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ORIGINAL ARTICLE

Karyotype and leaf epidermis histology traits of *Digitaria abyssinica* (Hochst. Ex A. Rich.) (Poaceae)

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Abstract

Digitaria abyssinica (African couchgrass) has economic potential as forage. This species is taxonomically complex, with a high number of synonyms, basic chromosome number x = 9, and diploid and tetraploid cytotypes. Anatomical, histological, and cytotaxonomic studies may contribute to the taxonomic description of this species and reveal its polyploidization and hybridization events. To this end, this study aimed to characterize the karyotype and leaf epidermis histology of two *D. abyssinica* genotypes. Chromosome characterization was performed based on chromosome banding using chromomycin A (CMA) and 4,6-diamidino-2-phenylindole (DAPI) and identification of 35S and 5S rDNA sites by fluorescent in situ hybridization. Nuclear DNA quantification was performed by flow cytometry. Paradermal sections were obtained for the leaf epidermis study by light and electron microscopy. The results confirmed that both genotypes are tetraploid (2n = 36). The number and distribution of 35S and 5S rDNA sites suggest the occurrence of postpolyploidization structural chromosomal changes or hybridization processes. Despite intraspecific variation in the number of 5S rDNA sites and $CMA^+/DAPI^+$ bands, no changes were identified in karyotypical symmetry or genome size. Leaf epidermis histology traits and cytogenetic data can support breeding programs and germplasm banks in identifying species or cultivars.

Keywords Polyploidy · Heterochromatin · African couchgrass · Cytotaxonomy · Chromosomal polymorphism

Abbreviations

AR	Arm ratio
CMA	Chromomycin A
CM	Chromosomal morphology
DAPI	4,6-diamidino-2-phenylindole
FISH	Fluorescent in situ hybridization
GISH	Genomic in situ hybridization
LA	Long arm length
SA	Short arm length
SEM	Scanning electron microscopy
TL	Total chromosome length
TLH	Total length of the haploid set

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Introduction

Digitaria Haller (Crabgrass), a genus in the family Poaceae, subfamily Panicoideae, and tribe Paniceae (Boonsuk et al. 2016), contains approximately 300 species, including annual and perennial plants, and has a cosmopolitan distribution (Vega and Rúgolo de Agrasar 2001; Govaerts 2011). In Brazil, 26 native, nine endemic, and 12 exotic species have been described, with greatest richness in the South and Southeast regions, mainly in Cerrado areas, rocky fields, and sites modified by human activities (Do Canto-Dorow and Longhi-Wagner 2001). Despite the paucity of studies on *Digitaria*, these plants have considerable economic importance due to their potential for producing high-nutrition food for livestock and humans (Boonman 1993; Aronovich et al. 1996; Jideani 1999; Chukwu and Abdul-kadir 2008).

The molecular phylogeny proposed by Giussani et al. (2001) suggests that the subfamily Panicoideae is divided into three clades, which correspond to groups with a constant basic number of x = 9 or x = 10. However, x = 15 and x = 17 have

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also been reported (Pozzobon et al. 2006). *Digitaria* is predominantly diploid (2n = 18) or tetraploid (2n = 36) (Rice et al. 2014), but there are records of 2n = 108 (Gould and Soderstrom 1970). The genus was previously subdivided into four subgenera, defined based on morphological variations in the spikelet structure (Henrard 1950), but cladistic analysis did not support this organization and provided evidence for a new division (Vega et al. 2009). Although the taxon shows monophyly, the kinship relationships among its species are not fully understood (Duvall et al. 2001; Giussani et al. 2001).

The complex inflorescence morphology is responsible for the challenge in establishing taxonomic limits (Verloove 2008). This difficulty may have contributed to the large number of synonyms and the inclusion of *Digitaria* species in different genera, such as *Axonopus* P. Beauv., *Cynodon* Rich., *Paspalidium* Stapf., and *Eriochloa* Kunth. (Govaerts 2011; The Plant List 2013). Similarly, *Digitaria abyssinica* has synonyms such as *Digitaria scalarum* (Schweinf.) Chiov. (Kok 1984; Rice et al. 2014), *Digitaria vestita* Fig. & De Not. (USDA 2019), and *Cynodon dactylon* (L.) Pers. (The Plant List 2013).

Digitaria abyssinica is a perennial, creeping grass with long, thin, and branched rhizomes (CABI - INVASIVE SPECIES COMPENDIUM 2019). The species has a wide geographical distribution (Boonman 1993; CABI - INVASIVE SPECIES COMPENDIUM 2019), mainly on the African continent, its center of origin (Hedberg and Edwards 1995), and presents diploid and tetraploid cytotypes with 18 and 36 chromosomes (Davidse and Pohl 1974; Gould and Soderstrom 1974; Morton 1993). The determination of chromosome number, ploidy level and description of morphological traits are essential for plant breeding and characterizing germplasm (Schifino-Wittmann 2001). Thus, this study aimed to characterize the karyotype and leaf epidermis histology of two *D. abyssinica* genotypes, which are essential for evolutionary studies, taxonomic circumscriptions, and genotypes selection in breeding programs.

Materials and methods

Plant material

Two genotypes of *D. abyssinica* yielded by the breeding program conducted by the Embrapa Gado de Leite, located in the municipality of Juiz de Fora, Minas Gerais State, Brazil, were evaluated. Plant identification was performed by Professor Tarciso de Souza Filgueiras (in memoriam) of the Botany Institute of São Paulo, State of São Paulo, Brazil, and Professor Thaíse Scotti do Canto Dorow, Universidade Franciscana (Franciscan University), municipality of Santa Maria, State of Rio Grande do Sul, Brazil.

Chromosome preparation for karyotyping

Roots were collected at 11 am, pretreated with cycloheximide (0.0025%) for 2 h at room temperature, fixed in Carnoy's solution (3:1 alcohol:acetic acid), and stored at −20 °C. Cell wall digestion was performed using an enzymatic mixture (10 μL of cellulase/pectinase 4:2, pH 4.8 + 5 μL of 5% pectolyase + 5 μL of 5% cytohelicase) for 1 h and 32 min at 37 °C. The slides were prepared using the cell dissociation and air-drying technique (Carvalho and Saraiva 1993).

CMA/DAPI banding

The slides were immersed for three days in 45% acetic acid solution to reduce the background and then subjected to 40 μL of McIlvaine buffer, pH 7.0, for 20 min and 20 μL of chromomycin A (0.5 mg/mL) with 2.5 M $MgCl_2$ for 1 h. Subsequently, the excess of CMA solution was removed with McIlvaine buffer, pH 7.0, and mounted with Vectashield® and DAPI (Guerra and Souza 2002).

Fluorescent in situ hybridization (FISH)

The 35S and 5S rDNA sequences (clones pTa71 and pTa794 from *Triticum aestivum*, respectively) were used. Indirect labeling of probes was performed with digoxigenin and biotin by nick-translation reaction (Reide 2002). The slides were denatured in 70% formamide in 2x SSC for 1.5 min at 85 °C and dehydrated through an alcohol series. The probe mixture was denatured at 95 °C for 8 min, and the hybridization was performed at 37 °C for 48 h. The probes were detected with anti-dig and anti-biotin using tris/sodium chloride (TNB) buffer for 1 h at 37 °C.

The slides were analyzed under a fluorescence microscope with a refrigerated monochrome camera (AxioCam HRM, Zeiss) at excitation/emission wavelengths of 358/461 (DAPI), 490/525 (fluorescein), and 550/575 (TRITC). The images were processed using AxioVision software, Release 4.8.2 (Zeiss).

Assembly of karyotypes and idiograms

Five metaphases of each genotype were measured with KaryoType software (Yu et al. 2015; Altinordu et al. 2016). The lengths of the short arms (SA) and long arms (LA) of the chromosomes, total chromosome length (TL), arm ratio (AR), and total length of the haploid set (TLH) were determined. The chromosome morphology and karyotypic symmetry were defined according to Levan et al. (1964) and Stebbins (1958), respectively. The karyograms and idiograms were assembled using Adobe Photoshop software.

Table 1 Karyotype data of *Digitaria abyssinica* genotypes 1 and 2, separated by slashes. Total chromosome length (TL), short arm (SA) and long arm length (LA), arm ratio (AR), total length of the haploid (TLH), and chromosomal morphology (CM) according to Levan et al. (1964), where m = metacentric

Pairs	TL	SA	LA	AR	CM
1	2.57 / 0.98	1.16 / 0.45	1.41 / 0.52	1.21 / 1.16	m
2	2.40 / 0.91	1.07 / 0.41	1.32 / 0.50	1.23 / 1.20	m
3	2.28 / 0.87	0.98 / 0.39	1.29 / 0.47	1.30 / 1.21	m
4	2.12 / 0.83	0.96 / 0.39	1.16 / 0.45	1. 21 / 1.16	m
5	1.97 / 0.80	0.88 / 0.36	1.10 / 0.44	1.25 / 1.24	m
6	1.92 / 0.76	0.88 / 0.35	1.04 / 0.42	1.17 / 1.18	m
7	1.79 / 0.72	0.81 / 0.34	0.98 / 0.39	1.21 / 1.17	m
8	1.65 / 0.66	0.75 / 0.31	0.91 / 0.36	1.21 / 1.13	m
9	1.49 / 0.60	0.68 / 0.27	0.81 / 0.33	1.18 / 1.23	m
TLH	18.19 / 7.13				

Genome size

Three samples of each species were quantified by flow cytometry to estimate the genome size (Doležel et al. 1998). Young leaves tissue (20–30 mg) of *D. abyssinica* genotypes were macerated together with *Pisum sativum* L. (internal reference standard - DNA quantity 2C = 9.09 pg) in a Petri dish containing 1 mL of ice-cold LB01 buffer to obtain the nuclear suspension. Subsequently, 15 μL of propidium iodide was added.

For each sample, at least 10,000 nuclei were measured. The analysis was performed using a FACSCalibur cytometer (Becton Dickinson), and the histograms were obtained using Cell Quest software and analyzed with WinMDI 2.9 software. The nuclear DNA content of the plants was estimated in picograms (pg).

Leaf epidermis analysis

Ten adult leaves of each genotype were collected from the sixth stem node above the soil surface. The material was fixed in a solution containing 90% ethanol, 5% formaldehyde, and 5% acetic acid (F.A.A.) at a ratio of 18:1:1 for 72 h at room temperature and then preserved in 70% ethanol (Johansen 1940).

Paradermal sections of the middle region of the leaves were obtained using the epidermis scraping technique developed by Nicolini (1967). The samples were stained with 5% safranin for 10 s. Semipermanent slides were mounted in glycerinated water and sealed with a coverslip. The samples were observed using a light-field microscope (Carl Zeiss, Axiolab A1) equipped with an AxioCam camera and AxioVision software.

For scanning electron microscopy (SEM), the leaf samples were prepared according to a method adapted from Bozzola and Russel (1999). Leaf fragments of approximately 2 cm^2 were fixed in Karnovsky fixative at pH 7.2 for 24 h. Subsequently, the samples were washed three times in 0.1 M cacodylate buffer for 10 min. Dehydration was achieved in an increasing series of

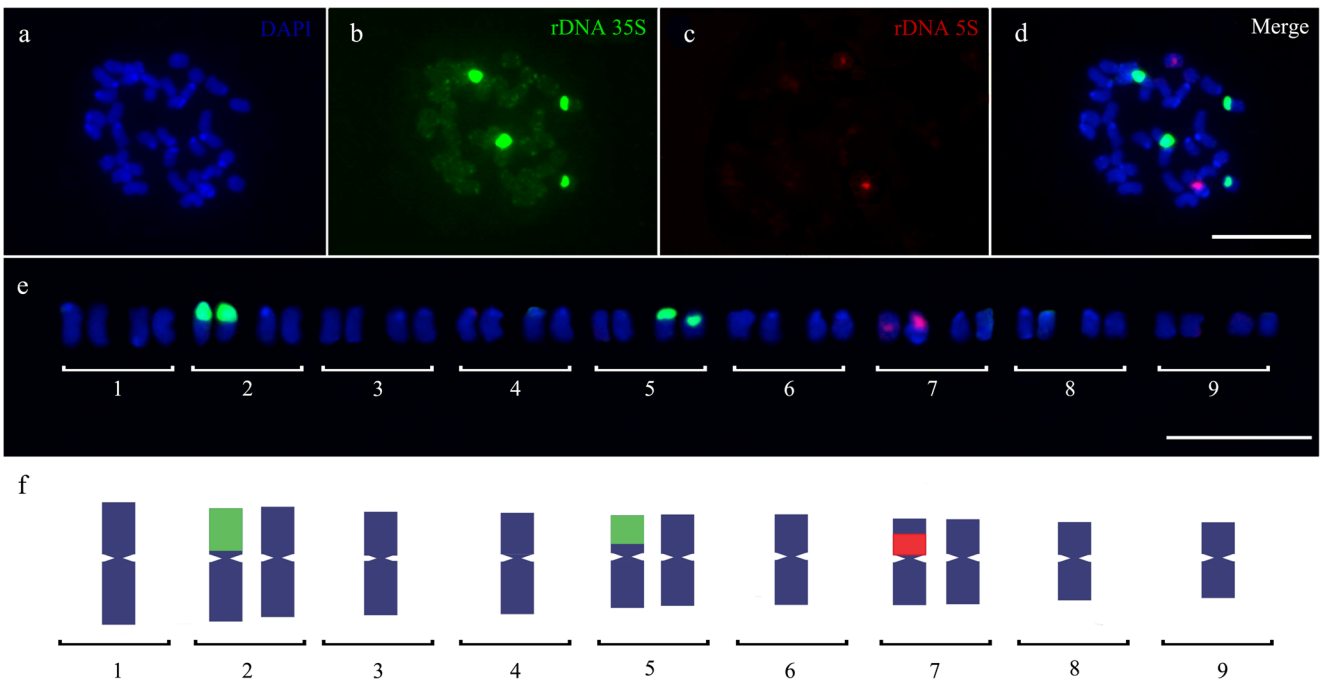

Fig. 1 Mitotic metaphase of *Digitaria abyssinica* genotype 1 with 2n = 4x = 36 chromosomes **a-d**; karyogram **e**; idiogram **f**. In green, pTa 71 probe (rDNA 35S site). In red, pTa 794 probe (rDNA 5S site). Scale bar: 10 μm

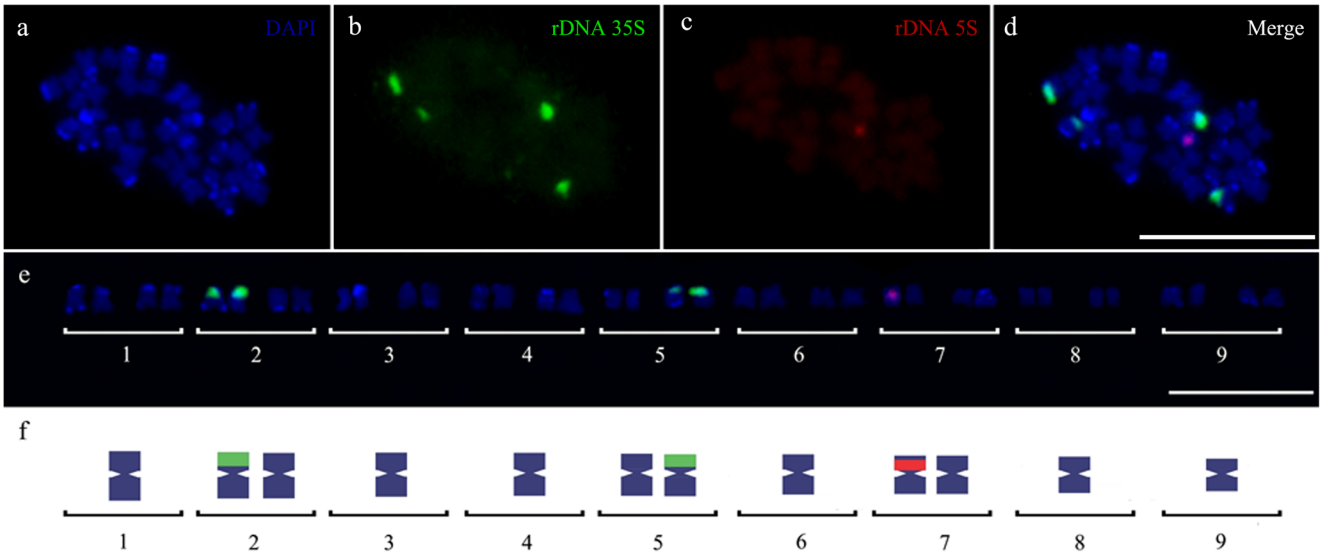

Fig. 2 Mitotic metaphase of *Digitaria abyssinica* genotype 2 with 2n = 4x = 36 chromosomes **a-d**; karyogram **e**; idiogram **f**. In green, pTa 71 probe (rDNA 35S site). In red, pTa 794 probe (rDNA 5S site). Scale bar: 10 μm

acetone (25, 50, 75, 90, and 100%) for 10 min each. The material was then subjected to critical point drying with CO_2 using BAL-TEC equipment, CPD-030. The specimens were fixed on metal supports and covered with gold in an SCD-050 BAL-TEC apparatus, and the electron micrographs were obtained using MEVLEO EVO 40 XVP (Carl Zeiss).

Results

Cytogenetic analysis

Considering the basic number of chromosomes as x = 9, *D. abyssinica* genotypes were considered as tetraploid and

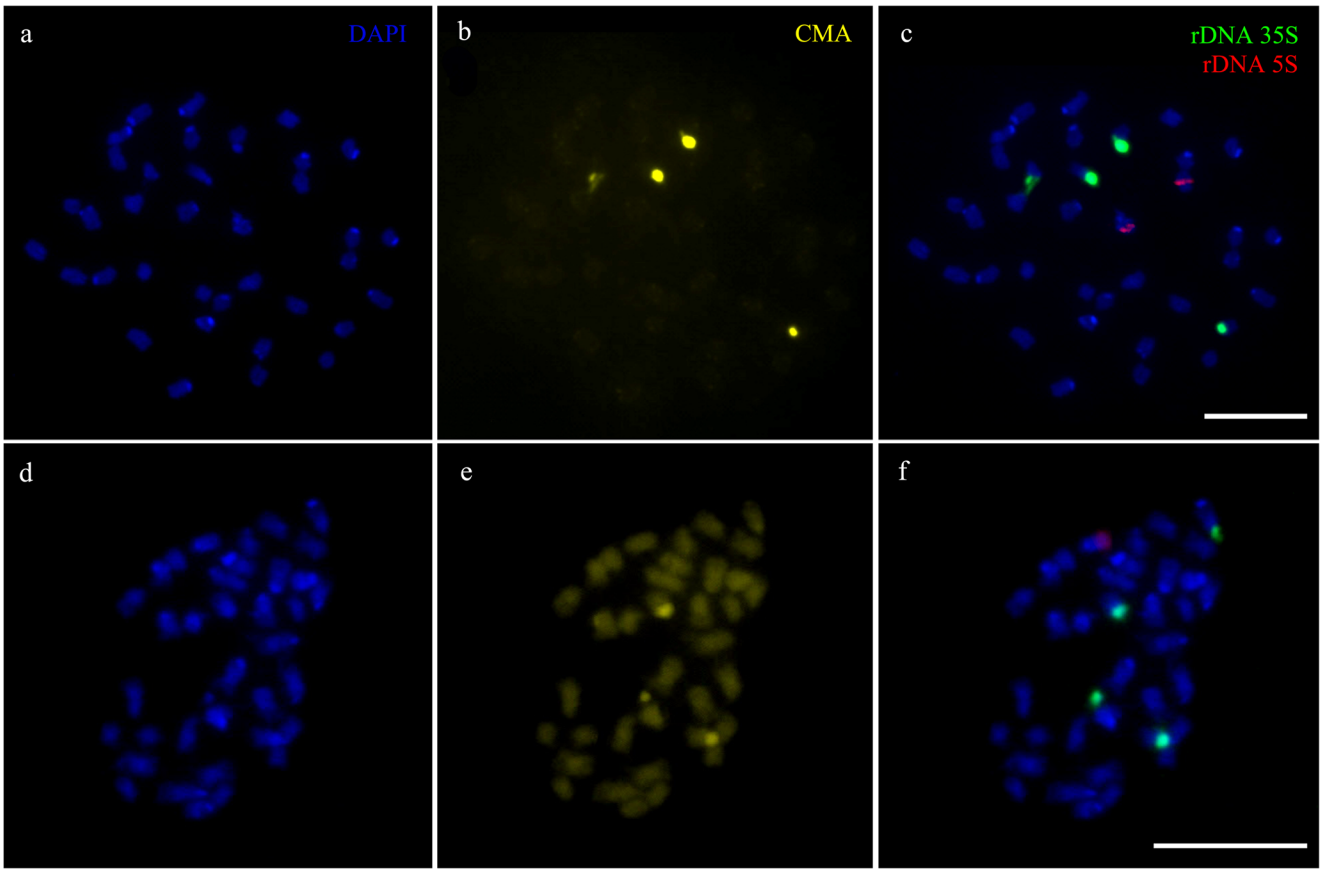

Fig. 3 CMA banding in the chromosomes of *Digitaria abyssinica* genotype 1 **a-c**; and genotype 2 **d-f**. Scale bar: 10 μm

36 m karyotype formulas (Table 1). In this sense, the chromosomes were divided into nine groups (Figs. 1 and 2). The relative length of the largest (group 1) and smallest chromosomes (group 9) corresponded to approximately 22% of the TLH for both genotypes. All chromosomes had AR values smaller than 2:1, and the ratio between the largest and the smallest chromosomes was 1.21 for genotype 1 and 1.15 for genotype 2. Therefore, the karyotype symmetry was classified into category 1A according to Stebbins (1958).

In both genotypes, four 35S rDNA sites were conspicuously observed in the short arms of the chromosomes belonging to groups 2 and 5 at the terminal position. (Figs. 1 and 2). For genotype 1, two sites of 5S rDNA were observed in group 7, at the proximal position (Fig. 1). However, in genotype 2, only one 5S rDNA signal was observed (Fig. 2). Four CMA^+ bands (CG-rich regions) were collocated with the 35S rDNA sites on genotype 1 (Fig. 3b-c). However, in genotype 2, only three conspicuous CMA^+ bands were visualized (Fig. 3e-f). The mean FISH 35S/CMA^+ and 5S signal sizes were 0.89 μm and 0.47 μm, respectively, in both genotypes. The nuclear DNA content was 2.5 and 2.4 pg for genotypes 1 and 2, respectively (Fig. 4).

Leaf epidermis analysis

Both genotypes of *D. abyssinica* exhibited hypostomatic leaves with stomata classified as dumbbell shaped and paracytic according to the arrangement of the subsidiary cells. Siliceous cells and tectorial uniseriate trichomes with superficial and dilated bases were observed on both sides of the epidermis. Epidermal papillae were not observed by SEM (Fig. 5).

Discussion

We report for the first time the assembly of karyotypes based on cytogenetic markers for *Digitaria*, which contributes to cytotaxonomy of the genus. Despite a large number of synonyms and taxonomic dubiety between *D. abyssinica* and *C. dactylon*, cytogenetic data and leaf epidermis histology were useful parameters with which to discriminate these species. Govaerts (2011) also demonstrated morphological traits for this differentiation.

Data analysis of *D. abyssinica* genotypes compared to the cytogenetic study performed in *C. dactylon* by Chiavegatto

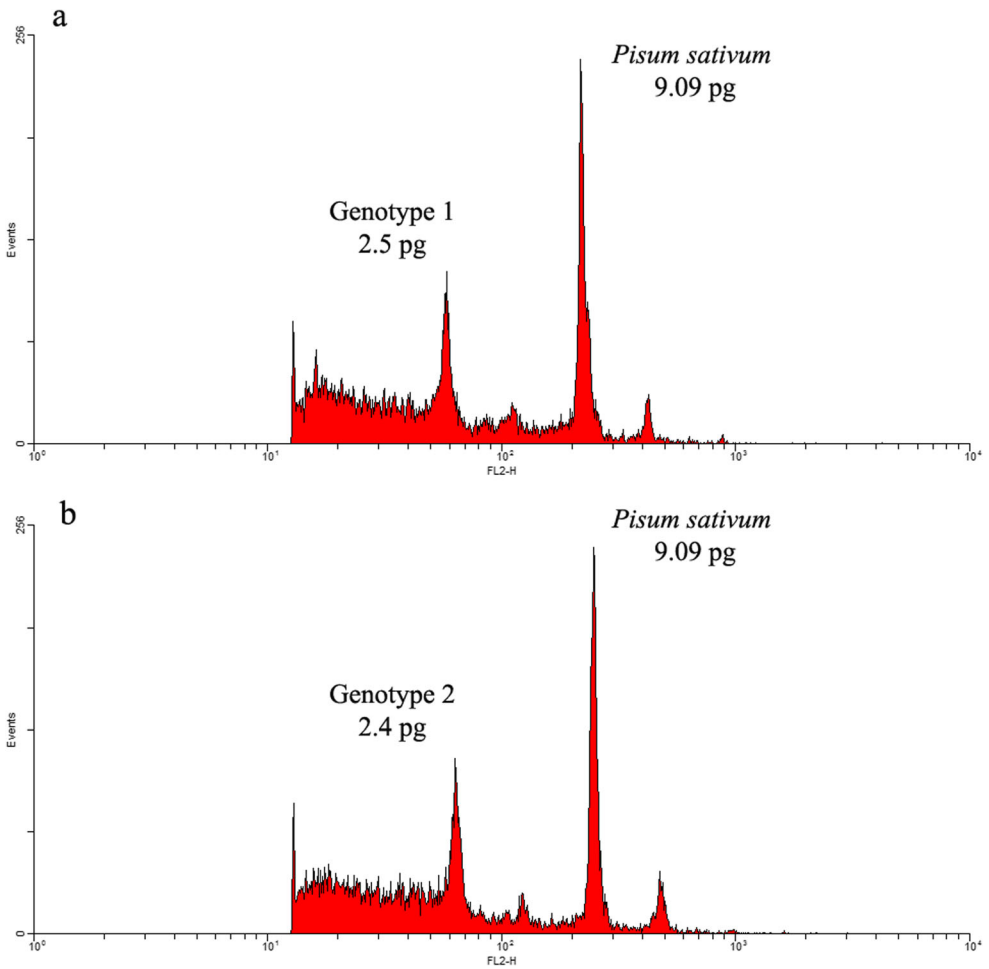

Fig. 4 Histograms with nuclear DNA quantification in *Digitaria abyssinica* genotypes. Genotype 1 **a**; genotype 2 **b**

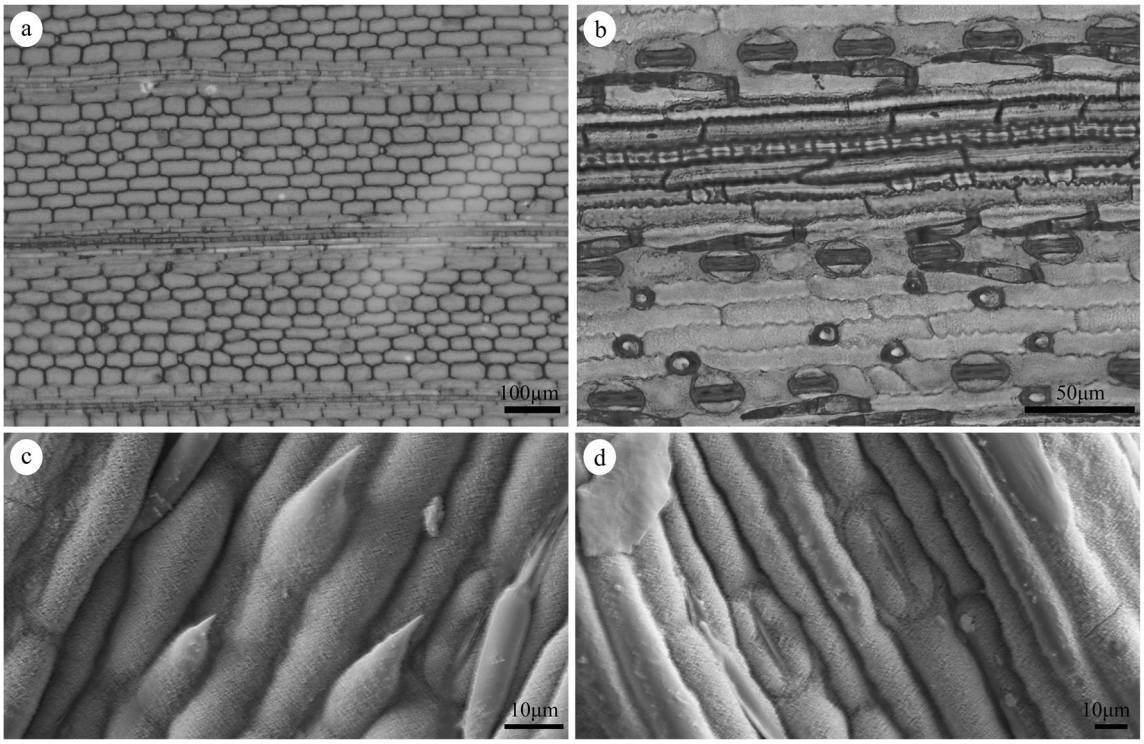

Fig. 5 Paradermal sections of the adaxial **a**; and abaxial **b** surfaces and electron micrographs of leaves representing tectorial trichomes **c**; and stomata **d** of *Digitaria abyssinica*

et al. (2016, 2019) revealed differences in chromosome size, karyotypical symmetry, and the position of ribosomal DNA sites. In *Digitaria abyssinica,* the 35S/CMA$^+$ rDNA sites were located at terminal positions in chromosome groups 2 and 5, while in *C. dactylon,* the position was (peri)centromeric in pairs 2 and 2′ (Chiavegatto et al. 2019). On the other hand, the 5S rDNA sites were located at the proximal position in group 7 in *D. abyssinica* and at the terminal position in pair 5′ in *C. dactylon* (Chiavegatto et al. 2019).

The presence of only one 5S rDNA site in genotype 2 of *D. abyssinica* may be due to a deletion or a lower number of ribosomal gene copies that is undetectable by FISH at the homologous chromosome. Another hypothesis for this variation could be the presence of transposable elements contiguous with the 5S rDNA sequence. The mechanism mediated by transposons can contribute to amplification, movement, or, in this case, loss of sequences (Bennetzen 2000; Topp and Dawe 2006; Sigman and Slotkin 2016).

Intraspecific variation in the number of CMA$^+$ bands was observed for *D. abyssinica* and *C. dactylon,* as described by Chaves et al. (2019). These polymorphisms at heterochromatic regions have been shown to be an important karyotypic feature for cytotaxonomic analyses. Once conserved in terms of number and position, the CMA$^+$ bands can serve as cytogenetic markers in different taxa. However, variations may be evidence of chromosomal rearrangements (Nardy et al. 2010; Laura et al. 2010; Roa and Guerra 2012; Bernardes et al. 2013; Abdeddaim-Boughanmi et al. 2019). Chaves et al. (2019) proposed that the variation in the number of CMA and DAPI bands in *Cynodon dactylon* is potentially due to polyploidization and hybridization events.

The differences in size among chromosomes carrying 35S rDNA sites and the numeric variation of heterochromatic bands (CMA and DAPI) could be another indicator of structural chromosomal changes or a possible allopolyploid origin of *D. abyssinica*. In polyploids, cytomolecular markers may indicate the occurrence of hybridization process, as demonstrated for several species of Poaceae (Hodkinson et al. 2002; Paštová et al. 2019; Lucía et al. 2018), such as *Cynodon* (Zhi-Yun et al. 2013; Chiavegatto et al. 2019).

Based on the heterozygous profiles found in molecular marker studies, Adoukonou-Sagbadja et al. (2010) reported the hybrid genomic nature of *Digitaria* species. Furthermore, Shambulingappa (1967) suggested that comparisons of pachytene chromosome morphology between diploid and tetraploid species of *Digitaria* could be used to identify its polyploid progenitors. However, the exact discrimination of chromosomes/genome is better resolved through genomic in situ hybridization (GISH) with candidate species (Silva and Souza 2013) or by genomic sequencing data comparison and the use of multiple cytomolecular markers (Yao et al. 2010; Li et al. 2015).

From this perspective, the difference between the two genotypes of *D. abyssinica* for the number of 5S rDNA sites and CMA bands could be explained as the result of postpolyploidization genomic changes (Roa and Guerra

2015; Majka et al. 2019) and chromosomal rearrangements (Barros et al. 2017). Although genotype 2 exhibited only one 5S rDNA site and three CMA^+ bands, no significant changes were observed in chromosomal morphology and karyotypic asymmetry in relation to genotype 1. Moreover, the genome size of both genotypes was similar to that of other *Digitaria* species (Adoukonou-Sagbadja et al. 2007). In contrast, *Cynodon* presents a great variation in the nuclear DNA content, including within *C. dactylon*, which has been associated with different heterochromatin compositions (Chiavegatto et al. 2016; Chaves et al. 2019).

Regarding the histology of the leaf epidermis, the genotypes of *D. abyssinica* could not be discriminated by these characteristics. However, the absence of papillae on *D. abyssinica* was the most divergent characteristic from *C. dactylon* (Chaves et al. 2018). Khan et al. (2017) considered the epidermis appendages to be a significant trait with which to identify grasses, whose papillae are an important taxonomic feature used to discriminate species. It should be noted that the absence of papillae is an attractive feature for breeding programs in developing cultivars with improved animal digestibility since these structures can accumulate lignin compounds (Houston et al. 2016). Nevertheless, papillae are also associated with resistance to adverse climates and unfavorable environmental conditions (Rodriguez et al. 2017), such as high temperatures (Barthlott et al. 2017) and pathogens (Žárský et al. 2013; Voigt 2014; Houston et al. 2016).

Other essential characteristics that corroborate the distinction between *D. abyssinica* and *C. dactylon* were the less sinuous epidermal cells, hypostomatic leaves, and absence of salt glands in the former, whereas *C. dactylon* exhibits more sinuous epidermal cells, different trichome structures, amphistomatic leaves, and salt glands (Chaves et al. 2018).

Therefore, the cytogenetic results obtained for *D. abyssinica* are useful for cytotaxonomy and could provide evidence of the structural chromosomal changes due to intraspecific variations. The origin of allopolyploidy in this species is still under debate, and in situ hybridization approaches such as GISH and other molecular markers can shed light on this matter.

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Author contributions ALAC carried out the study and data analysis and wrote the manuscript. RBC, MTMF, and LCR assisted in the cytogenetic analysis and reviewed the manuscript. ALSA carried out flow cytometry analyses; FRGB provided the genotypes for analysis and reviewed the manuscript. VHT conceived the study, participated in its design and coordination, helped draft and revise the manuscript, and provided financial support for the research.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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