Nuclear DNA quantification and biometric characterization of *Bixa arborea and Bixa orellana* fruits

Quantificação do DNA nuclear e caracterização biométrica dos frutos de *Bixa arborea* e *Bixa orellana*

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Thatielen Furini
Mestre em genética e melhoramento de plantas (PPGMP)
Este trabalho faz parte da dissertação de mestrado da primeira autora. Trabalho financiado pela CAPES
Universidade do Estado do Mato Grosso, Laboratório de Citogenética e Cultura de Tecidos, Alta Floresta, Mato Grosso, Brasil.
E-mail: thatyfurini2003@hotmail.com

Isane Vera Karsburg
Doutora em genética e melhoramento pela Universidade Federal de Viçosa (UFV)
Universidade do Estado do Mato Grosso, Laboratório de Citogenética e Cultura de Tecidos, Alta Floresta, Mato Grosso, Brasil.
E-mail: isane.karsburg@unemat.br

José Martins Fernandes
Doutor em Botânica pela universidade Federal de Viçosa (UFV)
Universidade do Estado do Mato Grosso, Alta Floresta, Mato Grosso, Brazil.
E-mail: fernanbio@bol.com.br

Samiele Camargo de Oliveira Domingues
Mestre em Biodiversidade e Agroecossistemas Amazônicos (PPGBioAgro)
Universidade do Estado do Mato Grosso, Alta Floresta, Mato Grosso, Brazil.
E-mail: samieledomingues@gmail.com

Joelson de Oliveira Barros
Mestre em genética e melhoramento de plantas (PPGMP)
Universidade do Estado do Mato Grosso, Laboratório de Citogenética e Cultura de Tecidos, Alta Floresta, Mato Grosso, Brasil.
E-mail: joel_bio10@hotmail.com

Maicon de Souza Pecegueiro
Mestre em genética e melhoramento de plantas (PPGMP)
Universidade do Estado do Mato Grosso, Laboratório de Citogenética e Cultura de Tecidos, Alta Floresta, Mato Grosso, Brasil
E-mail: maiconpc1905@hotmail.com

Wellington Ronildo Clarindo
Doutor em Genética e Melhoramento pela universidade Federal de Viçosa (UFV)
Universidade Federal de Viçosa, Laboratório de Citogenética e Citometria de Plantas, Viçosa, Minas Gerais, Brazil
E-mail: well.clarindo@ufv.br

Douglas Machado Leite
Mestre em genética e melhoramento de plantas (PPGMP)
Universidade do Estado do Mato Grosso, Laboratório de Citogenética e Cultura de Tecidos, Alta Floresta, Mato Grosso, Brasil
E-mail: douglasmachado_95@hotmail.com

ABSTRACT
Bixa arborea and *Bixa orellana* are rich in carotenoids, antioxidant compounds and vitamins, and therefore widely used by the population as a source of food and medicines. These two species are very similar morphologically, making genetic characterization necessary by analyzing the amount of nuclear DNA. Thus, this work aimed to quantify the nuclear DNA of *Bixa arborea* and *Bixa orellana*. For this purpose, the biometrics of the fruits of the two were carried out, in addition to analysis of the DNA content by means of flow cytometry. The amount of nuclear DNA showed no significant difference between the two species and between individuals of *B. arborea*. Thus, we can conclude that the two species have no significant difference in the size of the genome, with the shape of the fruit and the quantity of seeds being the most suitable to separate the two species.

Keywords: Flow cytometry, Chromosomes, Genetic material

RESUMO
Bixa arborea e *Bixa orellana* são ricas em carotenoides, compostos antioxidantes e vitaminas, e por isso muito utilizadas pela população como fonte de alimento e remédios. Estas duas espécies são muito semelhantes morfologicamente, tornando-se, necessária, a caracterização genética por meio da análise da quantidade de DNA nuclear. Assim, esse trabalho teve como objetivo fazer a quantificação do DNA nuclear de *Bixa arborea* e *Bixa orellana*. Para isso foi realizada a biometria dos frutos das duas, além de análise do conteúdo de DNA por meio de citometria de fluxo. A quantidade de DNA nuclear não apresentou diferença significativa entre as duas espécies e entre os indivíduos de *B. arborea*. Dessa forma, podemos concluir que as duas espécies não possuem diferença significativa no tamanho do genoma, sendo o formato do fruto e a quantidade de sementes o mais indicado para separar as duas espécies.

Palavras-chave: Citometria de fluxo, Cromossomos, Material genético

1 INTRODUCTION

In Brazil there are about 33,099 species of angiosperms, many of them used as food, medicinal plants or herbal medicines (CALIXTO, 2005; CRAGG and NEWMAN, 2013; MENEGUELLI ET AL., 2017; BFG, 2018). Among the species of the genus Bixa

L. (Bixaceae), known as annatto, some are used by the population for food and medicinal purposes (GARCIA et al., 2012; PILLAI et al., 2018), such as *Bixa arborea* Huber and *Bixa orellana* L Although the two species are native to Brazil, they are not endemic to our territory, occurring throughout South America (RADDATZ-MOTA et al., 2017). They are morphologically close and explored by the population, which uses the fruits for the extraction of the pigment and the leaves as a herbal medicine (Castro *et al.*, 2009; RADDATZ-MOTA et al., 2017).

The species differ in addition to the morpho-agronomic aspects, by distinct genotypic patterns (ALMEIDA et al., 2006; LOMBELLO and PINTO-MAGLIO, 2014). Most cytogenetic studies for the genus Bixa have shown that *B. arborea* differs from B. orellana, as it has 2n = 14 chromosomes (LOMBELLO and PINTO-MAGLIO, 2014). In this a pair of chromosome twice the size of the others, which characterizes a bimodal karyotype for the species (MORAWETZE, 1986; LOMBELLO and PINTO-MAGLIO, 2014). *B. orellana* has 2n = 16 chromosomes (KRISHNAN and AYYANGAR, 1987) and also has a bimodal karyotype. However, cytogenetic studies are still needed to increase the knowledge of the species karyotype due to the wide geographical distribution that they present (JHA and NATH, 2016).

Fruit biometrics is a process that is widely used in studies of genetic improvement (PINÃ-RODRIGUES, 2002). This is because this methodology allows to verify the relationship of variability with the environmental factors to which the plant is subjected (GONÇALVES et al., 2013). Allied to this, the study of fruit biometrics allows to outline strategies for germination and production of seedlings for species with wood potential or forest restoration (PINÃ-RODRIGUES, 2002), study of the propagation of species (BASKIN and BASKIN, 1998; ALVES et al., 2007) ensuring the rational use of flora (GUSMÃO et al., 2006; BARROSO et al., 2016). In summary, the study of fruit biometrics provides a means to differentiate species that are part of a same genus that present very varied anatomical and morphological similarities (CRUZ et al. 2001), even among specimens.

In turn, flow cytometry is an advantageous method that allows quantifying the size of the genome in plants (COSTA, 2018). This method has been shown to be a good alternative for several reasons: it is possible to prepare and analyze several samples in the same day, it does not need cells in division and it is non-destructive (BENET and LEITCH, 1995; FERNANDES, 2015). In this way, the amount of DNA of a species can be measured through this process, which is often used to count, examine and classify

microscopic particles in addition to analyzing the physical and chemical characteristics of cellular liquids (FALEIRO, 2012). This allows finding, besides the karyotype of the species, possible anomalies and cellular aberrations in the analyzed material (BENET andLEITCH, 1995; DOLEZEL, 1997).

In addition, Dolezel (1997) points out that the use of flow cytometry can be an important ally in plant breeding, in the following processes: establishment of the level of ploidy, identification of haploids and double-haploids in cultures of anthers and ovaries, verification of new levels of ploidy in crossbreeding results, detection of aneuploids, use in the study of apomixis, the identification of sex in dioic plants, in the identification of hybrids, in the identification of polysomatia, in the monitoring of the development of the seed and in the identification of the product of protoplast fusion.

In this context, the purpose of this chapter is to carry out a study on two botanical species widely used in food and also as a herbal medicine, *B. arborea* and *B. orellana*, to help their characterization, allowing to differentiate one from the other. and to create bases to develop studies of genetic improvement of these species. In this way we seek to quantify the nuclear DNA and the biometrics of the fruits.

2 MATERIAL AND METHODS

2.1 STUDY AREA

The work was developed at the Laboratory of Cytogenetics and Culture of Plant Tissues, of the State University of Mato Grosso Carlos Alberto Reyes Maldonado - University Campus of Alta Floresta. To carry out this study, leaves and fruits of *B. arborea* and *B. orellana* collected in Alta Floresta-MT were used as samples.

2.2 OBTAINING PLANT MATERIAL

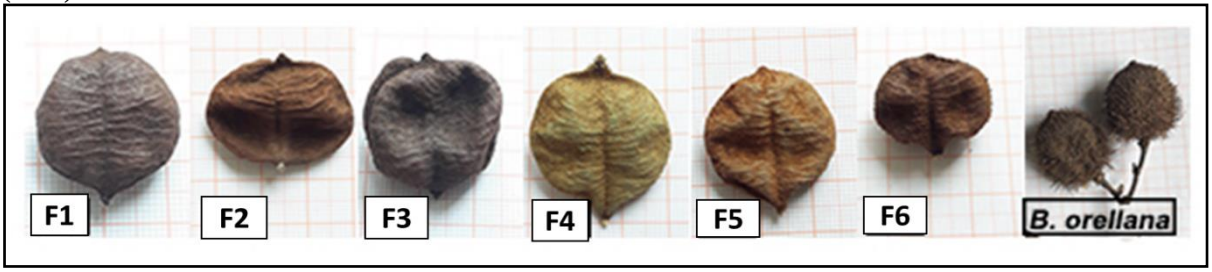
The two species of *Bixa* belonging were collected in the city of Alta Floresta-MT, located in the southern region of the Amazon (56º 05 '10 "W and 09º 52' 32" S). The region has a hot humid tropical climate, with high average temperatures (23 to 26ºC) during the year, with daily maximums of 34 to 37ºC. The annual rainfall indices show values from 1,700 to 1,800 mm and there are two well-defined climatic seasons, dry winter and rainy summer (PEREIRA, 1995; ZAPPI et al., 2011). The vegetation is formed predominantly by open and dense rain forest, seasonal forest and Cerrado (ZAPPI et al., 2011; PMAF, 2021). The main economic activities are services, agriculture and agriculture (IBGE, 2021; PMAF, 2021).

The coordinates of the collection site for the two species were marked with GPS. The varieties of *B. arborea* were collected on the property of Terezinha Ruella de Oliveira (T.R.O), located on Estrada Vicinal 1 Oeste, 1 Rural Zone approximately 13 km from the municipality of Alta Floresta - MT. As for the species *B. orellana* L., it was collected within the urban perimeter of Alta Floresta, located at Rua Perimetral NW, nº 1.300, Bairro Boa Esperança. The material was incorporated into the Herbário da Amazônia Meridional- (HERBAM) of the University of the State of Mato Grosso, located in the municipality of Alta Floresta - MT.

2.3 BIOMETRIC STUDY OF FRUITS AND SEEDS

Thirty fruits of each species were analyzed. The fruits were dried under natural conditions before starting the methodological procedures. At the Laboratory of Cytogenetics and Tissue Culture of the University of the State of Mato Grosso, Campus of Alta Floresta we selected eight characteristics that were observed with the aid of a precision scale and a digital caliper, they are: total length (cm), total width (cm), fruit thickness (cm), pericarp thickness (mm), fruit weight (mg), seed weight (mg), pericarp weight (mg) and number of seeds per fruit
(Figure 1).

Figure 1: Morphological aspects of *Bixa arborea* (F1 to F6) and *Bixa orellana* fruits. Alta Floresta – MT (2020).



Source: Thatielen Furini

The grouping of phenotypes was performed using the UPGMA grouping method (ARRIEL et al., 2006), using a similarity matrix for the characters analyzed, which proved to be the most appropriate in relation to the Ward and Closest Neighbor (LS) methods. The relative importance of the characters in relation to the genetic divergence between the subsamples was studied according to Singh (1981). Data analysis was performed with the aid of the statistical program R.

2.4 COLLECTION OF VEGETATIVE MATERIAL FOR FLOW CYTOMETRY

Flow cytometry analysis followed the protocol proposed by Otto (1990) and Galbraith et al., (1983). Samples of fresh leaves from the accessions of *Bixa arborea* and *B. orellana* were collected, and later stored in plastic luer lock bags, moistened with distilled water and conditioned in styrofoam with dry ice and transported to the Laboratory of Cytogenetics and Plant Cytometry at the Federal University of Viçosa UFV for analysis of flow cytometry.

2.5 EXTRACTIONS OF LEAF FRAGMENTS

Extractions of 2 cm^2 leaf disc fragments were carried out from young leaves of the species of *Bixa arborea* and *Bixa orellana*, both from the samples and from the internal standard. The internal standard used was *Solanum lycopersicum* L.

The samples and the reference standard S. lycopersicum with the known DNA content of 2C = 2.00 picograms, (PRAÇA-FONTES et al., 2011) were jointly chopped up (GALBRAITH et al., 1983) with assistance of a scalpel slide in a Petri dish, including 0.5 mL of OTTO I core isolation buffer at 4ºC (OTTO, 1990) for 30 seconds. This isolation buffer is supplemented with 2.0 mM dithiothreitol-DTT (Sigma®) and 50 µg mL-1 RNAse (Sigma®) (PRAÇA-FONTES et al., 2011).

2.6 EXTRACTION OF NUCLEAR DNA CONTENT

According to this protocol (PRAÇA-FONTES et al., 2011), samples of leaves of *B. arborea* and *B. orellana* were placed in distilled water at 4 ºC, then removed, dried and cut. Then the samples were cut into smaller pieces in a Petri dish containing 0.5 ml of OTTO-I lysis buffer. After incubation for 3 minutes, the suspension was filtered through a 30 µm nylon filter, placed in a test tube and sent to the centrifugation for 5 minutes at 1100 rpm. After removing the supernatant, the pellet was stained and placed for 30 minutes in the dark at room temperature to react with the dye. Then it was filtered and analyzed on the flow cytometer for DNA quantification.

2.7 DNA PROCESSING AND QUANTIFICATION

The size of the genome of the genus Bixa was calculated according to the following:

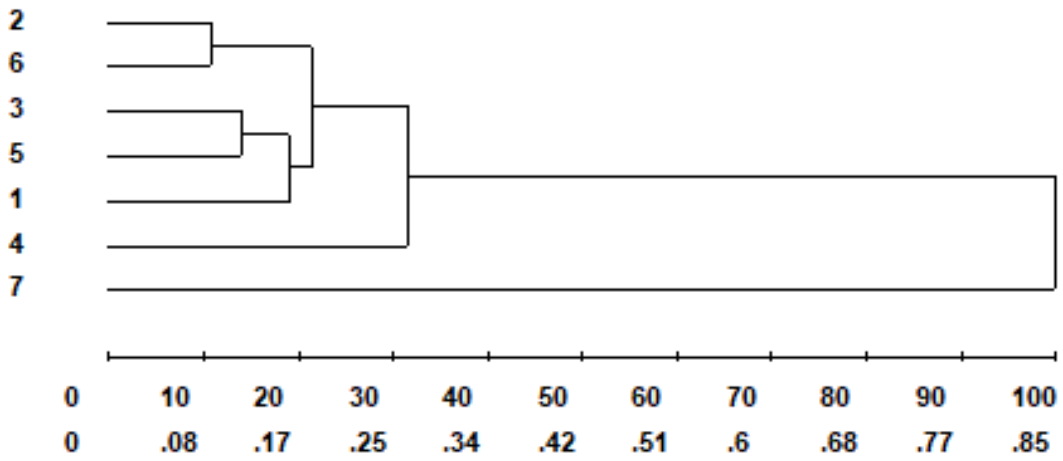
$$2C_B = \left(\frac{C1}{C2}\right) \times 2C_S$$

Where: 2CB: value of the content of DNA 2C (pg) of each species of Bixa; C1: mean of the G0 / G1 peak channel of Bixa species; C2: mean of the peak of the G0 / G1 peak of *S. lycopersicum*; 2CS: value of the 2C DNA content of S. *lycopersicum* (2.00 pg).

3 RESULTS AND DISCUSSION

When analyzing the results, it was observed that the amount of nuclear DNA between *B. arborea* (mean values ranged from 0.575 pg to 0.624 pg) and *B. orellana* (0.589 pg ± 0.005) did not differ significantly, although the two species showed morphological differences. The results showed that there is formation of two distinct populations, which are related to the two different species analyzed (figure 2).

Figure 2: Dendrogram obtained by the UPGMA method for six Bixa arborea phenotypes and one Bixa orellana phenotype in Alta Floresta – MT, (2020)



Source: Thatielen Furini

Likewise, the 6 phenotypes of *B. arborea* analyzed did not show any significant difference between them, with phenotype 1 being the one with the highest amount of nuclear DNA (0.624 pg ± 0.009), phenotype 5 being the one with the least amount of nuclear DNA, (0.575 pg ± 0.005) (Table 1). We cannot affirm that there is variability and existence of hybrids between the analyzed phenotypes, although they present marked phenotypic differences.

Tabela 1: Conteúdo de DNA (em pg) apresentado por cada um dos genótipos analisados através da Citometria de Fluxo, Alta Floresta – MT (2020).

Genótipos	Canal	Conteúdo de DNA (pg) (média)	Desvio Padrão	CV (%)
Bixa arborea 1	79,75	0.624a	0.009	1.62
Bixa arborea 1	85,81			
Bixa arborea 2	83,48	0.609ab	0.005	1.62
Bixa arborea 2	83,39			
Bixa arborea 3	83,54	0.617a	0.009	1.62
Bixa arborea 3	81,09			
Bixa arborea 4	79,75	0.586ab	0.004	1.62
Bixa arborea 4	79,99			
Bixa arborea 5	80,49	0.575b	0.005	1.62
Bixa arborea 5	78,26			
Bixa arborea 6	78,00	0.619a	0.007	1.62
Bixa arborea 6	77,95			
Bixa orellana 7	83,30	0.589ab	0.005	1.62
Bixa orellana 7	88,22			

Source: Thatielen Furini

The amount of nuclear DNA depends on the organism's evolutionary and adaptive history and how it adapted to the environment, reflecting on the phenotype (BENNET, 1972; SCHIFINO-WITTMANN, 2001). Although, it is normal to find a difference in the amount of DNA between individuals of the same species, as this is due to the appearance of structures in the genetic material, such as new nitrogenous bases, resulting from small adjustments that the organism undergoes due to contact with the environment (DAMATTA et al., 2010; FREITAS et al., 2017; OLIVEIRA et al., 2013), our results showed no significant difference, which may be related to other factors that were not explored in this work, such as the loss of genetic variability due to the increase in inbreeding and genetic drift, due to the small size of the studied population (CUSHMAN et al., 2016).

Unfortunately, studies focused on the genetic characterization of species of the genus *Bixa* are very scarce. However today it is known that Bixa orellana is a species that has a number of chromosomes that can vary from 2n = 14 (MUKHERJEE, 1975; ALMEIDA et al., 2006; HANSON et al., 2001; OHRI et al., 2004), up to 2n = 16 (SIMMONDS, 1954; KRISHNAN and AYYANGAR, 1987; RIVERA-MADRID et al., 2006).

Other studies show that *B. orellana* has 0.78 pg of nuclear DNA (OHRI et al., 2004), which differs considerably from our results. *B. arborea*, in turn, has a number of chromosomes 2n = 14 (MORAWETZ, 1986) and very few studies, which may be related to the fact that it is not exploited economically or in a traditional way by the population,

which uses *B. orellana* as a source of seeds and leaves (LOMBELO and PINTO-MAGLIO, 2014; DEQUIGIOVANNI et al., 2018). *B. orellana* is widely used because it has large amounts of chemical compounds that are only found in high content in this species, such as apocarotenoids, bixin and norbixin, phenolic compounds among others that are targeted and used mainly as a dye, in industry and by the population (PACHECO et al., 2018).

The coefficient of variation (CV) for all analyzes was 1.62%, which shows that the analyzes had good quality and, therefore, high data reliability. The CV shows sharpness and peak resolution between the G0 / G1 phases and points out the efficiency of the dye used (GONÇALVES et al., 2007). Some authors point out that a CV less than 5% is ideal (DOLEZEL and BARTOS, 2005).

It is necessary to point out that studies that quantify the nuclear DNA of *Bixa* species are scarce (OHRI et al., 2004). Most genetic characterization studies involving species of this genus are restricted to *B. orellana*, as demonstrated in the studies mentioned in this work (SEE MUKHERJEE, 1975; ALMEIDA et al., 2006; HANSON et al., 2001; RIVERA-MADRID et al., 2006; MORAWETZ, 1986).

Genetic studies involving *B. arborea* are non-existent and, therefore, this work is at the forefront. Thus, more detailed studies are needed to increase the genetic knowledge of these species, such as cytogenetic and reproductive studies. Cytogenetic studies will make it possible to assess whether there is a significant genetic difference between the two species analyzed, as the current analysis has not shown significant differences (PINTO-MAGLIO and PIEROZZI, 2015). Reproductive studies, in general, in turn, will make it possible to know if there is an exchange of genetic material between these two species in environments where they coexist (KAGEYAMA et al., 2003; HEREFORD, 2010) and this will increase the understanding of the relationships of kinship and evolutionary biology of *B. arborea* and *B. orellana*.

The dendrogram, constructed using the UPGMA method, based on a Euclidean distance matrix using fruit morphometry, showed the formation of two main groups by the Toucher method: one formed by phenotype 7 and another formed by phenotypes 2 and 6, 3, 5 and 1 and 4. In this case, phenotype 7 refers to *B. orellana*, while the second group is formed by the phenotypes of *B. arborea* (figure 2).

The variables that most explained this grouping were in decreasing order: number of seeds, total length, width and thickness of the fruit (Table 2). These results showed that between species the genotypic variation can accompany the phenotypic variation,

although in our results it was not possible to verify this. This fact may be related to the low number of samples that it was possible to collect. However, this same pattern does not happen with intraspecific variation, as there does not seem to be a relationship between genotypic variation and phenotypic variation. However, further statistical analysis is necessary, with a larger number of samples.

Tabela 2: Contribuição relativa de oito variáveis para a divergência genética conforme Singh (1981) dos frutos de *Bixa arborea* e *Bixa orellana* em Alta Floresta – MT. Alta Floresta – MT (2020)

Variável	Contribuição (%)
Número de Sementes	67,46
Comprimento total do fruto	16,133
Largura do fruto	6,183
Espessura do Fruto	9,851
Peso do Fruto	0,229
Peso da Semente	0,060
Peso do Pericarpo	0,053
Espessura do Pericarpo	0,025

Source: Thatielen Furini

Cluster analysis has been increasingly used and seeks, by means of classification criteria, to group varied genotypes based on greater or lesser similarity of characters (CRUZ et al., 2012). The UPGMA method is the most used and most popular method among cluster analyzes, being used in biology, in genetic, phylogenetic, evolutionary and ecological analyzes (DHAESELEER, 2005). In this context, a dendrogram can show the evolutionary relationships between the individuals analyzed based on physical characteristics (HUA et al. 2017). The UPGMA method was the most suitable, as it was the one that presented the highest cohenetic correlation (0.98) for the biometric data analyzed.

Some research shows that the study of the genetic diversity of plant species can be carried out based on analysis of the morphological characteristics of the specimens (SHYMOYA et al., 2002), because in this way, one can contribute to the genetic improvement, based on the selection increasingly productive or resistant genotypes. This is because the genotype of the species is strictly dependent on environmental characteristics (MENEZES et al., 2011), such as greater availability of nutrients, water and light.

4 CONCLUSIONS

Our analysis showed no significant difference with regard to the amount of nuclear DNA between species, nor intra-species. These results may be related to the characteristics of the groups of individuals used as a source of material: both were formed

by small populations and in the case of *B. orellana*, a single individual was used as a sample.

In contrast, the biometrics of the fruits and the cluster analysis were efficient in forming two distinct groups, therefore, the fruit proved to be a good structure for interspecific differentiation.

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