor Island in 1927. HT 'CIFC 4106', which represents the first HT plant, possesses 2n = 3x = 33 chromosomes and 1C DNA value equal 1C = 2.10 pg. Chromosomal number, nuclear 1C DNA content and geographical evidences support a possible allotriploid origin from fusion of a reduced reproductive cell of *Coffea arabica* (2n = 4x = 44) and *Coffea canephora* (2n = 2x = 22). *C. arabica*, another allopolyploid, accumulates studies that seek to unravel its progenitors. Molecular and karyotype data suggests that this true allotetraploid was formed from a reduced reproductive cell of *C. canephora* (CC) and another *C. eugenioides* (EE) followed by a polyploidization event. In this way, *C. arabica* genome is represented as $C^aC^aE^aE^a$. Based on this evidences, we formulate the following hypothesis: HT ‘CIFC 4106’ genome is CC^aE^a? The present study aimed to cytogenetically characterize *C. eugenioides*, *C. canephora*, *C. arabica* and HT 'CIFC 4106'. The combination of morphometric data, flow and image cytometries and 5S rDNA fluorescente in situ hybridization (FISH) expanded knowledge about the evolutive origin and genome structure of HT ‘CIFC 4106’. HT 'CIFC 4106' karyogram evidenced chromosomes group delimited according to the class and DNA value similar. Based on these karyotype features was possible show the presence of two identical genomes in HT 'CIFC 4106', possibly of *C. canephora* (CC) and a distinct genome (*C. eugenioides*, E). HT 'CIFC 4106' chromosomes presented class, DNA content identical to the chromosomes of *C. eugenioides*, *C. canephora* and *C. arabica*. 5S rDNA sites were detected in two HT ‘CIFC 4106’ chromosomes. Distribution patterns of 5S markings found in *C. eugenioides* and *C. canephora* revealed in this study corroborate with HT ‘CIFC 4106’ CC^aE^a genome hypothesis. The

distribution of the 5S rDNA sites found in three *Coffea* allied to the chromosomal DNA content and class shows chromosomes similar to *C. eugenioides* and *C. canephora* in HT 'CIFC 4106'. In addition, the data reveal possible structural chromosomal rearrangements along the evolution of HT 'CIFC 4106'.

Keywords: Coffee, allopolyploid, cytogenetic, image cytometry, FISH.

Introduction

Allopolyploids are polyploids formed from hybridization, following or no by duplication of the two divergent genomes. As well as autopolyploidy (polyploidy within or between populations of a single specie), the allopolyploidy is considered an important mechanism of speciation in plants (Stebbins 1950, Soltis and Soltis 2009, 2012). This relevance is corroborated by the abundance of species allopolyploids in nature (Liu and Wendel 2003). Informations based on origin and karyotype changes in natural allopolyploid is scarce (Mallet 2007, Soltis et al. 2009; 2016). Cytogenetic studies provide information on the evolution and diversification of the species, identifying potential progenitors and numerical and structural chromosome changes in allopolyploids (Chester et al 2012).

Coffea genus, Rubiaceae family, has been target of evolution researches, mainly about the natural allopolyploid genomes (Hamon et al. 2009, 2015). This genus is composed of approximately 120 species, which show natural distribution in Africa and the Indian Ocean Islands, southern and southeast Asia, and Australasia (Hamon et al 2015, 2017). Between these species, one allopolyploid speciation event is recognized, such as the formation of the true allotetraploid *Coffea arabica* L. (2n = 4x = 44 chromosomes; Clarindo and Carvalho 2009, Lashermes et al. 1999, Yu et al. 2011,). In addition, the formation of the natural triploid ‘‘Híbrido de Timor’’ (HT) ‘CIFC 4106’ (2n = 3x= 33) has recently been appointed (Capucho et al. 2009, Setotaw et al. 2010, Clarindo et al. 2013).

All *Coffea* species deposited in Chromosome Counts Database and Index to Plant Chromosome Numbers are diploids (2n = 2x = 22), except *C. arabica* (Rijo 1974), *Coffea jenkinsii* Hook (2n = 8x = 88, Mehra and Bawa 1969; CCDB 2017 –

http://ccdb.tau.ac.il/search/) and *Coffea excelsa* A. Chev. (2n = 4x = 44, Selvaraj 1987; IPCN 2017 – http://www.tropicos.org). Nuclear 2C value in *Coffea* varies between the diploid species from 1.03 pg for *Coffea racemosa* Lour to 1.80 pg for *Coffea humilis* A. Chev, reflecting also the polyploid condiction, as 2C = 2.62 pg to *C. arabica* (Noirot et al. 2003; Bennett and Leitch 2011) and 1C = 2.10 pg for HT 'CIFC 4106' (Clarindo et al. 2013). The chromosome number and nuclear 1C value remain constant in all HT 'CIFC 4106' individuals, which have vegetatively been propagated by cutting (Clarindo et al. 2013) and tissue culture (Sattler et al. 2016; Sanglard et al. 2017). Nuclear genome size of HT "CIFC 4106" will be referred to as 1C because its homologous pairs are not defined. Karyotypic studies in HT 'CIFC 4106' are necessary to confirm their origin and characterize possible homologous pairs.

Coffea diploid species have a monophyletic origin, from an ancestor (Lashmeres et al. 1996; Mahé et al. 2007; Yu et al. 2011) with basic chromosome number of x = 11, identical to the one currently represented by the genus (Mahé et al. 2007). *Coffea* diploid species diversification has been estimated about 5 – 25 Mya (Lashermes et al. 1996) or ~4.2 Mya (Yu et al. 2011). True allotetraploid *C. arabica* was originated in ~0.665 Mya (Yu et al. 2011). *Coffea arabica* was probably formed from a natural hybridization between two diploid *Coffea* species, followed by a polyploidization event (Lashermes et al. 1999; Cenci et al. 2012; Fig. 1).

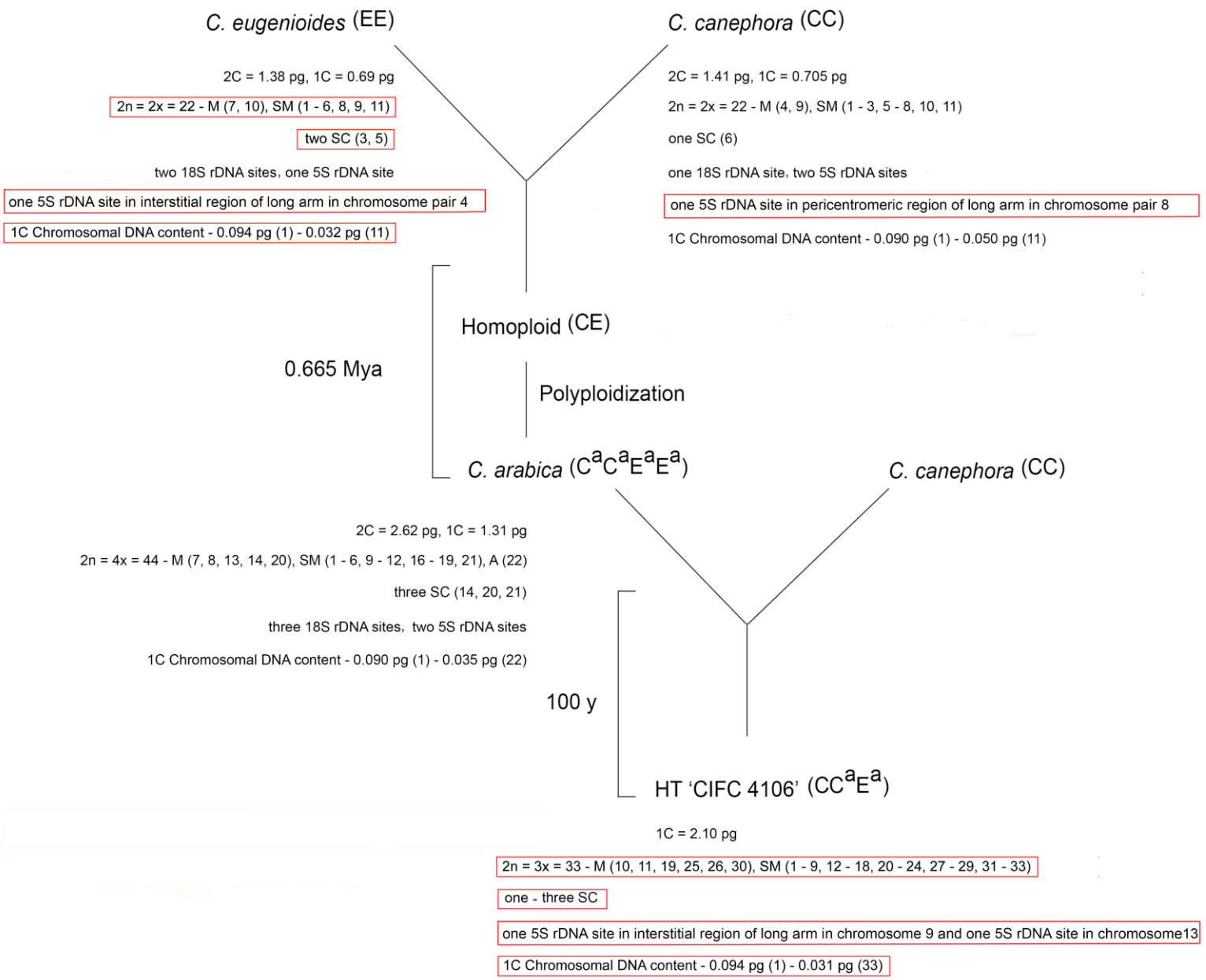

C. eugenioides (EE)
2C = 1.38 pg, 1C = 0.69 pg
2n = 2x = 22 - M (7, 10), SM (1 - 6, 8, 9, 11)
two SC (3, 5)
two 18S rDNA sites, one 5S rDNA site
one 5S rDNA site in interstitial region of long arm in chromosome pair 4
1C Chromosomal DNA content - 0.094 pg (1) - 0.032 pg (11)
C. canephora (CC)
2C = 1.41 pg, 1C = 0.705 pg
2n = 2x = 22 - M (4, 9), SM (1 - 3, 5 - 8, 10, 11)
one SC (6)
one 18S rDNA site, two 5S rDNA sites
one 5S rDNA site in pericentromeric region of long arm in chromosome pair 8
1C Chromosomal DNA content - 0.090 pg (1) - 0.050 pg (11)
Homoploid (CE)
0.665 Mya
Polyploidization
C. arabica (C^aC^aE^aE^a)
C. canephora (CC)
2C = 2.62 pg, 1C = 1.31 pg
2n = 4x = 44 - M (7, 8, 13, 14, 20), SM (1 - 6, 9 - 12, 16 - 19, 21), A (22)
three SC (14, 20, 21)
three 18S rDNA sites, two 5S rDNA sites
1C Chromosomal DNA content - 0.090 pg (1) - 0.035 pg (22)
100 y
HT 'CIFC 4106' (CC^aE^a)
1C = 2.10 pg
2n = 3x = 33 - M (10, 11, 19, 25, 26, 30), SM (1 - 9, 12 - 18, 20 - 24, 27 - 29, 31 - 33)
one - three SC
one 5S rDNA site in interstitial region of long arm in chromosome 9 and one 5S rDNA site in chromosome13
1C Chromosomal DNA content - 0.094 pg (1) - 0.031 pg (33)

Fig. 1 Karyotype features and origin of allopolyploids *C. arabica* and HT 'CIFC 4106'. Karyotype data about these *Coffea* were recorded from cytogenetic bibliographies (Pinto-Maglio and Da Cruz 1998; Noirot et al. 2003; Clarindo et al. 2006; Clarindo et al. 2009; Hamon et al. 2009) and from the unprecedented results of this study (red box). For these *Coffea* were reported: 2C and/or 1C nuclear DNA content measured by flow cytometry; 2n chromosome number; ploidy level; class of each chromosome of the karyotype (M – metacentric, SM – submetacentric, A – acrocentric); number and chromosome with secondary construction (SC); number of 18S and 5S rDNA sites; 1C chromosomal DNA content. The number de SC found in the HT 'CIFC 4106' prometaphases and metaphases varied of one (12 mitotic cells, Fig. 2k) to 3 (one mitotic cell, Fig. S1).

Due to evolutive and agronomic relevance, studies have focused to know the *C. arabica* progenitors. Some species have been pointed as possible progenitors: *Coffea eugenioides* S. Moore (Berthou et al. 1983; Orozco-Castillo et al. 1996; Cros et al. 1998; Raina et al. 1998; Lashermes et al. 1999), *Coffea canephora* Pierre ex A. Froehner (Berthou et al. 1983; Lashermes et al. 1997; Lashermes et al. 1999; Clarindo and Carvalho 2009), *Coffea congensis* A. Froehner (Berthou et al. 1983; Lashermes et al. 1997; Raina et al. 1998), and *Coffea brevipes* Hiern (Lashermes et al. 1997). *Coffea canephora* (CC) and *C. eugenioides* (EE) are the more probably ancestors of *C. arabica* ($C^aC^aE^aE^a$). Corroborating to this hypothesis, the sum of the number of 18S rDNA (two sites in *C. eugenioides,* one site in *C. canephora*) corresponds to the number of *C. arabica* (three sites) (Hamon et al. 2009). From morphometry (total and arms lenghts, and class) and DNA content, *C. arabica* chromosomes were compared with the of *C. canephora* (Clarindo e Carvalho 2009), one of the potential ancestors (Berthou et al. 1983; Lashermes et al. 1997; Lashermes et al. 1999). Chromosomes 1, 19 and 21 (submetacentrics, with 1C = 0.090, 0.050 and 0.050 pg, respectively) of *C. arabica* were considered identical to chromosomes 1, 10 and 11 of *C. canephora*, respectively

(Clarindo e Carvalho 2009). An accurate comparison between *C. arabica*, *C. canephora* and *C. eugenioides* karyotypes would deepen the knowledge of the probable relationship of these three *Coffea* and HT ‘CIFC 4106’.

Supposed first HT plant was found in Timor Island in 1927 and can be represented by access 'CIFC 4106'. HT ‘CIFC 4106’ triploid condiction (2n = 3x = 33 chromosomes; 1C = 2.10 pg; Clarindo et al. 2013) is consistent with the hypothesis of the first plant of HT originated from the fusion of a reproductive reduced cell of true allotetraploid *C. arabica* ($C^aC^aE^aE^a$) and another, also reduced, of *C. canephora* (CC), generating a semi-fertile allotriploid (Fig. 1). This hyphotesis is corroborated by historical evidences, since some HT plants were found in an area where these two *Coffea* species were cultivated (Rijo 1974). Besides, this germplasm has 1C = 2.10 pg of nuclear DNA content equivalent to the sum of the 1C value of the reproductive cells of *C. canephora* (1C = 0.73 pg, n = x = 11 chromosomes) and *C. arabica* (1C = 1.355 pg, n = 2x = 22 chromosomes) (Clarindo et al. 2013). However, the genomic composition HT 'CIFC 4106' is not known.

Based on previous karyotype and genome size data of HT ‘CIFC 4106’ (Clarindo et al. 2013; Sattler et al. 2016; Sanglard et al. 2017) and *C. arabica* (Noirot et al. 2003; Clarindo and Carvalho 2008, 2009; Hamon et al. 2009), our hypothesis is appointed: the allotriploid genome HT 'CIFC 4106' is possibly composed of one chromosomal set of *C. eugenioides* (E^a) and two of *C. canephora* (CC^a), representing a CC^aE^a genome (Fig. 1).

This study researched data that answer the following question: HT ‘CIFC 4106’ genome is CC^aE^a? Considering this hypothesis, the combination of cytogenetic (morphometry and chromosome class), flow and image cytometries (nuclear and chromosomal DNA content) and fluorescente in situ hybridization (FISH) data will

enlarge knowledge about the evolutionary origin and genome composition of HT 'CIFC 4106'. Thus, the aims of this study were to: a) measure the nuclear genome size, b) confirme the chromosome number, c) characterize the karyotype, d) calculate the chromosomal DNA content of the close related *Coffea* (*C. eugenioides*, *C. canephora*, *C. arabica* and HT 'CIFC 4106'), e) map 5S rDNA site by FISH in HT 'CIFC 4106' and possible ancestors *C. eugenioides* and *C. canephora*. Besides, this study also provided new insights about *C. arabica* origin and karyotype evolution.

Material and methods

Biological material

In vitro regenerated plantlets of *C. eugenioides*, *C. canephora*, *C. arabica* and HT 'CIFC 4106' were used as explant donors (Fig. 2a) for friable callus establishment (Fig. 2b), somatic embryo (Fig. 2c) and seedling regeneration (Fig. 2d). These biological materials were source of mitotic cells for cytogenetic and cytometry procedures.

Nuclear 2C value

Nuclei suspensions were obtained from simultaneously chopping (Galbraith et al. 1983; Otto 1990; Praça-Fontes et al. 2011) of the leave fragment (2 cm^2) of *S. licopersycum* 'Stupicke' (internal standard, 2C = 2.00 pg, Praça-Fontes et al. 2011) and of each one of the ten plantlets (Fig. 2e) of *C. eugenioides*, *C. canephora*, *C. arabica* and HT 'CIFC 4106' (samples). Staining of the nuclear suspensions was performed following international criteria (intercalant fluorochrome – propidium iodide Sigma®)

associated to methodological steps of Praça-Fontes et al. (2011). The suspensions were analyzed in a Partec PAS® flow cytometer (Partec® Gmbh, Munster, Germany) equipped with a laser source (488 nm). Relative fluorescence intensity histograms (Fig. 2f) were analyzed by the FlowMax Partec® software (Partec Gmbh, Munster, Germany). G_0/G_1 nuclei peak of *S. lycopersicum* was the reference for nuclear 2C value measurement of the *Coffea* (Fig. 2f).

Tissue culture biological material

For friable calli establishment (Fig. 2b), leaf fragments (1 cm²) from in vitro plantlets of each *Coffea* were excised and inoculated (Fig. 2a), with the abaxial surface facing upwards, in Petri dishes containing callus induction medium (Table S1). After 3 months, 0.5 g of friable calli was transferred to Erlenmeyers with 30 ml of callus proliferation medium without Phytagel (Fig. 2h, Table S1). The tissue culture conditions were modified by van Boxtel and Berthouly (1996). Four subcultures, every 15 days, were performed during the first 60 days. The Erlenmeyers were maintained on shaker at 100 rpm, under a 16/8 h light/dark regime, with 36 µmol m^{-2} s^{-1} light radiation and 24°C ± 2°C (Fig. $2h^1$). After the stabilization period of the culture, the cell aggregate suspensions were used for chromosome obtaining (Fig. $2h^2$).

Roots of distinct *Coffea* plantlets (Fig. 2g) were also used to expose at the different cytogenetic treatments. In order to regenerate the plantlets, friable embryogenic calli (Fig. 2b) were transferred to conversion and maturation medium (Fig. 2c; Table S1). Subsequently, the mature cotyledonary somatic embryos (Fig. 2d left) were transferred for germination medium (Fig. 2d right, Table S1). After the first root removing, the same seedlings were maintained in medium of the same prior

composition, but without GA_3. From the regenerated plantlets, root meristems were routinely excised and cytogenetically treated (Fig. 2g).

Prometaphases and metaphases obtention

Roots and cell aggregate suspensions were treated with 4 µM amiprophos-methyl (APM, Sigma®) (Clarindo and Carvalho 2009) or with 4 µM APM/95.0 µM cycloheximide (Sigma®, dos Reis et al. 2014), for 1 – 8 h at 25°C (Fig. 2g, h^2). Cell aggregate suspensions were treated between the period of 7 to 10 days after four subcultures, equivalent to 60 days (Fig. $2h^2$). The biological materials were washed in distilled water (dH_2O), fixed three times in 3:1 methanol:acetic acid (Sigma®), and stored for 24 h at -20°C. About ten Erlenmeyers of cell aggregate suspensions and 50 roots of each *Coffea* were used for each treatment.

Next, each root meristem (Fig. $2g^1$) or a sample of 20 – 30 aggregates (~100 µL volume) was transferred to microtubes (2 ml), washed three times with dH_2O, and enzymatically macerated for 1 – 2 h, at 34°C (Fig. $2g^2$, h^3). The enzymatic pool was composed of 4% cellulase (C-1184, Sigma®), 0.4% hemicellulase (H-2125, Sigma®) and 1% macerozyme (R10, Yacult Pharmaceutical®) diluted in pectinase (E-6287, Sigma®). The pool was diluted in dH_2O in the proportion 1:40 – 1:100. The dilution was adjusted according to the thickness of the roots and aggregates. After, the biological materials were washed for 10 min in dH_2O, fixed and stored at -20°C (Clarindo and Carvalho 2009).

Slides, from root meristems or aggregates, were prepared by dissociation and air-drying techniques (Fig. 2i, Carvalho and Saraiva 1997). The slides were previously

evaluated and selected under a Nikon Eclipse Ci phase contrast microscope (Nikon, Japan), following the criteria:

a) mitotic cells with little or no cytoplasmic background,

b) chromosomes with well-defined telomere and centromere,

c) chromosomes without overlaps and structural deformations of the chromatin.

For ICM (Fig. 2j,k), the selected slides of all *Coffea* were fixed in 17:5:1 methanol:37% formaldehyde:acetic acid (Sigma®), at 25°C for 24 h (Carvalho et al. 2011). For FISH (Fig. 2k) (*C. eugenioides, C. canephora and* HT 'CIFC 4106') the slides were fixed in 70% ethanol at -20°C for at least 15 days, or maintained at 37°C for five days.

Morphometric analysis and chromosomal DNA content

Feulgen's reaction procedure was performed in the chromosome preparations following guideline by Carvalho et al. 2011. For this, the slides were washed in dH_2O, promptly air-dried and hydrolyzed in 5 M HCl (Merck®) for 12 – 22 min at 25°C. Posteriorly, the slides were stained with Schiff's reagent for 12 h at 4°C, washed three times (3 min each time) in 0.5% SO_2 water.

In order to ensure optical conditions suitable for ICM, the Nikon Eclipse 80i model (Nikon, Japan) microscope and digital analysis system (CCD digital video camera DS-Fi1c of 8-bits gray and Nis Elements 3.0 imaging software – Nikon, Japan) were calibrated and configured by means stability, linearity and uniformity tests (Carvalho et al. 2011). Prometaphases and metaphases were scattered using microscope Nikon 80i model (Nikon, Japan – equipped with a stabilized light source, a 100x Nikon Pan Fluor oil immersion objective with 1.30 numerical aperture, aplanat achromat

condenser with 0.7 aperture, ND6 neutral density filter, and 550 – 570 nm interference green color filter) coupled to a monochromatic CCD digital video camera DS-Fi1c of 8-bits gray (Nikon, Japan), which is coupled to a Pentium Intel Core i5 (Termaltake – Asus) computer featuring the Nis Elements 3.0 imaging software (Nikon, Japan). At least twenty prometaphases and/or metaphases of each *Coffea* were chosen for morphometric analysis and chromosomal DNA content measurement.

DNA content of each chromosome of the *Coffea* was measured by distributing the mean value of the 1C nuclear DNA content (pg), obtained by FCM, proportionally to the mean integrated optical density (IOD) values of each chromosome, calculated by ICM (Carvalho et al. 2011). Besides, the chromosomes were characterized as to the total length, length of the long and short arms, and class. The chromosome class was determined as proposed by Levan et al. (1964) and reviewed by Guerra (1986): r = length of the long arm/length of the short arm.

5S rDNA site mapping

The probe 5S rDNA used for FISH was generated by PCR using forward and reverse primers of 5S rDNA (F:5'-CCTGGGAAGTCCTCGTGTTG-3' and R:5'-CTTCGGAGTTCTGATGGGAT-3') previously selected (Ribeiro et al. 2016). Genomic DNA were extracted according Doyle and Doyle (1990), using leaves from four HT 'CIFC 4106' plantlets in vitro regenerated. DNA concentration and purity were determined by NanoDrop (Thermo Scientific® 2000c). DNA integrity was verified by agarose gel electrophoresis.

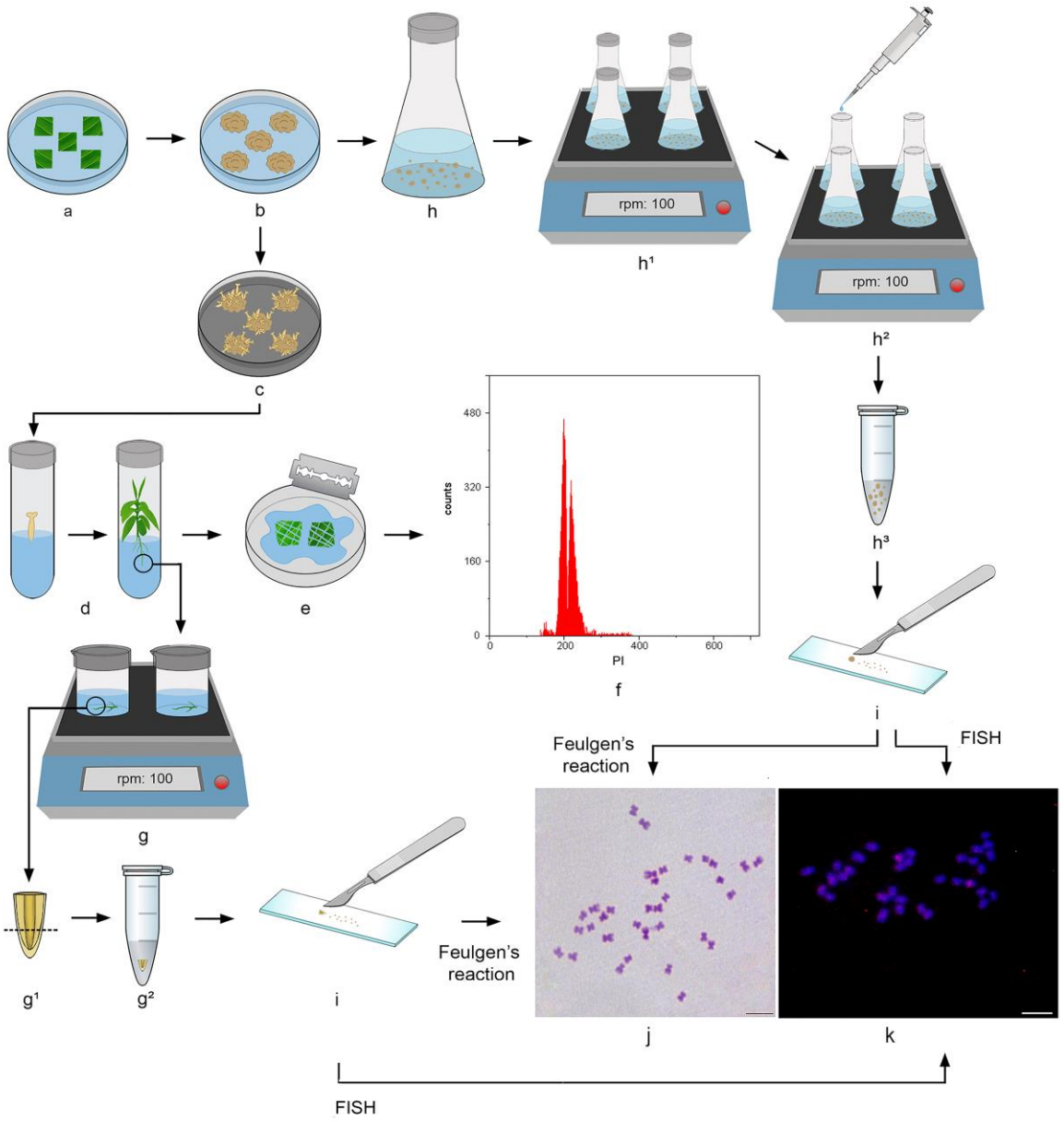
Polymerase chain reaction (PCR) was performed with a final volume of 25 µl, containing 200 ng DNA, 1x buffer (GoTaq®), 0.5 µM of each primer, 1.3 mM of each

dNTP, 1.25 U of Taq DNA polymerase (GoTaq®) and 3.0 mM $MgCl_2$. The amplification conditions were initial denaturation for 5 min at 95°C; 35 amplification cycles at 95°C for 1 min, 60°C for 1 min and 72°C for 1 min 30 sec; and final extension at 72°C for 5 min. The reactions were conducted in an Applied Biosystems Veriti™ 96-Well Thermal Cycler. PCR products were separated by electrophoresis in agarose (1.2%) gel with buffer 1X TBE (Tris/Borate/EDTA). The gel was visualized by staining with GelRed™.

5S rDNA probe was prepared using 200 ng of the HT 'CIFC 4106' PCR product amplification (Supplement Fig. 2); 2 mM of each dATP, dCTP and dGTP, 0.0005µM tetramethil-rhodamine 5-dUTP (Roche®); 0.5 µM 5S rDNA primers; 3.0 mM de $MgCl_2$; 1 U GoTaq® DNA polymerase (Promega Biotechnology®); 1× GoTaq® buffer in a final volume of 25 µL. PCR reactions were performed with the same above condictions. The labeling products were analyzed by 1.2% agarose gel electrophoresis.

Slides were treated in 1X PBS buffer for 5 min, 3% formaldehyde solution in PBS buffer for 15 min, followed by 1X PBS buffer for 5 min and ethanol series (70, 85, 100%) at -20°C. Hybridization mix consisted of 300 ng of each probe, 50% formamide (Sigma-Aldrich®), 2X SSC buffer and dH_2O in final volume of 35 µL. The mixture was placed on the slide and covered with HybriSlip™ plastic cover slip (Sigma®) and sealed with Rubber Cement (Elmer's). The hybridizations were conducted in thermociclador (SlideCycler Loccus®) with initial denaturation at 71°C for 4 min and 37°C for 24 h. After, the stringency washes were conducted in 2X SSC solution at 58°C. The slides were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich®) and sealed with colorless enamel. Prometaphases and metaphases were captured by a monochromatic CCD digital video camera DS-Fi1c of 8-bits gray

(Nikon, Japan) coupled to a epifluorescence microscope Nikon 80i model (Nikon, Japan) with a 100X objective lens, UV-2E/C (DAPI) and G2A (5S rDNA probe).


a
b
h
rpm: 100
h¹
rpm: 100
h²
c
h³
d
e
counts
480
320
160
0
0
200
400
600
PI
f
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Feulgen's reaction
FISH
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g
g¹
g²
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Fig. 2 Guide for obtention of *Coffea* prometaphasic/metaphasic chromosomes and flow cytometry from regenerated seedlings and cell aggregate suspensions provided via indirect somatic embryogenesis. **a)** Five leaf fragments (~1 cm^2) of *Coffea* inoculated, with the abaxial surface facing upwards, in Petri dishes containing callus induction medium. **b)** Calli established after 3 months in callus induction medium, exhibiting friable aspect and pale yellow color. **c)** Friable embryogenic calli in conversion and maturation medium. During the 180 days, friable calli showed globular, heart, torpedo, pre-cotyledonary and mature cotyledonary somatic embryos. **d)** Seedlings recovered in germination medium from the mature cotyledonary somatic embryo. **e – f)** Representative histogram, which was obtained from flow cytometry procedure (**e**), exhibiting fluorescence peaks of G_0/G_1 nuclei of the standard *S. lycopersicum* (chanel 200) and HT 'CIFC 4106' (chanel 210) (**f**). **g – g^2)** Antitubulin exposuring (**g**) and enzymatic maceration (g^1 and g^2) of the excised *Coffea* roots, highlighting that root meristem should be isolately macerated. **h – h^1)** Erlenmeyers on shaker at 100 rpm with the cell aggregate suspension maintained in callus induction medium without Phytagel. **h^2 – h^3)** Antitubulin exposuring (h^2) and enzymatic maceration (**h^3)** of the of the cell aggregation suspensions of the four *Coffea*, emphasizing that 20 – 30 aggregates (~100 µL volume) should be macerated. **i)** Slides prepared by dissociation and air-drying techniques from root meristems (**c** – g^2) or aggregates (**h** – h^3). **j)** HT 'CIFC 4106' karyotype showing 2n = 3x = 33 chromosomes stained from Feulgen's reaction, with one chromosomes exhibiting the SC in the short arm. **k)** FISH of the 5S rDNA in HT 'CIFC 4106'showed two 5S markings. Bars = 5µm.

Results

Tissue culture biological material

Friable calli (Fig. 2b) were obtained after 3 months from leaves of all *Coffea*. The calli exhibited friable aspect and pale yellow color in callus induction medium (Table S1). After transferring of these calli to callus proliferation medium, the cell aggregate suspensions yielded proliferative cells (Table S1; Fig. 2h). Aggregates showed an increase in 0.5 g their biomass every 15 days, indicating the cell proliferation occurrence in the aggregates. Cell division was confirmed by the presence of cells at different stages of the cell cycle (early, middle and late prophases and prometaphases, and metaphases) on the cytogenetic slides.

During 180 days in embryo conversion and maturation medium, friable calli showed globular, heart, torpedo, pre-cotyledonary and mature cotyledonary somatic embryos. From the mature cotyledonary somatic embryo, seedlings were recovered in germination medium (Fig. 2d left). Thus, cell aggregate suspensions (Fig. 2h) and plantlets (Fig. 2d right) for all *Coffea* continuously provided enough biological material for nuclear DNA measurement (leaves, Fig. 2e, f), chromosome number determination (Fig. 2j), karyotype characterization, chromosomal DNA content estimation, and 5S rDNA site mapping (Fig. 2).

Nuclear 2C value

Mean nuclear 2C value was 2C = 1.38 ± 0.012 pg (1C = 0.690 pg) for *C. eugenioides*, 2C = 1.41 ± 0.037 pg (1C = 0.705pg) for *C. canephora*, 2C = 2.62 ± 0.004 pg (1C = 1.310 pg) for *C. arabica* and 1C = 2.100 ± 0.008 pg for HT ‘CIFC 4106’ (Fig.

2). The mean nuclear DNA content of the regenerated plantlets was the same to the explant donor plants.

Prometaphases and metaphafases obtention

Considering the criteria assumed in the present study to chose the slides, the treatments that generated prometaphases and metaphases for *C. eugenioides*, *C. canephora*, *C. arabica* and HT 'CIFC 4106' from cell aggregates and root meristems were: 7 h in 4 µM APM and 2 – 3 h in 4 µM APM/95 µM cycloheximide. Times over 8 h in 4 µM APM or over 3 h in 4 µM APM/95 µM cycloheximide resulted cells with chromosomes highly compacted, making it impossible to distinguish the chromosomes in morphometric analyzes.

Enzymatic maceration and dissociation of the cell aggregates and root meristems, and air drying technique were crucial for the obtaining of chromosomes scattered, without overlapping and free of cytoplasm. These aspects were obtained from cell aggregates and ~1 mm root meristems macerated with 1:40 (enzymatic pool: dH_2O), and also from ~0.5 mm root meristems macerated in 1:70 – 1:80 (enzymatic pool: dH_2O), for 2 h at 34°C. For all the *Coffea*, stoichiometrically stained chromosomes were only found in the slides fixed in 17:5:1 methanol:37% formaldehyde:acetic acid for 24 h at 25°C, hydrolyzed in 5 M HCl for 18 min at 25°C, and stained with Schiff's reagent for 12 h at 4°C (Fig. 2j).

Morphometric analysis and chromosomal DNA content

For the first time, the karyotypes of *C. eugenioides* and HT 'CIFC 4106' were characterized and assembled. *C. eugenioides* and *C. canephora* showed 2n = 2x = 22 chromosomes, *C. arabica* 2n = 4x = 44 and HT 'CIFC 4106' 2n = 3x = 33 (Fig. 3). *C. eugenioides* karyogram (Fig. 3a, Table 1) is composed by nine chromosome pairs classified as submetacentrics (1 – 6, 8, 9 and 11) and two metacentric (7 and 10). Two chromosome pairs (3 and 5) highlighted due to their secondary constrictions in the short arm (Fig. 3a). *C. canephora* displayed nine submetacentric chromosome pairs (1 – 3, 5 – 8, 10, 11) and two metacentric (4, 9), and presence of secondary constriction on chromosome pair 6 (Fig. 3b, Table 1). *C. arabica* exhibited sixteen submetacentric chromosome pairs (1 – 6, 9 – 12, 15 – 19, 21), five metacentric (7, 8, 13, 14 and 20) and one acrocentric (22) (Fig. 3c, Table 1). HT 'CIFC 4106' possesses six chromosomes (10, 11, 19, 25, 26, 30) classified as metacentrics, and the other twenty-seven (1 – 9, 12 – 18, 20 – 24, 27 – 29, 31 – 33) as submetacentrics (Fig. 3d, Table 1). The number of secondary constrictions varied between the HT 'CIFC 4106' prometaphases/metaphases of one to three (Fig. 1, Fig. S1).

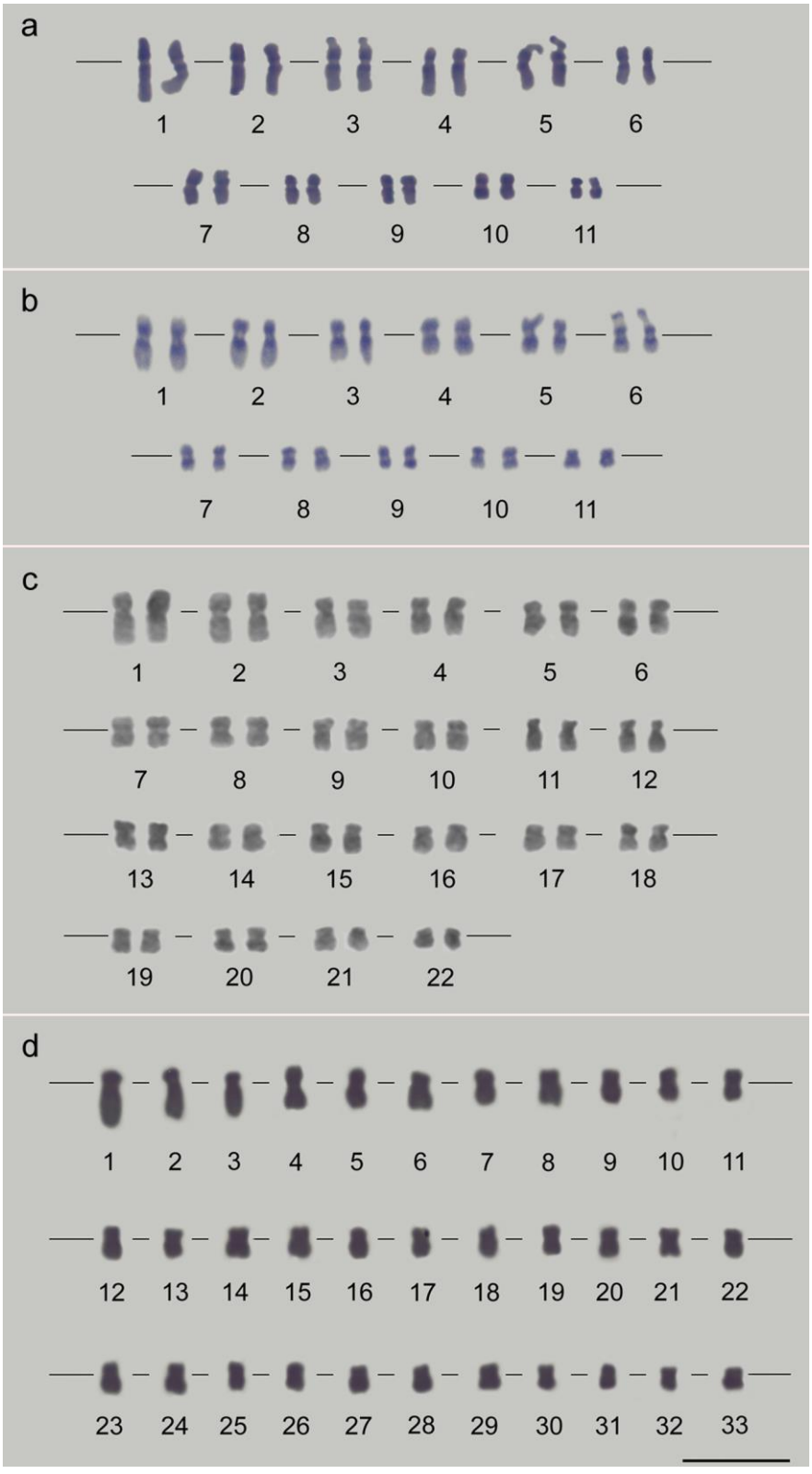

Fig. 3 Karyogram from mitotic chromosomes of *C. eugenioides* **a**, *C. canephora* **b**, *C. arabica* **c**, and HT 'CIFC 4106' **d**. **a** *C. eugenioides* showed two metacentric chromosome pairs (7, 10), nine submetacentrics

(1 – 6, 8, 9 and 11) and two chromosomes pairs with SC (3 and 5). **b** *C. canephora* presented two metacentric chromosome pairs (4 and 9), nine submetacentric (1 – 3, 5 – 8, 10 and 11) and one with SC (6). **c** *C. arabica* exhibited 5 metacentric chromosome pairs (7, 8, 13, 14 and 20), sixteen submetacentric (1 – 6, 9 – 12, 15 – 19 and 21) and one acrocentric (22). **d** HT 'CIFC 4106' showed six metacentric (10, 11, 19, 25, 26, 30) and twenty-seven submetacentric chromosomes (1 – 9, 12 – 18, 20 – 24, 27 – 29, 31 – 33). *Bar = 5 µm*.

Table 1 Cytogenetic and chromosomal DNA content (pg) of ancestors *C. eugenioides*, *C. canephora* and allopolyploids *C. arabica* and HT 'CIFC 4106'.

C. eugenioides			*C. canephora*		*C. arabica*		HT 'CIFC 4106'	
Chromosome	Class	1C DNA (pg)	Class	1C DNA (pg)	Class	1C DNA (pg)	Class	1C DNA (pg)
1	S	0.094	S	0.090	S	0.090	S	0.094
2	S	0.085	S	0.080	S	0.085	S	0.090
3	S	0.070	S	0.075	S	0.075	S	0.090
4	S	0.069	M	0.065	S	0.075	S	0.085
5	S	0.065	S	0.065	S	0.065	S	0.081
6	S	0.062	S	0.060	S	0.065	S	0.080
7	M	0.059	S	0.060	M	0.060	S	0.077
8	S	0.054	S	0.055	M	0.060	S	0.076
9	S	0.052	M	0.055	S	0.060	S	0.070
10	M	0.048	S	0.050	S	0.060	M	0.066
11	S	0.032	S	0.050	S	0.055	M	0.066
12					S	0.055	S	0.066
13					M	0.055	S	0.066
14					M	0.055	S	0.060
15					S	0.055	S	0.060
16					S	0.055	S	0.060
17					S	0.050	S	0.060
18					S	0.050	S	0.060
19					S	0.050	M	0.060
20					M	0.050	S	0.059
21					S	0.050	S	0.059
22					A	0.035	S	0.059
23							S	0.059
24							S	0.055
25							M	0.055
26							M	0.055
27							S	0.055
28							S	0.050
29							S	0.050
30							M	0.050
31							S	0.048
32							S	0.048
33							S	0.031
Total nuclear 1C/2C		0.69/1.38 pg		0.705/1.41 pg		1.31/2.62 pg		2.10 pg

Mean values of chromosomal DNA content ranged from 0.094 (chromosome 1) to 0.032 pg (chromosome 11) in *C. eugenioides*, 0.090 (chromosome 1) to 0.050 pg (chromosome 11) in *C. canephora*, 0.090 (chromosome 1) to 0.035 pg (chromosome 22) in *C. arabica*, and 0.094 (chromosome 1) to 0.031 pg (chromosome 33) in HT 'CIFC 4106' (Table 1).

HT 'CIFC 4106' chromosomes were separated into groups or individually according to total length, class, centrometric index and 1C chromosomal DNA content (Fig. 4).

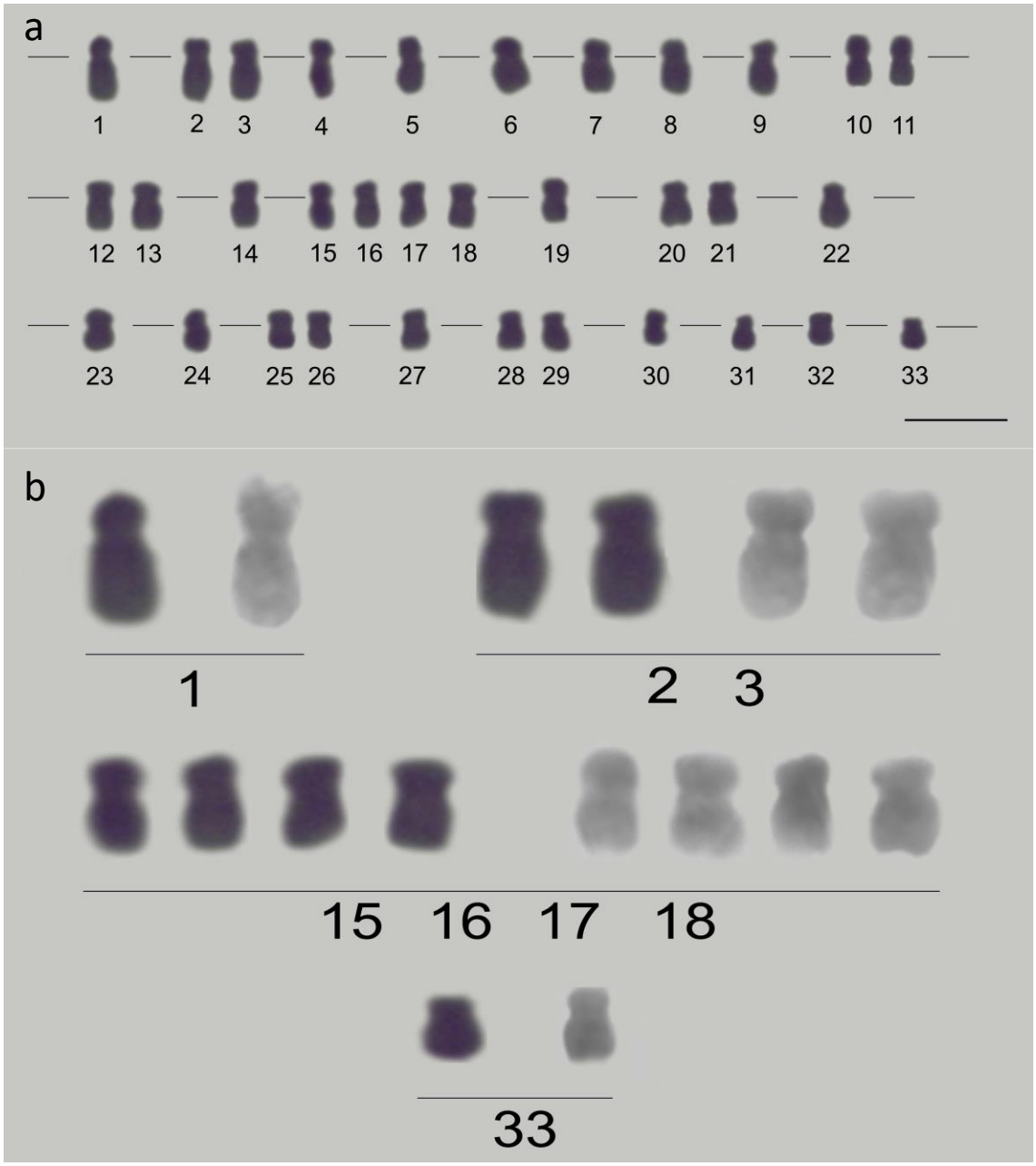

Fig. 4. HT 'CIFC 4106' karyogram (above) **a)** evidencing the individual and chromosome groups defined according to total lenght, class, morphometry (centromeric index – data not shown) and 1C chromosomal DNA content. 17 chromosomes (1, 4 – 9, 14, 19, 22 – 24, 27, 30 – 33) showed at least one particular cytogenetic feature. The grouped chromosomes were 2 – 3, 10 – 11, 12 – 13, 15 – 18 (four chromosomes), 20 – 21, 25 – 26, and 28 – 29. Spotlight (below) **b)** of individual (1, 33) and grouped chromosomes (2 and 3, 15 – 18) scaterred of distinct prometaphases of the HT 'CIFC 4106'. Bar = 5 µm.

5S rDNA site mapping

Amplification conditions allowed to obtain amount of DNA, ~686.00 ng/µL for individual 1 of HT 'CIFC 4106' and ~700 ng/µL for individual 2. Product of amplification of 5S rDNA showed a clear and well defined band of approximately 500 base pairs (bp) (Fig. S2).

Signals of FISH with 5S rDNA were evidenced from 8 to 20 metaphases of *C. eugenioides*, *C. canephora* and HT 'CIFC 4106'. The 5S rDNA loci were observed in the interstitial portion of the long arm on chromosomes 4 in *C. eugenioides*, in *C. canephora* on in pericentromeric region of the long arm of the chromosome 8 and HT 'CIFC 4106' interstitial long arm of the chromosomes 9 and 13 (Fig. 5, Fig. S3).

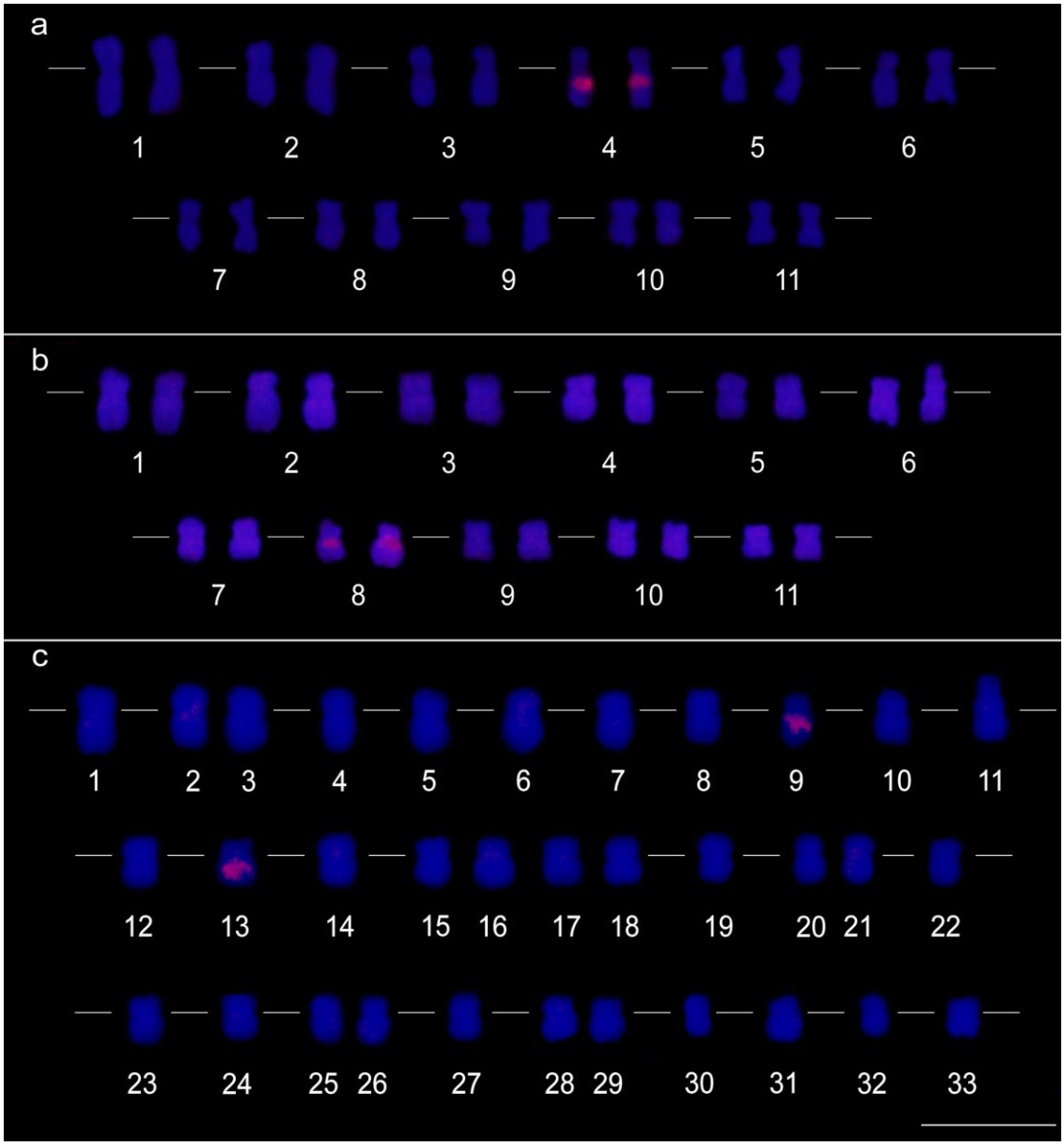

Fig. 5. Mapping of 5S rDNA regions (red signals) using 5S gene probe detected with tetramethil-rhodamine 5-dUTP on karyotypes counterstained with 40 ,6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue). **a)** *C. eugenioides* in interstitial long arm of the chromosome 4. **b)** *C. canephora* in pericentromeric region of the long arm of the chromosome 8. **c)** HT 'CIFC 4106' in interstitial long arm of the chromosomes 9 and 13. Bars = 5 µm.

Discussion

Based on previous studies, *Coffea* chromosomes are considered relatively small and homomorphic (Lashermes et al. 1999; Pinto-Maglio 2006; Hamon et al. 2009). These aspects are presented as obstacles to the assembly and analysis of karyotypes (Hamon et al. 2009, Clarindo and Carvalho 2009). In this sense, the characteristics of the preparations containing mitotic chromosomes are a crucial factor for the advancement of studies of karyotype evolution of *Coffea*. Although these aspects, our results demonstrated that the methodologies utilized in this work provided slides with adequate chromosomes according to the criteria mentioned above for *Coffea* cytogenetic analyzes.

Roots (Fig. 2d) and cell aggregate suspensions (Fig. 2h) presented proliferative cells, which are a biological material fundamental for the cytogenetic procedures. Approximately 100 slides of each with prometaphases and metaphases were generated, allowing to test all subsequent steps, mainly the enzymatic maceration, HCl hydrolysis in image cytometry and FISH procedure. Of these slides, about 10% for each *Coffea* showed chromosomes according to criteria determined in this study, and, then, were used in the prometaphase/metaphase capture step. Cell aggregate suspensions and root meristems provided cells with a stable chromosome number in each *Coffea*. The biological materials from tissue culture were confirmed as an alternative source for obtaining mitotic cells in *Coffea* (Clarindo and Carvalho 2006, 2009). Therefore, the in vitro condition guarantees the availability of *Coffea* biological material for procedures during a long time.

In addition to this care, three bottlenecks were considered for morphometry and DNA content measurement of the chromosomes: the antitubulin treatment with APM or

APM/cycloheximide, the enzymatic maceration, and the hydrolysis exposure time in 5 M HCl. The best antitubulin treatments of 4 µM APM or 4 µM APM/ 95 µM cycloheximide presented prometaphases/metaphases with chromosomes in sufficient amount, at least 20 per slide. APM/cycloheximide combination for prometaphase/metaphase arrest was an unprecedented cytogenetic result for *Coffea*. This combination associated the effect of two different chemical compounds. APM is a phosphoric amide compound that inhibits the polymerization of microtubules (Morejohn and Fosket 1984; Planchais 2000). Cycloheximide is an inhibitor of eukaryotic translation, blocking the elongation phase of the nascent polypeptide, specifically acting under the E-site of the 60S ribosomal subunit (Schneider-Poetsch 2010). Thus, proteins, such as the α- and β-tubilins, are not synthesized with the addition of cycloheximide. However, due to this cycloheximide cellular effect, different stages of the cell cycle can be blocked, depending on the momentum of the cell cycle in which that substance is added (Rose 1970). Therefore, the success of using a compound that inhibits protein synthesis, such as cycloheximide, is related to the proliferative state of the biological material. In this sense, in biological materials with higher rates of cell division, as in this study, this substance will act on a larger number of cells in prometaphase and metaphase. In this way, as observed here, the combination of APM with cycloheximide accumulated a higher number of prometaphase and metaphase cells than the treatment with APM.

The maceration of the cell aggregations or root meristems involved an enzymatic pool containing four types of enzymes (cellulase, hemicellulase, macerozyme and pectinase), unlike previous protocols for *Coffea* using only pectinase (Clarindo and Carvalho 2006, 2008, 2009). This pool was effective due to specific act on different components of the cell wall. This strategy resulted slides with few or without

cytoplasmic traces, facilitating to adjust the time for HCl hydrolysis in image cytometry and accessibility of the 5S rDNA probe during FISH procedure.

Duration of the HCl hydrolysis treatment was identical for all *Coffea*. The time of 18 min in 5 M HCl, associated to 12 h of the staining in Schiff's reagent, provided stoichiometrically stained chromosomes without chromatin damage. HCl hydrolysis, during Feulgen's reaction, removes purine bases of the deoxyribose, giving free aldehyde groups when the sugar ring opens. Pararosaniline present in the Schiff's reagent binds to the aldehyde groups becoming purple (Greilhuber 2008).

The reproducibility of fluorescent markings of 5S rDNA probe analyzed of three *Coffea* metaphases showed that the FISH procedure was adequate (Fig. 5). The success of this technique was attributed to the cytogenetic preparations according to the selection criteria established in the present study such as chromatin integrity, absence of cytoplasmic vestiges added to stringency conditions during FISH. High stringency conditions was ensured by probe concentration, 50% formamide and 2X SSC solution buffer at 58°C. Formamide is a denaturing agent capable of interacting with the hydrogen bonds present in the double helix, destabilizing the DNA (Blake and Delcourt 1996, Fuchs et al. 2010). Once the DNA duplex was destabilized, the hybridization efficiency was decreased at high concentrations of formamide, increasing stringency. These conditions enable a specific marking by reducing the background.

The CC^aE^a hypothesis about HT 'CIFC 4106' genome was raised based on historical evidences (Rijo 1974), chromosome number, nuclear 2C value (Clarindo et al. 2013), and origin of its possible progenitor *C. arabica* (Lashermes et al. 1999; Hamon et al. 2009). Historical evidences suggest the origin of the HT from *C. canephora* and *C. arabica*, because the first plants were found in a plantation of *C. arabica* 'Typica' ($C^aC^aE^aE^a$) which also contained some *C. canephora* (CC) plants (Rijo 1974). Besides,

chromosome number (2n = 3x = 33) determined for HT ‘CIFC 4106’ (Fig. 1, 2j, 3d) reflects a triploid condition, also from the basic chromosome number x = 11 of *Coffea* (Hamon et al. 2009). Nuclear 1C value of HT ‘CIFC 4106’ is equivalent to 2.10 pg (Fig. 2f; Clarindo et al. 2013). Then, the 2n chromosome number and 1C value of the HT ‘CIFC 4106’ is equivalent to the sum of the reduced reproductive cells of its possible progenitors.

Considering these evidences, the genomic origin of HT ‘CIFC 4106’, as well as of its possible progenitors of the true allotetraploid *C. arabica*, goes back from the monofiletism of the *Coffea* genus (Fig. 1, Yu et al 2011). From the diversification of a common ancestor, all *Coffea* diploid species were originated (Hamon et al. 2017), as *C. eugenioides* (EE) and *C. canephora* (CC) (Lashermes et al. 1999; Yu et al. 2011; Cenci et al. 2012). The fusion of reduced reproductive cells from these two species, following a polyploidization event, resulted in the *C. arabica* ($C^aC^aE^aE^a$) formation (Yu et al. 2011). More recent, the fusion of two reduced reproductive cell between *C. canephora* (C) and of *C. arabica* (C^aE^a) possibly gave rise to HT (Fig. 1).

HT ‘CIFC 4106’ chromosomal number corresponded to 2n = 3x = 33 (Fig. 2j, 3d; Fig. S1, Fig. S3a), the same reported in previous studies (Clarindo et al. 2013; Fig. 1). Therefore, the allotriploidy is a stable karyotype condition in all HT ‘CIFC 4106’ that has been vegetatively propagated: the plants in greenhouse, and the cell aggregate suspensions and regenerated seedlings propagated in vitro. The allotriploid condition supports to the fusion of one reduced reproductive cell of *C. arabica* (C^aE^a; n = 2x = 22) with another also reduced cell of *C. canephora* (C; n = x = 11). Corroborating, nuclear DNA content of the HT 'CIFC 4106' (1C = 2.10 pg, Fig. 2f) reinforces the CC^aE^a hypothesis. This value is equivalent to the sum of the mean nuclear genome size of *C. canephora* (CC; 2C = 1.41 pg, Fig. 1) and half of *C. eugenioides* (E; 1C = 0.690 pg).

In addition to the 2n chromosome number, this study expanded the karyotype data in *Coffea* through the characterization of all chromosomes of the allotriploid HT 'CIFC 4106' and of the diploid *C. eugenioides* (Fig. 3a, d). In order to recount the history of HT 'CIFC 4106' origin, the karyotype characterization also was performed for the diploid *C. canephora* and true allotetraploid *C. arabica*. The karyotypes of the four *Coffea* have morphologically identical and similar chromosomes (Table 1, Fig. 3). The ancestors *C. canephora* and *C. arabica* (also in Clarindo and Carvalho 2009), and *C. eugenioides* predominantly showed metacentric and submetacentric chromosomes, with a single exception for the acrocentric pair (22) of *C. arabica*. Likewise, only metacentric and submetacentric chromosomes were observed in HT 'CIFC 4106' karyotype, supporting the CC^aE^a hypothesis.

DNA content, obtained by the image cytometry, of all chromosomes of the four *Coffea* increased the knowledge about the relation to the ancestors and HT "CIFC 4106'. Image cytometry is a reliable, accurate and relatively affordable (Carvalho et al. 2011) technique for specialized microscopy laboratories. Differently of other techniques that allow to measure the chromosomal DNA content, image cytometry needs of relatively small number of prometaphases/metaphases, at least 10 (Carvalho et al. 2011).

Based on the DNA content and class of the chromosomes of HT 'CIFC 4106' formed pairs as: 2 – 3, 10 – 11, 12 – 13, 20 – 21, 25 – 26, 28 – 29 (six groups). One group of four chromosomes was formed (15 – 18), and the other chromosomes was isolated in class and/or DNA content (Fig. 4, Table 1). The chromosomes in pairs (six groups) can represent the CC^a subgenomes, being C of *C. canephora* and C^a of *C. arabica*. A look at the HT 'CIFC 4106' karyogram shows that the possible pairs 2 – 3, 10 – 11, 25 – 26 and 28 – 29 may represent the chromosomes 1, 4, 9 and 10 or 11 of *C.*

canephora, respectively (Table 1). HT 'CIFC 4106' group 15 – 18 is similar to pair 6 and 7 of *C. canephora* (Fig. 4; Table 1).

Other evidence supporting the $CC^{a}E^{a}$ genome hypothesis, specifically in relation to the E genome, is due to chromosome 1, 4 and 9 of HT 'CIFC 4106' being equal (morphometry and DNA content) to 1, 2 and 3 of *C. eugenioides*, respectively. In addition, chromosomes 7 and 11 of *C. eugenioides* are similar to 19 and 33 of HT 'CIFC 4106', respectively (Fig. 3, Table 1).

Morphometric and chromosomal DNA content data also provided insights about origin of *C. arabica* from *C. canephora* and *C. eugenioides*, which have been pointed as the most probably progenitors (Berthou et al. 1983; Lashermes et al. 1999; Clarindo and Carvalho 2009). These *Coffea* species showed very similar karyotype features, which point to a close relationship between the *C. arabica* and with the *C. canephora* and *C. eugenioides*. Some chromosome pairs of *C. canephora* and of *C. eugenioides* present the same chromosome class and DNA content of some chromosome pairs or of chromosome pair groups of *C. arabica* (Table 1, Fig. 3). The *C. canephora* pair 9 was equal *C. arabica* pair 13 and 14. *C. canephora* pair 10 and 11 were identical *C. arabica* pairs 17, 18, 19 and 21 respectively. This result corroborates that after hybridization at the *C. arabica* origin, the hybrid genome was followed by a genome duplication event. Chromosome class and DNA content provide insights about the participation of the genomes of the *C. canephora* and *C. eugenioides* progenitors in the formation of the allopolyploid *C. arabica*.

rDNA sites are highly conserved genomic regions used to investigate genome origin and organization in several plant species (Cai et al. 2006, Hamon et al. 2009, Karafiátová et al 2013), especially in evaluation of the effects of polyploidy in relation to the number and position of these sequences (Roa and Guerra 2012). In this study,

changes in the position of 5S rDNA site were observed in relation to the ancestors of the HT 'CIFC 4106'. The number of 5S rDNA markers is same for the *Coffea* analyzed, the association of this data assiciated to chromosomal class and DNA content provides evidence of the ancestry of HT 'CIFC 4106'. *C. eugenioides* chromosome 4 and HT 'CIFC 4106' chromosome 9 were both labeled with 5S rDNA in the interstitial long arm and presented similar DNA content of 1C = 0.069 and 1C = 0.070 pg, respectively.. Possibly, HT 'CIFC 4106'chromosome 9 was inherited from a reduced cell of *C. arabica* (C^aE^a), precisely from E^a (*C. eugenioides* ancestral). Number of rDNA 5S sites found in *C. eugenioides* were same reported by Hamon et al. (2009).

Considering the CC^aE^a hypothesis and numbers rDNA sites found in HT 'CIFC 4106' and in *C. eugenioides* and *C. canephora*, chromosome rearragements occurred during the diversification of *C. arabica* and/or HT 'CIFC 4106'. According to HT 'CIFC 4106' allotriploidy, at least 3 rDNA sites were expected. However, one site on chromosome 9 and another on chromosome 13 were observed. In *Tragopogo*n allopolyploids occurred losses of genetic markers correlated with karyotype changes (Tate et al. 2006, 2009).

Similar investigations in synthetic lines and natural population allotetraploids of *Tragopogon mirus* evidenced losses of one 45S rDNA site (Lim et al. 2008). Similar studies were conducted with 5S rDNA probes discriminating genomes from allopolyploid progenies, investigating genetic relationship between the progenitors and their hybrid progenies (Fulneček et al. 2002, Lim et al. 2008, Zhang et al. 2015), studying genome structure in uniform karyotypes (Cai et al. 2006, Hamon et al. 2009).

Conclusions

The data from this study corroborate with CC^aE^a hypothesis of the HT ‘CIFC 4106’ in the following points:

a) the nuclear DNA content of the HT 'CIFC 4106' (1C = 2.10 pg) is equivalent to the sum of the mean nuclear genome size of *C. canephora* (2C = 1.41 pg) and half of *C. eugenioides* (1C = 0.690 pg);

b) HT 'CIFC 4106' chromosome number (2n = 3x = 33) is correspondent to the fusion of one reproductive cell of *C. arabica* (C^aE^a; n = 2x = 22) and one of *C. canephora* (C; n = x = 11);

c) the four *Coffea* have morphologically similar karyotypes, in which predominate metacentric and submetacentric chromosomes;

d) chromosomal class and DNA content allow to infer presence of two identical genomes, possibly of *C. canephora*;

e) FISH with rDNA 5S added to data of chromosome number, class and chromosomal DNA content corroborated with the participation of the genome of *C. eugenioides* and *C. canephora* in the formation of HT ‘CIFC 4106’.

For the first time was describe morphologically the karyograms of *C. eugenioides* and HT ‘CIFC 4106’. From the methodologies adopted in the present study it was possible confirm the basic number of x = 11 for the four *Coffea* and level of ploidy. These data had an impact not only on understanding of HT ‘CIFC 4106’ origin but also on origin of *C. arabica*. The information generated represents the basis and extension of evolutionary studies related to hybrid speciation in *Coffea*.

Supplementary material

Table S1 Composition of the tissue culture media used for roots and cell aggregate suspensions establishement for all *Coffea*.

Compounds	**Culture media**			
	Callus induction medium	**Callus proliferation medium**	**Conversion and maturation medium**	**Germination medium**
MS (Sigma®)	2.15 g L^{-1}	2.15 g L^{-1}	4.3 g L^{-1}	4.3 g L^{-1}
Gamborg's B5 vitamins	10 ml L^{-1}	10 ml L^{-1}	10 ml L^{-1}	10 ml L^{-1}
Sucrose (Sigma®)	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}	30 g L^{-1}
2,4-D (Sigma®)	9.06 µM	9.06 µM	-	-
BAP (Sigma®)	4.44 µM	4.44 µM	4.44 µM	-
GA_3 (Sigma®)	-	-	-	2.89 µM
Malt extract (Sigma®)	0.4 g L^{-1}	0.4 g L^{-1}	0.8 g L^{-1}	-
Casein (Sigma®)	0.1 g L^{-1}	0.1 g L^{-1}	0.2 g L^{-1}	-
L-cysteine (Sigma®)	0.08 g L^{-1}	0.08 g L^{-1}	0.04 g L^{-1}	-
Phytagel (Sigma®)	2.8 g L^{-1}	-	2.8 g L^{-1}	2.8 g L^{-1}
Activated charcoal (Isofar®)	-	-	2.0 g L^{-1}	-
pH	5.6	5.6	5.6	5.6

All media were autoclaved for 20 min at 121°C. The leave fragments in callus induction medium was stored in Petri dishes and maintained in the dark at 24 ± 2°C. Callus proliferation medium for suspensions of cell aggregates establishemnt was maintened in Erlenmeyers, on shaker at 100 rpm, subcultured at each every 15 days for 60 days. Cotiledonary somatic embryos was regenerated in conversion and maturation medium in Petri dishes, and plantlets recovered in germination medium in tubes. These medium, except callus induction medium, were maintained at 24 ± 2°C, under a 16/8 h (light/dark) regime with 36 µmol m^{-2} s^{-1} light radiation provided by two fluorescent lamps (20 W, Osram®). MS: Murashige e Skoog (1962); Gamborg et al. (1968), 2,4-D: 2,4-dichlorophenoxyacetic acid; BAP: 6-benzilaminopurina; GA_3: gibberellic acid.

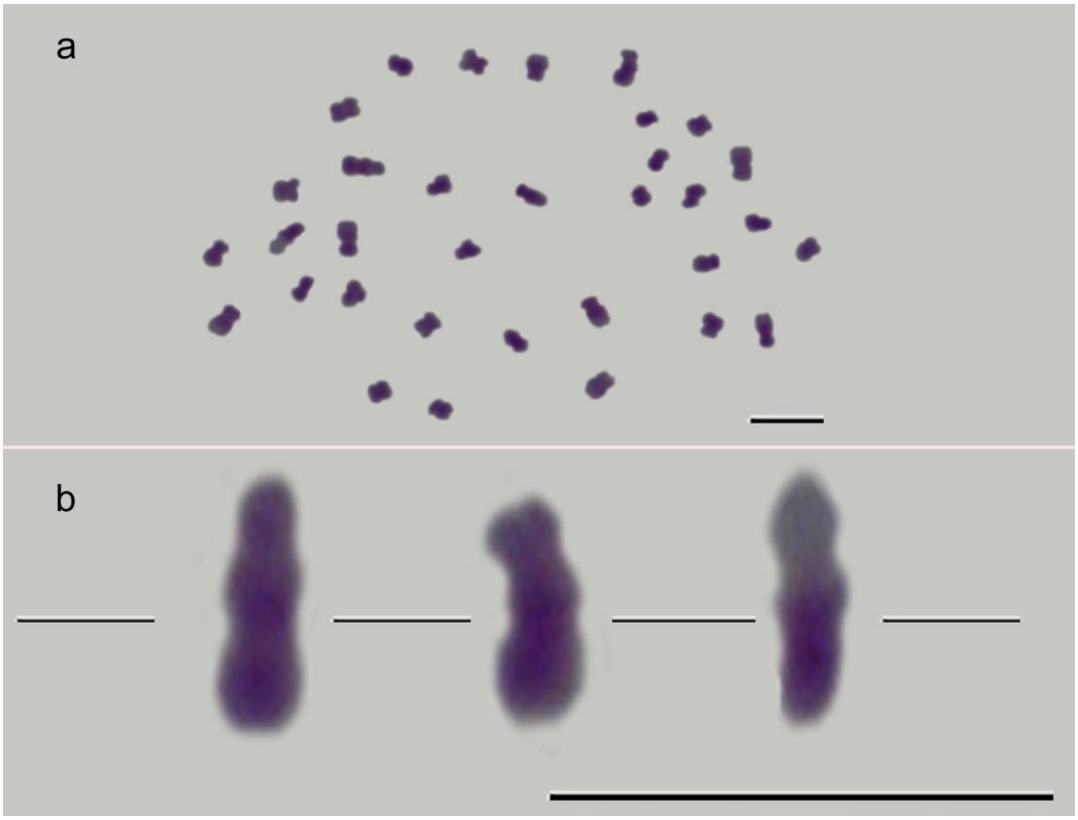

Fig. S1. **a** Karyotype of HT 'CIFC 4106' showing the 2n = 3x = 33 and displaying three chromosomes with well-defined secondary constrictions in the short arm of three chromosomes. **b** Spotlight of the three chromosomes with secondary constriction in the short arm. *Bars = 5µM*.

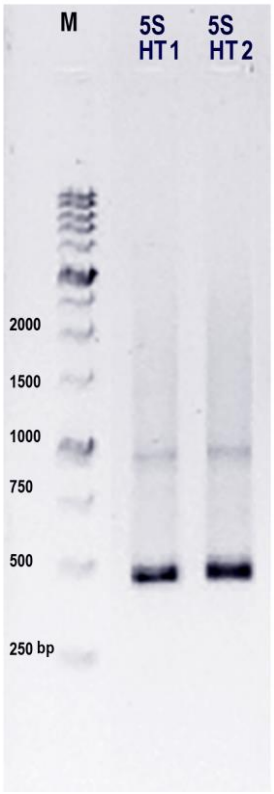

Fig. S2. PCR amplification of 5S rDNA gene in two individuals of HT 'CIFC 4106' for construction of probe from these amplified products. M: 1000 bp ladder marker.

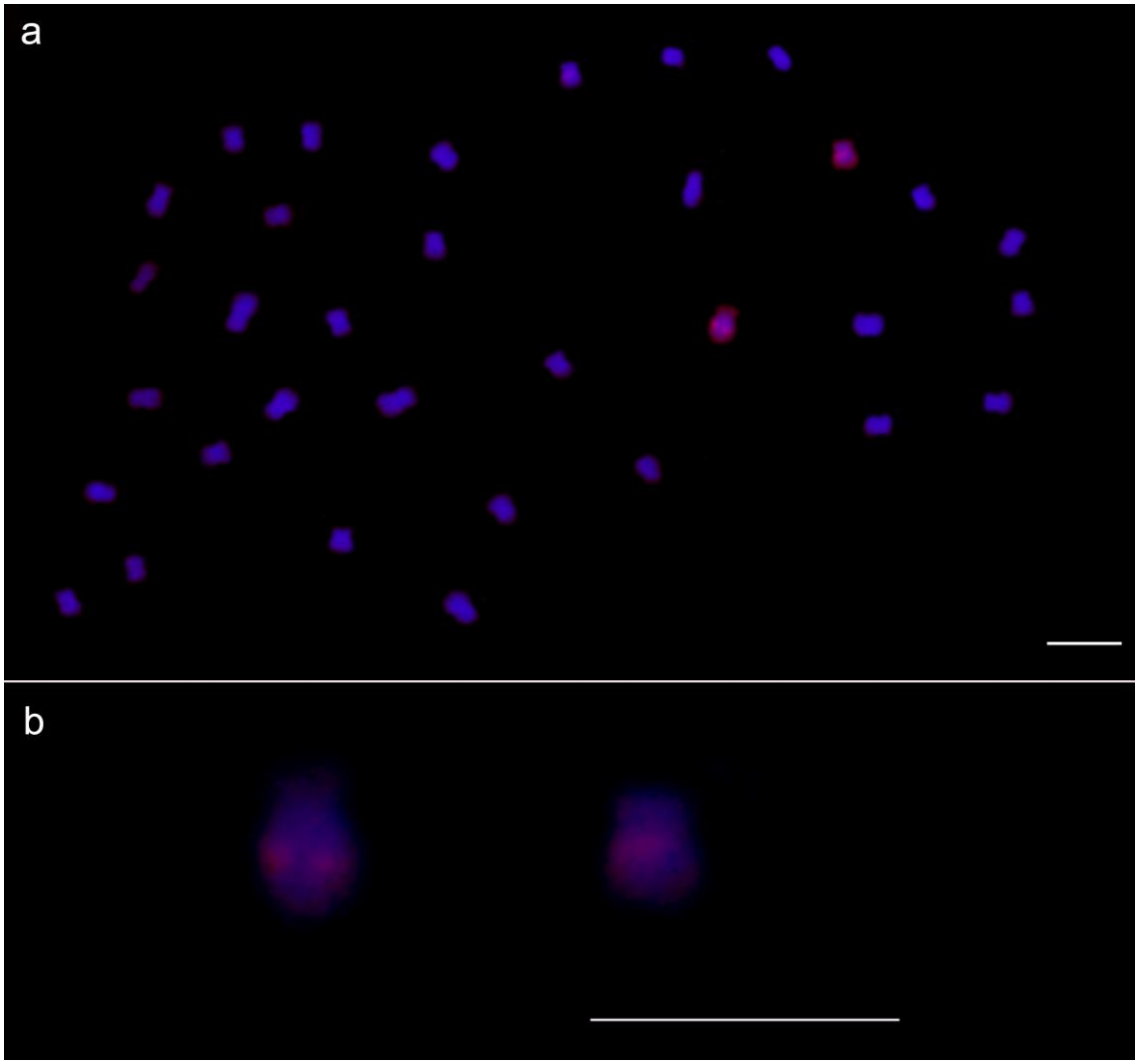

Fig. S3. a) HT 'CIFC 4106' karyotype showed two chromosomes with 5s rDNA (red) marking. **b)** Chromosomes evidencing the 5S rDNA signal in the interstitial region of the long arm of two chromosomes. Bar = 5 µm.

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