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rDNA mapping, heterochromatin characterization and AT/GC content of *Agapanthus africanus* (L.) Hoffmanns (Agapanthaceae)

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ABSTRACT

Agapanthus (Agapanthaceae) has 10 species described. However, most taxonomists differ respect to this number because the great phenotypic plasticity of the species. The cytogenetic has been an important tool to aid the plant taxon identification, and to date, all taxa of *Agapanthus* L'Héritier studied cytologically, presented $2n = 30$. Although the species possess large chromosomes, the group is karyologically little explored. This work aimed to increase the cytogenetic knowledge of *Agapanthus africanus* (L.) Hoffmanns by utilization of chromosome banding techniques with DAPI / CMA₃ and Fluorescent *in situ* Hybridization (FISH). In addition, flow cytometry was used for determination of DNA content and the percentage of AT / GC nitrogenous bases. Plants studied showed $2n = 30$ chromosomes, ranging from 4.34 - 8.55 μm , with the karyotype formulae (KF) = $10m + 5sm$. Through FISH, one 45S rDNA signal was observed proximally to centromere of the chromosome 7, while for 5S rDNA sites we observed one signal proximally to centromere of chromosome 9. The 2C DNA content estimated for the species was $2C = 24.4$ with 59% of AT and 41% of GC. Our data allowed important upgrade for biology and cytotaxonomy of *Agapanthus africanus* (L.) Hoffmanns.

Key words: African lily, chromosome banding, cytotaxonomy, flow cytometry, genome size, molecular cytogenetics.

INTRODUCTION

The genus *Agapanthus* L'Héritier (Agapanthaceae) is a monocotyledonous, herbaceous and perennial plant group endemic to southern Africa, with ten species in which three are considered evergreen and seven deciduous plants (Leighton 1965). Nevertheless, *Agapanthus* L'Héritier taxonomy

is considered very difficult once there are few unique characters and a high plasticity depending on circumstances of growing. This feature takes some authors to believe in the existence of only one species with innumerable varieties (McNeil 1972). So, the obtaining and exploring of new characters, less plastic to identification of these species are of great importance for *Agapanthus* L'Héritier taxonomy.

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The use of karyological data in taxonomy, contribute widely to evaluate the genetic relationship among species or populations and to a better understanding of the way they diverged from each other (Guerra 2008, Sousa et al. 2013, Reis et al. 2014). In *Agapanthus* L'Héritier, all plants measured until the moment have the same chromosome number, $2n = 30$ (Riley and Mukerjee 1960, Sharma and Mukhopadhyay 1963), with little differences only in chromosome length. Since chromosome length can vary with condensation, this parameter in plastic plants, studied without another chromosome marker is not a good tract for detection of speciation. For this, more detailed karyological studies are very important to increment the utilization of chromosome length like a parameter of species identification in *Agapanthus* L'Héritier.

Agapanthus africanus (L.) Hoffmanns, an endemic South African species, highly desired as ornamental, has been grown around the world, being adapted to conditions in Europe, Australia, New Zealand and North as well as South America, since the late seventeenth century (Win Snøeijer 2004). Besides its ornamental attributes the species are traditionally used by local communities in South Africa as medicinal for various disorders: during pregnancy to induce labour (Kaido et al. 1997); to treat constipation in pregnancy (Van Wyk et al. 1997); as antenatal or post-natal treatment of the mother and for high blood pressure (Duncan et al. 1999). Additionally some studies have shown that *Agapanthus africanus* (L.) Hoffmanns has an important antifungal potential against plant pathogens (Singh et al. 2008, Tagegne et al. 2008).

Due its varied application and importance around the world, the aim of the present work was to obtain new chromosomal markers for *Agapanthus africanus* (L.) Hoffmanns (an important informations for taxonomy and breeding of the species) utilizing molecular cytogenetics,

chromosomes banding and flow cytometry which are scarce approaches for the genus.

MATERIALS AND METHODS

PLANT MATERIAL AND PREPARATION OF MITOTIC SPREADS

Seeds of *Agapanthus africanus* (L.) Hoffmanns were collected at three different sites: Bom Pastor Neighborhood (21°46'31.6"S and 43°20'42.3"W), São Pedro Neighborhood (21°46'19.9"S and 43°22'39.1"W), and in the Campus of the Federal University of Juiz de Fora (21°46'23.3"S and 43°22'09.3"W), in Juiz de Fora Minas Gerais, Brazil. For each locality around 50 seeds were collected and herbarium voucher specimens were deposited at the CESJ Herbarium of the Federal University of Juiz de Fora (available at <http://www.splink.org.br>).

After that, seeds were germinated and roots tips were pre-treated with 8-hydroxyquinoline solution (0.003 M) at room temperature for 9h and then fixed in ethanol and acetic acid (3:1 v/v) for 24h at -20°C. Root meristems were submitted to enzymatic maceration (2% Celullase: 20% Pectinase) for 5h at 37°C. The slides were prepared according to Carvalho and Saraiva (1993, 1997).

CHROMOSOME MORPHOMETRY

Chromosome length, short and long arms length and ratio between chromosome arms were measured and chromosome classification was done according to Levan et al. (1964). The ideogram was mounted according to decreasing size order of chromosomes short arms.

CHROMOSOME BANDING

Chromosome banding was performed according Schweizer (1976). Five aged slides from each localities were stained with chromomycin A₃ (0.5 mg/mL) for 1h, dystamicyn (0.1 mg/mL) for 30 min and 2-4 diamidino-2-phenylindole (2 µg/mL) for 30 min. The slides were mounted in Mcllvaine's

pH 7.0 buffer-glycerol (1:1 v/v). For this analysis ten metaphases from plants of each locality were observed and captured in an Olympus DP72 digital camera. The chromosomes were observed using an epifluorescence microscope (Olympus BX 51) with appropriate filter set.

After images documentation the slides were discolored in 100% ethanol for 24h at room temperature and used for molecular cytogenetic processes.

MOLECULAR CYTOGENETIC

Fluorescence *in situ* hybridization (FISH) was performed using 45S rDNA probes from *Triticum aestivum* L. (Gerlach and Bedbrook 1979) and 5S probes from *Zea mays* L. (courtesy Koo and J. Jiang). Each probe was labeled with digoxigenin by nick translation and then hybridized according to Jiang et al. (1995) with minor modifications. The hybridization mixture was denatured at 85°C for 10 min and immediately transferred to an icebox. The slides were denatured at 85°C for 1 min and treated with a series of ethanol washes (70%, 90%, and 100% for 5 min each). The hybridization mixture was then added to the slides and the chromosomes allowed to hybridize at 37°C for 48h. Posthybridization washes were carried out using 2 × SSC buffer for 15 min and 1 × PBS buffer for 5 min. Probes were detected with anti-DIG conjugate with rhodamine and postdetection washes were performed using 1 × TNT buffer and for 15 min and 1 × PBS buffer for 5 min at room temperature. Chromosomes were counter-stained with 2 µg/mL of DAPI. The slides were mounted in Vectashield (Vector) and samples were rehybridized after slides discoloration in 100% ethanol for 24h. Images with 45S and 5S signals were merged using CellSens software (Olympus). Chromosomes were observed using an epifluorescence microscope (Olympus BX 51) with appropriate filter set.

FLOW CYTOMETRY (FCM)

Nuclear DNA content was determined according to the method of Galbraith et al. (1983). Approximately 20–30 mg of young and fresh leaves of 3 plants of *Agapanthus africanus* (L.) Hoffmanns from each locality and the same amount of young foliar tissue of standard references (*Pisum sativum* L.) were chopped on ice with 1 mL of OTTO I lysis buffer solution (0.1 M C₆H₈O₇; 0.5% Tween 20) (Otto 1990) supplemented with 50 µg/mL⁻¹ RNase. The suspension was filtered through 40nm mesh into 2 mL microcentrifuge tube and centrifuged at 1,100 rpm for 5 minutes. The pellet was incubated in 100 µL OTTO I lysis buffer for 10 minutes and then was added 300 µL of OTTO I: OTTO II (0.4 M Na₂HPO₄·12H₂O) (1:2, v/v) buffers. The sample were homogenized and stained with 50 µg/mL⁻¹ of propidium iodide (PI) to determine the total DNA content.

AT/GC composition was determined by adding DAPI (4,6-diamidino-2-phenylindole) 4 µg/mL⁻¹ to the samples. At least 10,000 nuclei were analyzed per sample in a FACSCantoII (Becton Dickinson) flow cytometer. The histograms were analyzed using Flowing 2.5.1 software (www.flowingsoftware.com).

DNA nuclear content (pg) of each sample was estimated by the relative fluorescence intensity of the sample and the internal reference standard (*Pisum sativum* L. 9.09 pg). Three samples, of each accession, were measured according to the following equation (Dolezel 2003):

$$DNA\ content\ of\ sample = \frac{PIFI\ of\ sample \times DNA\ content\ of\ standard}{PIFI\ of\ standard}$$

(PIFI is the fluorescence intensity of propidium iodide in G₁ cells).

The AT percentage of *Agapanthus africanus* (L.) Hoffmanns was measured in relation to *P. sativum* L. reference standard, following the formula described by Godelle et al. (1993):

$$AT\ sample = AT\ standard \left(\frac{R_{DAPI}}{R_{IP}} \right)^{\frac{1}{r}}$$

TABLE I
Karyology of *A. africanus* and estimative of DNA content for each chromosome.

Chromosome	Relative length (%)	Absolute length (µm)	Short arm length (µm)	Long arm Length (µm)	Arm ratio	Classification	DNA pg/chromosome	Mpb/chromosome
1	9.46	8.55	3.93	4.62	1.17	m	1.15	1128.72
2	8.91	8.05	3.65	4.39	1.20	m	1.08	1063.10
3	8.91	8.05	3.42	4.63	1.35	m	1.08	1063.10
4	6.71	6.07	2.93	3.14	1.07	m	0.81	800.61
5	7.17	6.48	2.71	3.77	1.38	m	0.87	855.49
6	7.42	6.70	2.64	4.06	1.53	m	0.90	885.32
7	7.55	6.82	2.40	4.42	1.84	sm	0.92	900.83
8	5.81	5.25	2.21	3.04	1.37	m	0.70	693.22
9	6.44	5.82	2.14	3.68	1.71	sm	0.78	768.39
10	5.49	4.96	2.07	2.79	1.34	m	0.66	655.04
11	5.37	4.86	2.05	2.90	1.41	m	0.65	640.72
12	4.92	4.44	1.86	2.58	1.38	m	0.60	587.03
13	5.40	4.88	1.66	3.22	1.93	sm	0.66	644.30
14	5.57	5.03	1.56	3.47	2.22	sm	0.67	664.59
15	4.80	4.34	1.46	2.88	1.97	sm	0.58	572.71

pg = picograms; Mpb = Megabase pairs; µm = micrometer; m = median; sm = submedian.

(*R* is the ratio of fluorescence intensity between the peak of *Agapanthus africanus* (L.) Hoffmanns and *Pisum sativum* L., and *r* (binding length) = 3 for DAPI dye).

The percentage of the complementary bases was calculated as GC% = 100 – AT%.

Taking into account that 1 pg = 978 Mpb (Dolezel et al. 2003), the combination of cytometric and cytogenetic data were provided.

RESULTS AND DISCUSSION

Samples of *Agapanthus africanus* (L.) Hoffmanns from the three different localities studied here showed symmetrical karyotype with $2n = 30$, of which ten chromosomes with centromeres at the median region (m, Arm Ratio = 1 - 1.7), and five of them at submedian (sm, Arm Ratio = 1.71 - 3.0) (Table I). No secondary constrictions were observed and absolute chromosome length ranged from 4.34 - 8.55 µm (Table I) while the

relative chromosome length revealed that the largest chromosome represented around 9.46% of the genome size and the shortest one 4.80% (Table I). Our results are in conformity with the cytogenetics morphometric data in the literature for *Agapanthus* L'Héritier (Riley and Mukerjee 1962, Sharma and Mukhopadhyay 1963, Yaacob and Taha 2014), however, no cytogenetics data for the taxa *Agapanthus africanus* (L.) Hoffmanns has been described. In the most recent revision of the genus (Win Snoeijer 2004) *Agapanthus africanus* (L.) Hoffmanns is considered like synonymy of *Agapanthus minor* Lodd., *Agapanthus umbellatus* L'Héritier, *Agapanthus umbelliferus* Poir., *Agapanthus tuberosus* L. ex DC. and *Agapanthus variegatus* Steud. Sharma and Mukhopadhyay (1963) studying the cytogenetics of varieties of *Agapanthus umbellatus* L'Hér. observed a very similar chromosomal morphology with the data observed for us, principally when we compare it with

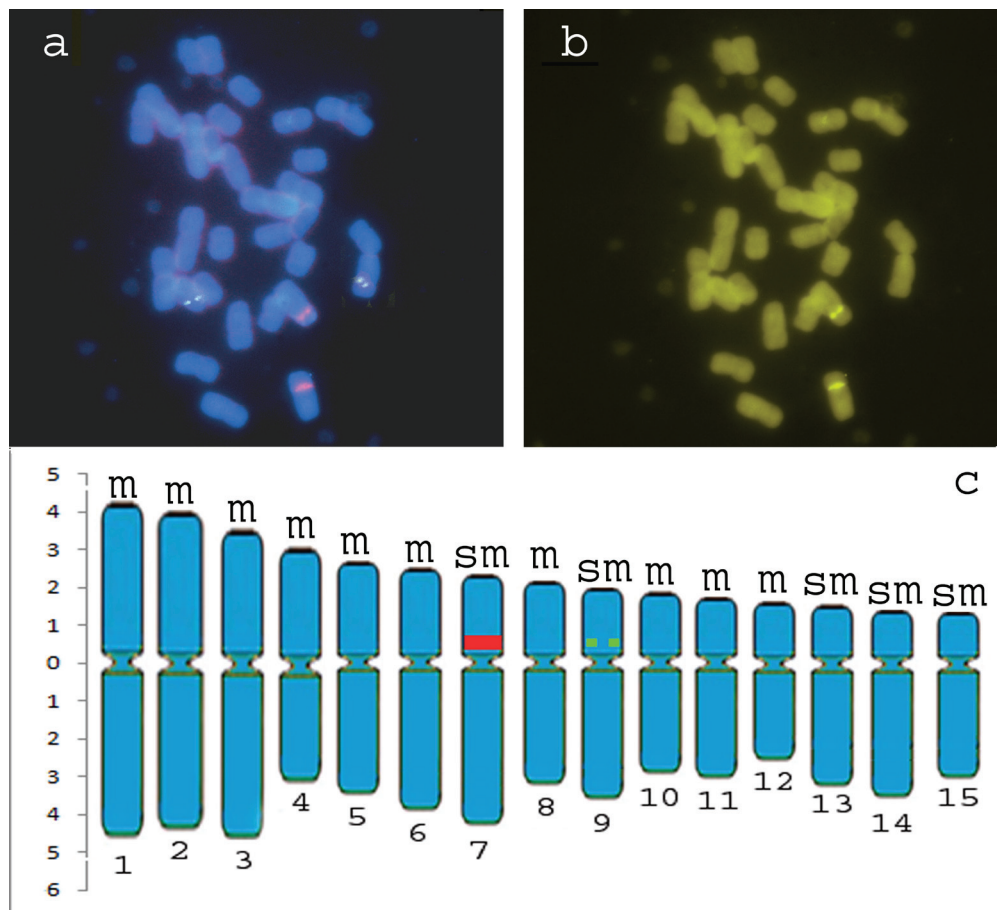


Figure 1 - Representative metaphases with 5S rDNA and 45S rDNA mapping (a); CMA₃ banding pattern (b) and Ideogram of *Agapanthus africanus* (L.) Hoffmanns (c) (green squares= 5S rDNA, red rectangle = 45S rDNA), m = metacentric and sm = submetacentric.

the taxa *Agapanthus umbellatus* var. *intermedius* Tubergen where the size of chromosomes varied from 4.1-8.9 μm , with all chromosomes with centromeres at median and sub-median regions. Nevertheless, these authors observed one pair of chromosomes with secondary constrictions, not observed in our work with DAPI coloration.

Although chromosomes in *Agapanthus* L'Héritier are very large and of easy obtainment, the cytogenetics of the group is very little explored. Our observations are the first data related to molecular cytogenetics and with the mapping of heterochromatin for the genus. The 45S rDNA signal was observed in one chromosome pair on the interstitial portion, proximally to the centromere

(in the short arm of chromosome 7) (Fig. 1a, c), while 5S rDNA signals were observed in interstitial portion, proximally to the centromere, in the short arm of chromosome 9 (Fig. 1a, c). The 45S rDNA sites showed greater bands when compared with those ones observed for 5S rDNA sites, which showed pairs of little dots (Fig. 1a, c). This experiment was very important to determine the number of rDNA sites in this species once no secondary constrictions were observed in the slides prepared here. Once again our data are in conformity with observations of Sharma and Mukhopadhyay (1963) for the *Agapanthus umbellatus* var. *intermedius* Tubergen since these authors observed one pair of secondary constrictions (these data indicate that at

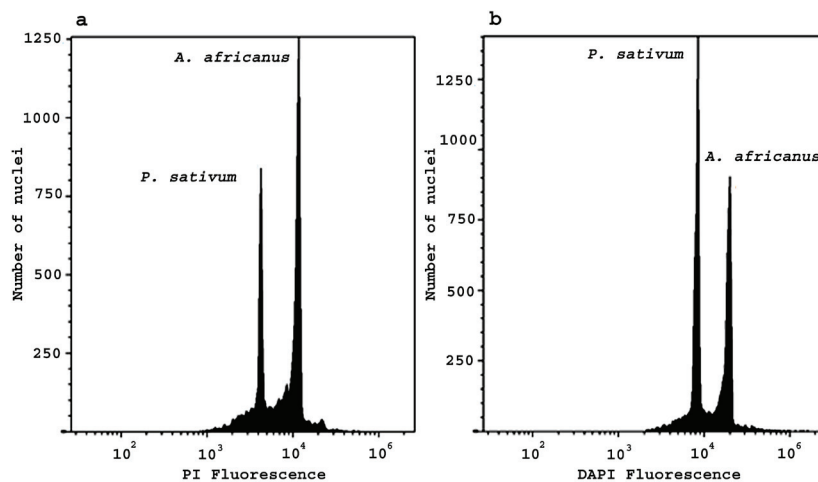


Figure 2 - Flow cytometry histograms representing *Agapanthus africanus* (L.) Hoffmanns using *Pisum sativum* L. (9.09 pg) as internal standard. **(a)** The fluorochrome propidium iodide (PI) was used to measurement of DNA amount (24.4 pg; CV=2.47) (2C values). **(b)** The fluorochrome 4',6-diamidino-2-phenylindole (DAPI) was used to determine the AT base percentual (59%; CV = 1.64).

least one pair of 45S rDNA is present in this species once that in major plants this sites are coincident). Nevertheless, like us, Panzera et al. (1996) also observed in *Allium cepa* L. sites of active 45S rDNA no coincident with secondary constrictions, confirming that these sites can or can't form secondary constrictions.

For the chromosome banding, no centromeric, interstitial or terminal DAPI bands were observed. However, fluorochrome staining with CMA₃ revealed bands on chromosome pair 7, which were also DAPI negative (Fig. 1b). The observed bands in homologues chromosomes showed similar size and bright being coincident with 45S rDNA (Fig. 1a, b), a common feature in a wide variety of plants (Sumner 1990, Guerra and Felix 2000, Sousa et al. 2013). No additional CMA bands were observed in any of the chromosome pairs (Fig. 1b).

Regarding to the nuclear genome size estimation, the FCM provided high quality histograms with G₀/G₁ peaks showing CV = 2.47. The 2C DNA content estimated for the species was 2C = 24.4 pg. Taking into account that 1pg = 978 Mpb (Dolezel et al. 2003), and combining cytometric and cytogenetic

data, the largest chromosome (chromosome 1) corresponds to 1128.72 Mpb (~9.5% of the genome), while the smallest one (chromosome 15) corresponds to 572.71 Mpb (~4.8% of the genome) (Table I). Genome size, obtained by flow cytometry has been successfully used to investigate *Agapanthus* L'Héritier (Zonneveld 2004). When all species in a genus have the same chromosome number, as in *Agapanthus* L'Héritier, differences in nuclear DNA content have proven to be very effective in delimiting of infrageneric divisions in a number of taxa (Ohri 1998, Zonneveld 2004). With this approaches, Zonneveld and Duncan (2003) suggest that there are six species in *Agapanthus* L'Héritier and not ten as described by Leighton (1965). The species in this context would be: *Agapanthus campanulatus* F. M. Leight. (2C = 22.3 pg), *Agapanthus caulescens* Spreng. (2C = 23.2 pg), *Agapanthus coddii* F.M. Leight. (2C = 24.0 pg), *Agapanthus praecox* Willd. (2C = 25.5 pg), *Agapanthus inapertus* Beauverd (2C = 25.1 pg), and *Agapanthus africanus* (L.) Hoffmanns (2C = 31.6 pg), while the other four, according to the new proposal, would receive a status below

species rank. When compared with the value observed for Zonneveld and Duncan (2003), the DNA content obtained by us ($2C = 24.4$ pg) is very next of *Agapanthus coddii* F. M. Leight. ($2C = 24.0$ pg) while the content obtained for *Agapanthus africanus* (L.) Hoffmanns for these authors was different of our ($2C = 31.6$ pg). Once more, this observation shows the great intraspecific variation observed in the genus.

In addition to the DNA content, PI and DAPI fluorochromes index allowed, for the first time, the estimation of base composition of the genome of *Agapanthus africanus* (L.) Hoffmanns. The percentage of base was 59% for AT and 41% for GC. The representative histograms of DNA content and base composition can be seen in figure 2.

Finally, our data constitute an important upgrade in the biology of the *Agapanthus africanus* (L.) Hoffmanns, and can be used to future taxonomic, phylogenetic studies and for the chromosome evolution of the genus.

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RESUMO

Agapanthus (Agapanthaceae) possui 10 espécies descritas. No entanto a maioria dos taxonomistas difere relativamente a este número, devido à grande plasticidade fenotípica das espécies. A citogenética tem sido uma ferramenta importante para auxiliar a identificação de taxa de plantas, e até o momento, todas as espécies de *Agapanthus* L'Héritier estudadas citologicamente, apresentaram $2n = 30$. Embora as espécies possuam grandes cromossomos, o grupo é cariológicamente pouco explorado. Este trabalho teve como objetivo aumentar o conhecimento citogenético de *Agapanthus*

africanus (L.) Hoffmanns pela utilização de técnicas de bandeamento cromossômico com DAPI / CMA₃ e Hibridização *in situ* Fluorescente (FISH). Além disso, a citometria de fluxo foi utilizada para a determinação do conteúdo de DNA e do percentual de bases nitrogenadas AT e GC. As plantas estudadas apresentaram $2n = 30$ cromossomos, variando de $4,34 - 8,55 \mu m$, com a fórmula cariotípica (KF) = $10m + 5sm$. Através da FISH, um sinal para rDNA 45S foi observada proximalmente ao centrômero do cromossomo 7, enquanto que para os sítios de rDNA 5S, observamos um sinal proximal ao centrômero do cromossomo 9. O conteúdo 2C de DNA estimado para a espécie foi de $2C = 24,4$ com 59% de AT e 41% de GC. Nossos dados possibilitaram uma atualização importante para a biologia e citotaxonomia de *Agapanthus africanus* (L.) Hoffmanns.

Palavras-chave: lírio Africano, bandeamento cromossômico, citotaxonomia, citometria de fluxo, tamanho do genoma, citogenética molecular.

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