UNIVERSIDADE FEDERAL DE VIÇOSA

ISADORA LEMOS GOMES

NUCLEAR DNA CONTENT AND KARYOTYPE OF *Agalinis marianae*: A NEW AND ENDANGERED SPECIES

VIÇOSA - MINAS GERAIS

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Monografia, apresentada ao Curso de Ciências Biológicas da Universidade Federal de Viçosa como requisito para obtenção do título de bacharel em Ciências Biológicas.

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Isadora Lemos Gomes
Autora

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Wellington Ronildo Clarindo
Orientador

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ABSTRACT

Agalinis marianae V.C. Souza (Orobanchaceae) is an endemic and critically endangered species from Minas Gerais, Brazil, restricted to the ironstone rocky outcrops of the Quadrilátero Ferrífero region. *A. marianae* was recently described (2022), therefore, little is known about its biology. This study presents the first genomic characterization of *A. marianae*, including nuclear DNA content, chromosome number, karyotype structure, and karyogram assembly. Genome size was measured using flow cytometry. *A. marianae* has a mean nuclear DNA content of 2C = 12.59 ± 0.33 pg (1C = 6.295 pg, equivalent to 6156 Mbp), representing a moderate genome size within the family. This is the first data reported about nuclear DNA content for the genus. Roots from *A. marianae* plantlets were used for cytogenetic analyses. Metaphases were captured for 2n chromosome counting and karyogram assembly. *A. marianae* presents 2n = 32 chromosomes (x = 16). *A. marianae* karyotype is composed of chromosomes with a total length varying from 4.87 (chromosome 1) to 3.13 µm (chromosome 16). Seven chromosome pairs were classified as metacentric and nine pairs as submetacentric. Secondary constrictions were also observed in some chromosomes. The karyotype also possesses morphologically similar chromosomes, suggesting a polyploid origin for the species This result provides insights into the genome organization and evolution of *A. marianae*. Additionally, the results contribute essential information for future genomic and epigenetic studies while supporting the development of effective conservation strategies.

Keywords: *Agalinis marianae*; karyogram; chromosome characterization; nuclear DNA content.

RESUMO

Agalinis marianae V.C. Souza (Orobanchaceae) é uma espécie endêmica e criticamente ameaçada de Minas Gerais, Brasil, restrita aos campos rupestres ferruginosos da região do Quadrilátero Ferrífero. A espécie foi recentemente descrita (2022) e, portanto, pouco se conhece sobre sua biologia. Este estudo apresenta a primeira caracterização genômica de *A. marianae*, incluindo o conteúdo de DNA nuclear, o número cromossômico, a estrutura do cariótipo e a montagem do cariograma. O tamanho do genoma foi medido por citometria de fluxo. *A. marianae* apresenta um conteúdo médio de DNA nuclear de 2C = 12,59 ± 0,33 pg (1C = 6,295 pg, equivalente a 6156 Mbp), representando um tamanho genômico moderado para a família. Este é o primeiro relato sobre o conteúdo de DNA nuclear para o gênero. Raízes de plântulas de *A. marianae* foram utilizadas para análises citogenéticas. Metáfases foram capturadas para a contagem cromossômica 2n e a montagem do cariograma. A espécie apresenta 2n = 32 cromossomos (x = 16). O cariótipo de *A. marianae* é composto por cromossomos com comprimento total variando de 4,87 µm (cromossomo 1) a 3,13 µm (cromossomo 16). Sete pares de cromossomos foram classificados como metacêntricos e nove pares como submetacêntricos. Constrições secundárias também foram observadas em alguns cromossomos. O cariótipo ainda apresenta cromossomos morfologicamente semelhantes, sugerindo uma origem poliplóide para a espécie. Este resultado fornece informações sobre a organização e evolução do genoma de A. marianae. Além disso, os resultados contribuem com informações essenciais para futuros estudos genômicos e epigenéticos, ao mesmo tempo que subsidiam o desenvolvimento de estratégias eficazes de conservação.

Palavras-chave: *Agalinis marianae*; cariograma; caracterização cromossômica; conteúdo de DNA nuclear.

SUMMARY

1 INTRODUCTION

Agalinis genus (Orobanchaceae) presents ~40 species of herbs, shrubs and sub-shrubs. According to a study accomplished by Souza, V.C. for the Brazilian Flora Report in 2020, 13 *Agalinis* species occur in Brazil, nine of which are endemic, with hotspots in Minas Gerais and Rio Grande do Sul states. In 2022, one more species was described: *Agalinis marianae* V.C.Souza (Souza *et al*. 2022). Some *Agalinis* species are considered endangered and have been included in protected preservation areas. For these, in addition to botanical studies in *Agalinis* species, genomic research is needed to understand the *Agalinis* diversity and origin, as well as to design the conservation programs.

In this sense, karyotype and nuclear genome size data about *Agalinis* are scarce, even the genomic data being relevant for developing more efficient preservation strategies and conserving biodiversity. Previous studies (Kondo, Musselman & Mann Jr. 1978; Canne-Hilliker 1988; Neel & Cummings 2004) provided informations about the 2n chromosome number and karyotype of some *Agalinis* species, but were not able to determine the ploidy level or genome size. While most *Agalinis* species native from North America present 2n = 26 and 28 chromosomes, South American species present mostly 2n = 32 chromosomes. Raven (1975) suggested that Scrophulariaceae genera – previous taxonomic Family *Agalinis* was included – were usually characterized by descending aneuploidy, but was unsure if the original diploids still existed, positing X = 7 as the original basic chromosome number for the family.

The latest described species, *A. marianae*, is an endemic and rare species that occurs in a restricted area of the municipalities of Mariana and Ouro Preto, in Minas Gerais, Brazil (Souza *et al*. 2022). *A. marianae* occurs within the Iron Quadrangle region, which is considered one of the most important mineral provinces in the world with intense activity of iron ore extraction (Salles *et al*. 2018; Spier *et al*. 2003). This region was also affected by the collapse of a waste containment dam in 2015, which has been appointed as the biggest environmental tragedy in Brazil (Lopes 2016). *A. marianae* is classified as critically endangered B1, according to the IUCN Red List Criteria (IUCN 2022). As *A. marianae* is new to science, few data about its biology and evolution are known.

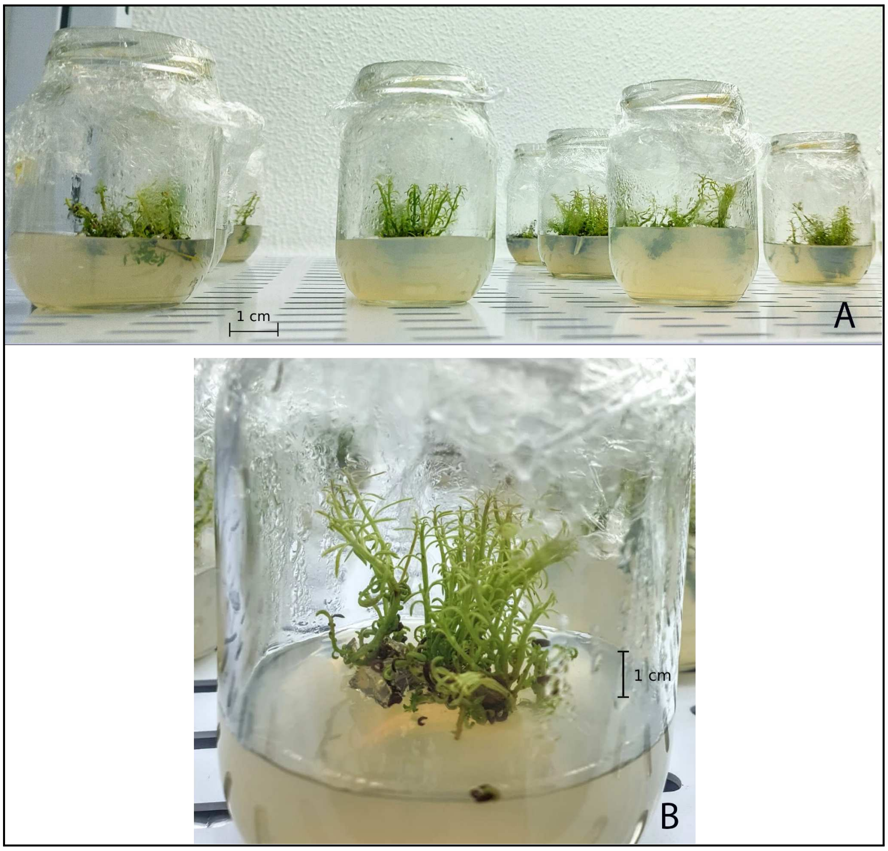
Thus, we aimed to provide the first data about *A. marianae*'s genome: (a) 2C/1C nuclear value, (b) 2n chromosome number, (c) chromosome characterization, and (d) karyogram assembly.

2 MATERIALS AND METHODS

2.1 Biological material

A. marianae seeds and leaves were collected from 34 individuals in Mariana (Minas Gerais, Brazil) in the region Alegria Norte, Campo Rupestre Ferruginoso (Ironstone Rocky Outcrop), coordinates 20º 09′ 30″ S, 43º 31′ 00″ W, 1353 m. *A. marianae* seeds were disinfected with 70% ethanol (Merck®) for 1 min, kept in 2.5% $NaOCl_2$ supplemented with 0.1% Tween 20 (Merck®) for 20 min, washed five times with autoclaved dH_2O and dried in filter paper. Then, the seeds were inoculated into flasks containing ½MS culture medium (Murashige and Skoog 1962) supplemented with 10 mL L^{-1} MS vitamins, 3.0% sucrose and 0.7% agar type A. The flasks were kept under 16/8 h light/dark at 25ºC (Figure 1). After ~30 days, plantlets were recovered and some roots were extracted.

Figure 1 – *A. marianae* plantlets *in vitro*.



Source: Autor. 1A) Flasks containing *A. marianae* plantlets cultivated *in vitro* in a growth room under controlled temperature and luminous condition. 1B) *A. marianae* plantlet *in vitro* 30 days after inoculation.

2.2 Nuclear 1C value measurement

A. marianae 1C value was measured using flow cytometry. *A. marianae* leaves collected *in situ* were used for the analysis. The leaves were stored in plastic bags, which were properly labeled and closed for transportation. Flow cytometry procedures were performed ~20 h after collection of the leaves. *Solanum lycopersicum* L. 'Stupické' leaves were used as an internal standard for 1C nuclear value measurement. *S. lycopersicum* presents well-documented genome size and stability in cytometric analyses (2C = 2.00 pg, Praça-Fontes *et al.* 2011), necessary qualities for a good internal standard.

The procedure used for flow cytometry was adapted from what is described by Praça-Fontes *et al.* (2011). Leaf fragments (~2 cm^2) from each individual of *A. marianae* and the internal standard were simultaneously chopped (Galbraith *et al*. 1983) for ~30 s in 0.5 mL of OTTO-I extraction buffer (OTTO, 1990), supplemented with 50 µg mL^{-1} RNAse (Sigma®) and 2 mM dithiothreitol (Sigma®). 0.5 mL of OTTO-I was added to each sample, and the suspensions were homogenized and filtered through 30 µm nylon mesh (Partec®). Subsequently, the suspensions were centrifuged at 1,100 rpm for 5 min, the supernatant was discarded, and the pellet was resuspended in 100 µL of OTTO-I and incubated for 10 min. The nuclear suspensions were stained with 1.5 mL of OTTO-II buffer (OTTO, 1990), containing 75 µM propidium iodide (Sigma®), 2.0 mM dithiothreitol (Sigma®), and 50 µg mL^{-1} RNAse (Praça-Fontes *et al*., 2011). The suspensions were incubated in the dark at room temperature for at least 30 min, filtered through 20 µm nylon mesh (Partec®) and analyzed. At least 10,000 nuclei were analyzed for each sample.

The nuclei suspensions were analyzed using a BD Accuri C6 flow cytometer (Accuri cytometers, Belgium) outfitted with a 50 mW 488 nm laser to detect wavelengths emissions using FL2 (585/40 nm) and FL3 (650 LP) filters. The histograms were analyzed with BD CSampler™. 2C nuclear value was measured by dividing the mean channel of the fluorescence peak corresponding to the G_0/G_1 nuclei of the standard for each sample. The nuclear 1C value in pg and Mbp were calculated by using the mean 2C value.

2.3 2n chromosome number

Roots collected from *in vitro A. marianae* plantlets were treated with 4 µM amiprophos-methyl (Sigma®) supplemented with 0,3% dimethyl sulfoxide (Sigma®) during 3 – 5 h at 30ºC. Subsequently, the roots were washed three times with dH_2O on a shaker for 10 min, and fixed with 3:1 methanol and acetic acid (Sigma®). Three changes of the fixative solution were performed, one every 10 min (Carvalho *et al*. 2007). Finally, the roots were stored at -20ºC for at least 24 h.

The fixed roots were washed three times with dH_2O on a shaker for 10 min. The root meristems were excised and macerated in enzymatic pool (4% cellulase Sigma®, 0.4% hemicellulase Sigma®, 1% macerozyme Onozuka R10 Yakult diluted in pectinase Sigma®) at 1:10 (enzymatic pool: dH_2O), 1:15, 1:20 and 1:25 ratio. The maceration was conducted in a dry bath for 2 h at 30ºC and 36.5ºC. The meristems were washed three times with dH_2O, fixed with 3:1 methanol and acetic acid in three changes of 10 minutes each, and stored at -20ºC for at least 12 h (Carvalho *et al*. 2007).

Slides were prepared from the macerated root meristems using cell dissociation and air-drying techniques (Carvalho & Saraiva 1993). The slides were stained with 5% Giemsa solution (Merck®) and analyzed under an Olympus BX-60 photomicroscope. Metaphases and prometaphases were scattered using a 12-bit CCD digital video camera (Olympus®) coupled to the microscope under a 100× objective with a numerical aperture of 1.4 and used for chromosome counting and karyogram assembly.

2.4 Chromosome morphometry and karyogram assembly

Chromosome morphometry and, consequently, karyogram assembly were performed from one metaphase. The metaphase was analyzed and edited using Photoshop® version 25.2. The chromosomes were first segmented and had their total length and long arm measured; the short arm length was determined by the difference between the total and long arm sizes. The arm ratio (long/short) was calculated and the chromosomes were classified, according to the reference values established by Guerra (1986). The total length and arm ratio values were used for chromosome pairing.

3 RESULTS AND DISCUSSION

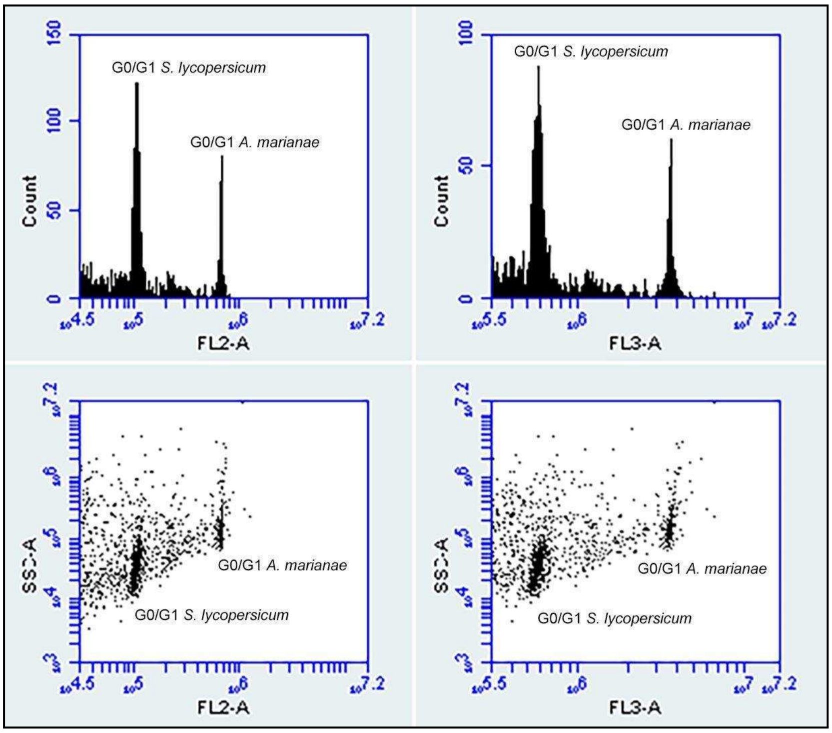
3.1 Genome size

For nuclear genome size measurement, the flow cytometry procedure provided histograms presenting well-defined and narrow G_0/G_1 peaks, which exhibited coefficient of variation (CV) below 5% (Figure 2). This CV value indicates that the procedure ensured enough intact, isolated and stoichiometrically stained nuclei, proper for flow cytometry analysis. Therefore, the flow cytometry procedure may be considered appropriate for nuclear

DNA content measurement for *A. marianae* samples using *Solanum lycopersicum* (2C = 2.00 pg) as an internal standard.

A. marianae has a mean nuclear DNA content of 2C = 12.59 ± 0.33 pg, equivalent to 1C = 6.295 pg and 1C = 6156 Mbp. For the first time, the nuclear DNA content value was reported for an *Agalinis* species. According to Bennett and Leitch (2000) nuclear DNA amounts parameters for angiosperms, *A. marianae* 1C value is classified as medium (3.5 - 14.0 pg).

Figure 2 – Flow cytometry histograms.



Source: Autor. Histograms presenting G_0/G_1 peaks of *S. lycopersicum* (2C = 2.00 pg) and *A. marianae* (2C = 12,59 pg).

3.2 Chromosome number

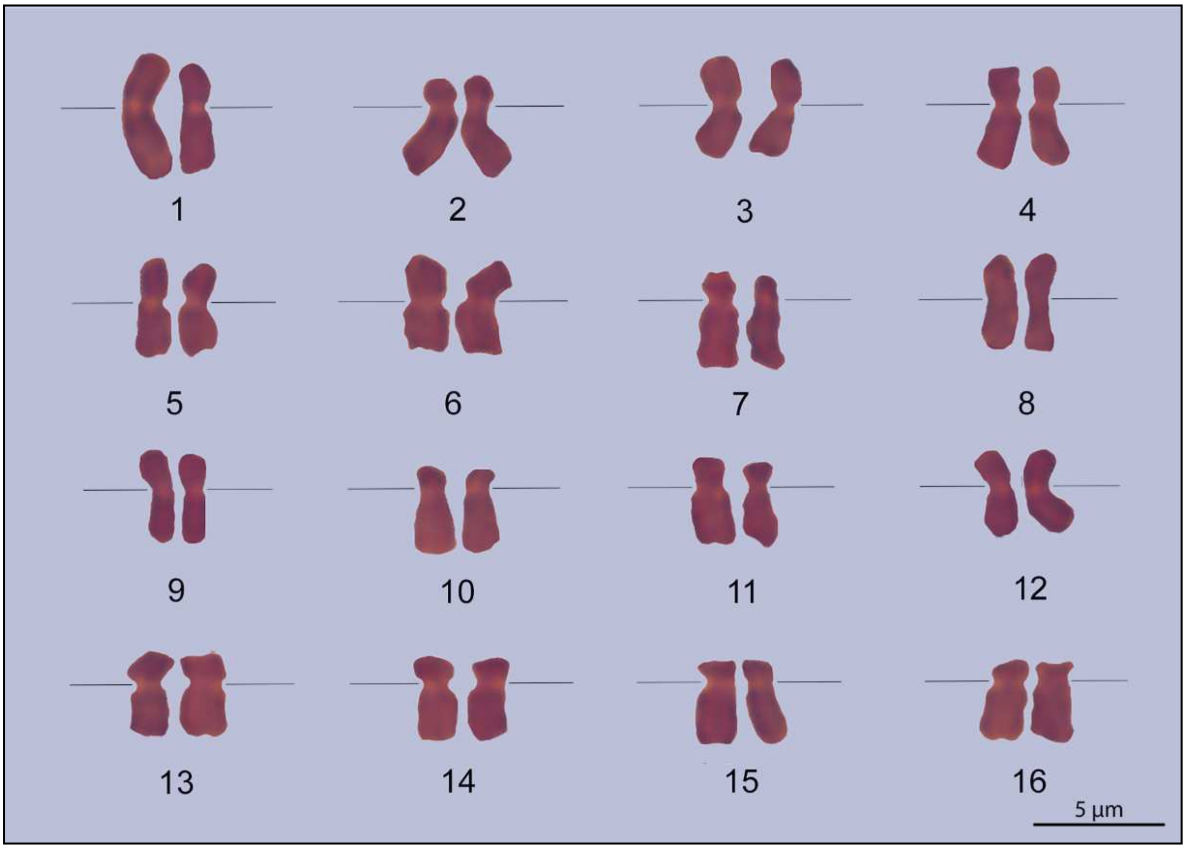
Cytogenetic procedure used for *A. marianae* provided adequate prometaphases and metaphases, without cytoplasm background or chromatin damage. The most suitable metaphases were obtained from root meristems treated with 4 µM amiprophos-methyl for 5 h at 30ºC, and enzymatically macerated at 1:20 (enzymatic pool: dH_2O) for 2 h at 30ºC. When treated with higher concentrations of enzymatic pool, metaphases were mostly incomplete or with chromosomes too scattered. Lower concentrations of the enzymatic pool provided metaphases with integral plasma membranes, resulting in overlapped chromosomes. In addition, the air-drying technique had to be very subtle to prevent the dispersion of the chromosomes. Therefore, the challenge was to obtain metaphases either complete and featuring isolated chromosomes, adequate for morphometry and karyogram assembly.

A. marianae plantlets present 2n = 32 chromosomes (x = 16). This 2n chromosome number is consistent with most studied *Agalinis* species in South America. Considering the 13 Brazilian *Agalinis* species, seven present chromosome number 2n = 32 (Canne-Hilliker 1988; Neel & Cummings 2004).

3.3 Karyogram and chromosome morphology

We propose the first karyogram for *Agalinis marianae* (Figure 3).

Figure 3 – First karyogram proposed for *Agalinis marianae* (2n = 32).



Source: Autor. Metaphasic chromosomes pre-treated with 4 µM APM for 5 h and stained with 5% Giemsa. The *A. marianae* karyogram showed 16 homologous pairs.

Morphometry analysis evidenced chromosomes measuring from 4.87 (chromosome 1) to 3.13 µm (chromosome 16). The species present seven metacentric chromosome pairs (1, 3, 5, 6, 8, 9 and 12) and nine submetacentric pairs (2, 4, 7, 10, 11, 13, 14, 15 and 16) (Table 1). Secondary constrictions (NOR) were observed in one chromosome pair, but they were not visible in the metaphase used for karyogram assembly (Figure 4). Consequently, it was not possible to identify which pair presents the NOR. Secondary constrictions have already been reported on chromosome 1 of some *Agalinis* species, such as *A. aphylla, A. fasciculata* and *A. setacea*. (Kondo, Musselman and Mann Jr. 1978).

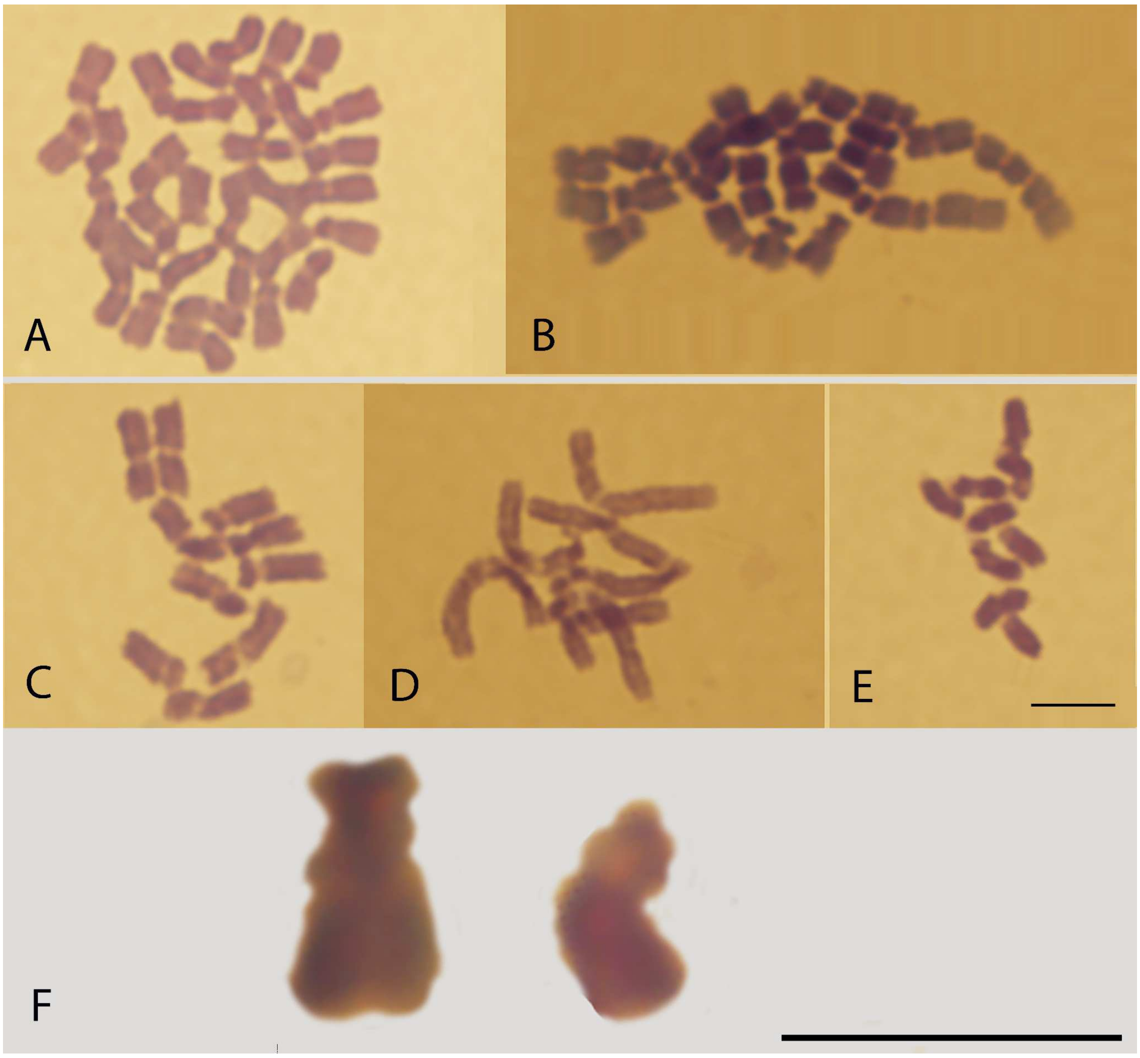
Table 1 – *A. marianae* chromosome morphometry data and class.

Chrom.	**Pair**	**Total (µm)**	**Arm**		**r**	**Class**	**Relative size (%)**
			Short	**Long**			
1	1	4,87	2,09	2,78	1,33	**M**	**4,25**
2	1	4,24	1,74	2,50	1,44	**M**	**3,70**
3	2	3,97	1,19	2,78	2,34	**SM**	**3,46**
4	2	3,90	1,15	2,75	2,39	**SM**	**3,40**
5	3	3,93	1,95	1,98	1,02	**M**	**3,43**
6	3	3,83	1,88	1,95	1,04	**M**	**3,34**
7	4	3,93	1,50	2,43	1,62	**SM**	**3,43**
8	4	3,79	1,46	2,33	1,60	**SM**	**3,31**
9	5	3,82	1,70	2,12	1,25	**M**	**3,33**
10	5	3,62	1,50	2,12	1,41	**M**	**3,16**
11	6	3,76	1,85	1,91	1,03	**M**	**3,28**
12	6	3,76	1,85	1,91	1,03	**M**	**3,28**
13	7	3,69	1,12	2,57	2,29	**SM**	**3,22**
14	7	3,65	1,08	2,57	2,38	**SM**	**3,19**
15	8	3,65	1,70	1,95	1,15	**M**	**3,19**
16	8	3,62	1,67	1,95	1,17	**M**	**3,16**
17	9	3,58	1,56	2,02	1,29	**M**	**3,12**
18	9	3,48	1,46	2,02	1,38	**M**	**3,04**
19	10	3,37	0,97	2,40	2,47	**SM**	**2,94**
20	10	3,20	0,94	2,26	2,40	**SM**	**2,79**
21	11	3,34	1,15	2,19	1,90	**SM**	**2,91**
22	11	3,27	1,08	2,19	2,03	**SM**	**2,85**
23	12	3,30	1,46	1,84	1,26	**M**	**2,88**
24	12	3,27	1,57	1,70	1,08	**M**	**2,85**
25	13	3,30	1,04	2,26	2,17	**SM**	**2,88**

26	13	3,20	1,01	2,19	2,17	**SM**	**2,79**
27	14	3,28	1,31	1,97	1,50	**SM**	**2,86**
28	14	3,20	1,18	2,02	1,71	**SM**	**2,79**
29	15	3,23	0,83	2,40	2,89	**SM**	**2,82**
30	15	3,27	0,87	2,40	2,76	**SM**	**2,85**
31	16	3,13	0,94	2,19	2,33	**SM**	**2,73**
32	16	3,13	0,91	2,22	2,44	**SM**	**2,73**
Total		**114,58**	**43,71**	**70,87**			**100,00**

Chrom. = chromosome number; Pair = number of the chromosome pair; Total = total length; Long/Short = arm length; r = arm ratio – long/short; M = metacentric; SM = submetacentric; Relative size = % size in relation to sum of the mean values of total length.

Figure 4 – Metaphasic chromosomes captured on microscope.



Source: Autor. A) Metaphase used for karyogram assembly, evidencing 2n = 32 chromosomes. B) Metaphase featuring 25 chromosomes. Chromosome exhibiting NOR located in the center-south. C) Dispersed group of chromosomes. Chromosome exhibiting NOR located in the center. D) Dispersed group of chromosomes. Chromosome exhibiting NOR located in the center-west. E) Dispersed group of chromosomes. Chromosome exhibiting NOR located to the north. F) Chromosomes exhibiting NOR from images B and E in evidence. Bar = 5 µm.

Groups of chromosomes are morphologically similar (5/6, 8/9, 11/13, 10/15) regarding total length, arm ratio and class. Homomorphic chromosomes have already been observed for the genus. Kondo, Musselman and Mann Jr. (1978) assembled the karyograms and chromosome morphometric data of six *Agalinis* species from North America: *A. aphylla* (2n = 26), *A. fasciculata* (2n = 28), *A. linijolia* (2n = 28), *A. purpurea* (2n = 28), *A. setacea* (2n = 28) and *A. virgata* (2n = 28). In their study, they also noted the morphological similarities between the chromosomes in *Agalinis* karyotypes. Homomorphism is frequently identified in angiosperms, and is observed in species such as *Coffea arabica* L., *Jatropha curcas* L. and *Glycine max* L., known to have a polyploid origin (Clarindo and Carvalho 2008; Carvalho et al. 2008; Clarindo et al. 2007).

Polyploidy is a common adaptive strategy among neotropical plants, and is especially important for species inhabiting hostile environments, such as the rocky outcrops. Studies prove that all angiosperm species experienced polyploidization events during evolution (Jiao *et al.* 2011; Madlung 2013). Though polyploid species have never been reported for *Agalinis* genus, it has been for genera phylogenetically close to *Agalinis*, such as *Castilleja* and *Orobanche* (Heckard 1968; Heckard and Chuang 1975). These observations suggest a polyploid origin of *Agalinis*.

4 CONCLUSION

We provided the first data about the *A. marianae* genome. The results reveal that *A. marianae* presents nuclear DNA content 2C = 12.59 ± 0.33 pg (1C = 6.295 pg = 6156 Mbp) and karyotype 2n = 32 (x = 16) composed of metacentric and submetacentric chromosomes. In addition, the homomorphic chromosome pairs suggest polyploidy, highlighting the potential role of polyploidy in the adaptation of this critically endangered plant to its habitat. For further studies, we suggest more analyses regarding the ploidy level of *Agalinis* species. These findings allow us to know more information not only about *A. marianae*, but also for other species of the genus; they represent the kickoff to understanding more about South American *Agalinis* species biology and evolution, contributing to further genomic and epigenetic studies and development of preservation programs.

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