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PAULO MARCOS AMARAL SILVA

IS THE DIOECY IN *Myrsine* (Primulaceae) **DEFINED BY SEX CHROMOSOMES?**

ALEGRE, ES

2015

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Dissertation presented to the Universidade Federal do Espírito Santo, as part of the requirements of the Genetics and Breeding Graduate Program, for the attainment of the title Magister Scientiae.

Adviser: DSc. Milene Miranda Praça Fontes.
Co-Adviser: DSc. Tatiana Tavares Carrijo

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Approved in August 14, 2015.

EXAMINERS

Milene Miranda PraçaFontes
Universidade Federal do Espírito Santo
Adviser

Tatiana Tavares Carrijo
Universidade Federal do Espírito Santo
Co- Adviser

Wellington RonildoClarindo
Universidade Federal do Espírito Santo

Carla Cristina Gonçalves Rosado
Universidade Federal do Espírito Santo

To my dear mother, Sirlene Amaral Silva, my example of faith, perseverance and courage.

I dedicate

“Eye has not seen, nor ear heard, nor have entered into the heart of man, the things which God has prepared for those who love Him.”

1 Corinthians 2:9

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To God for the gift of life and to know that "the Lord has big plans for us."

BIOGRAPHY

Paulo Marcos Amaral Silva, son of Genivaldo dos Santos Silva and Sirlene Amaral Silva, was born in Rio de Janeiro, May 10th, 1990.

In 2009, he joined the *Universidade Federal do Espírito Santo* (UFES), in Alegre, ES graduating Bachelor of Biological Sciences in March 2013.

During the graduation period was Cell Biology monitor and Biotechnology Laboratory, colleger PIBIC/FAPES, where he developed research activities in molecular biology.

In September 2013, he joined the Genetics and Breeding Graduate Program, at Master degree, in the line of research - Cytogenetic and Evolutionary Biology and area of knowledge - Genome Evolution, submitting to the defense of dissertation in August 2015.

SUMMARY

ABBREVIATION LIST

APM	amiprophos-methyl
CV	coefficient of variation
CIM	callogenesis induction medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EIM	embryos induction medium
EMM	embryo maturation medium
FCM	flow cytometry
FEC	embryogenic friable calli
GM	germination medium
IZE	immature zygotic embryos
MS	Murashige and Skoog
NAA	α- naphthaleneacetic acid
PEG	polyethylene glycol
RM	regeneration medium
SE	somatic embryogenesis

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GENERAL INTRODUCTION

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Figure 2: karyotypes of *M. coriacea* with 45 and 46 chromosomes respectively (a-b), *M. parvifolia* with 45 and 46 chromosomes respectively (c-d), *M. guianensis* showed 45 chromosomes (e) and *M. umbellata* exhibiting 46 chromosomes (f). Bar = 5 µm.

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GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

Dioecious species are characterized by individuals with separate sexes, as *Myrsine* genus (Bawa and Opler 1978). The dioecy occurs in 6% of the 250,000 Angiosperms species described (Renner and Ricklefs 1995). The sex in plants can be controlled by genetics, hormonal, environmental and/or chromosomal factors. However, the system of sex determination most derived, which culminates in dioecy, involve sexual chromosomes (Ming and Moore 2007). Thus, the study of sexual chromosomes contributes with the knowledge about evolutionary mechanisms fixing the sexual dimorphism in vegetal groups (Zang et al. 2014).

Genes found in autosomal chromosomes also may be related with the sex determination. In *Ecballium elaterium* (L.) A. Rich., the allelic constitution of only one chromosomal locus defines male or female characteristics (Lebel-Hardenack and Grant 1997). The identification of genes related with sex determination is a challenge due poor knowledge about how these genes interact among themselves and with other factors that determine the sex in plants (Ming and Moore 2007; Zang et al. 2014).

The determination of genre in plants also is influenced by hormones, insomuch that application of exogenous phytohormones or their inhibitors can promote sexual reversion (Zhang et al. 2014). In *Arabidopsis thaliana* L., the jasmonic acid is related with male characteristics, as elongation of stamen filaments and pollen maturation. The ethylene is associated to female particularities in *Cucumis melo* L. (Zhang et al. 2014). Environmental factors as temperature, salinity,

photoperiod, nutrients and availability of water also are involved with the sex determination (Barrett and Hough 2012).

Epigenetic mechanisms as post-transcriptional modifications and/or post-translational alterations in histones are associated with the development of male and female organs (Adam et al. 2011*).* Gorelick (2003) considers that the first step for sexual chromosomes evolution is the DNA methylation, may result in suppression of meiotic recombination between homologous pairs. The hypothesis most current about the origin of sexual chromosomes describes evolutionary stages wherein occur modifications in autosomal chromosomes, resulting in proto-chromosomes X or Z, either Y or W. These changes (numeric and/or structural) during the evolution may originate a heteromorphic chromosome pair. The chromosomal system of sex determination most derivative is constituent by one sexual chromosome (Figure 1) (Ming et al. 2011).

Among the current knowledge, about 0.01% of angiosperms exhibit experimental evidence by cytogenetic and/or molecular techniques of the presence of sexual chromosomes. Of these species, 17 (Table 1) showed heteromorphic sexual chromosomes and 20 (Table 2) displayed homomorphic sexual chromosomes (Ming et al. 2011). Considering the dioecy fixed in the genus, Myrsine is a model suitable for investigate the existence of sexual chromosomes.

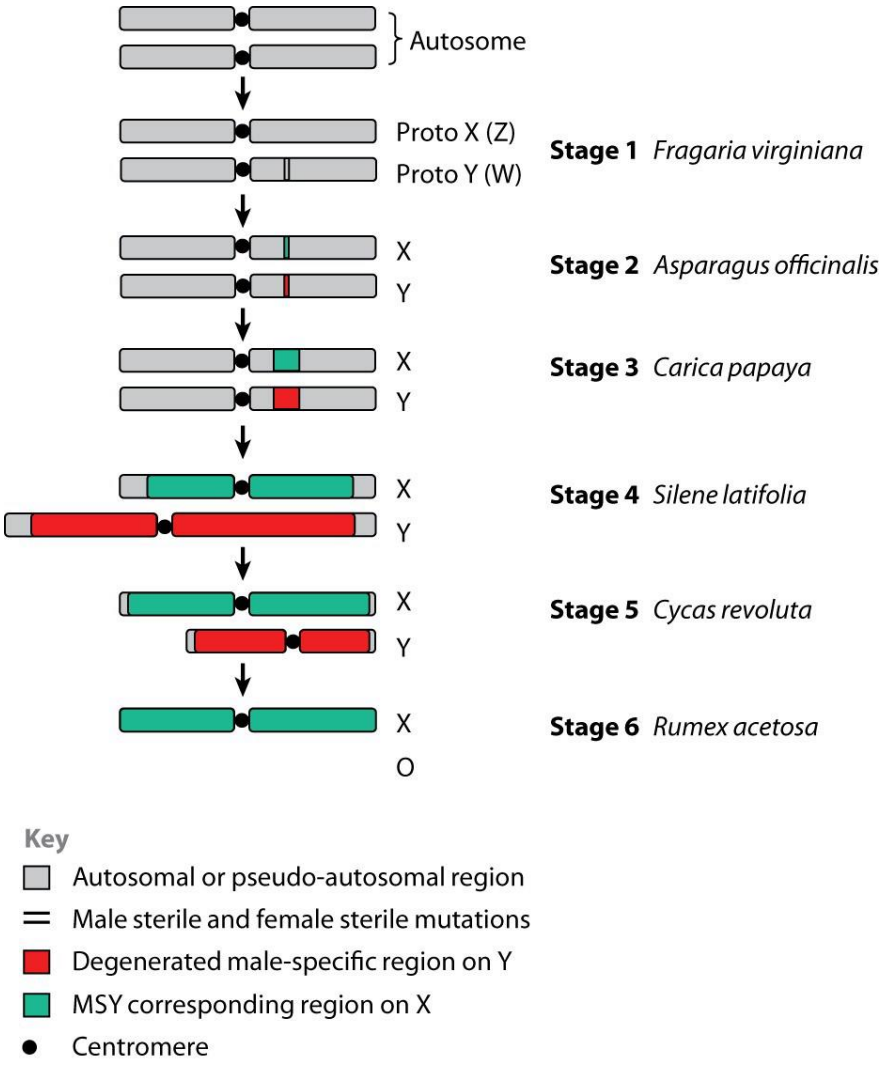

Figure 1: The six stages of sex chromosome evolution. Stage 1: unisexual mutation of two sex determination genes with complementary dominance. Stage 2: suppression of recombination between the two sex determination genes and YY genotype is viable. Stage 3: suppression of recombination spread to neighboring regions and a small male-specific region of the Y chromosome (MSY) region evolved. YY genotype is not viable. Stage 4: the MSY expands in size and degenerates in gene content via accumulation of transposable element insertions and intrachromosomal rearrangements. The X and Y chromosomes become heteromorphic. Stage 5: severe degeneration of the Y chromosome. Deletion of nonfunctional DNA sequences results in reduction of Y-chromosome size. Stage 6: suppression of recombination spreads to the entire Y chromosome. The Y chromosome is lost, and X-to-autosome ratio sex determination system has evolved. (Source: Ming et al. 2011).

Table 1: Species of plants with heteromorphic sex chromosomes under light microscopy

Family	Specie	Female	Male	Reference
Cannabaceae	*Cannabis sativa* L.	XX	XY	Sakamoto et al. (2000)
	Humulus lupulus L.	XX	XY	Grabowska-Joachimiak et al. (2006)
	H. lupulus var, cordifolius Maxim.	$X_1X_1X_2X_2$	$X_1Y_1X_2Y_2$	Ono (1995)
	H. japonicus Siebold & Zucc.	XX	XY_1Y_2	Grabowska-Joachimiak et al. (2006)
Caryophyllacea	*Silene latifolia* Poir.	XX	XY (Y about 1/3 longer than the X)	Nicolas et al. (2005)
	S. dioica (L.) Clairv.			
	S. diclinis (Lag.) M. Laínz	XX	$X\ Y_1Y_2$	Howell et al. (2009)
Cucurbitaceae	*Coccinia grandis* (L.) Voigt	XX	XY (Y is 2.5 times longer than the X)	Roy & Roy (1971)
	Trichosanthes dioica Roxb.			
	T. kirilowii Maxim.	XX	XY	Patel (1952)
	T. ovigera Blume			
Polygonaceae	*Rumex acetosa* L.	XX	XY_1Y_2 (the X is slightly larger than each of the two Ys)	Blocka-Wandas et al. (2007)
	R. acetosella L.	XX	XY	Cuñado et al. (2007)
	R. graminifolius Rudolph ex Lamb.	XX	XY	Love (1943)
	R.hastatulus Baldwin	XX	XY ou XY_1Y_2	Smith (1964)
	R. papillaris Boiss. & Reut.	XX	XY_1Y_2	Navajas-Pérez (2009)
	R. paucifolius Nutt.	XX	XY	Smith (1968)
	R. suffruticosus J. Gay ex Meisn.	XX	XY	Cuñado et al. (2007)

(Adapted from Ming et al. 2011)

Table 2: Species of plants with homomorphic sex chromosomes under light microscopy

Family	Specie	Female	Male	Reference
Actinidiaceae	*Actinidia chinensis* Planch.	XX	XY	Fraser et al. (2009)
Amaranthaceae	*Acnida tamariscina* (Nutt.) Alph. Wood	XX	XY	Murray (1940)
Arecaceae	*Phoenix dactylifera* L.	XX	XY	Siljak-Yakovlev et al. (1996)
Asparagaceae	*Asparagus officinalis* L.	XX	XY	Loptien (1979)
Caricaceae	*Carica papaya* L.	XX	XY	Liu et al. (2004)
	Vasconcellea cundinamarcensis V.M. Badillo, *V. goudotiana*Triana & Planch., *V. parviflora* A. DC., *V. pulchra* V.M. Badillo	XX	XY	Wu et al. (2010)
Caryophyllaceae	*Silene otites* (L.) Wibel	ZW? XX?	ZZ? XY?	Sansome (1938)
	S. colpophylla Wrigley	XX	XY	Warnke (1942)
Chenopodiaceae	*Spinacia oleracea* L.	XX	XY	Lan et al. (2006)
Cucurbitaceae	*Bryonia dioica* Jacq.	XX	XY	Oyama et al. (2009)
	Ecballium elaterium (L.) A. Rich.	XX	XY	Galán (1946)
Datiscaceae	*Datisca cannabina* L.	ZW	ZZ	Wolf (2001)
Dioscoreaceae	*Dioscorea tokoro* Makino	XX	XY	Terauchi & Kahl (1999)
Santalaceae	*Viscum fischeri* Engl.	$X_1X_2X_3X_4$	$Y_1Y_2Y_3Y_4Y_5$	Barlow & Wiens (1976)
Rosaceae	*Fragaria virginiana* Mill.	ZW	ZZ	Spigler et al. (2008)
	F. moschata Duchesne	ZW	ZZ	Ahmadi & Bringhurst (1991)
Salicaceae	*Populus trichocarpa* Torr. & A. Gray	ZW	ZZ	Yin et al (2008)

(Adapted from Ming et al. 2011)

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CHAPTER

Is the dioecy in *Myrsine* (Primulaceae) defined by sex chromosomes?

1. ABSTRACT

The dioecy occurs in 6% of the angiosperms, including *Myrsine* species (Primulaceae) that show male and female individuals distinguishable by differences that includes the morphology of sepals and petals. The sexual system fixed in this genus, was the motivation in research if the existence of sexual chromosomes culminate in dioecy for *Myrsine*. Employing tissue culture, flow cytometry and cytogenetic tools in *Myrsine coriacea* (Sw.) R.Br. ex Roem & Schult., *Myrsine umbellata* Mart., *Myrsine guianensis* (Aubl.) Kuntze and *Myrsine parvifolia* A.DC., the aimed this study was answer the question: is the dioecy in *Myrsine* is defined by sex chromosomes? From leaves of male and female individuals collected in the field and leaves and roots of unknown sex obtained of in vitro culture, were found mean values of DNA content statistically identical. These data were attributed to the presence of secondary metabolites reported for the genus. However, an intraspecific variation was observed in cytogenetic analysis in the four *Myrsine* species. Slides exhibited metaphases with 2n = 45 and 2n = 46 chromosomes in different individuals. These

results were observed consistently from different times of exposition to anti-mitotic agent and distinct treatments of enzymatic maceration. Thus, the chromosome number variation found, associated with the sexual system well defined, can be concerning to the sexual chromosomes in *Myrsine* genus. These data suggest a chromosomal system of sex determination with multiple X and/or Y described in some plant species. The system ZW also is possible, as well as X0 or Z0, systems still not reported in vegetal groups. The present work provided the first data about the nuclear DNA content by flow cytometry in *Myrsine* and supplied cytogenetics evidences that indicate the existence of sexual chromosomes.

Keywords: cytogenetic, flow cytometry, sex chromosomes, sex determination.

2. INTRODUCTION

Primulaceae comprises 58 genus involving species hermaphrodite, monoecious, dioecious and polygamous. Between them, *Myrsine* genus shows about 300 species represented by shrub and trees dioecious (Freitas and Kinoshita 2015). The dioecy is a characteristic fixed in this genus, being that the dimorphism is not limited to sexual organs, but also on the morphology of sepals and petals, which exhibit differences in size and form (Albuquerque et al. 2013). These particularities in *Myrsine*, contrasting to the other species taxonomically related, aroused the interest to investigate the factors that promote the dioecy in this genus.

Despite of the advances (Charlesworth 2013), is still not clear for the majority of plant taxa the factors (environmental, hormonal, genetic and/or chromosome) that determine the sexual system (Bachtrog et al. 2014). The studies have been accomplished using applications to identify and, posteriorly, characterize the chromosomal sex determination system. From these approaches, sex chromosomes have also found in distinct plant clade, as Bryophytes (*Marchantia polymorpha* L., *Ceratodon purpureus* (Hedw.) Brid.), Gymnosperms (*Cycas revoluta* Thunb., *Ginkgo biloba* L.) and Angiosperms (Ming 2011). In a review, Zhang et al. (2014) listed 19 Angiosperms species with homomorphic sex chromosomes, which were previously conceptualized by Vyskot and Hobza (2004) as chromosomes morphological similarly that possess distinct genes related with sex determination. Zhang et al. (2014) also reported 18 Angiosperms species exhibiting heteromorphic sex chromosomes – chromosomes morphologically different in male and female that carry genes of sex determination (Vyskot and Hobza 2004).

Cytogenetic approaches contribute with the knowledge concerning plant sexual chromosomes. Nevertheless, Armstrong and Filatov (2008) highlighted the need of challenges in classical cytogenetic procedures in order to clearly identify and characterize each chromosome of the karyotype. Methodologies that provide metaphase with chromosomes not overlap, centromere well defined, no damage to the chromatin structure and free of cytoplasm debris are fundamental for an accurate chromosomal characterization (Nunes et al. 2013). Cytogenetic techniques that result in images with high resolution enable the identification of heteromorphic chromosomes that can be related with the dioecy expressed in a taxon. In view of this, methodological subtleties can be crucial in elucidation of chromosomal aspects related to dioecious sexual system in *Myrsine*. However, only the chromosome number is reported for the genus. Among the 12 *Myrsine* species with chromosome number determined, 11 showed 2n = 46 chromosomes (Beuzenberg and Hair 1983; Dawson 1995; Dawson 2000; Bedalov et al. 2002) and only one with 2n = 48 chromosomes, *Myrsine guianensis* (Aubl.) Kuntze (Molero et al. 2006).

From the premise that karyotype differences provided by heteromorphic sex chromosomes culminate in variations of 2C nuclear DNA value (Mendonça et al. 2010), males and females individuals can be screen by the flow cytometry (FCM), a practice, rapid and accurate tool (Costich et al. 1991). For this purpose, the FCM has mainly been used in animals (Garner 2006; Vanthournout et al. 2014), as mammals (Garner et al. 2013) and birds (Loureiro et al. 2014), and minoritarily in plants (Costich et al. 1991; Doležel and Göhde 1995). For a reliable discriminatory analysis, adaptations in the FCM procedures are necessary to obtain G_0/G_1 peaks with values of coefficient of variation (CV) below than half difference of the nuclear genome size

between the individuals male and female (Rosado et al. 2009; Karsburg et al. 2009). Therefore, methodological alterations in FCM can help the screen of male and female individuals in *Myrsine*. This aspect has not been applied in *Myrsine* genus, and only data about nuclear 2C value is limited to *Myrsine africana* L. that presents 2C = 2.46 picograms (pg) (Hanson et al. 2003).

The cytogenetic and FCM approaches in *Myrsine* can help on understanding of sex determination system of the genus. Thus, the aim of this study was employ these tools to answer the question: is the dioecy in *Myrsine* defined by sex chromosomes?

3. MATERIAL AND METHODS

3.1. Plant material

Specimens of *Myrsine coriacea* (Sw.) R.Br. ex Roem & Schult. and *Myrsine umbellata* Mart. were collected within natural populations occurring at Atlantic Forest fragment located in Iúna, Espírito Santo – Brazil (20°21'6"S – 41°31'58"W). *Myrsine guianensis* (Aubl.) Kuntze and *Myrsine parvifolia* A.DC were collected in state park Paulo César Vinha, Guarapari, Espírito Santo – Brazil (20°36'15"S – 40°25'27"W). From plants of these species, leaves, immature and mature fruits were collected. The collections were conducted between October of 2012 and July of 2015.

Solanum lycopersicum L. and *Pisum sativum* L. (internal standards for FCM, 2C = 2.00 pg and 2C = 9.16 pg, respectively, Praça-Fontes et al. 2011) were kindly supplied by Dr. Jaroslav Doležel (Experimental Institute of Botany, Czech Republic).

3.2. In vitro plantlet recovering

The pericarp of the mature fruits of *M. coriacea*, *M. umbellata*, *M. guianensis* and *M. parvifolia* was manually removed, and the seeds were disinfested accordingly Oliveira et al. (2013). The seeds were inoculated in germination medium (GM) constituted of salts MS (Sigma®), vitamins MS (Musrashige and Skoog, 1962), 60 g L^{-1} sucrose, 7 g L^{-1} agar and 2.685 µM of acetic naphthaleneacetic acid (NAA). Seeds of *S. lycopersicum* and *P. sativum* were subjected to the same procedures described above and inoculated in GM without NAA. The germination was conducted at 25 °C under a 16/8 hours (light/dark) regime.

Due to larger availability of seeds in field, *M. coriacea* was also employed for somatic embryogenesis (SE). SE was realized with zygotic embryos obtained of immature fruits collected 45 days after the anthesis. Posteriorly the removal of pericarp, the seeds were disinfected (Oliveira et al. 2013). In asseptic conditions, the immature zygotic embryos (IZE) were extracted and placed in Petri dishes with callogenesis induction medium (CIM). The resulted friable embryogenic calli (FEC) were transferred to embryo induction medium (EIM), after the somatic embryos were placed to embryo maturation medium (EMM), and the mature cotiledonary somatic embryos were converted in plantlets in regeneration medium (RM).

The media employed in SE contained half strength MS basal salts, 10 mL L^{-1} B5 vitamins (Murashige and Skoog 1962), 0.08 g L^{-1} L-cystein, 0.4 g L^{-1} L-glutamine, 0.1 g L^{-1} myo-inositol, 30 g L^{-1} sucrose and 2.8 g L^{-1} Phytagel. The pH was adjusted to 5.7 prior autoclaving, and the culture was maintained in radiation provided by two

fluorescent lamps (20 W, Osram®, Brazil) with 36 µmol m^{-2} s^{-1} light at 25 °C. The particularities of each medium are summarized in Figure 1.

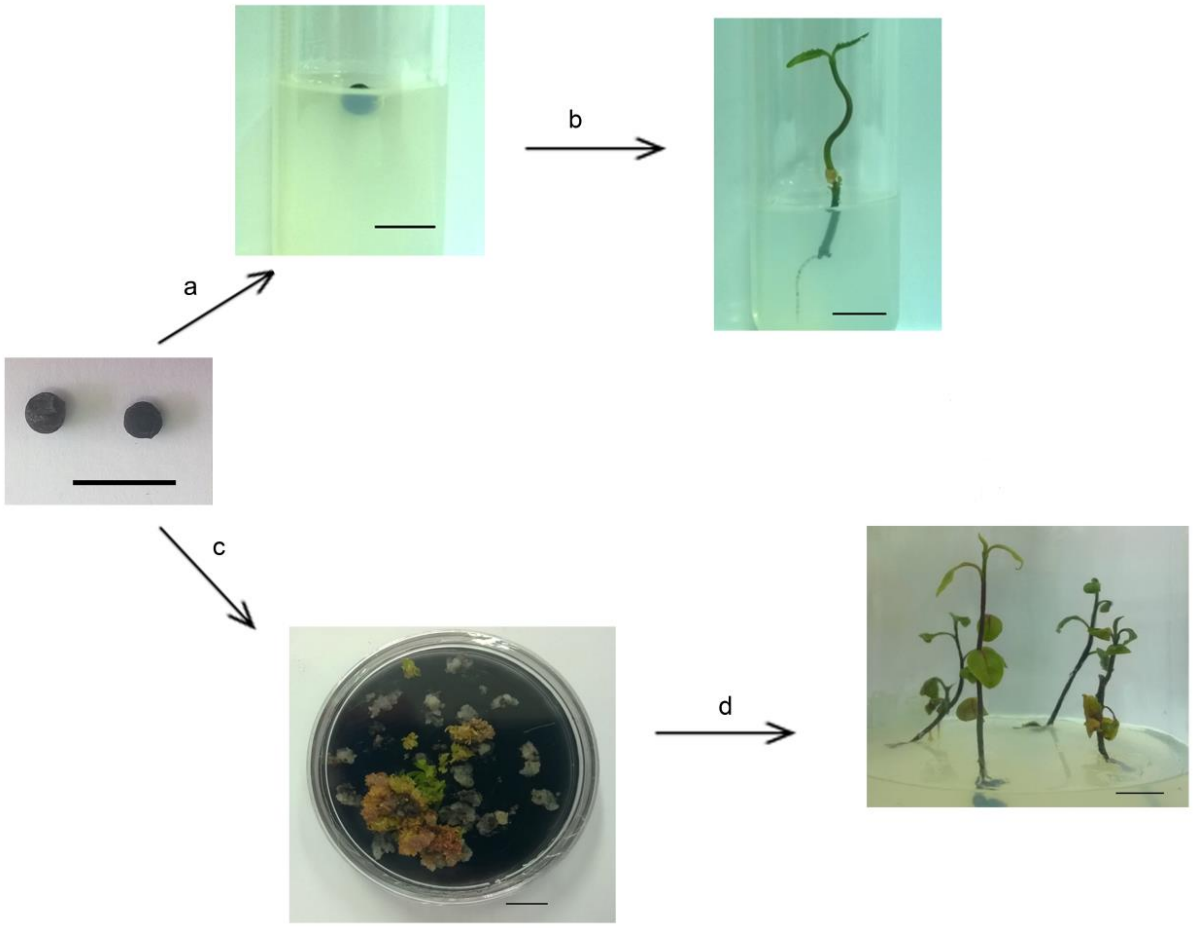

Figure 1: (a – b) Plantlet obtained from seed germination in GM; c) FEC result of IZE placed in CIM (kept in the dark at 25 ºC) with somatic embryos in distinct embryogenic stages; d) Plantlet regenerated from mature cotiledonary somatic embryos in RM. Bar = 1 cm.

3.3. Nuclear 2C value measurement

In order to determine the suitable biological material and nuclei isolation buffer, the FCM procedure was accomplished using: (a) leaves collected in field of male and female individuals of the four *Myrsine* species (samples) and the

standards, (b) leaves and roots of plantlets in vitro of *M. umbellata*, *M. coriacea* (samples) and *P. sativum* (standard), and (c) two distinct buffers, with or without polyethylene glycol (PEG), for nuclei isolation.

Nuclei suspensions were obtained from chopping (Galbraith et al. 1983; Praça-Fontes et al. 2011) of leaf fragments (1 cm^2) excised of each sample (*Myrsine* species) and standard (*S. lycopersicum* or *P. sativum*). Posteriorly, 500 µL nuclei isolation buffer was added, the suspensions were processed and stained following Praça-Fontes et al. (2011). The suspensions were analyzed in the flow cytometer Partec PAS II/III (Partec GmbH). The genome size of the *Myrsine* species was calculated according to the formula:

$$2C_M = \left(\frac{C1}{C2}\right) . 2C_P$$

Wherein:

$2C_M$*:* value of the 2C DNA content (pg) of each *Myrsine* species.

C1: average channel of G_0/G_1 peak of the *Myrsine* species.

C2: average channel of G_0/G_1 peak of standard.

$2C_S$: value of the 2C DNA content of standard.

Differences in genome size between the sexes and for leaves and roots were evaluated with test F at level of 5% of probability.

3.4. Cytogenetic analysis

Roots obtained from in vitro germination were treated with 5 µM amiprophos-methyl (APM) for 12, 15, 18 or 24 h at 4°C. Besides, roots of *M. coriacea* obtained from SE were exposed to 2 mM hydroxyurea (Sigma®) for 18 h at 25°C, kept under running water for 3 h, and treated with 5 µM APM for 12 h at 4°C.

After, the fixation was performed from protocol describe by Carvalho et al. (2007), the roots, after 24 h, were washed in dH_2O, and macerated in 1:5 pectinase solution (enzyme: dH_2O) for 3 h at 34°C, or 1:20 enzymatic (4% cellulase, 1% macerozyme and 0,4% hemicellulase) solution for 1 h 30 min or 1 h 45 min at 34°C. The material was washed in dH_2O, fixed again and kept at -20°C.

Slides were prepared and stained according to Carvalho et al. (2007). The slides were analyzed on a Nikon eclipse Ci-S microscope (Nikon). The capture of metaphases was performed using 100x objective and a CCD camera (Nikon Evolution™) coupled to a Nikon microscope 80i (Nikon).

4. RESULTS

4.1. In vitro plantlet recovering

Approximately 60 days after inoculation in GM, morphologically normal plantlets were obtained for *M. coriacea*, *M. umbellata*, *M. guianensis* and *M.*

parvifolia. All plantlets exhibited leaves and roots enough to FCM and/or cytogenetic approaches (Figure 1).

FEC were obtained from IZE of *M. coriacea* in CIM, consequently somatic embryos were recovered in different morphogenic stages, and cotyledonary somatic embryos were maturated in EIM. From these embryos plantlets were regenerated after 120 days in RM, and their roots were used for cytogenetic (Figure 1).

4.2. Nuclear 2C value measurement

The preliminary FCM measurements showed that the mean 2C value of the *Myrsine* species (2C = 6.65 – 7.96 pg) is closer of the 2C value of the standard *P. sativum* (2C = 9.16 pg) than *S. lycopersicum* (2C = 2.00 pg). Based on international criteria for FCM about linearity, *P. sativum* was considered the most adequate standard in relation to *S. lycopersicum*.

FCM analysis performed from leaves collected of plants in the field not resulted in histograms showing G_0/G_1 peaks. Due to this result, the buffer of nuclei isolation was supplemented with PEG. This change generated histograms with G_0/G_1 peaks for *M. umbellata* and the standard *P. sativum* exhibiting coefficient of variation (CV) less than 5%. The mean value was 2C = 6.65 ± 0.03 and 2C = 6.67 ± 0.07 for male and female plants of *M. umbellata*. The values found between the sexes were statistically identical by test F at level of 5% of probability.

Considering FCM results, leaves and root meristems of in vitro plantlets of *M. coriacea* and *M. umbellata* were separately employed for nuclei isolation, enabling the genome size measurement. The mean values were 2C = 6.60 ± 0.14 pg for

leaves and 2C = 6.74 ± 0.18 pg for roots in *M. coriacea*; 2C = 6.63 ± 0.13 pg for leaves and 2C = 6.82 ± 0.09 pg for roots in *M. umbellata*. The differences between leaves and roots were statistically identical by test F at level of 5% of probability.

4.3. Cytogenetic analysis

The treatment with APM for 18 or 24 h resulted only in metaphasic chromosomes. Already the roots exposed for 12 or 15 h provided metaphases and prometaphases with chromosomes in distinct chromatin compact level. Roots of *M. coriacea* previously exposed to hydroxyurea enabled to obtain prometaphases with telomeric regions well defined.

Root meristems macerated in 1:5 pectinase solution not provided the disorganization of cell wall, ensuring all chromosomes inside of cell. This aspect allowed an accurate chromosome number determination in 45 or 46 chromosomes (Figure 2 - b).

Roots treated in 1:20 enzymatic (4% cellulase, 1% macerozyme and 0.4% hemicellulase) solution for 1h 30 min enabled to obtain individualized chromosomes with preserved morphology (Figure 2 – c-f). The prometaphases and metaphases showed chromosomes did not overlap, centromere well defined, no damage to the chromatin structure and free of cytoplasm debris (Figure 2 – c- f).

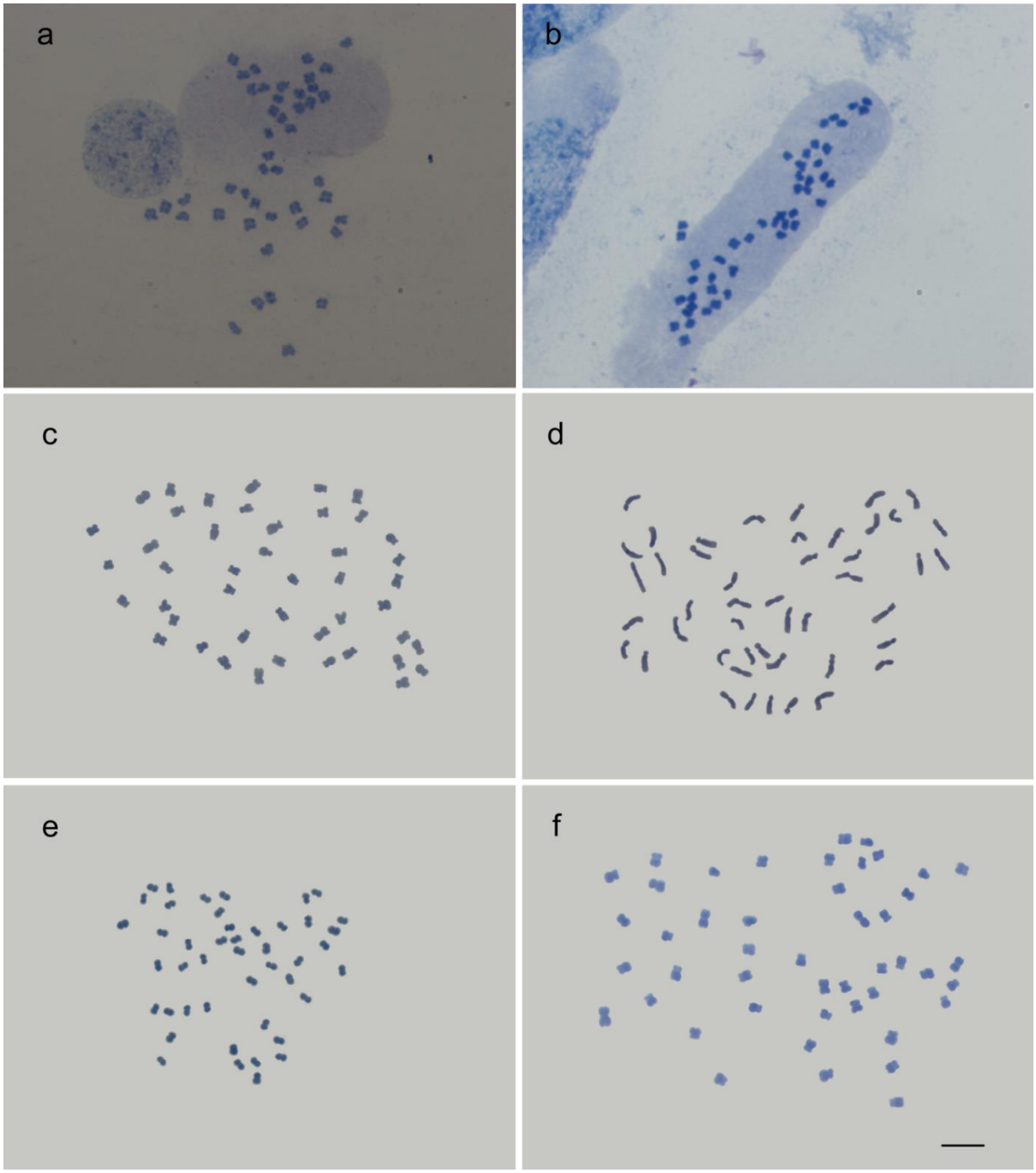

Figure 2: Karyotypes of *M. coriacea* with 45 and 46 chromosomes respectively (a-b), *M. parvifolia* with 45 and 46 chromosomes respectively (c-d), *M. guianensis* showed 45 chromosomes (e) and *M. umbellata* exhibiting 46 chromosomes (f). Bar = 5 µm.

5. DISCUSSION

In FCM, the first step was defined the best standard applied on measurement of DNA content of the *Myrsine* species. *P. sativum* was considered more adequate in relation to *S. lycopersicum* because it satisfies the international FCM criteria. *Myrsine* species presents 2C DNA value (2C = 6.65 – 7.96 pg) closer to the *P. sativum* (2C = 9.16 pg), without overlapping of the G_0/G_1 peaks. These aspects assured accurate FCM analysis due to avoiding nonlinearity errors (Praça-Fontes et al. 2011).

However, a challenge for FCM was the presence of secondary metabolites in the *Myrsine* leaves. Tannins, saponins, flavonoids and steroids are reported in this genus (Abbi et al. 2011). These compounds present in leaves of *Myrsine* species (Luna et al. 2014) probably prevented the measurement of 2C value in individuals of the field when the OTTO I buffer (Otto, 1990) was supplemented with DTT (dithiothreitol). Cytosolic compounds can reduce or inhibit the interaction of fluorochromes to the DNA molecule during the staining step of the nuclei (Noirot et al. 2003). The antioxidants, as the DTT, employed in nuclei isolation buffer, have function to inhibit the interference of cytosolic compounds to the DNA, preserving the chromatin structure (Shapiro 2003). Nevertheless, the addition of this reducing agent in nuclei isolation buffer was not efficient to provide nuclei suspensions suitable for FCM. Thus, the DTT, a compound more specific for molecules that possess free sulfhydryl groups (Life Technologies 2015), was replacement by PEG that shows a large spectrum for antioxidant activities, a mechanism named as PEGylation (Life Technologies 2015). In accordance, the PEG was more efficient in inhibit the action

of cytosolic compounds in nuclei isolation step in relation to DTT, resulting in G_0/G_1 peaks for *M. umbellata* and *P. sativum* with CV below 5%.

This change made feasible the measurement of DNA content only for *M. umbellata*, that presented 2C = 6.65 ± 0.03 for male and 2C = 6.67 ± 0.07 for female. The FCM result from leaves collected in the field may have been influenced by environmental conditions. *M coriacea* and *M. umbellata* leaves were collected in Atlantic Forest fragment at 600 and 1,100 m of elevation, respectively, and *M. guianensis* and *M. parvifolia* were collected in fragments of sandy coastal plain (*Restinga*). The production of secondary metabolic is influence by environmental factors, as humidity, temperature, light intensity, availability of water and nutrients (Akula and Ravishankar 2011). Thus, the elevation gradient can be associated with the FCM result, suggesting for *Myrsine* a differentiated production of secondary metabolic compounds in higher altitudes.

The mean 2C values obtained for each sex of *M. umbellata* were statistically identical. The technical constraint in delimit male and female individuals is related with the necessity of obtain G_0/G_1 peaks with CV values below than half difference of the 2C DNA value between the sexes (Mendonça et al. 2010). This limitation explains the small use (Costich et al. 1991; Doležel and Göhde 1995) of this tool for sexual selection in vegetal groups.

In view this, in vitro plantlets of unknown sex were also used for FCM measurements aiming to minimize the interference of metabolics secondary due to controlled environment on the tissue culture. Besides, based on presuppose that different vegetal tissues produces varied rates of secondary metabolites (Murthy et al. 2014), leaves and roots were used. Therefore, the mean 2C DNA values were

measured for leaves and roots of all species. The mean values for leaves and roots were statistically identical, as well as the values obtained from leaves in vitro and ex vitro for *M. umbellata*. Based on these results, plantlets in vitro were fundamental for FCM accomplishment and leaves or roots are recommended for measurement of 2C DNA in *Myrsine*. From these procedures, the mean 2C nuclear DNA content of *Myrsine* species was measured for the first time by FCM.

The only data about genome size in *Myrsine* was reported for *M. africana* with 2C = 2.46 pg (Hanson et al. 2003). These authors measured the DNA content by Feulgen microdensitometry and used *Vigna* sp. as standard. Pal et al. (2004) reported levels of endoreduplication in cells of *V. radiata*, varying of 2C to 64 C. The differences between the values found for *Myrsine* species in this study and the value observed for *M. africana* differ in about 200%.

2C DNA value was also reported for other Primulaceae species, values close to observed for *Myrsine* species in this study were reported for *Cyclamen purpurascens* Mill. (2C = 6.6 pg) and *Dodecatheon meadia* L. (2C = 5.58 pg). Higher DNA contents are described for *Cyclamen coum* Mill. (2C = 13.56 pg), *Soldanella pusilla* Baumg. (2C = 12.36 pg) and minors values for *Soldanella hungarica* Simonk (2C = 3.16 pg) and *Primula vulgaris* Huds (2C = 0.47 pg) (Bennett and Leitch 2012). The interspecific variation for 2C DNA value between the species employed in this study, and for other species of Primulaceae family, reported by Bennett and Leitch (2012), can suggest numerical and structural changes in the karyotype for this vegetal clade.

In relation to cytogenetics of *Myrsine*, only chromosome number is mentioned (Beuzenberg and Hair 1983; Dawson 1995; Dawson 2000; Bedalov et al. 2002;

Molero et al. 2006). Furthermore, the reports are limited to number and not display images that represent the chromosomes. In view this, the methodological cytogenetics parameters were revalued. For this, the establishment of *Myrsine* species in vitro was fundamental, which enabled the obtainment of root meristems for the cytogenetics analysis independently of reproductive period. The meticulous standardization of cytogenetics procedures was essential for an accurate chromosomal characterization. Thus, the distinct procedures with antimitotic agent and of enzymatic maceration allowed the counting and characterization of chromosomes.

The diversity of chromosome numbers among the slides analyzed was the base for exploring karyotype differences between the individuals. The number of 46 (Beuzenberg and Hair 1983; Dawson 1995; Dawson 2000; Bedalov et al. 2002) and 48 chromosomes (Molero et al. 2006) has been reported, but the number 45 was found for the first time in this study. A sexual system well defined (dioecy), in association with the occurrence of individuals with odd chromosome number, strengthen the hypothesis of a chromosomal system of sex determination in *Myrsine*. Identical characteristics were observed in *Humulus japonicus* Siebold & Zucc., females with 2n = 2x = 16 and males 2n = 2x = 17, that possesses the system XX/XY_1Y_2 (Grabowska-Joachimiak et al. 2006). A similar case occurs in *Frullania dilatata* (L.) Dumortier, females with 2n = x = 9 chromosomes (with two large sex) and males 2n = x = 8 (with only one sex chromosome), featuring a system X_1X_2/Y. The system ZW also is possible, once the sex heterogametic is not determined. This system still was not described in plants, but is reported in Lepidoptera species (Yoshido et al. 2005).

The chromosome number found for *Myrsine* species also suggest an X0 or Z0 system, that proposes a total suppression of recombination between the X and Y and loss of Y of these chromosomes. Since, the sex is determined by ratio between X and autosome chromosomes (Ming et al. 2011). The X0 system is reported in *Zyginidia pullula* (Insecta, Hemiptera) with 17 chromosomes in males and 18 in female (Negri et al. 2006), and the system Z0 is demonstrated in several species of Lepidoptera (Yoshido et al. 2006).

6. CONCLUSIONS

The sexual system fixed in *Myrsine* and the intraspecific variation found by cytogenetics suggests that a chromosomal system of sex determination culminated in dioecy of the genus. The number of 45 chromosomes is reported for the first time for *Myrsine*, even as the genome size measured by FCM. Methodological strategies fundamental on *Myrsine* are described in this study that provides support for research in tissue culture, FCM and cytogenetics. These data contribute with the understanding about systematic and evolution of sexual system for *Myrsine*.

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