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RENATA FLÁVIA DE CARVALHO

POLYPLOID ORIGIN AND KARYOTYPE EVOLUTION IN *Myrsine* L.

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Dissertação apresentada à Universidade Federal do Espírito Santo, como parte das exigências do Programa de Pós-Graduação em Produção Vegetal, para obtenção do título de *Magister Scientiae*.
Orientador: Prof. Dr Wellington Ronildo Clarindo.
Coorientadora: Profª. Drª. Tatiana Tavares Carrijo

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Aprovada em 29 de fevereiro de 2016.

COMISSÃO EXAMINADORA

Prof. Dr. Wellington Ronildo Clarindo
Universidade Federal do Espírito Santo
Orientador

Profª. Drª. Tatiana Tavares Carrijo
Universidade Federal do Espírito Santo
Coorientadora

Profª. Drª. Milene Miranda Praça-Fontes
Universidade Federal do Espírito Santo

Profª. Drª. Marcia Flores da Silva Ferreira
Universidade Federal do Espírito Santo

À minha amada mãe Geralda, pelo exemplo de força e coragem, amor e apoio incondicional.

Eu dedico

“Lembrando que sempre há uma outra chance, uma outra amizade, um outro amor, uma nova força. Para todo fim um recomeço.”

Antoine de Saint-Exupéry

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BIOGRAFIA

Renata Flávia de Carvalho, filha de Sebastião Flaviano de Carvalho e Geralda de Fátima Souza Carvalho, nasceu em Inhapim, Minas Gerais, no dia 01 de junho de 1989. Em 2009, ingressou na Universidade Federal do Espírito Santo, em Alegre, ES, graduando-se em Licenciatura Plena em Ciências Biológicas em Fevereiro de 2014. Durante o período de graduação foi monitora da disciplina Biologia Celular, monitora ambiental no Parque Estadual Cachoeira da Fumaça, e monitora do núcleo de acessibilidade da UFES. Foi bolsista de iniciação à docência (PIBID) e voluntária do PIVIC, onde desenvolveu atividades relacionadas à citogenética vegetal e cultura de tecidos. Em março de 2014 ingressou no curso de Mestrado pelo Programa de Pós-Graduação em Produção Vegetal, atuando na área de Biotecnologia e Ecofisiologia do Desenvolvimento de Plantas, sob orientação do Prof. Dr. Wellington Ronildo Clarindo, submetendo-se à defesa de dissertação em fevereiro de 2016.

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LISTA DE ABREVIATURAS

APM	amiprofos-methyl
CV	coefficient of variation
dH_2O	distilled water
FCM	flow cytometry
PEG	polyethylene glycol
UPGMA	Euclidean Distance and Unweighted Pair-Group Method Average

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RESUMO

A poliploidia desempenha um papel relevante na diversificação e especiação das Angiospermas, incluindo a família Primulaceae. Espécies diploides, tetraploides e octaploides são reportadas para os gêneros *Cyclamen*, *Dodecatheon* e *Primula*, mas os aspectos evolutivos que conduzem às mudanças cromossômicas são pouco compreendidos. Para expandir o conhecimento sobre esse assunto em Primulaceae, nós estudamos três espécies de *Myrsine* (*M. coriacea, M. umbellata* e *M. parvifolia*) que mostram diferentes habilidades para ocupar os variados tipos de vegetação dentro da Mata Atlântica brasileira. A caracterização citogenética evidenciou indivíduos com 2n = 45 cromossomos para *M. parvifolia* e *M. coriacea*, com a maioria dos indivíduos das três espécies tendo 2n = 46. Os cariogramas apresentaram pares de cromossomos morfologicamente semelhantes e distintos, sugerindo uma origem monofilética e alopoliploide verdadeiro para as três espécies. Além disso, um evento de pós-poliploidização relacionado a rearranjos cromossômicos estruturais foi evidenciado a partir de diferenças no valor médio nuclear 2C e morfometria dos cromossomos encontrados entre as espécies. Assim como tem sido feito para outras espécies, abordagens de citogenética e de tamanho do genoma acuradas representam um ponto de partida para o entendimento da origem e influência da poliploidização e mudanças pós-poliploidização no cariótipo das espécies de *Myrsine*. Além disso, a ocupação diversa das espécies de *Myrsine* em ambientes distintos pode ser atribuída à alopoliploidia e seus efeitos. Portanto, este estudo pioneiro de *Myrsine* fornece informações sobre o papel relevante da poliploidia na evolução do cariótipo e diversificação em Primulaceae.

Palavras-chave: Citogenética, citometria de fluxo, Floresta Atlântica, Myrsinaceae, poliploidia, Rapanea.

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ABSTRACT

Polyploidy plays a relevant role in the diversification and speciation of Angiosperms, including the family Primulaceae. Diploid, tetraploid and octaploid species are reported for the genera *Cyclamen*, *Dodecatheon* and *Primula*, but the evolutionary aspects that led to chromosomal changes are poorly understood. To expand the knowledge on this subject in Primulaceae, we studied three *Myrsine* species (*M. coriacea, M. umbellata* and *M. parvifolia*) that show different abilities to occupy the varied types of vegetation within the Brazilian Atlantic Forest. Cytogenetic characterization evidenced individuals with 2n = 45 chromosomes for *M. parvifolia* and *M. coriacea*, with most individuals of the three species having 2n = 46. Karyograms presented morphologically identical and distinct chromosome pairs, suggesting a true allopolyploid and monophyletic origin for the three species. In addition, a post-polyploidization event related to structural chromosome rearrangements was evidenced from differences in the mean 2C nuclear value and chromosome morphometry found between the species. As has been done for other species, accurate cytogenetic and nuclear genome size approaches represent a starting point for understanding the origin and influence of polyploidization and post-polyploidization changes in *Myrsine* species karyotypes. In addition, the diverse occupation of *Myrsine* species in distinct environments may be attributed to allopolyploidy and its effects. Therefore, this pioneering study of *Myrsine* provides insights about the relevant role of polyploidy in the karyotype evolution and diversification in Primulaceae.

Keywords: Atlantic Forest, cytogenetics, flow cytometry, Myrsinaceae, polyploidy, Rapanea.

1 INTRODUCTION

Polyploidy is a trigger for plant diversification, mainly in the core Eudicots, a monophyletic angiosperm group that underwent two or more whole-genome duplication events (Bodt et al. 2005; Vanneste et al. 2014) since the Albian (112 – 99.6 Mya, Cretaceous) (Bodt et al. 2005). Polyploidization and the ensuing genomic changes cause morphological, physiological, reproductive and behavioral differences in polyploid organisms compared to their ancestors. These changes may lead to reproductive isolation and the formation of new species (Tayalé and Parisod 2013), as well as increasing species abilities to colonize new habitats, occupy distinct environments and expand their geographic distribution (Kolář et al. 2013; Theodoridis et al. 2013). Therefore, genome changes may be related to differentiated spatial occupation by phylogenetically related species.

With regard to genomic origin, the polyploid is classified as either an autopolyploid (generated by polyploidization of the zygote or by fusion of unreduced or unreduced and reduced reproductive cells of the same species), true allopolyploid (formed by crossing different species) or segmental allopolyploid (hybridization between species with similar genomes, presenting a karyotype with homeologous chromosomes) (Stebbins 1947). Polyploids are also categorized based on their evolutionary age, being denominated paleopolyploids (Stebbins 1947), mesopolyploids (Mandáková et al. 2010; Tamayo-Ordóñez et al. 2016) or neopolyploids (Stebbins 1947), which respectively underwent ancient, less ancient and recent polyploidization events. With regard to the formation mechanism, natural polyploids arise from

spontaneous genome hybridization and duplication, while synthetic polyploids result from genome duplication induction with or without previous hybridization events (Yang et al. 2011). The genomic origin, formation mechanism and evolutionary age of the polyploid can be disentangled by accurate cytogenetic characterization.

Polyploid origins have been evidenced by classical cytogenetic approaches. The holoploid chromosome complement (n) of the reduced (haplophasic) meiotic cell was considered a parameter for investigating this issue (Stebbins 1947; Grant 1982; Masterson 1994). Fine adjustments in cytogenetic procedures, combining advances in microscopy and image analysis systems, have provided accurate karyotype characterizations for some species. The number and presence of morphologically similar and distinct chromosome pair groups are karyotypic signs of polyploid origin (Gonçalves et al. 2007; Freitas et al. 2007; Clarindo and Carvalho 2008; Nunes et al. 2013; Clarindo et al. 2013; Reis et al. 2014). In addition, accurate cytogenetic characterization allows for recognition of karyotype changes that occurred throughout evolution, making it possible to elucidate the mechanisms that led to taxa diversification (Sharma and Sharma 2014).

Studies combining cytogenetics and nuclear DNA content offer data for understanding evolutionary processes in different species, while also providing data supporting polyploid origins (Clarindo and Carvalho 2008; Kolář et al. 2013; Coulleri et al. 2014). Measurement of the nuclear DNA content is complementary to cytogenetic data and is useful for detecting genome size variations between related species, screening for possible polyploid individuals or even polyploidy populations (Marhold et al. 2010; McIntyre 2012; Kolář et al.

2013; Krejčíková et al. 2013). In addition to polyploidy, variations in the nuclear 2C value in the same taxa have also been caused by aneuploidy and structural chromosome rearrangements (Amaral-Silva et al. 2016).

Previous studies reported polyploidy in Primulaceae for *Primula* (Abou-El-Enain 2006; Casazza et al. 2012; Theodoridis et al. 2013), *Cyclamen* (Bennett and Grimshaw 1991; Ishizaka 2003), and *Dodecatheon* (Oberle et al. 2012), all of which are genera comprised of herbaceous species. Despite *Myrsine* being one of the largest and most important genera of Primulaceae, the polyploid origins of this species have never been confirmed. Twelve species, among the 300 known for this genus, were studied regarding cytogenetic aspects (Beuzenberg and Hair 1983; Dawson 1995, 2000; Molero et al. 2002; Hanson et al. 2003; Molero et al. 2006), and the chromosome number (2n = 46 or 2n = 48) was a single karyotype data reported. Thus, the evolutionary aspects that culminated in the diversification of *Myrsine* species are still poorly understood.

Studying polyploidy in *Myrsine* can provide further information about the processes that led to the emergence of polyploidy in Primulaceae. The genus is predominantly comprised of tree and shrub species and most previous cytogenetic research within Primulaceae focused on herbaceous species. Furthermore, studying the polyploid origins of this taxa may provide insights into the role of polyploidy for species distribution in the Brazilian Atlantic Forest. In this biome, *Myrsine* species show different abilities to occupy the various vegetation types (Restinga vegetation, High Altitude Campos, Rocky Outcrops, Ombrophyllous and Mixed Ombrophyllous Forests). As such, this study aimed

to confirm the polyploid origin and its consequences on three *Myrsine* species by chromosome number, karyotype morphometry and nuclear DNA content.

2 MATERIAL AND METHODS

2.1 Plant samples

The tree species selected for this study were *Myrsine coriacea* (Sw.) R.Br. ex Roem & Schult. (Voucher – T.T. Carrijo 1458, VIES) and *Myrsine umbellata* Mart. (Voucher – T.T. Carrijo 1467, VIES), which are widespread species found in all types of vegetation, including open areas within Ombrophyllous and Mixed Ombrophyllous Forests, and *Myrsine parvifolia* A.DC. (Voucher – T.T. Carrijo 2232, VIES), a species widely distributed along the Brazilian coast that primarily occurs in Restinga vegetation (BFG 2015). Fruits and leaves of all species were collected. *Myrsine coriacea* and *M. umbellata* were sampled from October 2012 to July 2015 in a forest remnant located in Iúna municipality, Espírito Santo (ES) State, Brazil (20°21'6''S – 41°31'58''W), characterized as Rocky Outcrops, at 600 (*M. coriacea*) and 1,100 m.s.m (*M. umbellata*). *M. parvifolia* was collected in a forest remnant located in Guarapari municipality, ES, Brazil (20°36'15''S – 40°25'27''W), characterized as coastal sandy plains vegetation (Restinga) at sea level. Leaves and fruits of *Solanum lycopersicum* L. and *Pisum sativum* L. (internal standards for flow cytometry – FCM, 2C = 2.00 pg and 2C = 9.16 pg, respectively, Praça-Fontes et al. 2011) were supplied by Dr. Jaroslav Doležel (Experimental Institute of Botany, Czech Republic).

2.2 In vitro plantlet recovering

Fruit pericarp was manually removed and the seeds were disinfested according to Oliveira et al. (2013) and germinated in a medium composed of MS salts (Sigma®) and vitamins (Musrashige and Skoog 1962), 30 g L^{-1} sucrose (Sigma®), 7 g L^{-1} agar and 2.685 µM naphthaleneacetic acid (NAA, Sigma®). *Solanum lycopersicum* and *P. sativum* seeds were subjected to the same disinfestation procedure and inoculated in medium without NAA. Germination was done at 25 °C under a 16/8 hours (light/dark) regime.

2.3 Nuclear 2C value measurement

In order to adapt the FCM for *Myrsine*, the following procedures were done: (a) initially, from leaves collected in the field of male and female individuals (samples) and of the two standards; (b) afterward, replacing the dithiothreitol antioxidant by polyethylene glycol (PEG) in nuclei isolation buffer; and (c) from leaves of the samples and *P. sativum* plantlets in vitro cultivated.

Nuclei suspensions were obtained by co-chopping (Galbraith et al. 1983) leaf fragments (1 cm^2) cut from each sample (*Myrsine* species) and standard (*S. lycopersicum* or *P. sativum*). The suspensions were processed and stained following Otto (1990) and Praça-Fontes et al. (2011) and analyzed with the flow cytometer Partec PAS II/III (Partec GmbH). *Myrsine* genome size was measured by multiplying the 2C value of the internal standard using the fluorescence intensity corresponding to G_0/G_1 nuclei peak. Mean 2C values were compared by the *F* test at 5% probability.

2.4 Cytogenetic analysis

Roots were cut from the in vitro plantlets, treated with 5.0 µM amiprofos-methyl (APM) (Agrochem KK Nihon Bayer®) for 12, 15, 18 or 24 h at 4°C, rinsed in distilled water (dH_2O) for 20 min and fixed in methanol:acetic acid (3:1) for 24 h. The fixative solution was changed three times and the material was stored at -20°C (Carvalho et al. 2007). The roots were washed, macerated in 1:5 pectinase solution (enzyme:dH_2O) for 3 h at 34°C, or 1:20 enzymatic pool (4% cellulase – Kinki Yakult MFG, 1% macerozyme – Kinki Yakult MFG, and 0.4% hemicellulase – Sigma®) for 1 h 30 min or 1 h 45 min at 34°C, washed in dH_2O, fixed, and stored at -20°C.

Slides were prepared and stained according to Carvalho et al. (2007) and analyzed on a Nikon eclipse Ci-S microscope (Nikon). Prometaphases and metaphases were captured using the 100x objective and a CCD camera (Nikon Evolution™) coupled to a Nikon microscope 80i (Nikon). About 100 slides were analyzed for each *Myrsine* species. Chromosome morphometry was characterized and the class was determined as proposed by Levan et al. (1964) and reviewed by Guerra (1986).

Using chromosome morphometric data (total, short and long arms length), the standardized Euclidean Distance and Unweighted Pair-Group Method Average (UPGMA) was applied to each species. In addition, the value of the relative size (% size in relation to sum of the mean values of total length, Table 1) of each chromosome was compared among species by the Scott-Knott test at 5% probability. Analyses were made using the software R 3.2.4 (R Core Team 2016).

3 RESULTS

3.1 In vitro plantlet recovering

Approximately 60 days after in vitro inoculation, plantlets were obtained for the three *Myrsine* species. All plantlets exhibited sufficient and morphologically normal leaves and roots for FCM and cytogenetic analyses, respectively.

3.2 Nuclear 2C value measurement

FCM analysis performed on leaves collected in the field did not result in histograms showing profile G_0/G_1 peaks. So, dithiothreitol antioxidant was replaced by PEG in the nuclei isolation buffer OTTO I. This change provided G_0/G_1 peaks, exhibiting a coefficient of variation (CV) less than 5% for *M. umbellata* and the two internal standards. The channel of the *P. sativum* G_0/G_1 peak however was closer to *M. umbellata* than *S. lycopersicum*. Thus, based on linearity international criteria for FCM, *P. sativum* was the standard chosen for the next measurements. The mean 2C value of the male (2C = 6.65 pg ± 0.02) and female (2C = 6.67 pg ± 0.11) *M. umbellata* individuals were statistically identical by the *F* test. Considering these previous results, the 2C value was measured from leaves of in vitro plantlets. The mean values were 2C = 4.81 pg ± 0.05 for *M. parvifolia*, 2C = 6.60 pg ± 0.14 for *M. coriacea* and 2C = 6.63 pg ± 0.13 for *M. umbellata*. The mean value of the *M. umbellata* in vitro plantlets was statistically identical to the males and females in the field. Therefore, the mean

value adopted for this species was 2C = 6.65 pg, which was statistically equal to the *M. coriacea*.

3.3 Cytogenetic analysis

Roots exposed to a 12 h APM provided prometaphases, exhibiting chromosomes at a distinct chromatin compact level, and metaphases. Enzymatic maceration in 1:5 pectinase solution ensured the chromosomes remained inside the cell, allowing an accurate determination of 2n = 45 or 2n = 46. Chromosome number of 2n = 45 was found for 12.60% individuals of *M. parvifolia* and 8.45% of *M. coriacea*, with 2n = 46 for the three species. Based on these results, the next slides were made from roots of particular seedlings with 2n = 45 or 2n = 46. Root maceration with 1:20 enzymatic pool for 1h 30 min supplied chromosomes no damage to the chromatin structure, without overlapping, with well-defined centromeres and free of cytoplasm debris (Fig. 1).

Karyotype characterization was possible only after carefully testing the time and concentration of the APM antitubulin and cell wall enzymes. *Myrsine parvifolia* presented a greater total sum of the length of the chromosomes despite having less nuclear DNA content. For this species only, we found prometaphase chromosomes showing low level of chromatin compaction (Fig. 2 a), resulting in a higher sum of the total length (Table 1). *Myrsine coriacea* and *M. umbellata* did not show pronounced variation in chromatin compaction, but the quality of the chromosomes allowed us to characterize the karyotype and to assemble the karyogram (Fig. 2 b – c, Table 1).

Morphometric analysis was used to classify the chromosomes and evidence similarities and differences among species karyotypes. *Myrsine parvifolia* presented three metacentric (2, 9 and 21) and 20 submetacentric (1, 3 – 8, 10 – 20, 22 and 23) chromosome pairs, *M. coriacea* showed five metacentric (1 – 5), 17 submetacentric (6 – 21 and 23) and one acrocentric (22) chromosome pairs, and *M. umbellata* displayed nine metacentric (1 – 3, 7, 15 – 17, 21 and 23) and 14 submetacentric (4 – 6, 8 – 14, 17, 18, 20 and 22) chromosome pairs (Fig. 2, Table 1).

Morphologically similar and identical chromosomes groups were found in all species. *Myrsine parvifolia* presented sets of two chromosome pairs (5 – 6, 13 – 14, 16 – 17 and 22 – 23), as did *M. coriacea* (4 – 5, 10 – 11, 13 – 14, 15 – 16 and 19 – 20), and *M. umbellata* presented three sets of two (11 – 12, 16 – 17 and 18 – 19) and one set of three chromosome pairs (8 – 10). The other chromosome pairs in each species were considered morphologically distinct (Fig. 2, Table 1, 2). Using morphometric data and applying the UPGMA statistical analysis, the chromosomes of each *Myrsine* species were grouped in three clusters in all species (Fig. 3 a – c, Table 2). Chromosome groups formed by qualitative analysis of all species were clustered by UPGMA, supporting previous findings.

As the mean 2C values of *M. coriacea* (6.60 pg) and *M. umbellata* (6.65 pg) were statistically identical, the Scott-Knott test was used to compare the relative size (Table 1) of each chromosome of these species. Chromosomes 1, 2, 6, 7, 11, 14, 19 and 23 differed between the species, while the others were statistically identical (Fig. 3 d, Table 2).

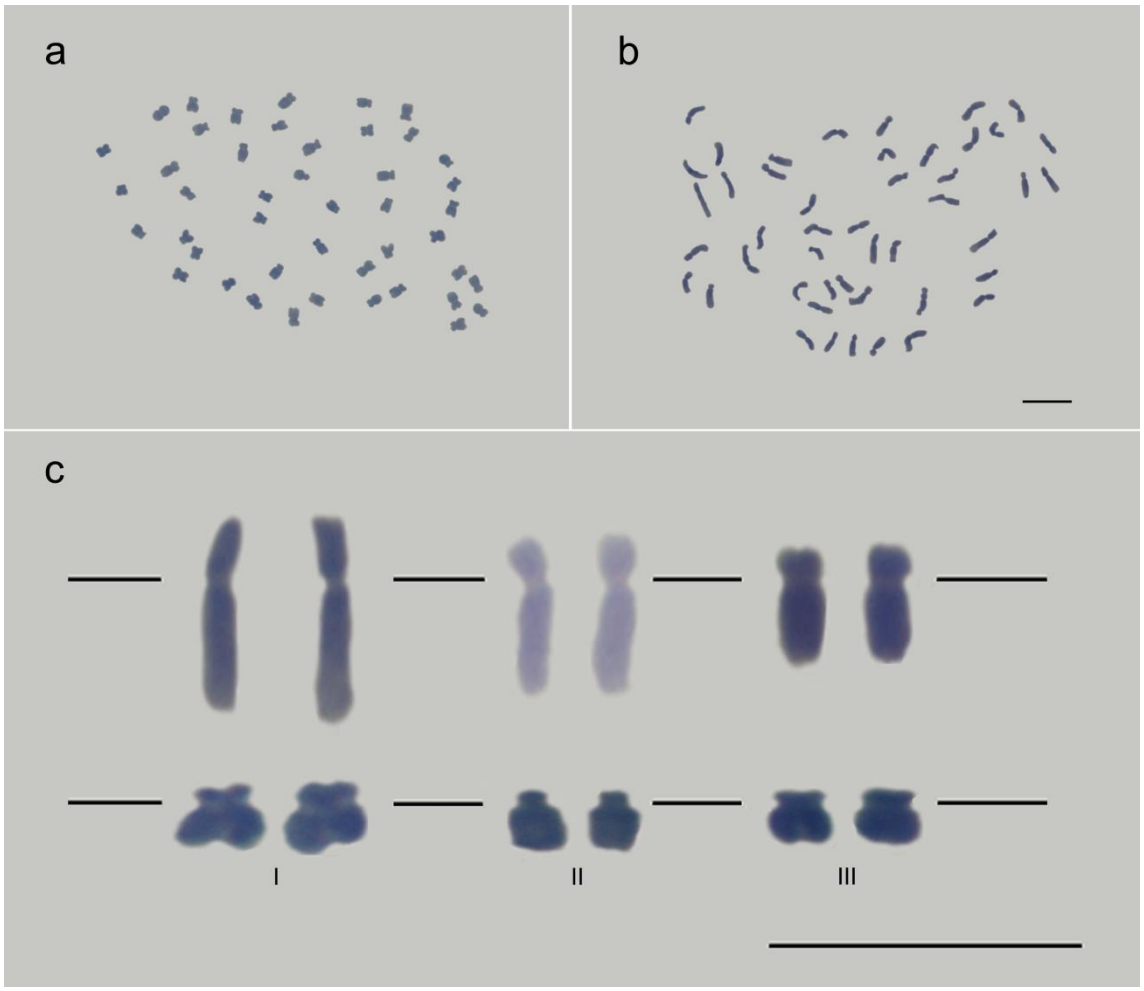

Figure 1. First images of the *Myrsine* chromosomes. Karyotype of a *M. parvifolia* individual with 2n = 45 (a) and other with 2n = 46 (b) chromosomes. Note the different levels of chromatin compaction between the chromosomes of the two karyotypes. The distinct chromatin compact level was highlighted in 'c', where the same submetacentric chromosome of *M. parvifolia* (above) and the same acrocentric chromosome of M. coriacea (below) were scattered of two different prometaphases (I and II) and one metaphase (III). Bar = 5 µm.

Table 1– Morphometry and chromosome class performed at least 10 prometaphases/metaphases. In all species were found chromosomes morphologically identical, similar and distinct, supporting the allopolyploidy.

	M. parvifolia						*M. coriacea*						*M. umbellata*					
Chrom.	**Total ± SD**	**Short**	**Long**	**r**	**Class**	**Relative size (%)**	**Total ± SD**	**Short**	**Long**	**r**	**Class**	**Relative size (%)**	**Total ± SD**	**Short**	**Long**	**r**	**Class**	**Relative size (%)**
1	2.64 ± 0.29	1.01	1.63	1.61	SM	5.60	2.79 ± 0.09	1.24	1.55	1.25	M	6.14	2.72 ± 0.06	1.14	1.59	1.39	M	6.60
2	2.47 ± 0.23	1.09	1.37	1.25	M	5.24	2.45 ± 0.11	1.02	1.42	1.38	M	5.38	2.67 ± 0.06	1.14	1.54	1.35	M	6.48
3	2.45 ± 0.22	0.86	1.59	1.85	SM	5.19	2.35 ± 0.10	1.09	1.26	1.15	M	5.17	2.13 ± 0.16	0.94	1.19	1.26	M	5.16
4	2.44 ± 0.27	0.68	1.75	2.55	SM	5.17	2.30 ± 0.05	1.02	1.27	1.24	M	5.06	2.13 ± 0.08	0.84	1.29	1.53	SM	5.16
5	2.24 ± 0.18	0.71	1.53	2.13	SM	4.76	2.29 ± 0.08	0.99	1.30	1.30	M	5.04	2.08 ± 0.14	0.74	1.34	1.80	SM	5.04
6	2.21 ± 0.17	0.73	1.48	2.00	SM	4.69	2.22 ± 0.17	0.86	1.36	1.57	SM	4.88	1.88 ± 0.11	0.64	1.24	1.92	SM	4.56
7	2.18 ± 0.25	0.81	1.37	1.68	SM	4.62	2.17 ± 0.12	0.78	1.39	1.77	SM	4.77	1.83 ± 0.11	0.79	1.04	1.31	M	4.44
8	2.16 ± 0.27	0.61	1.55	2.51	SM	4.59	2.12 ± 0.11	0.78	1.34	1.71	SM	4.67	1.83 ± 0.09	0.59	1.24	2.08	SM	4.44
9	2.15 ± 0.29	0.86	1.29	1.49	M	4.55	2.04 ± 0.15	0.81	1.23	1.50	SM	4.49	1.83 ± 0.09	0.59	1.24	2.08	SM	4.44
10	2.13 ± 0.25	0.61	1.51	2.45	SM	4.51	2.00 ± 0.10	0.78	1.23	1.56	SM	4.41	1.78 ± 0.12	0.59	1.19	2.00	SM	4.32
11	2.09 ± 0.22	0.79	1.31	1.65	SM	4.44	2.00 ± 0.17	0.75	1.26	1.67	SM	4.41	1.68 ± 0.13	0.59	1.09	1.83	SM	4.08
12	1.99 ± 0.16	0.75	1.23	1.63	SM	4.22	1.89 ± 0.10	0.71	1.18	1.64	SM	4.16	1.68 ± 0.08	0.59	1.09	1.83	SM	4.08
13	1.97 ± 0.23	0.66	1.31	1.96	SM	4.19	1.89 ± 0.07	0.57	1.32	2.31	SM	4.16	1.68 ± 0.10	0.49	1.19	2.40	SM	4.08
14	1.95 ± 0.14	0.65	1.30	2.00	SM	4.14	1.84 ± 0.06	0.55	1.29	2.32	SM	4.06	1.68 ± 0.14	0.66	1.02	1.52	SM	4.08
15	1.93 ± 0.16	0.72	1.21	1.67	SM	4.11	1.81 ± 0.11	0.65	1.16	1.78	SM	3.98	1.58 ± 0.06	0.64	0.94	1.46	M	3.84
16	1.85 ± 0.13	0.65	1.20	1.82	SM	3.93	1.81 ± 0.08	0.57	1.24	2.17	SM	3.98	1.58 ± 0.06	0.69	0.89	1.29	M	3.84
17	1.85 ± 0.23	0.72	1.13	1.56	SM	3.93	1.78 ± 0.04	0.66	1.11	1.66	SM	3.91	1.58 ± 0.09	0.69	0.89	1.29	M	3.84
18	1.84 ± 0.19	0.70	1.15	1.63	SM	3.92	1.71 ± 0.13	0.65	1.06	1.63	SM	3.77	1.58 ± 0.11	0.59	0.99	1.67	SM	3.84
19	1.82 ± 0.22	0.63	1.20	1.89	SM	3.87	1.68 ± 0.11	0.55	1.13	2.03	SM	3.70	1.58 ± 0.08	0.59	0.99	1.67	SM	3.84
20	1.75 ± 0.18	0.68	1.06	1.55	SM	3.71	1.67 ± 0.09	0.58	1.09	1.85	SM	3.69	1.58 ± 0.13	0.49	1.09	2.20	SM	3.84
21	1.68 ± 0.14	0.79	0.89	1.13	M	3.56	1.55 ± 0.16	0.49	1.06	2.17	SM	3.42	1.43 ± 0.14	0.59	0.84	1.42	M	3.48
22	1.66 ± 0.16	0.58	1.08	1.85	SM	3.53	1.55 ± 0.04	0.35	1.20	3.33	A	3.42	1.38 ± 0.11	0.49	0.89	1.80	SM	3.36
23	1.66 ± 0.30	0.58	1.08	1.86	SM	3.53	1.52 ± 0.07	0.39	1.13	2.88	SM	3.34	1.28 ± 0.10	0.59	0.69	1.17	M	3.13
Sum	47.22	16.99	30.23			100.00	45.53	16.96	28.57			100.00	41.30	15.79	25.51			100.00

Chrom = chromosomes; Total = total length; SD = standard deviation; Long/Short = arm length; r = arm ratio – long/short; M = metacentric; SM = submetacentric; A = acrocentric; Relative size = % size in relation to sum of the mean values of total length; Sum = sum of the mean values.

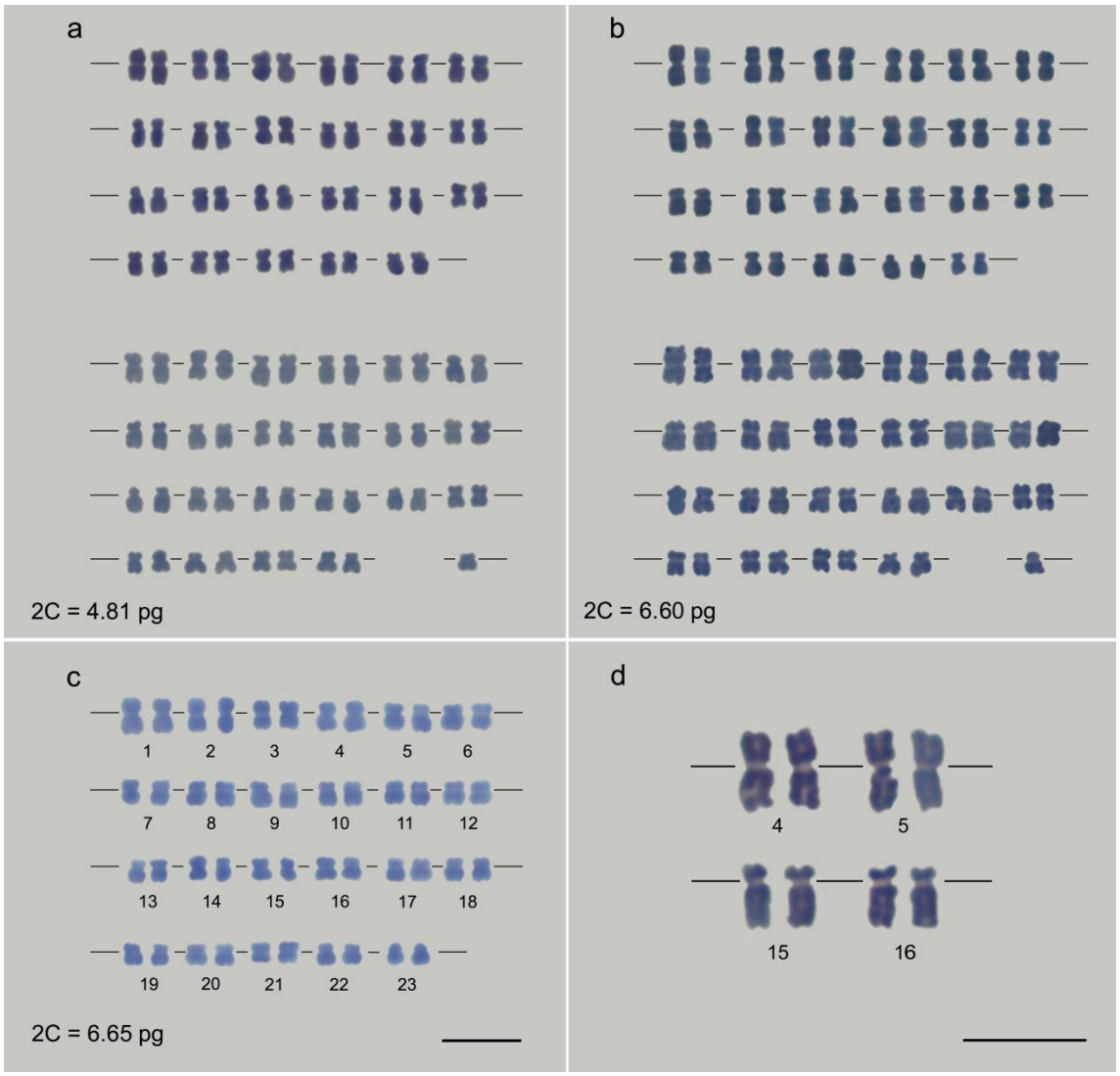

Figure 2. *Myrsine* karyograms displaying 2n = 45 (a – *M. parvifolia* and b – *M. coriacea*) or 2n = 46 chromosomes (a – c, the three species). In all *M. parvifolia* (a) and *M. coriacea* (b) individuals with 2n = 45, the odd chromosome number was well-marked by absence of the homologue pair of the chromosome 23. Metacentric and submetacentric chromosomes prevailing in the karyograms of the three species, being that only one acrocentric chromosome was identified in *M. coriacea* (b – chromosome 22). Although showing approximately 2C = 1.50 pg less DNA, *M. parvifolia* (a) displayed the same chromosome number in relation to the other species (b – *M. coriacea*, c – *M. umbellata*). For all species, morphometric analyses evidenced identical, similar and distinct chromosome pairs with regard to morphometry and class. The similarity of some chromosomes was highlighted from the metacentric chromosome pairs 4 and 5 (d – above) and submetacentric 15 and 16 (d – below) of *M. coriacea*. In contrast, other chromosomes showed singular morphology, as the chromosome 1 and 2 of all species, the 22 of *M. coriacea*, which is the single acrocentric chromosome, and the chromosome 23. Bar = 5 µm.

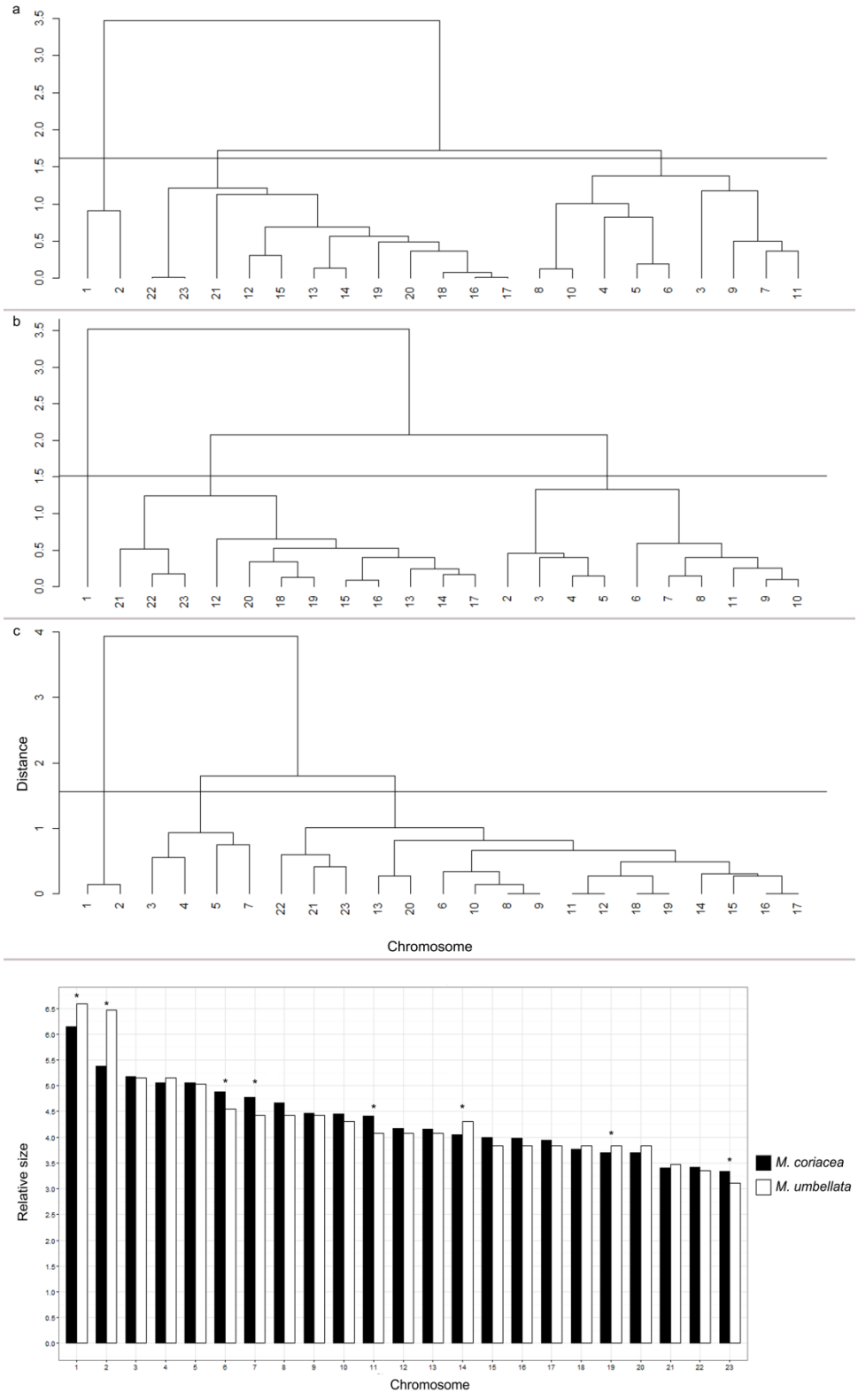

Figure 3. (a – c) Multivariate clustering generated from chromosome morphometric data (total, long and short arms length). Mojena's criteria showed three clusters for *M. parvifolia* (a), *M. coriacea* (b) and *M. umbellata* (c) with cut point between 1.5 to 1.8. This analysis confirmed the morphological discrepancy of the chromosome 1, and the similarity of other chromosomes. (d) Graphic provided by comparison between mean

relative size (% size in relation to sum of the mean values of total length, Table 1) of each chromosome of *M. coriacea* and *M. umbellata*. The chromosomes 1, 2, 6, 7, 11, 14, 19 and 23 (*) between the species are statistically different in relation to mean relative size according to Scott Knott test at 5% of probability.

Table 2 – Chromosome groups of the *Myrsine* karyotype suggested from karyogram evaluation (Fig. 2 and Table 1) and confirmed by UPGMA clustering (Fig. 3 a – c).

Species	***Karyogram evaluation**	****UPGMA clustering**	*****Confirmed chromosome groups**
M. parvifolia	5–6; 13–14; 16–17; and 22–23	1 and 2; 3–11; and 12–23	5–6; 13–14; 16–17; and 22–23
M. coriacea	4–5; 9–10; 13–14; 15–16; and 19–20	1; 2–11; and 12–23	4–5; 9–10; 13–14; 15–16; and 19–20
M. umbellata	8–10; 11–12; 16–17; and 18–19	1 and 2; 3–5, 7; and 6, 8–23	8–10; 11–12; 16–17; and 18–19

* Chromosome groups morphologically identical or similar defined from all morphometric data (total length, short and long arms, r = ratio long/short arm, chromosomal class; relative size) and observation of the karyogram.

** Chromosome groups formed by UPGMA clustering method using data about total, short and long arms length.

*** Common chromosome groups evidenced by two analyses (qualitative *x* quantitative).

4 DISCUSSION

4.1 2C value and karyotype

The first step in FCM was to define the best antioxidant and internal standard. The presence of secondary metabolites in the *Myrsine* leaves, such as tannins, saponins, flavonoids and steroids (Abbi et al. 2011) made this challenging. These compounds probably prevented us from measuring the 2C value in individuals from the field when the OTTO I buffer (Otto, 1990) was supplemented with dithiothreitol. Cytosolic compounds can reduce or inhibit the interaction of the fluorochromes and DNA during the nuclei staining step (Noirot et al. 2003). Antioxidants inhibit this interference, preserving the chromatin structure (Shapiro 2003). Nevertheless, the dithiothreitol was not efficient at providing nuclei suspensions suitable for FCM. Thus, this compound, which is more specific for molecules that possess free sulfhydryl groups, was replaced by PEG because of its wide spectrum for antioxidant activities, an effect called PEGylation (Life Technologies 2015). Due to this effect, PEG was more efficient at inhibiting the action of cytosolic compounds, resulting in G_0/G_1 peaks for *M. umbellata* and *P. sativum* with CV below 5%. Owing to the linearity parameter, *P. sativum* was a more adequate standard relative to *S. lycopersicum*, which reduced measurement errors.

Secondary metabolite interference was completely resolved for other *Myrsine* species using in vitro plantlets propagated in a controlled environment. FCM measurements from leaves collected in the field may have been influenced by environmental conditions. Secondary metabolite production is influenced by humidity, temperature, light intensity and the availability of water

and nutrients (Akula and Ravishankar 2011). Thus, the conditions at each elevation gradient can be associated with the FCM result, suggesting a differentiated production of secondary metabolic compounds for *Myrsine* at distinct altitudes.

Male and female *M. umbellata* showed the same mean 2C values. Technical constraints in discriminating male and female individuals are related to the necessity of obtaining G_0/G_1 peaks with CV less than half the difference of the 2C DNA value between them (Mendonça et al. 2010). This limitation explains the small application (Costich et al. 1991; Doležel and Göhde 1995) of the FCM for sexual selection in plant taxa.

Genome size in *Myrsine* had only been reported for *M. africana* as 2C = 2.46 pg (Hanson et al. 2003), which was measured by Feulgen microdensitometry using *Vigna* sp. as standard. Levels of endoreduplication in cells of *V. radiata*, varying from 2C to 64C, were reported by Pal et al. (2004). Thus, the differences, which were about 200% between the values found for *Myrsine* species in this study and the value observed for *M. africana,* can be related with the C value of *V. radiata* used as reference.

Values close to *M. umbellata* and *M. coriacea* species were reported for *Cyclamen purpurascens* Mill. (2C = 6.60 pg) and *Dodecatheon meadia* L. (2C = 5.58 pg). Higher DNA contents were described for *Cyclamen coum* Mill. (2C = 13.56 pg), *Soldanella pusilla* Baumg. (2C = 12.36 pg), and lower 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 the 2C DNA value used in this study and for other species of Primulaceae (Bennett and

Leitch 2012), suggests the occurrence of numerical and structural changes in the karyotype.

As well as for FCM, karyotype data about *Myrsine* species in the literature are very limited. The chromosome number had been reported (Beuzenberg and Hair 1983; Dawson 1995; Dawson 2000; Molero et al. 2002; Molero et al. 2006), but images of the chromosome had not. In vitro *Myrsine* plantlets were fundamental to providing sufficient quantities of roots for the cytogenetic study independent of the reproductive period. Meticulous standardization of the antimitotic agent and enzymatic maceration were also essential for accurate chromosomal characterization.

Chromosome number 2n = 46 (Beuzenberg and Hair 1983; Dawson 1995; Dawson 2000; Molero et al. 2002; present study) and 2n = 48 (Molero et al. 2006) had been reported, but this was the first record of 2n = 45. The odd chromosome number 2n = 45 may be related to a chromosomal system of sex determination, since dioecy is predominant within the genus (Albuquerque et al. 2013). Another hypothesis for the existence of an odd chromosome number in *Myrsine* is the occurrence of aneuploidy. Post-polyploidization genome restructuring events, such as aneuploidy, have been reported in polyploids (Chester et al. 2012; Reis et al. 2014). This change will lead to a gene dosage imbalance and often causes severe phenotypic alterations. The presence of duplicate genes due to polyploidy however may have a buffering effect on the genome, allowing these species to survive in the environment (Edger and Pires 2009; Xiong et al. 2011).

4.2 Polyploidy in *Myrsine*

Karyotype data provided evidence that supported the polyploid origin of *Myrsine*. Some classical cytogenetic approaches have also evidenced a polyploid origin for distinct species, such as *Glycine max* (L.) Merr. (Clarindo et al. 2007), *Paulinia cupana* Kunth (Freitas et al. 2007), *Coffea arabica* L. (Clarindo and Carvalho 2008) and *Pitcairnia flammea* Lindley (Nunes et al. 2013). A breakthrough came when statistical analyses were used to support the qualitative data and confirm the previous polyploid hypothesis for *Myrsine*. Therefore, all chromosome groups defined by karyogram evaluation were confirmed by UPGMA clustering (Table 2).

Some chromosome groups determined by statistical analysis are morphologically distinct, such as chromosomes 22 and 23 of *M. coriacea*. Although clustered (Fig. 3 b), these chromosomes are cytogenetically distinct, with 22 being acrocentric and 23 submetacentric (Fig. 2 b, Table 2). Likewise, distinct chromosomes clustered in *M. parvifolia* (Fig. 3 a, Table 2) and *M. umbellata* (Fig. 3 c, Table 2). Chromosome 1 of *M. coriacea* and 1 and 2 of *M. parvifolia* and *M. umbellata* presented the highest contrast, considering the morphology and Euclidean distances (Fig. 2, Fig. 3). The occurrence these chromosomes represents evidence that the three *Myrsine* species are true allopolyploids. Associated with this karyotype aspect, other *Myrsine* characters, such as reproductive viability (Albuquerque et al. 2013) and wide distribution (Freitas et al. 2016), corroborate the allopolyploid nature of the species.

The basic chromosomal number for *Myrsine* species is x = 23, based on the chromosome numbers 2n = 45 and 2n = 46. Nevertheless, polyploidy, which was evidenced by the karyotypes, suggests that the basic number is below x =

23. In addition, groups of two morphologically identical chromosome pairs in *Myrsine* support a tetraploid origin. Other Primulaceae species have shown different ploidy levels. *Primula* has tetraploid, hexaploid, octaploid and dodecaploid species (Abou-El-Enain 2006; Calesazza et al. 2012; Theodoridis et al. 2013), *Cyclamen* presents triploids and tetraploids (Bennett and Grimshaw 1991, Ishizaka 2003) and *Dodecatheon* has tetraploid species (Oberle et al. 2012). Thus, polyploidy is recurrent in Primulaceae and plays a key role in the diversification of this taxa.

Regarding the temporal distinction for *Myrsine*, there are no studies of sequence comparison and the ancestors that gave rise to the species are not known. Paleopolyploidy traces in plant genomes are difficult to detect due to diplodization process, where the paleopolyploid species present diploid segregation (Soltis et al. 2015). The *Myrsine* species showed morphologically identical and several distinct chromosome pairs. These karyotype aspects are similar to the paleopolyploid *G. max* (Clarindo et al. 2007). On the contrary, true allopolyploid species, such as *Coffea arabica* L., present karyotypes prevailing the morphologically similar chromosome groups. Therefore, the karyotype aspects found for the *Myrsine* species are more closely related to a paleopolyploid. This hypothesis is relevant as a parameter for phylogenetic studies in Primulaceae based on karyotypic comparisons between the species.

As the *Myrsine* species have 2n = 45/46 chromosomes and presented nuclear DNA content variation, the polyploidization probably represented a status change of the karyotype before chromosome rearrangements occurred. Polyploidy is considered the main phenomenon that causes the formation of new plant species. In addition, polyploids also undergo genomic restructuring

that may lead to reproductive isolation in new lineages and hence drive speciation (Tayalé and Parisod 2013). Therefore, the cytogenetic and FCM results offered evidence to understand karyotype origin and evolution in *Myrsine*.

Similarities and differences regarding relative size (% size in relation to sum of the mean values of total length, Table 1) were evidenced between *M. coriacea* and *M. umbellata* through the Scott-Knott test. In addition to the chromosome number (2n = 45/46), the homogeneity, which was evidenced for some chromosomes, implies that these species originated from a common ancestor. The distinct chromosomes are likely to be attributed to structural changes that happened throughout their evolution, altering the chromosome relative size and contributing to taxa diversification. Comparative investigations of the karyotype of related species have usually been applied to infer the evolutionary role of karyotypic modifications in different taxa and to describe the pattern and directions of chromosomal evolution within a group (Stebbins 1971; Shan et al. 2003; Sharma and Sharma 2014).

Based on the constant chromosome numbers 2n = 45 and 2n = 46 displayed by *Myrsine* species, interspecific variation of the nuclear 2C value between *M. parvifolia* compared to *M. coriacea* and *M. umbellata* was also caused by structural chromosome rearrangements. The changes to the nuclear DNA content have also been attributed to structural rearrangements during karyotypic evolution (Pellicer et al. 2014; Coulleri et al. 2014; Amaral-Silva et al. 2016). Changes that culminate in genome expansion are attributed to repetitive sequence amplification, transposable element activation (Yang et al 2011), chromosomal duplications (Gaeta et al. 2007), aneuploidy (Chester et al. 2012),

transposon and retrotransposon insertions (Madlung et al. 2005). Conversely, illegitimate recombination, unequal homologous recombination (Suda et al. 2015), aneuploidy (Xiong et al. 2011) and deletions (Gaeta et al. 2007) are responsible for the decreased nuclear 2C value (Stebbins 1971). These changes probably occurred throughout the evolution of *Myrsine* genome, characterizing the differences in DNA content and karyotype.

The occupation of *Myrsine* species in distinct environments may be attributed to the redundancy of genes caused by allopolyploidy. This redundancy has a buffering effect on the genome, masking deleterious mutations by the presence of multiple copies of the same gene in the genome. Another consequence of gene redundancy is the possibility of functional change, where the genes can diversify in function without compromising essential functions (Comai 2005; Yang et al. 2011; Soltis et al. 2014). In addition to duplicate genes, the merging of divergent genomes in true allopolyploids results in a high level of heterozygosity and hybrid vigor, allowing for adaptation to new environments and conditions (Comai 2005; Yang et al. 2011; Beest et al. 2012).

Unlike the segmental allopolyploid and autopolyploid, the broad distribution of the true allopolyploid is directly related to reproductive viability (Beest et al. 2012). The disomic inherence presented by true allopolyploids ensures a larger percentage of viable reproductive cells. Consequently, more offspring are generated and the species can take new environments. These factors may partially contribute to the wide spatial distribution of *M. coriacea* and *M. umbellata*, compared to the restricted *M. parvifolia*. Despite being polyploid, *M. parvifolia* has a lower nuclear 2C value than other species, which

may imply it has a lower number of gene copies than other species and this in turn may have led to restrictions of its niche. Complex polyploids have provided insights into the evolutionary history of different taxa, contributing to the understanding of the role of polyploidy in spatial distribution of species. Spatial distribution differences are consistent with the hypothesis that polyploid organisms inhabit distinct environments from their ancestors (Marhol et al. 2010; McIntyre 2012; Krejčíková et al. 2013). Some polyploids such as the *Galium pusillum* complex (Kolář et al. 2013) and species of *Primula* sect. Aleuritia (Theodoridis et al. 2013) however have shown restricted patterns of spatial distribution. Our results point to the need for future studies designed specifically to test this issue in *Myrsine*. Therefore, reconstruction is critical for the phylogeny of the genus but is not yet available in the literature.

In conclusion, karyotype and nuclear genome size of three *Myrsine* species pointed to a true allopolyploid origin and post-polyploidization changes. In addition, we summarized some consequences of this true allopolyploid origin for *Myrsine* (Fig. 4), the key role of this allopolyploid origin and its genomic and ecologic outcomes provide a useful guide for taxonomic, evolutive and phylogenetic studies in Primulaceae and other Angiosperms.

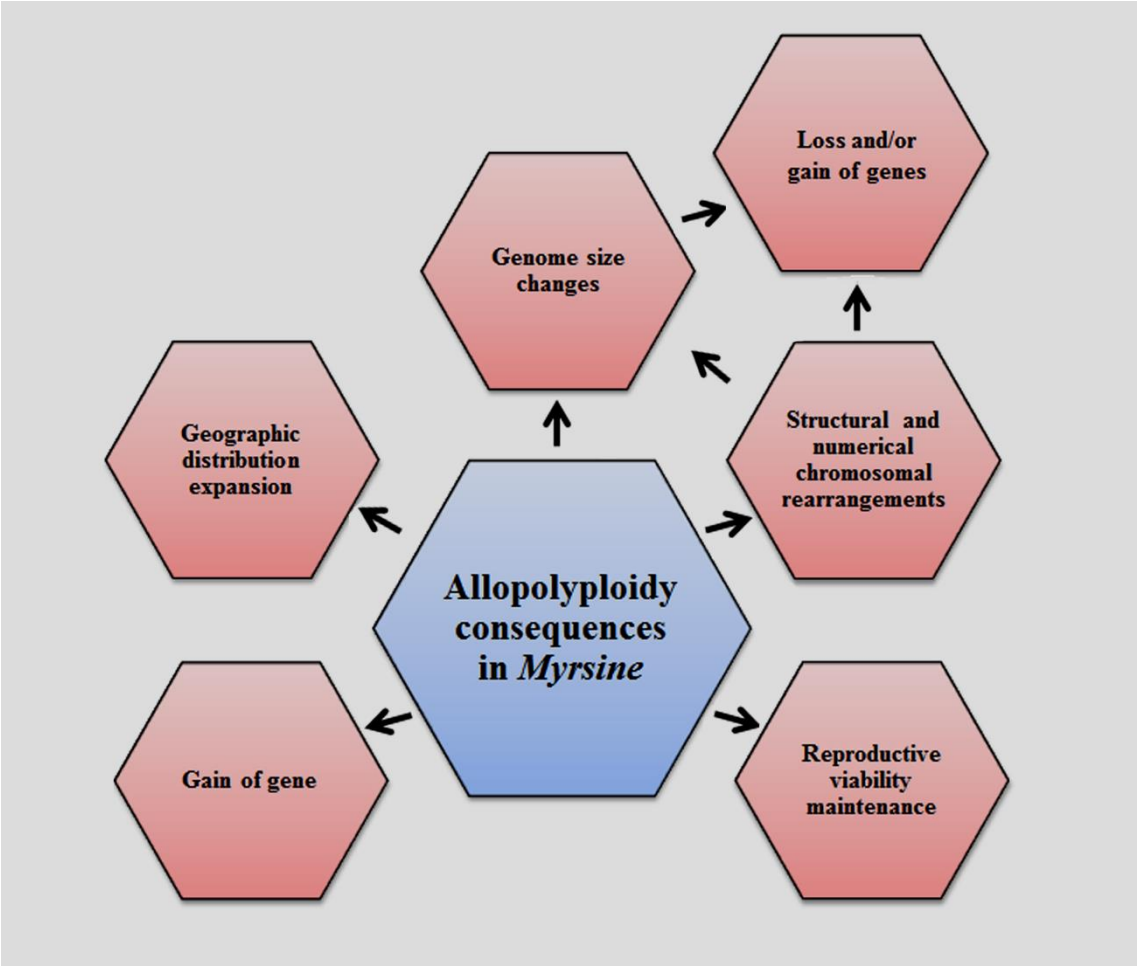

Figure 4. Scheme summarizing the outcomes considering a true allopolyploid origin of the three *Myrsine* species. Some post-polyploidization modifications were evidenced by karyotype and nuclear 2C value data, as the genome size differences, and structural and numerical chromosomal rearrangements. The gene gain is considered a direct effect of the polyploidization, as well as of some chromosomal rearrangements (e.g. duplication) after a polyploidization event. Besides of gain, the chromosomal rearrangements, as the deletions, can lead to loss of genes. In addition to genomic changes, the allopolyploid in *Myrsine* may have promoted the geographic distribution expansion and the reproductive viability maintenance.

6 References

Abbi V, Joseph L, George M (2011) Phytochemical analysis of fruit extract of *Myrsine africana*. International Journal of Pharmacy and Pharmaceutical Research 3(4):427–430.

Abou-El-Enain MM (2006) Chromosomal variability in the genus *Primula* (Primulaceae). Botanical Journal of the Linnean Society 150(2):211–219. doi: 10.1111/j.1095-8339.2006.00464.x

Akula R, Ravishankar GA (2011) Influence of abiotic stress signals on secondary metabolites in plants. Plant Signaling & Behavior 6(11):1720–1731. doi: 10.4161/psb.6.11.17613

Albuquerque AAE, Lima HA, Gonçalves-Esteves V, Benevides CR, Rodarte ATA (2013) *Myrsine parvifolia* (Primulaceae) in sandy coastal plains marginal to Atlantic rainforest: a case of pollination by wind or by both wind and insects? Brazilian Journal of Botany 36(1):65–73. doi:10.1007/s40415-013-0007-4

Amaral-Silva PM, Clarindo WR, Carrijo TT, Carvalho RC, Praça-Fontes MM (2016) The contribution of cytogenetics and flow cytometry for understanding the karyotype evolution in three *Dorstenia* (Linnaeus, 1753) species (Moraceae). Comp Cytogen 10(1): 97–108. doi:10.3897/CompCytogen.v10i1.6719

Bedalov M, Matter B, Küpfer P (2002) IOPB chromosome data 18. International organization of plant biosystematists newsletter. Utrecht, Reading, Copenhagen, 20.

Beest M, Le Roux JJ, Richardson DM, Brysting AK, Suda J, Kubešová M, Pyšek P (2012) The more the better? The role of polyploidy in facilitating plant invasions. Annals of Botany 109(1):19–45. doi:10.1093/aob/mcr277

Bennett MD, Leitch IJ Angiosperm DNA C-values database (release 8.0, Dec. 2012) Available in < http://www.kew.org/cvalues/ > Access: December 2, 2015

Bennett ST, Grimshaw JM (1991) Cytological studies in *Cyclamen* subg. *Cyclamen* (Primulaceae). Plant Syst Evol 176(3–4) 135–143. doi: 10.1007/BF00937904

Beuzenberg EJ, Hair JB (1983) Contributions to a chromosome atlas of the New Zealand flora—25 Miscellaneous species. New Zealand Journal of Botany 21(1):13–20. doi: 10.1080/0028825X.1983.10428520.

BFG [THE BRAZIL FLORA GROUP] (2016) Growing knowledge: an overview of Seed Plant diversity in Brazil. Rodriguésia 66:1085–1113.

Bodt S, Maere S, Van de Peer Y (2005) Genome duplication and the origin of angiosperms. Trends in Ecology & Evolution 20(11):591–597. doi:10.1016/j.tree.2005.07.008

Carvalho CR, Clarindo WR, Almeida PM (2007) Plant cytogenetics: still looking for the perfect mitotic chromosomes. The Nucleus 50(3):453–462.

Casazza G, Granato L, Minuto L, Conti E (2012) Polyploid evolution and Pleistocene glacial cycles: a case study from the alpine primrose *Primula marginata* (Primulaceae). BMC Evolutionary Biology 12:56. doi:10.1186/1471-2148-12-56

Chester M, Gallagher J, Symonds VV, Silva AVC, Mavrodiev EV, Leitch AR, Soltis PS, Soltis DE (2012) Extensive chromosomal variation in a recently formed natural allopolyploid species, *Tragopogon miscellus* (Asteraceae). Proceedings of the National Academy of Sciences 109(4):1176–1181. doi: 10.1073/pnas.1112041109

Clarindo WR, Carvalho CR (2008) First *Coffea arabica* karyogram showing that this species is a true allotetraploid. Plant Syst Evol 274(3-4):237–241. doi:10.1007/s00606-008-0050-y

Clarindo WR, Carvalho CR, Alves BMG (2007) Mitotic evidence for the tetraploid nature of *Glycine max* provided by high quality karyograms. Plant Syst Evol 265(1–2):101–107. doi:10.1007/s00606-007-0522-5

Comai L (2005) The advantages and disadvantages of being polyploid. Nature 6(11):836–846. doi:10.1038/nrg1711

Costich DE, Meagher TR, Yurkow EJ (1991) A rapid means of sex identification in *Silene latifolia* by use of flow cytometry. Plant Molecular Biology Reporter 9(4):359–370. doi: 10.1007/BF02672012

Coulleri JP, Urdampilleta JD, Ferrucci MS (2014) Genome size evolution in Sapindaceae at subfamily level: a case study of independence in relation to karyological and palynological traits. Botanical Journal of the Linnean Society 174(4):589–600. doi: 10.1111/boj.12145

Dawson MI (1995) Contributions to a chromosome atlas of the New Zealand flora — 33. Miscellaneous species. New Zealand Journal of Botany 33: 477–787. doi: 10.1080/0028825X.1995.10410619

Dawson MI (2000) Index of chromosome numbers of indigenous New Zealand spermatophytes. New Zealand Journal of Botany 38(1):47–150. doi: 10.1080/0028825X.2000.9512673

Doležel J, Göhde W (1995) Sex determination in dioecious plants *Melandrium album* and *M. rubrum* using high-resolution flow cytometry. Cytometry 19(2):103–106. doi: 10.1002/cyto.990190203

Edger PP, Pires JC (2009) Gene and genome duplications: the impact of dosage-sensitivity on the fate of nuclear genes. Chromosome Research 17(5):699–717. doi 10.1007/s10577-009-9055-9

Freitas DV, Carvalho CR, Filho FJN, Astolf-Filho S (2007) Karyotype with 210 chromosomes in guaraná (*Paullinia cupana* “Sorbilis”). J Plant Res 120(3):399–404. doi: 10.1007/s10265-007-0073-4

Freitas, MF (2016) *Myrsine* in Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro. Available in: <http://floradobrasil.jbrj.gov.br/jabot/floradobrasil/FB10238>. Access: February 13th.

Gaeta RT, Pires JC, Iniguez-Luy F, Leon E, Osborn TC (2007) Genomic changes in resynthesized *Brassica napus* and their effect on gene expression and phenotype. The Plant Cell 19(11):3403–3417. doi: 10.1105/tpc.107.054346

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220(4601):1049–1051. doi: 10.1126/science.220.4601.1049

Guerra MS (1986) Reviewing the chromosome nomenclature of Levan et al. Revista Brasileira de Genética 9(4):741–743.

Hanson L, Brown RL, Boyd A, Johnson AT, Bennett MD (2003) First Nuclear DNA C-values for 28 Angiosperm Genera. Annals of Botany 91(1):31–38. doi: 10.1093/aob/mcg005

Ishizaka H (2003) Cytogenetic studies in *Cyclamen persicum*, *C. graecum* (Primulaceae) and their hybrids. Plant Syst Evol 239(1–2):1–14. doi 10.1007/s00606-002-0261-6

Kolář F, Lučanová M, Vít P, Urfus T, Chrtek J, Fér T, Ehrendorfer F, Suda J (2013) Diversity and endemism in deglaciated areas: ploidy, relative genome size and niche differentiation in the *Galium pusillum* complex (Rubiaceae) in Northern and Central Europe. Annals of Botany 111:1095–1108. doi:10.1093/aob/mct074

Krejčíková J, Sudová R, Lučanová M, Trávníček P, Urfus T, Vít P, Weiss-Schneeweiss H, Kolano B, Oberlander K, Dreyer LL, Suda J (2013) High ploidy diversity and distinct patterns of cytotype distribution in a widespread species of *Oxalis* in the Greater Cape Floristic Region. Annals of Botany 111:641–649. doi:10.1093/aob/mct030

Levan A, Fredga A, Sanderberg AA (1964) Nomenclature for centromeric position in chromosome. Hereditas 52(2):201–220. doi: 10.1111/j.1601-5223.1964.tb01953.x

Madlung A, Tyagi AP, Watson B, Jiang H, Kagochi T, Doerge RW, Martienssen R, Comai L (2005) Genomic changes in synthetic *Arabidopsis* polyploids. The Plant Journal 41(2): 221–230. doi: 10.1111/j.1365-313X.2004.02297.x

Mandáková T, Joly S, Krzywinski M, Mummenhoff K, Lysak MA (2010) Fast diploidization in close mesopolyploid relatives of *Arabidopsis*. The Plant Cell 22:2277–2290. doi: 10.1105/tpc.110.074526

Marhold K, Kudoh H, Pak J-H, Watanabe K, Španiel S, Lihová (2010) Cytotype diversity and genome size variation in eastern Asian polyploid *Cardamine* (Brassicaceae) species. Annals of Botany 105(2):249–264. doi: 10.1093/aob/mcp282

Masterson J (1994) Stomatal size in fossil plants: evidence for polyploidy in majority of angiosperms. Science 264(5157):421–423.

McIntyre PJ (2012) Cytogeography and genome size variation in the *Claytonia perfoliata* (Portulacaceae) polyploid complex. Annals of Botany 110(6):1195–1203. doi:10.1093/aob/mcs187

Mendonça MAC, Carvalho CR, Clarindo WR (2010) DNA content differences between male and female chicken (*Gallus gallus domesticus*) nuclei and Z and W chromosomes resolved by image cytometry. Journal of Histochemistry & Cytochemistry 58(3):229–235. doi: 10.1369/jhc.2009.954727

Molero J, Daniña JR, Honfi AI, Franco D, Rovira A (2006) Chromosome studies on plants from Paraguay II. Candollea 61(2):373–392.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15(3):473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Noirot M, Barre P, Duperray C, Louarn J, Hamon S (2003) Effects of caffeine and chlorogenic acid on propidium iodide accessibility to DNA: consequences on genome size evaluation in coffee tree. Annals of Botany 92(2):259–264. doi: 10.1093/aob/mcg139

Nunes ACP, Nogueira EU, Gontijo ABPL, Carvalho CR, Clarindo WR (2013) The first karyogram of a Bromeliaceae species: an allopolyploid genome. Plant Syst Evol 299(6):1135–1140. doi 10.1007/s00606-013-0784-z

Oberle B, Montgomery RA, Beck JB, Esselman EJ (2012) A morphologically intergrading population facilitates plastid introgression from diploid to tetraploid *Dodecatheon* (Primulaceae). Botanical Journal of the Linnean Society 168(1):91–100. doi: 10.1111/j.1095-8339.2011.01191.x

Oliveira SC, Nunes ACP, Carvalho CR, Clarindo WR (2013) In vitro polyploidization from shoot tips of *Jatropha curcas* L.: a biodiesel plant. Plant Growth Regulation 69(1):79–86. doi:10.1007/s10725-012-9749-4

Otto F (1990) DAPI staining of fixed cells for high-resolution flow cytometry of nuclear DNA. Methods in Cell Biology 33:105–110.

Pal A, Vrána J, Doležel J (2004) Flow cytometric analysis of variation in the level of nuclear DNA endoreduplication in the cotyledons amongst *Vigna radiata* cultivars. Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics 57(3): 262–266. doi:10.1080/00087114.2004.10589402

Pellicer J, Kelly LJ, Leitch IJ, Zomlefer WB, Fay MF (2014) A universe of dwarfs and giants: genome size and chromosome evolution in the monocot family Melanthiaceae. New Phytologist 201(4):1484–1497. doi: 10.1111/nph.12617

Praça-Fontes MM, Carvalho CR, Clarindo WR, Cruz CD (2011) Revisiting the DNA C-values of the genome size-standards used in plant flow cytometry to choose the ‘‘best primary standards’’. Plant Cell Rep 30(7):1183–1191. doi:10.1007/s00299-011-1026-x

R Core Team (2016). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Avaible in<https://www.R-project.org/> Access: June 6, 2016

Reis AC, Sousa SM, Vale AA, Pierre PMO, Franco AL, Campos JMS, Vieira RF, Viccini LF (2014) *Lippia alba* (Verbenaceae): a new tropical autopolyploid complex? American Journal of Botany 101(6):1002–1012. doi:10.3732 /ajb.1400149

Shan F, Yan G, Plummer JA (2003) Karyotype evolution in the genus *Boronia* (Rutaceae). Botanical Journal of the Linnean Society 142(3):309–320. doi: 10.1046/j.1095-8339.2003.00163.x

Shapiro HM (2003) Practical flow cytometry, 4th edn. New York: Wiley-Liss.

Sharma G, Sharma N (2014) Cytology as an important tool for solving evolutionary problems in Angiosperms. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences 84(1):1–7. doi 10.1007/s40011-013-0203-9

Soltis DE, Visger CJ, Soltis PS (2014) The polyploidy revolution then…and now: Stebbins revisited. American Journal of Botany 101(7):1057–1078. doi: 10.3732/ajb.1400178

Soltis PS, Marchant DB, Van de Peer Y, Soltis DE (2015) Polyploidy and genome evolution in plants. Current Opinion in Genetics & Development 35:119–125. doi: 10.1016/j.gde.2015.11.003

Stebbins GL (1947) Types of polyploids: their classification and significance. Advances in Genetics 1:403–429.

Stebbins GL (1971) Chromosomal evolution in higher plants. London: Addison-Wesley.

Suda J, Meyerson LA, Leitch IJ, Pyšek P (2015) The hidden side of plant invasions: the role of genome size. New Phytologist 205(3):994–1007. doi: 10.1111/nph.13107

Tamayo-Ordóñez MC, Espinosa-Barrera LA, Tamayo-Ordóñez, YJ, Ayil-Gutiérrez B, Sánchez-Teyer LF (2016) Advances and perspectives in the generation of polyploid plant species. Euphytica 1:1–22. doi: 10.1007/s10681-016-1646-x

Tayalé A, Parisod C (2013) Natural pathways to polyploidy in plants and consequences for genome reorganization. Cytogenetic and Genome Research 140(2–4): 79–96. doi: 10.1159/000351318

Term Fisher Scientific (2016) <https://www.thermofisher.com/br/en/home.html> Accessed in 15 January 2016

Theodoridis S, Randin C, Broennimann O, Patsiou T, Conti E (2013) Divergent and narrower climatic niches characterize polyploid species of European primroses in *Primula* sect. Aleuritia. Journal of Biogeography 40(7): 1278–1289 doi:10.1111/jbi.12085

Vanneste K, Maere S, Van de Peer Y (2014) Tangled up in two: a burst of genome duplications at the end of the Cretaceous and the consequences for plant evolution. Phil. Trans. R. Soc. B 369:20130353. doi:10.1098/rstb.2013.0353

Xiong A, Gaeta RT, Pires JC (2011) Homoeologous shuffling and chromosome compensation maintain genome balance in resynthesized allopolyploid *Brassica napus*. Proceedings of the National Academy of Sciences 108(19):7908–7913. doi: 10.1073/pnas.1014138108

Yang X, Ye CY, Cheng ZM, Tschaplinski TJ, Wullschleger SD, Yin W, Xia X, Tuskan GA (2011) Genomic aspects of research involving polyploidy plants. Plant Cell Tiss Organ Cult 104(3):387–397. doi 10.1007/s11240-010-9826-1