GUILHERME MENDES DE ALMEIDA CARVALHO

DETERMINAÇÃO DO TAMANHO GENÔMICO, DA RELAÇÃO AT/CG E DO CARIÓTIPO EM EUCALIPTOS (*Eucalyptus* spp.) POR CITOMETRIA DE FLUXO E CITOGENÉTICA

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: 30 de junho de 2016.

Wellington Ronildo Clarindo

Sílvia das Graças Pompolo

Milene Miranda Praça Fontes

Sérgio Yoshimitsu Motoike

Carlos Roberto de Carvalho
(Orientador)

“Naquele prego
Eu já pendurei meu laço,
O arreio do Picasso,
Cavalo de estimação;
E um par de esporas
Que custou muito dinheiro
E o chapéu de boiadeiro
Que eu lidava no sertão.
Naquele prego
Pendurei muito cansaço,
Muito suor do mormaço
E poeira do estradão;

[...]

De agora em diante
Vou tirar dele meu laço,
O arreio do Picasso
E as esporas vou guardar.
Naquele prego
Pendure uma sacola
Cheia de livros da escola
E vontade de estudar;
Quando amanhã
Você estiver aqui sentado
Lembrando nosso passado
Olhando o prego pioneiro,
Quero que seja
Um doutor bem afamado
E diga sempre em alto brado:
Sou filho de um boiadeiro!”

História de um prego – João Pacífico

À minha família e ao meu laboratório.

Dedico.

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BIOGRAFIA

Guilherme Mendes de Almeida Carvalho, filho de Elvio Lúcio de Carvalho e Maria Socorro Mendes Almeida Carvalho, nasceu em Salinas-MG, no dia 06 de outubro de 1988.

Em 2007, ingressou no curso de Ciências Biológicas na Universidade Federal de Viçosa (UFV), em Viçosa-MG. Já no início do curso se tornou bolsista do Programa de Educação Tutorial (PET-Biologia), onde permaneceu trabalhando por dois anos e meio com as áreas de Ensino, Pesquisa e Extensão. Após esse período, iniciou seus trabalhos como bolsista da FAPEMIG, em projeto intitulado: Determinação do tamanho do genoma, da relação AT/GC e do cariótipo de macaúba (*Acrocomia aculeata*) por citometria de fluxo e citogenética, onde permaneceu até a formatura.

Todos os seus trabalhos de pesquisa foram desenvolvidos no Laboratório de Citogenética e Citometria (Departamento de Biologia Geral), onde iniciou como estagiário em 2008. Em paralelo, às atividades de bolsista, participou do grupo de pesquisa que tinha como objetivo a aplicação de técnicas citométricas no esclarecimento de questões reprodutivas do fungo *Hemileia vastatrix*. Em julho de 2011, se formou como bacharel em Ciências Biológicas, recebendo votos de louvor pelo desempenho acadêmico entre os formandos.

Em agosto de 2011, iniciou o mestrado no Programa de Pós-Graduação em Genética e Melhoramento onde pode seguir trabalhando com a aplicação da técnica de citometria de fluxo no estudo do fungo *H. vastatrix.*

No início de 2013, iniciou o doutorado no mesmo programa. Em junho de 2014 ingressou como professor do ensino básico, técnico e tecnológico no Instituto Federal do Norte de Minas Gerais e desde então vem

desempenhando, simultaneamente, as atividades do doutorado e da docência. Enquanto pesquisa, pode aliar trabalhos na área metodológica para avaliação de genotoxicidade em plantas e citogenética e citometria aplicadas ao estudo genômico em espécies de eucaliptos.

SUMÁRIO

RESUMO

CARVALHO, Guilherme Mendes de Almeida, D.Sc., Universidade Federal de Viçosa, junho de 2016. **Determinação do tamanho genômico, da relação AT/CG e do cariótipo em eucaliptos (*Eucalyptus* spp.) por citometria de fluxo e citogenética**. Orientador: Carlos Roberto de Carvalho.

O gênero *Eucalyptus* é um grupo extremamente bem sucedido de plantas arbóreas, compreendendo mais 700 espécies. Além de sua importância em regiões endêmicas como a Austrália, o eucalipto é importante na economia global devido a sua alta taxa de crescimento, adaptabilidade a várias condições ecológicas (elevação, clima e solo) e uso variado (matéria prima, carvão, fibra, polpa e papel). O estudo do genoma contribui para o entendimento de aspectos evolutivos e outros aspectos biológicos básicos do grupo. O entendimento da natureza de um genoma requer informação sobre o conteúdo de DNA e deveria ser considerada crucial em qualquer programa de análise genômica comparativa. O presente estudo determinou e reavaliou o tamanho do genoma e a composição de bases de 25 espécies de *Eucalyptus.* Além disso, o estudo comparou cariogramas de diferentes espécies, por citogenética clássica e molecular, em busca de possíveis alterações ou regiões não homólogas nos cromossomos de espécies que apresentavam maior diferença no conteúdo de DNA nuclear. No primeiro artigo, foi desenvolvido um protocolo citogenético para a obtenção de cromossomos com uma melhor resolução longitudinal. Assim, foi possível a montagem do cariograma de *E. citriodora* com 2n = 22 cromossomos. No segundo artigo, o valor 2C e a relação AT/CG foram estimados para as 25 espécies de *Eucalyptus*. A partir dos valores do tamanho do genoma os quais variaram entre 2C = 0,91 pg e 2C = 1,37 pg, foi feita uma análise comparativa do cariograma de quatro espécies e nenhuma diferença foi identificada. Em uma abordagem citomolecular, com o uso da hibridização *in situ* do genoma nenhuma região de não homologia cromossômica foi discriminada entre as espécies *E. baileyana* (1,36 pg) e *E. citriodora* (1,01 pg). Os resultados alcançados no presente trabalho corroboram para considerar pequenas alterações do conteúdo de DNA dispersas no genoma, possivelmente provenientes da atividade de elementos transponíveis, como a principal causa da variação do tamanho do genoma em *Eucalyptus.*

ABSTRACT

CARVALHO, Guilherme Mendes de Almeida, D.Sc., Universidade Federal de Viçosa, June, 2016. **Karyogram, genome size and AT/CG base composition in eucalypts (*Eucalyptus* spp.) by cytogenetic and flow cytometry**. Adviser: Carlos Roberto de Carvalho.

The genus *Eucalyptus* represents an extremely successful group of woody plants covering more than 700 species. Besides its importance in the Australian environment, eucalypts are important in the global economy due to their high growth rates, adaptability to various ecological conditions (e.g. elevation, climates, soils) and multiple uses (e.g. raw material, energy wood, timber, pulp and paper). The study of genome contributes to understanding evolutionary aspects of the group and others basic biological processes. A basic understanding of the nature of a given genome requires information regarding the amount of DNA and it should be considered a crucial aspect of any truly comprehensive program of comparative genomic analysis. The present study determinate, as well as revaluate, the size and genomic base composition of 25 *Eucalyptus* species. Furthermore, this study compared karyotypes of different species by classical and molecular cytogenetic looking for possible chromosomal alterations or chromosomal non-homologous regions correlated with the genome size variation among the species. In the first paper, a cytogenetic protocol was developed to obtain of chromosomes with improved longitudinal resolution. Thus, *E. citriodora* karyogram was assembly confirming a karyotype with 2n = 22 chromosomes. In the second paper 2C value and base composition were measured for 25 *Eucalyptus* species. From the genome size differences that range from 2C = 0.91 pg to 2C = 1.37 pg comparative karyological analysis were conducted and no remarkable differences were indentified. In a molecular cytogenetic approach, a genome in situ hybridization experiment was performed and it was not possible discriminate any non-homologous chromosomal regions, between *E. baileyana* (1.36 pg) and *E. citriodora* (1.01 pg). The results achieve in the present work corroborate to considerate small and dispersed DNA content changes, possible due transposable elements activity, as the mainly cause of genome size variation in *Eucalyptus*.

GENERAL INTRODUCTION

Eucalyptus genus represents an extremely successful group of woody plants (Kersting et al., 2015) covering more than 800 taxa (Slee et al., 2006). Besides its importance in the Australian environment (Poke et al. 2006), *Eucalyptus* are important in the global economy, with a worldwide plantation estimated at 19.6 Mha (Iglesias-Trabado and Wilstermann, 2009). The species of the genus posses high growth rates, adaptability to various ecological conditions (e.g. elevation, climates, soils) and multiple uses (e.g. raw material, energy wood, timber, pulp and paper) (Silva et al., 2016).

Latterly, significant increases in productivity have been obtained through *Eucalyptus* pre-selection and breeding, plant protection and management practices (Gonçalves et al., 2013). However, the need for greater productivity have been lead genome-assisted breeding and transgenic technology as strategies (Grattapaglia and Kirst 2008). Biotechnological tools such as artificial polyploidization may represent a remarkable gain in the genetic improvement potentially aiming high performance clones (Lin et al. 2010; Han et al. 2011).

Flow cytometry (FCM) represents a technique with excellent accuracy and dynamic range, high sample throughput rates and relative low cost analysis. Due to this, FCM is accepted as the main method of choice for genome size measurements (Galbraith and Lambert, 2012). Besides its use in C-value estimation, the FCM has been applied to determinate the genomic AT/CG ratio of plant species (Meister and Barow, 2007). Most reports about *Eucalyptus* C-value were provided by FCM analysis (Marie and Brown, 1993; Grattapaglia and Bradshaw, 1994; Pinto et al., 2004; Morgan and Westoby, 2005; Praça et al., 2009; Ribeiro et al., 2016), while some species had their

genome size even measured by image cytometry (Praça et al., 2009), or complete-genome sequencing (Myburg et al., 2014).

Genome study contributes to understanding evolutionary aspects of the group and others basic biological processes (Wendel et al., 2016). A basic understanding of the nature of a given genome requires information regarding to the DNA amount and it should be considered a crucial aspect of any truly comprehensive program of comparative genomic analysis (Gregory, 2005).

Instead of the importance of cytogenetic information in basic knowledge, Sybenga (1992) stated that the information provided by cytogenetic researches is useful in many stages of a breeding program. With respect to the difficulties in phylogenetic studies of the genus, some authors have made efforts to elucidate the evolutionary biology of the *Eucalyptus*, focusing on the genomic data (Myburg et al. 2014; Hudson et al. 2015).

Despite the great economic importance, few studies have engaged in determining the nuclear DNA content in *Eucalyptus* taxa. Thus, this research used FCM technique for determination as well as the revaluation of the size and genomic base composition of 25 *Eucalyptus* species, available in seed banks Research Institutes and Forestry Companies in Brazilian territory. Furthermore, this study compared karyotypes of different species by classical and molecular cytogenetic aiming to identify possible variation on chromosomes morphology or non-homologous regions at chromosomal level correlated with the genome size variation among the species.

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RESEARCH PAPER 1: THE EUCALYPT KARYOGRAM RESOLVED

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ABSTRACT

The genus *Eucalyptus* is the most cytologically studied group of plants in the family Myrtaceae. Despite the ecological and economic importance of this genus much of its biology remains poorly understood. Cytological methods are useful in evolutionary studies; however, karyomorphological descriptions of the *Eucalyptus* species are limited. In the present study, we developed a cytogenetic methodology that facilitates karyogram assembly and chromosome classification of *Eucalyptus citriodora*. The protocol includes treatment of root tips with amiprophos-methyl in Hoagland's solution, slide preparation by cell dissociation, and an air-drying technique. Using this method, well-resolved metaphasic chromosomes with different compaction levels were obtained. A chromosome number of 2n = 22 was confirmed. On the basis of metaphasic chromosomes with limited condensation, *E. citriodora* showed six metacentric and four submetacentric chromosomes, and one submetacentric chromosome with a nucleolus organizer region (NOR). Thus, the informative chromosomes obtained in the present study have enabled the most precise karyotype description of a *Eucalyptus* species, to date.

Key words: cytogenetics; eucalyptus; karyotype; chromosome.

INTRODUCTION

The genus *Eucalyptus* L'Herit. comprises approximately 700 species (Brooker 2000), and is the best studied group of plants at the cytological level in the family Myrtaceae (Grattapaglia et al. 2012). Despite the importance of the genus, many biological aspects of this group remain poorly understood (Jackson et al. 1999).

Cytogenetic information is useful in many stages of a breeding program (Sybenga 1992). In addition, cytogenetic data are essential for the application of biotechnological tools, such as *in vitro* polyploidy induction, that may facilitate significant advances in *Eucalyptus* breeding programs (Lin et al. 2010; Han et al. 2011).

Some authors have made efforts to elucidate the evolutionary biology of the genus *Eucalyptus*, focusing on genomic data (Bayly et al. 2013; Myburg et al. 2014; Hudson et al. 2015). Cytogenetic studies on *Eucalyptus* can provide valuable information on the evolution of the group (Matsumoto et al. 2000). However, the modal chromosome number (2n = 22) of eucalypts (Grattapaglia et al. 2012) limits the use of cytotaxonomy (Atchison 1947).

To date, the chromosome numbers of 135 *Eucalyptus* species have been described (Oudjehih and Abdellah 2006), in which, only seven have their karyotype assembled (Matsumoto et al. 2000). Improvement in the cytogenetic protocols achieved to date for *Eucalyptus* (Gamage and Schmidt 2009), has not generated any information beyond chromosome counting. This indicates the limitation of a karyomorphological approach in cytogenetic studies for *Eucalyptus*. For complete cytogenetic research of the genus *Eucalyptus*, it is imperative to develop a methodology that enables karyogram assembly and comprehensive chromosomal descriptions.

A few cytogenetic studies have been conducted on *Eucalyptus citriodora* Hook. There has been no consensus on the chromosome number for this species; it had first been reported as 2n = 20 (Sugiura 1931) and later as 2n = 22 (Atchison 1947; Haque 1984; Oudjehih and Abdellah 2006). Moreover, the chromosome number represents the only cytogenetic information reported for *E. citriodora*, which is based on the absence of a karyomorphological approach for chromosome study. To the best of our knowledge, the present study is the first to propose a methodology that describes the *E. citriodora* karyotype assembly and classification of chromosome pairs with regard to the centromere position. This procedure provides an improved method for the cytogenetic analysis of *Eucalyptus*.

MATERIAL AND METHODS

Plant Material

Eucalyptus citriodora seeds were kindly provided by Dr. Dário Grattapaglia (Plant Genetics Laboratory, Embrapa Recursos Genéticos e Biotecnologia, Brasília, Distrito Federal, Brazil) and stored at 4°C until use. The cytogenetic analyses were conducted at the Laboratory of Cytogenetics and Cytometry, Department of General Biology, Universidade Federal de Viçosa, Minas Gerais, Brazil.

Cytogenetics

The seeds were placed in Petri dishes with moistened filter paper and maintained in the dark until germination. For chromosome blocking, the root tips of the germinated seeds were incubated in Hoagland's solution (Hoagland and Arnon, 1938) supplemented with 0.5% (w/w) dimethyl sulfoxide (DMSO, Sigma, St. Louis, EUA) and the microtubule inhibiting agent amiprophos-methyl (APM, Nihon Bayer Agrochem K. K., Tokyo, Japan) at a final concentration of 2.6, 3.0, or 3.4 μM for a period of 2:30, 3:00, or 3:30 h at 30°C, totaling nine treatments.

The root tips (2–5 mm) were subsequently washed in distilled water thrice, for 10 minutes each, and then fixed in fresh methanol – acetic acid solution (3:1 v/v; Merck, Darmstadt, Germany). The fixative was changed thrice and the samples were stored at -20°C (Carvalho and Saraiva 1997). The root tips were washed and incubated for 2–3 h at 34°C in an enzymatic digestion solution, at a ratio of 1:8, 1:10, 1:12, or 1:14 v/v of an enzyme mix (cellulase 4%; pectolyase 1%; and hemicellulose 0.4%) and water. Then, the root tips were washed thrice for 10 min in distilled water, fixed again, and stored at -20°C (Clarindo and Carvalho 2006).

The *E. citriodora* slides were prepared by cell dissociation and air-drying techniques, as described by Carvalho et al. (2007), and immediately stained with a 5% Giemsa (Merck) solution in a phosphate buffer (pH 6.8) for 7 min, washed twice in distilled water and air-dried.

Microscopy and digital image

Metaphases were captured with a DP-71 video camera (Olympus, Tokyo, Japan), mounted on a BX-60 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a stabilized light source, an MPlanApo 100X/1.40 objective oil lens, an aplanatic–achromatic condenser with a 1.4 diaphragm aperture, and a neutral density filter (ND6). Image analysis of the digitalized frame was performed using the Image Pro-Plus 6.1 software (Media Cybernetics, MD, USA). The morphometry of the *E. citriodora* chromosomes was characterized according to the arm size (μm) as described by Levan et al. (1964) and reviewed by Guerra (1986). Five high quality metaphases from the best treatment were used for this analysis.

RESULTS

Root tips treated with Hoagland's solution supplemented with 0.5% DMSO and 3 μM APM for 3 h at 30°C showed the best metaphasic indices and chromosomes with variation for chromatin condensation. The procedure of cell dissociation of root meristems incubated in the enzymatic solution (1:10 v/v) for 2 h at 34°C, combined with the air-drying technique generated well-preserved chromosomes, without any cytoplasmic background or overlaps (Fig. 1a, b, and c). Cytogenetic preparations of *E. citriodora* provided well-defined metaphasic chromosomes to be used in image analyses, chromosomal counting, and karyogram assembly (Fig. 1d, e, and f).

Three *E. citriodora* karyograms with different condensation levels were assembled, confirming a karyotype with 2n = 22 chromosomes (Fig. 1). Although well-defined primary constrictions could be identified in all karyograms, an improved longitudinal resolution was obtained by observing metaphasic chromosomes with decreased condensation. These karyograms with different condensation levels were used for ideogram construction and chromosome classification, based on the centromeric position (Table 1, Fig. 2). The chromosome pair that had the secondary constriction, where the nucleolar organizing region (NOR) is found, was arbitrarily positioned as pair n.6, the last of the chromosome pairs considered as large.

As a result, the most condensed karyogram (Fig. 1d; Fig. 2a) presents nine metacentric (1, 2, 3, 4, 5, 7, 8, 9, and 10) and two submetacentric (6 and 11) chromosomes, ranging in size from 0.99 to 2.03 μm. The karyogram with an intermediate condensation level (Fig. 1e; Fig. 2b) shows seven metacentric (1, 2, 3, 5, 7, 8, and 9) and four submetacentric (4, 6, 10, and 11) chromosomes, varying in size from 1.63 to 2.85 μm. The least condensed karyogram (Fig. 1f;

Fig. 2c) shows six metacentric (1, 2, 3, 5, 7, and 9) and five submetacentric (4, 6, 8, 10, and 11) chromosomes, with a size variation of 1.80 to 4.13 μm. The chromosomes 4, 8, and 10 varied in their classification, considering the three different ideograms.

DISCUSSION

Cytogenetic treatments used in this experiment produced clear metaphasic chromosomes with different condensation levels. Previous studies have shown subtle ranges in chromatin condensation (Clarindo and Carvalho 2006, Carvalho et al. 2007) which is common in cytogenetic protocols without the synchronization step.

The use of herbicides such as amiprophosmethyl (APM) as a microtubule-inhibiting agent has proven useful in plant cytogenetics (Carvalho et al. 2007), because of its efficiency at inhibiting mitosis at micromolar concentrations in a short time (Dolezel et al. 2004). Chromosome breaks (Sharma and Sharma 1999), over-condensation of the metaphasic chromosomes (Schwarzacher and Leitch 1994), and abnormal mitosis (Planchais et al. 2000) are avoided. In *Eucalyptus* cytogenetics, chromosome breaks have been reported as the potential reason for the incorrect determination of chromosome number in some species (Oudjehih and Abdellah 2006). Previous cytogenetic studies in *Eucalyptus* used 8-hydroxyquinoline as an inhibitor of mitosis (Matsumoto et al. 2000; Oudjehih and Abdellah 2006; Gamage and Schmidt 2009) and probably resulted in chromosomes with an over-condensed condition. Thus, cytogenetic analysis most of the eucalypt species studied was limited to chromosome counting.

Cell dissociation combined with an air-drying technique was used successfully in the present study. This procedure has been considered indispensable and universal in plant cytogenetic studies (Carvalho et al. 2007). Since this approach avoids the squashing step, it is advantageous for use in *Eucalyptus* cytogenetics because squashing has been related to chromosome breakage (Oudjehih and Abdellah 2006).

The 2n = 22 chromosomes determined for *E. citriodora* here is the same as that stated by Atchison (1947), Haque (1984) and Oudjehih and Abdellah (2006). The karyotype assembly, performed for the first time in this study, enables the identification and classification of all chromosome pairs, based on the centromere position. Some authors have highlighted the small size of *Eucalyptus* chromosomes. According to Atchison (1947), *Eucalyptus* chromosomes vary between 1.2–2.5 μm, while Matsumoto et al. (2000) reported a chromosome size ranging from 0.6 to 1.4 μm for seven *Eucalyptus* species. For *E. citriodora*, Haque (1984) observed homogeneity in the size of all chromosomes (approximately 2.0 μm), except the one with secondary constriction. These values are comparable to our results on the metaphasic chromosomes of highly or intermediary condensed metaphases (Fig. 1a, b). Even in the highly condensed metaphases, the identification and pairing of the homologous chromosomes was possible. This led to the conclusion that the limitations in the cytogenetic analysis of *Eucalyptus* are not only due to the small size of the chromosomes but also related to the structural quality of the chromatin.

Three chromosomes pairs (4, 8, and 10) had different arm ratio classifications among the three *E. citriodora* karyotypes/ideograms. These results indicate that highly compact homomorphic chromosomes – typical of over-condensed metaphases – hamper a precise arm ratio estimate and hide the minor differences between chromosomes (Ohmido et al. 1998; Abreu et al. 2011). With respect to the least-condensed ideogram (Fig 2c), *E. citriodora* possesses six metacentric and five submetacentric chromosomes, which show a certain type of karyotype asymmetry. This result is not consistent with the

karyotype symmetry and chromosome metacentric nature observed in the species observed by Matsumoto et al. (2000) and Haque (1984).

Molecular cytogenetic procedures are excellent tools in structural, comparative and functional genomics studies (Figueroa and Bass 2010) and have been used in comparative karyotypic analysis of other species of trees, such as *Pinus* (Hizume et al. 2002). Observing clear metaphasic chromosomes is imperative for producing molecular cytogenetic data in *Eucalyptus*, as well-spread and well-preserved chromosomes are essential features for chromosome in *in situ* hybridization experiments (Andres and Kuraparthy 2013).

CONCLUSIONS

Using the cytogenetic methodology developed in the present study, we obtained well-defined metaphasic chromosomes of *E. citriodora*, which were less-condensed and showed better longitudinal resolution than those reported previously. Thus, this methodology may be applied for improved cytogenetic characterization of other *Eucalyptus* species.

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FIGURES AND TABLES

Table 1: Classification of the 2n = 22 mitotic chromosomes of *Eucalyptus citriodora* based on the centromeric position.

Chromosome	Fig. 2a	Fig. 2b	Fig 2c
	Class*		
1	M	M	M
2	M	M	M
3	M	M	M
4	M	SM	SM
5	M	M	M
6	NOR	NOR	NOR
7	M	M	M
8	M	M	SM
9	M	M	M
10	M	SM	SM
11	SM	SM	SM

* Class: M – metacentric; SM – submetacentric.

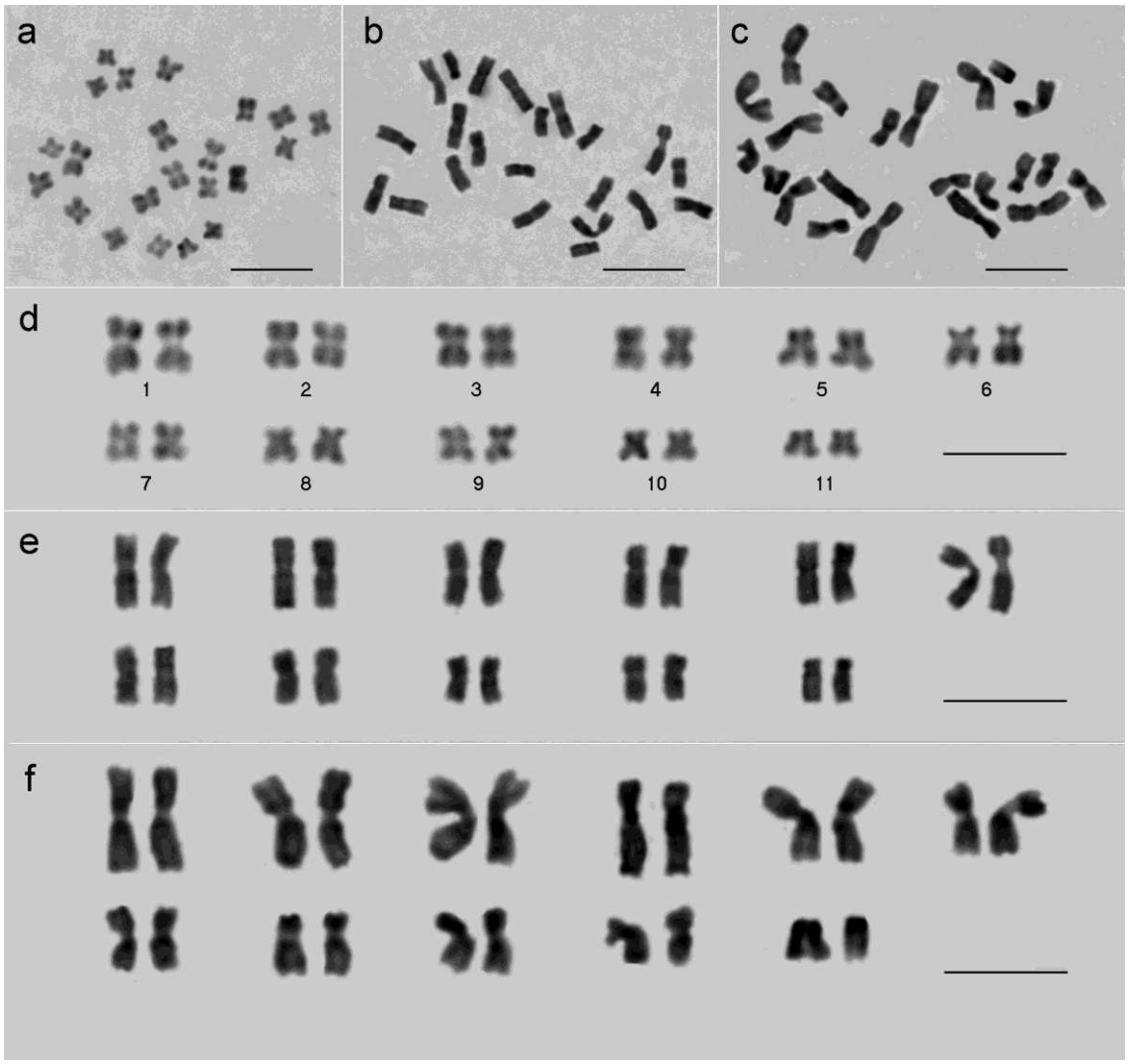

Figure 1: *Eucalyptus citriodora* metaphases, stained with Giemsa solution, in different compaction levels and without a cytoplasmic background or overlaps (a–c); Karyograms showing 2n = 22 chromosomes. A decrease in extent of compaction level is observed (d–f). Note the presence of secondary constriction on pair six. Bar = 5 µm.

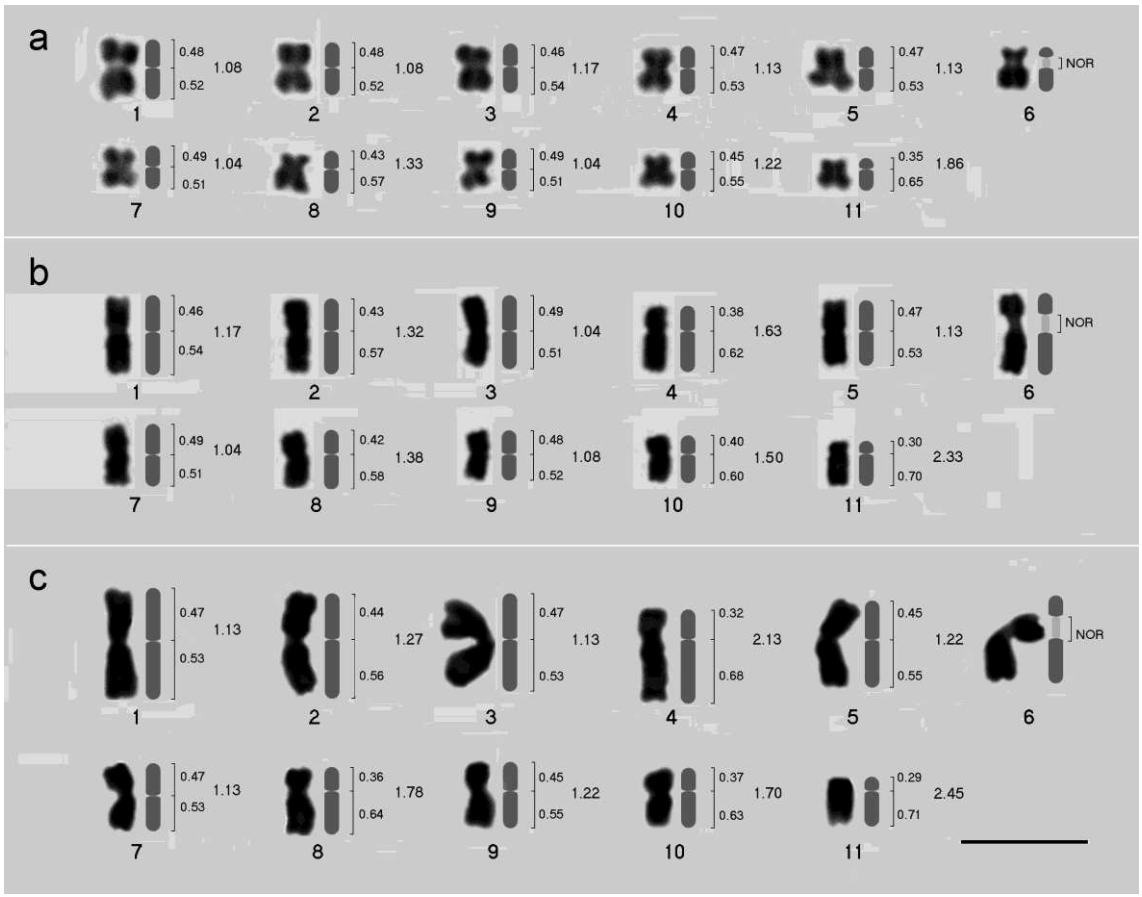

Figure 2: *Eucalyptus citriodora* ideograms based on karyotypes with different condensation levels. Relative arm length and arm ratio are shown, respectively, to the right of chromosome representation. Bar = 5 µm.

RESEARCH PAPER 2: GENOME SIZE VARIATION IN EUCALYPTUS: WHERE IS THE DIFFERENCE?

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ABSTRACT

Eucalyptus genus represents a group of woody plants that are used in commercial forest plantations due to their high growth rates, adaptability to various ecological conditions and multiple uses. Although the huge amount of molecular data for *Eucalyptus,* a basic understanding of the nature of a given genome requires information regarding the amount of DNA and it should be considered a crucial aspect of any truly comprehensive program of comparative genomic analysis. The genome study at cytogenetic level is an important approach to understand the species evolution in plant group. An integrate view of molecular and cytogenetic data is an excellent approach in structural, comparative, and functional genomics studies. Here, the genome size and base composition of 25 *Eucalyptus* species were measured. Besides, in a comparative karyotype approach, possible chromosomal alterations correlated with the genome size variation were evaluated. For this purpose, classical cytogenetic and genomic in situ hybridization (GISH) experiments were conducted. *Eucalyptus* species show a genome size range from 2C = 0.91 pg for *E. intermedia* to 2C = 1.37 pg for *E. paniculata*, and AT/CG ratios varying from AT= 57.1% for *E. paniculada* and *E. baileyana* to AT = 59.4% for *E. benthamii*. Comparative karyotype analysis reveals no remarkable differences in chromosome number (2n = 22) or morphology among *Eucalyptus* species with considerable difference in DNA nuclear content. GISH experiment did not discriminate non-homologous chromosomal regions of *E. baileyana* and *E. citriodora*, despite the difference of 0.45 pg between their genome size. The results achieve in the present work corroborate to consideration small and dispersed DNA changes as the mainly cause of genome size variation in *Eucalyptus*.

INTRODUCTION

The genus *Eucalyptus* represents an extremely successful group of woody plants (Kersting et al., 2015) that are used in commercial forest plantations due to their high growth rates, adaptability to various ecological conditions and multiple uses (Silva et al., 2016). Native from Australian and North Islands, these species present a high level of ecological and morphological diversity which provides the opportunity to investigate the process of lineage divergence at different taxonomic levels (Holman et al., 2003).

The study of genome contributes to understanding these evolutionary aspects of the group, others basic biological processes and are a prelude to engineering these processes for the betterment of humankind (Wendel et al.; 2016). Although the huge amount of molecular data for *Eucalyptus*, as genome sequencing (Myburg et al., 2014), microarray-based Diversity Arrays Technology (DArT) (Hudson et al., 2015) and genetic linkage maps (Grattapaglia et al., 2015), a basic understanding of the nature of a given genome requires information regarding the amount of DNA and it should be considered a crucial aspect of any truly comprehensive program of comparative genomic analysis (Gregory, 2005).

The first report of genome size of *Eucalyptus* species was made by Marie and Brown (1993) for *E. globulus* (2C = 1,13 pg). To date, 16 species had their DNA nuclear content estimated (Bennett and Leitch, 2012; Ribeiro et al., 2016), which represent a very small number if compared with the 894 described *Eucalyptus* taxa (Slee et al., 2006). Most reports about *Eucalyptus* C-value were provided by flow cytometry (FCM) analysis (Marie and Brown, 1993; Grattapaglia and Bradshaw, 1994; Pinto et al., 2004; Morgan and Westoby,

2005; Praça et al., 2009; Ribeiro et al., 2016), while some species had their genome size even measured by image cytometry (Praça et al., 2009), or complete-genome sequencing (Myburg et al., 2014). Besides its use in genome size estimation, the FCM has been applied to determinate the genomic AT/CG ratio of plant species (Meister and Barow, 2007; Favoreto et al., 2012). The DNA base composition of an organism may provide a closer insight into genome organization as compared to the amount of nuclear DNA (Doležel et al., 2007).

Flow cytometry is an analytical and preparative technique offering high throughput and precision (Vrána et al., 2014) and is accepted as the method of choice for genome size measurements (Galbraith and Lambert, 2012). Of the 8,510 prime estimates listed today in the Kew C-value database (http://data.kew.org/cvalues/), 4,844 were produced using flow cytometry (Bennett and Leitch, 2012). Despite this, the use of FCM produced different 2C value for *E. globulus* – 1,13 pg (Marie and Brown, 1993; Azmi, 1997); 1,09 pg (Grattapaglia and Bradshaw, 1994); 1,40 pg (Pinto et al., 2004) and 1,10 pg (Praça et al., 2009). Thus, FCM in species of the genus depends on the development of appropriate protocols that minimize the effects of secondary metabolites and are in accordance with international recommendations for this kind of analysis.

The study of the genome at cytogenetic level is an important approach to understand the species evolution in plant group (Cai et al., 2014; Čížková et al., 2015). Instead of this, the knowledge of *Eucalyptus* cytogenetic is very scale focusing on chromosome counting. Only few works aimed to describe chromosomes profile in *Eucalyptus* species using classical (Matsumoto et al., 2000; Carvalho and Carvalho, 2016) or molecular cytogenetic (Ribeiro et al.;

2016). An integrate view of molecular and cytogenetic data is considered an excellent approach in structural, comparative, and functional genomics studies (Figueroa and Bass, 2010). Therefore, genomic in situ hybridization (GISH) is considered a remarkable tool for plant genome analysis, which may allows the discrimination of non-homologous regions or entire chromosome of compared species (Gao et al., 2016; Liu et al., 2016).

In this study we estimated the genome size and base composition of 25 *Eucalyptus* species. Karyotypes of species with low (*E. intermedi*a and *E. microcorys*) and high nuclear DNA content (*E. botryoides* and *E. baileyana*) were compared aiming to identify possible chromosomal alterations correlated with the genome size variation. Besides, we applied GISH technique looking for non-homologous regions at chromosomal level on *E. baileyana* karyotype (high nuclear DNA content) when hybridized with *E. citriodora* genome (low nuclear DNA content).

MATERIAL AND METHODS

Plant material

Eucalyptus seeds were donated by forestry companies and research institutes (Table 1), and stored at 4°C. Seeds of the standard, *Solanum lycopersicum* 'Stupické' (2C = 2.0 pg, Praça-Fontes et al., 2011 and AT = 64.5%, Doležel et al., 1992), were provided by Dr. Jaroslav Doležel (Experimental Institute of Botany, Czech Republic). The cytogenetic and cytometric analysis were conducted at the Laboratory of Cytogenetics and Cytometry, Department of General Biology, Universidade Federal de Viçosa, Minas Gerais, Brazil.

In vitro culture

Eucalyptus spp. and *S. lycopersicum* seeds were cultivated *in vitro.* Surface disinfection was accomplished by immersion (20 s) in 70% (v/v) ethanol, followed by immersion (20 min) in 2.5% (v/v) commercial sodium hypochlorite containing one drop of Tween-20 per 100 mL of solution, and rinsed three times in deionized, autoclaved water. Germination medium contained Murashige and Skoog's (MS) basal salts (Murashige and Skoog 1962; Sigma®), B5 vitamins (Gamborg et al., 1968), 0.01% (w/v) myo-inositol (Sigma®), 2% (w/v) sucrose, 0.0004% L-cysteine (Sigma®) and 0.6% (w/v) agar (Sigma®). The pH was adjusted to 5.7 prior to autoclaving. Under aseptic conditions, in laminar air flow, three seeds were inoculated per tube, and tubes were kept in the dark until germination. Cultures were maintained at 25°C ± 2°C, under a 16/8-h light/dark regime, with 36 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light radiation for greening of the leaves, for 60 days.

FCM analysis

Nuclei isolation

Young leaves from each *Eucalyptus* species and standard plant were separately or simultaneously chopped (Galbraith et al., 1983) for 30 s, with a razor blade in a Petri dish containing 0.1 mL OTTO-I lysis buffer (Otto, 1990), supplemented with 2.0 mM of dithiothreitol (DTT; Sigma®), 7% of Polyethylene-glycol 2000 (PEG, Merck®). For propidium iodide (PI) staining, the same buffer was supplemented with 50 $mg \cdot mL^{-1}$RNAse (Sigma®). Subsequently, a volume of 0.9 mL of the same buffer was added to the nuclei suspension and the homogenate was filtered through 30-mm nylon mesh (Partec®) into a 2.0-mL microcentrifuge tube (Eppendorf®) and centrifuged at 100g for 5 min. The supernatant was poured off and the pellet was resuspended and incubated in 100 mL OTTO-I lysis buffer for 10 min (room temperature).

Genome size determination

The nuclear suspensions were stained in 1.5 mL OTTO-II buffer, supplemented with 2.0 mM of DTT (Sigma®), 75 mM PI (Sigma®, excitation/emission wavelengths: 480–575/550–740 nm) and 50 $mg \cdot mL^{-1}$ RNAse (Sigma®), pH 8.6 (Doležel et al. 1992; Meister, 2005), for 30 min in the dark. The G_0/G_1 nuclei peak of *S. lycopersicum* was positioned on channel 200 of the relative DNA content histogram. Genome size of each *Eucalyptus species* was determined by multiplying the genome size of *S. lycopersicum* by the ratio of their fluorescence intensities. The average 2C value in pg was converted to bp by considering that 1 pg of DNA corresponds to $0.978 \cdot 10^9$ bp (Doležel et al. 2003).

Base composition

The base-specific dye 4',6-diamidino-2-phenylindole (DAPI, Sigma®, excitation/emission wavelengths: 320–385/400–580 nm) was used to determine AT composition. Staining was accomplished by incubating the nuclei suspension in 1.5 mL OTTO II buffer, supplemented with 2.0 mM of DTT (Sigma®) and 15 mM DAPI, for 20 min in the dark. As for genome size determination, AT percentage of each *Eucalyptus* species was measured in relation to *S. lycopersicum* reference standard, comparing the peaks of fluorescence of the DAPI stained G_0/G_1 nuclei, following the formula described by Godelle et al. (1993): $AT_{sample} = AT_{standard} \times (R_{DAPI}/R_{PI})^{1/r}$, where *R* is the ratio of fluorescence intensity from the peak of *Eucalyptus* species to that of *S. lycopersicum* and r (binding length) = 3 (Meister and Barow, 2007). The percentage of the complementary bases was calculated as GC% = 100 - AT%.

Equipment handling

Nuclei suspensions were analyzed with a Partec PAS® flow cytometer (Partec®), equipped with a Laser source (488 nm), used for PI excitation and a mercury arc lamp (388 nm), for DAPI excitation. PI fluorescence (excitation: 610 nm) was collected through a RG band-pass filter, whereas DAPI fluorescence (excitation: 435 nm) was collected through a GG 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 analysis. Five independent repetitions were performed in different days, accounting for over 10,000 nuclei at a time. Peaks with coefficient of variation (CV) below 5% were considered the limit for adequate analysis in FCM.

Cytogenetics

Cytogenetic procedure was conducted as described by Carvalho and Carvalho (2016). Seeds from two *Eucalyptus* species with low or high DNA nuclear content were chosen and placed in Petri dishes with moistened filter paper and maintained in the dark until germination. Root tips were incubated in Hoagland's solution (Hoagland and Arnon, 1938) supplemented with 0.5% (w/w) dimethyl sulfoxide (DMSO, Sigma®) and the microtubule inhibiting agent amiprophos-methyl (APM, Nihon Bayer Agrochem®) at a final concentration of 3.0μM for a period of 3:00, at 30°C.

Root tips (2 – 5 mm) were washed in distilled water thrice, for 10 min each, and then fixed in fresh methanol:acetic acid (Merck®) solution (3:1). The fixative was changed thrice and the samples were stored at -20°C (Carvalho and Saraiva, 1997). Root tips were washed and incubated for 2 – 3 h at 34°C in an enzymatic digestion solution, at a ratio of 1:10 or 1:12 of an enzyme mix (cellulase 4%; pectolyase 1%; and hemicellulose 0.4%) and water. Then, the root tips were washed three times for 10 min in distilled water, fixed again, and stored at -20°C (Clarindo and Carvalho, 2006).

The *Eucalyptus* slides were prepared by cell dissociation and air-drying techniques (Carvalho et al. 2007), and immediately stained with a 5% Giemsa (Merck®) solution in a phosphate buffer (pH 6.8) for 7 min, washed twice in distilled water and air-dried.

GISH procedure

Genomic DNA of the *E. citriodora* (probe) and *E. baileyana* (DNA competitor) was extracted by using GenElute™ Plant Genomic DNA Miniprep kit (Sigma®). DNA concentration and purity were determined

spectrophotometrically using Nanodrop, and its integrity was further checked by agarose-gel electrophoresis. The probe was constructed by random primer PCR reaction using Thermo Sequenase™ DNA Polymerase (GE®). The labeling reactions consisted of 200 ng of *E. citriodora* genomic DNA, 0.2 mM dATP, dCTP and dGTP each, 0.1 mM dTTP, 0.1 mM tetramethyl-rhodamine-5-dUTP (Roche Diagnostics), 1X polymerase buffer, 4 µM of degenerated oligonucleotide primer (DOP-PCR), in 20 µL-volume. The labeled genomic probe was quantitated at the Qubit 2.0 Fluorometer (Invitrogen®), and evaluated by electrophoresis in 1.5 % agarose gels. Slides with chromosome preparations without cytoplasm background, no overlaps and with adequate chromatin compaction level were selected for GISH experiments. Slides were washed in phosphate-buffered saline (PBS) solution for 5 min, fixing with 2 % formaldehyde solution in PBS at room temperature for 10 min and washing again. Chromosome denaturation was carried out in 70 % formamide, 2X saline-sodium citrate (SSC) buffer, at 72°C for 4 min and after the slides were dehydrated in a cold ethanol series. The hybridization mixture was denatured in thermocycler at 85°C for 5 min and immediately transferred to ice. After receive the hybridization mixture, the slides were covered with plastic coverslip HybriSlip™ (Sigma®) and sealed with Rubber Cement (Elmer's®). Hybridization process was conducted in ThermoBryte™ (ThermoFisher®) at 37°C for 24 hours. After this period the stringency, washes were conducted at three solutions of 50 % formamide/2X SSC and a 2X SSC at 45°C for 5 min each. Subsequently, the slides were counterstained with DAPI. Images of *E. baileyana* metaphases were captured by a DP-71 video camera (Olympus®) attached to a BX-60 fluorescence microscope (Olympus®), with a 100X objective lens, WG filters for GISH analysis and WU filter for DAPI staining. The

frame was digitized using the Image Pro-Plus 6.1 software (Media Cybernetics®). The merging and final adjustments of the images were done with the tools of the same software.

RESULTS

Flow cytometry analysis

In vitro culture produces plantlets with health leaves appropriate for FCM analysis. For nuclear genome size and base composition measurement, the FCM procedure, including OTTO-I buffer for 10 min and OTTO-II buffer for 30 min, provided histograms with G_0/G_1 peaks exhibiting CV below 5%. The mean 2C value was lowest in *E. intermedia* (2C = 0.91 pg) and highest in *E. paniculata* (2C = 1.37 pg) (Table 1). Regarding base composition the mean values oscillated from AT% = 61.30 for *E. urophylla* to AT% = 62.85 for *E. intermedia*. Table 1 summarizes the mean genome size (pg and Mbp) and base composition (AT%) for each FCM-analyzed species. Eight representative histograms are shown in Fig. 1. Each one corresponding to genome size or base composition estimation of two species with low (*E. microcorys* and *E. intermedia*) and two with high DNA nuclear content (*E. botryoides* and *E. baileyana*).

Cytogenetics

E. intermedia (2C = 0.91 pg), *E. microcorys* (2C = 1.04 pg), *E. botryoides* (2C = 1.35 pg) and *E. baileyana* (2C = 1.36 pg) were chosen for cytogenetic analysis. Root tips treated with Hoagland's solution supplemented with 0.5% DMSO and 3 µM APM for 3 h at 30°C showed satisfactory metaphasic indices. Cell dissociation of root meristems enzymatically treated for 2 h at 34°C, combined with the air-drying technique generated well-preserved chromosomes, without any cytoplasmic background or overlaps. *Eucalyptus* cytogenetic preparations provided well-defined metaphasic chromosomes to be used in chromosomal counting and karyogram assembly (Fig. 2).

Assembled karyograms confirmed a karyotype with 2n = 22 chromosomes, with the presence of a secondary constriction (in chromosomes arbitrarily positioned as pair n.6), for these four analyzed species (Fig. 2a, b, c and d). Besides constancy in chromosome number, the karyotypes show remarkable similarity, present no significant differences in chromosomes morphology, or in classification based on centromeric position (data not shown).

Genome in situ hybridization

The methodology for genomic probe construction by DOP-PCR and hybridization process was adequate for GISH experiment. Even with the stringency of 85 – 90% from GISH conditions all the 22 chromosomes of *E. baileyana* were hybridized homogeneously with genomic DNA probe of *E. citriodora* (Fig. 3). Therefore, it was possible to evidence the high genomic homology between these two *Eucalyptus* species.

DISCUSSION

FCM Procedure

The first step undertaken to fulfill the aim of the present study was to adapt a FCM protocol for analysis in *Eucalyptus*. The nuclei extraction of in vitro culture young leaves on OTTO-I isolation buffer supplemented with 2 mM DTT and with 7 % PEG provided histograms with lowest CVs (below 5 %) and over 10,000 particles during the FCM analysis. PEG has become commonly used in FCM studies because of its wide spectrum for antioxidant activities, an effect called PEGylation (Thermo Fisher Scientific, 2016). Due to this effect, PEG was efficient at inhibiting the action of cytosolic compounds, resulting in G_0/G_1 peaks for some plant species. Woody species are generally considered more recalcitrant due to the presence of secondary metabolites that may interfere with DNA staining (Loureiro et al. 2006). Among the diversity of secondary metabolites produced by the genus, the tannins, as gallic and ellagic acid, have been isolated from several *Eucalyptus* species (Brezáni and Šmejkal, 2013). Tannins are known to be good PEG-binding agents (Kim et al., 2015) and several works reported the potential of PEG to inhibit the action of these compounds (Nsahlai et al., 2011; Mahlo and Chauke, 2012).

The in vitro culture of *Eucalyptus* species and standard was conducted as a way to ensure a controlled environment for plants growth. Pinto et al. (2004) reported a notorious decrease in PI fluorescence from both *Eucalyptus* and *S. lycopersicum* nuclei in samples obtained from leaves of the field plants, which was not observed in leaves of in vitro germinated plantlets. Besides, *Eucalyptus* seedlings cultivated in vitro provide softer leaves if compared with those cultivated in greenhouse or in the field. This aspect allows time and

intensity reduction of chopping procedure, as conducted by Ribeiro et al. (2016) for improvement of FMC analysis in *Eucalyptus*.

PI is recommended as the fluorochrome of choice for genome size studies (Leitch and Bennett, 2007). In this study, the use of PI as intercalating fluorochrome enabled to review the DNA nuclear content of *Eucalyptus* species that had C-value previously determined using DAPI. As a base-specific fluorochrome, DAPI was used here to access AT/CG ratio of all studied species for the first time by FMC.

Genome size and base composition in *Eucalyptus*

The *Eucalyptus* species studied in this work show a genome size (2C) range of 0.91 – 1.37 pg. This values represent a shorter interval if compared with the 2C value range reported by Grattapaglia and Bradshaw (1994) of 0.77 – 1.47 pg, even our work covering more *Eucalyptus* subgenus and species. Regarding the subgenus studied, *Corymbia* species present a relatively low DNA nuclear content varying from 0.91 pg for *E. intermedia* to 1.01 pg for *E. citriodora*, if compared with *Symphyomyrtus* species that vary from 1.22 pg for *E. benthamii* to 1.37 pg for *E. paniculata*. The same observation was made by Grattapaglia and Bradshaw (1994), in which *E. citriodora* and *E. torelliana* present C-values considerably below *Symphyomyrtus* analyzed species. These authors also observed a correlation between phylogenetic relationship and C-value similarity. This statement is in disagreement with our finds when genome size data are compared with subgenus phylogeny. The subgenus *Alveolata* (*E. microcorys* = 1.04 pg) and *Minutifructus* (*E. deglupta* = 0.99 pg), comprise species presenting DNA nuclear content more similar to *Corymbia*, while species from subgenus *Eucalyptus* (*E. tindaliae* = 1.19 pg) and *Eudesmia* (*E. baileyana* = 1.36 pg) show high genome size as *Symphyomyrtus*. However,

Alveolata and *Minutifructus* are included in the same clade of *Symphyomyrtus* (Brooker, 2000) and the subgenus *Eucalyptus* and *Eudesmia* belong to more phylogenetic distant clades (Ladiges et al., 2003). Instead of DNA nuclear content may represents a tendency within the subgenus, it is clear that genome size similarity of itself should not be considered in establishment of phylogenetic relationship. The diversification and evolution processes in *Eucalyptus* seem to have not had unidirectional way in the genome size variation, as an increase in DNA nuclear content in more derivate clades for example. This view is in accordance with recent stated by Puttick et al. (2016) that rates of genome size evolution, not C-value, correlate with speciation in angiosperms.

The AT/CG ratios estimated here represent a subtle range varying from AT= 61.30% for *E. urophylla* to AT = 62.85% for *E. intermedia*. Base-pair composition generally varies only slightly among closely related taxa (Meister and Barow, 2007) and in this case seems to have no correlation with genome size differences in *Eucalyptus*. Nevertheless, base composition estimative by FCM remains as an important genomic data that may used as reference for sequencing projects. Regarding molecular cytogenetic the evidence of DAPI banding patterns variation in *Eucalyptus* (Ribeiro et al., 2016) may be associated to AT% measured for studied species.

Comparative karyotype analysis

Karyograms of four *Eucalyptus* species were compared with the aim of identify possible chromosomal alteration that may be related to the variation of DNA nuclear content. Regarding classical cytogenetic approach karyograms of *E. microcorys* and *E. intermedia* (both with low DNA nuclear content) do not vary in number (2n = 22) or chromosome profile when compared with karyograms of *E. botryoides* and *E. baileyana* (both with high DNA nuclear

content). So the major difference (0.41 pg) among 2C value of these *Eucalyptus* species (almost a half of *E. intermedia* genome size) is not related to a remarkable variation on chromosomal morphology or rearrangement. A vast karyological continuum has been reported for *Eucalyptus* genus by several classical cytogenetic research (Matsumoto et al., 2000; Bachir and Abdellah, 2006; Carvalho and Carvalho, 2016). Eventual differences in chromosome morphologies rather than be due to large rearrangements seem to be dependent of local expansions of possible repetitive clusters (Ribeiro et al., 2016).

With increase of genomic data by the spread of sequencing genome techniques, authors have pointed to the role of transposable elements (TE) as one of mainly driving forces on genome evolution in plants (Wang et al., 2016; Wendel et al., 2016; Zhao et al., 2016). The genome size variation in *Eucalyptus* species, elucidated in this work, may reflect small and numerous DNA changes that are spread throughout the genome. Myburg et al. (2014) stated that the difference in 2C value of *E. grandis* and *E. globulus* is associated with thousands of small dynamic changes (loses and gains) across the genomes of both species. In this view, the homogeneity on hybridization profile in GISH experiment could be related to the lack of technique resolution for discriminate such small DNA differences. Besides, if the overall sequence homology between the two genomes exceeds 85%, the specificity of the GISH procedure may be insufficient to discriminate between them (Poggio et al., 2005). From an integrative approach allying molecular cytogenetic and DNA nuclear content estimation, Ribeiro et al. (2016) also evidenced a high conservation of the genomes in six *Eucalyptus* species.

CONCLUSIONS

We estimate the genome size and base composition of 25 *Eucalyptus* species by FCM with improved protocol, that even species with minor differences could be discriminated. Here we estimate a range of 0.91 – 1.37 pg for 2C value in *Eucalyptus* species, which represents a difference of 50 %. The present study seems have any relation between 2C value and AT/CG ratio. Comparative karyotype analysis reveals no remarkable differences in chromosome number (2n = 22) or morphology among *Eucalyptus* species with considerable difference in DNA nuclear content. GISH experiment evidenced the genomic homology at of *E. baileyana* and *E. citriodora*, despite the difference of 0.45 pg between their genome sizes. The results achieve in the present work corroborate to considerate small and dispersed DNA content changes as the mainly cause of genome size variation in *Eucalyptus*.

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TABLES AND FIGURES

Table 1: Nuclear DNA content and base composition of *Eucalyptus* species. Taxonomic classification according to Brooker (2000).

		Mean DNA content		
Species	**Origin**	**2C/pg ± SD**	**1C/Mpb**	**%AT ± SD**
Subgenus *Symphyomyrtus*				
Section Adnataria				
E. paniculata	IPEF-Brazil	1,37 ± 0,010	670,5	61,38 ± 0,225
Section Exsertaria				
E. brassiana	IPEF-Brazil	1,31 ± 0,014	641,9	61,94 ± 0,244
E. camaldulensis	UFV-Brazil	1,33 ± 0,009	651,3	61,77 ± 0,255
E. exserta	IPEF-Brazil	1,32 ± 0,012	643,5	61,87 ± 0,435
E. tereticornis	UFV-Brazil	1,27 ± 0,008	622,1	62,57 ± 0,243
Section Maidenaria				
E. benthamii	Klabin-Brazil	1,22 ± 0,006	595,0	62,42 ± 0,118
E. dorrigoensis	Klabin-Brazil	1,25 ± 0,008	610,0	62,01 ± 0,260
E. dunnii	UFV-Brazil	1,32 ± 0,006	643,7	61,90 ± 0,231
E. globulus	UFV-Brazil	1,31 ± 0,011	641,9	61,57 ± 0,308
E. viminalis	Klabin-Brazil	1,26 ± 0,011	614,1	62,05 ± 0,308
Section Latoangulatae				
E. botryoides	IPEF-Brazil	1,35 ± 0,004	658,0	61,76 ± 0,262
E. grandis	SIF-Brazil	1,33 ± 0,011	651,0	61,89 ± 0,187
E. pellita	SIF-Brazil	1,35 ± 0,004	657,8	61,76 ± 0,279
E. propinqua	IPEF-Brazil	1,27 ± 0,013	622,1	62,10 ± 0,293
E. resinifera	IPEF-Brazil	1,34 ± 0,004	657,0	61,47 ± 0,261
E. robusta	IPEF-Brazil	1,30 ± 0,014	636,3	61,88 ± 0,342
E. saligna	SIF-Brazil	1,33 ± 0,012	649,7	62,27 ± 0,293
E. urophylla	SIF-Brazil	1,30 ± 0,011	636,3	61,30 ± 0,296
Subgenus Eucalyptus				
E. tindaliae	IPEF-Brazil	1,19 ± 0,014	580,7	62,26 ± 0,202
Subgenus *Eudesmia*				
Section Reticulatae				
E. baileyana	Klabin-Brazil	1,36 ± 0,012	665,9	61,40 ± 0,229
Subgenus *Alveolata*				
E. microcorys	IPEF-Brazil	1,04 ± 0,012	508,9	61,50 ± 0,449
Subgenus *Minutifructus*				
E. deglupta	IPEF-Brazil	0,99 ± 0,014	485,5	62,29 ± 0,283
Subgenus *Corymbia*				
Section Septentrionales				
E. citriodora	IPEF-Brazil	1,01 ± 0,011	494,1	61,78 ± 0,226
E. maculata	Klabin-Brazil	0,93 ± 0,006	455,2	62,46 ± 0,368
Section Dorsiventrales				
E. intermedia	Klabin-Brazil	0,91 ± 0,008	445,0	62,85 ± 0,435

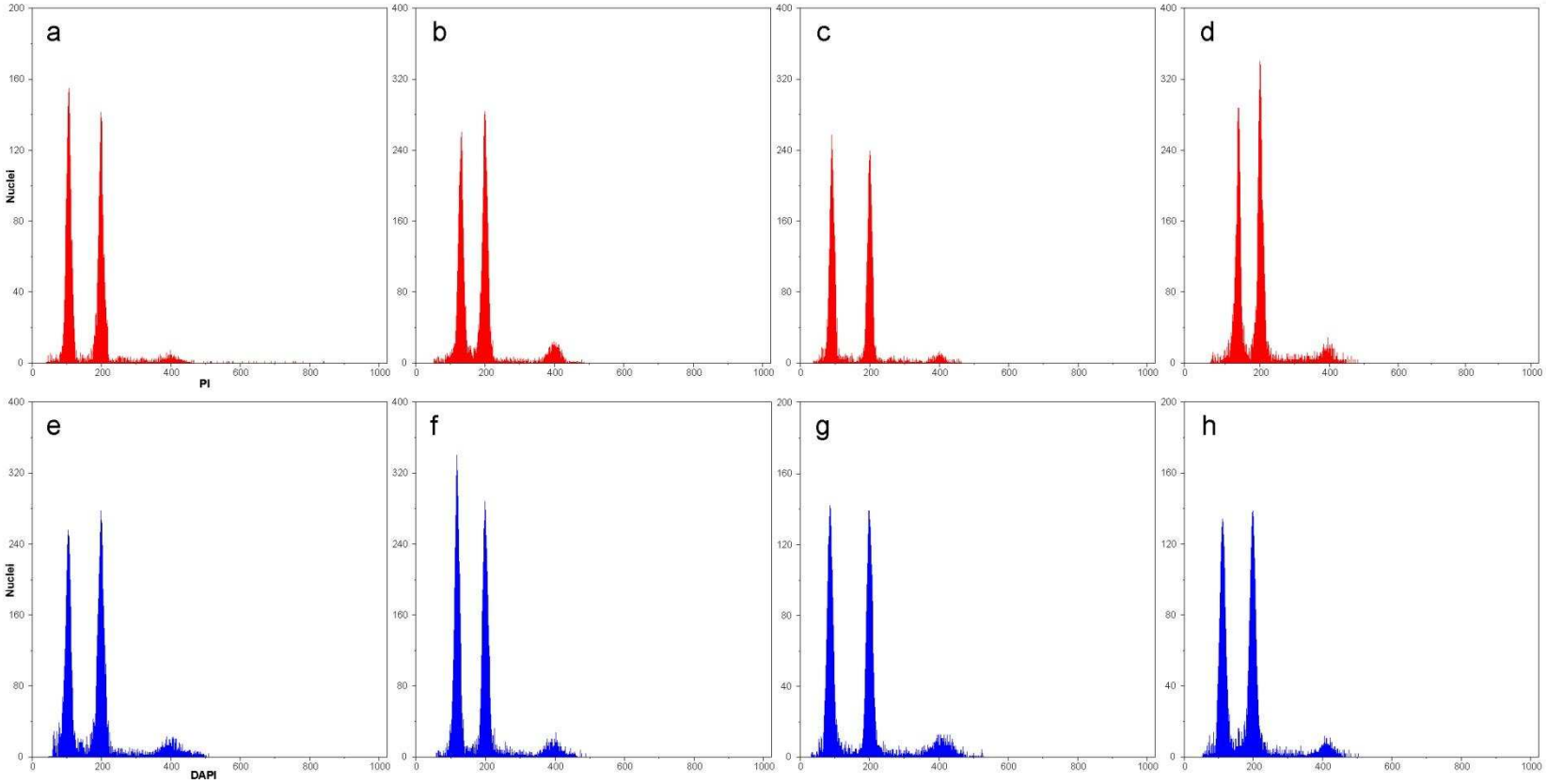

Figure 1: Eight representative FCM-histograms obtained from nuclei suspensions stained with PI (a, b, c, d) or DAPI (e, f, g, h). In all histograms G_0/G_1 nuclei peak of *S. lycopersicum* (internal standard, 2C = 2.00 pg and AT = 64.5%) was set to channel 200. (a) Histogram showing G_0/G_1 nuclei peak *E. microcorys* (2C = 1.01 pg, channel 101). (b) Histogram showing G_0/G_1 nuclei peak of *E. botrioydes* (2C = 1.35 pg, channel 135). (c) Histogram showing G_0/G_1 nuclei peak of *E. intermedia* (2C = 0.91 pg, channel 91). (d) Histogram showing G_0/G_1 nuclei peak of *E. baileyana* (2C = 1.36 pg, channel 136). (e) Histogram showing G_0/G_1 nuclei peak *E. microcorys* (AT = 61.50%, channel 90). (f) Histogram showing G_0/G_1 nuclei peak of *E. botrioydes* (AT = 61.76%, channel 118). (g) Histogram showing G_0/G_1 nuclei peak of *E. intermedia* (AT = 62.85%, channel 85). (h) Histogram showing G_0/G_1 nuclei peak of *E. baileyana* (AT = 61.40%, channel 117).

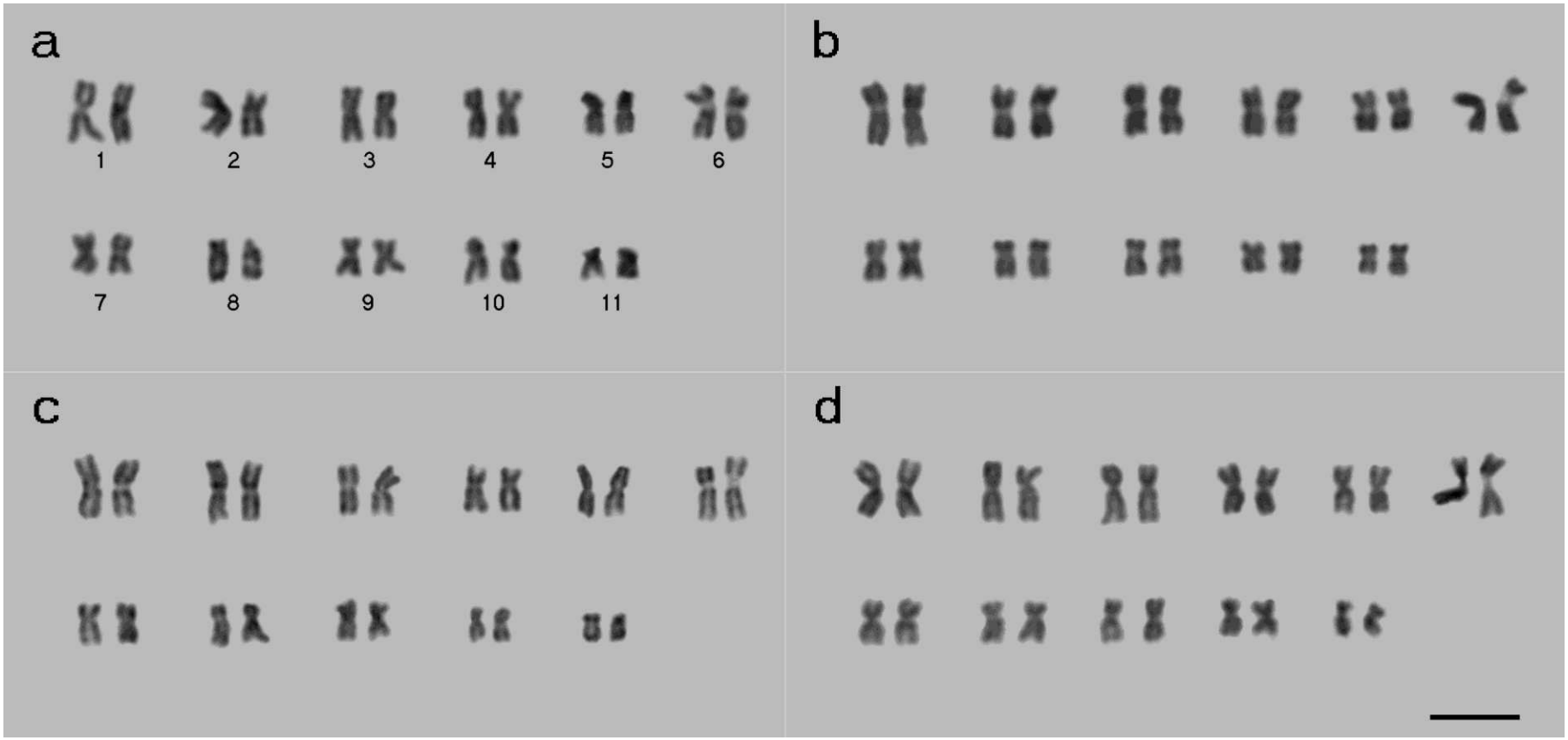

Figure 2: Karyograms of *Eucalyptus* species (2n = 22 chromosomes) evidencing the high level of similarity. (a) *E. botryoides*. (b) *E. baileyana*. (c) *E. microcorys*. (d) *E. intermedia*. In all karyograms chromosome with the presence of secondary constriction was arbitrarily positioned as pair n.6. Bar = 5 µm.

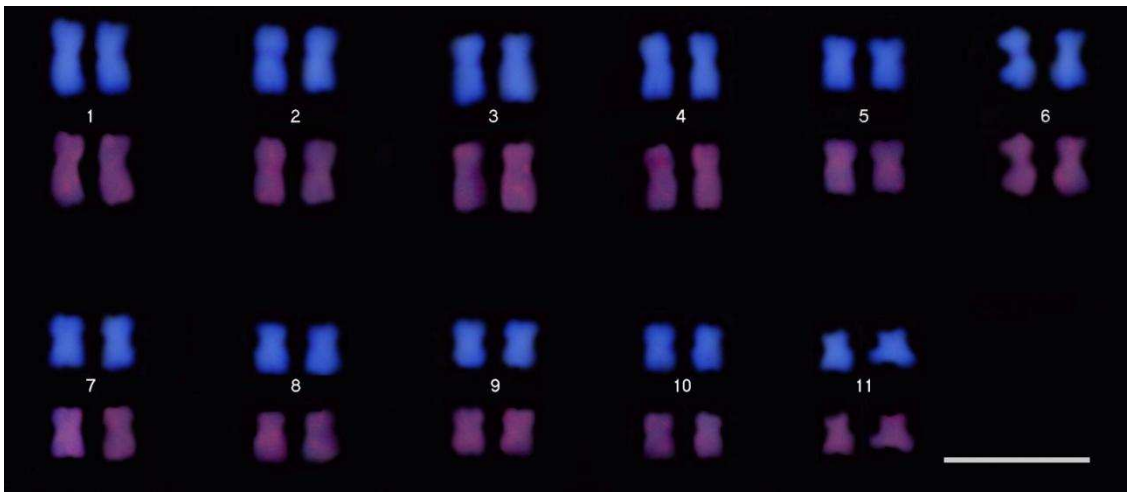

Figure 3: *E. baileyana* assembled karyogram counter-stained with DAPI (in blue), and hybridized with genomic probe of *E. citriodora* (in red), showing a homogeneous hybridization pattern. Bar = 5 µm.

OVERALL CONCLUSION

We estimate the genome size and base composition of 25 *Eucalyptus* species by FCM with improved protocol, that even species with minor differences could be discriminated. With the remarkable difference in 2C value within the studied group raise the question which is the possible karyotype characteristic that maybe be related with this genome size variation.

The classical cytogenetic methodology developed here, enable obtain well-defined metaphasic chromosomes and with better longitudinal resolution of *E. citriodora* (2n = 22), which ensure the assembly of the best *Eucalyptus* karyotype so far. Besides, this methodology enables a comparative cytogenetic analysis of four *Eucalyptus* species that did not review any considerable difference among the karyotypes. GISH experiment was carry out aiming discriminate any chromosomal difference not reviewed by classical cytogenetic approach. As result was not possible discriminate non-homologous chromosomes regions of *E. baileyana* and *E. citriodora*, despite the difference of 0.45 pg between their genome sizes. The results achieve in the present work corroborate to considerate small and dispersed DNA content changes as the mainly cause of genome size variation in *Eucalyptus*.