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Interploid hybrids in the '*brizantha*' complex of *Urochloa* P. Beauv.: identification, genome size, cytogenetic characterization and perspectives

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ABSTRACT. Crossing between sexual artificial tetraploids of *Urochloa ruziziensis* and apomictic polyploids of *Urochloa decumbens,* and *Urochloa brizantha* is a breeding strategy. Crosses are carried out without prior emasculation of the plants; therefore, confirmation of hybridization and identification of pollen donors require reliable techniques such as microsatellite markers. Information on the reproductive mode, chromosome number, and genome size is also important. The aims of this study were (i) to identify the male parents and mode of reproduction of the three hybrids using molecular markers, (ii) to define the chromosome number and ribosomal DNA (rDNA) sites using fluorescent in situ hybridization (FISH), and (iii) to estimate genome size using flow cytometry. The simple sequence repeats (SSR) markers identified *U. brizantha* cv. Marandu, *U. brizantha* genotype 2 and *U. brizantha* cv. Xaraés as the male parents of hybrids 1F/21, 7F/153, and 10F/v5, respectively. The 1F/21 hybrid was aneuploid (2n = 36+1, 2C = 3.61 pg), whereas the 7F/153 (2n = 40, 2C = 3.79 pg) and 10F/v5 (2n = 41, 2C = 3.88 pg) hybrids resulted from interploid crosses involving *U. brizantha* (2n = 5*x* = 45). The p779/p780 apospory marker was not amplified in the hybrids, indicating sexual reproduction. The number of rDNA sites shows a dynamic behavior and inheritance of these sequences, while the position of the sites on the chromosomes is conserved within the '*brizantha*' complex. Identifying interploid hybrids provides new perspectives for *Urochloa* breeding, with the possibility of exploring the genetic variability of other apomictic polyploids. These results are promising in terms of inferences and highlight the need for further studies on interploid hybridization in *Urochloa*.

Keywords: aneuploidy; *Brachiaria*; FISH; polyploidy; SSR markers.

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Introduction

The increasing demand for agricultural products due to population growth has been accompanied by the adoption of more sustainable systems. Pastures composed of improved and properly managed forage are more efficient because they contribute to increased productivity without expanding the cultivated areas (Jank et al., 2021). The genus *Urochloa* P. Beauv. [syn. *Brachiaria* (Trin.) Griseb.] has important tropical forages with good yield, nutritional quality, and adaptability to low fertility soils (Jank et al., 2014; Ferreira et al., 2021), as the species of the '*brizantha*' complex: *Urochloa brizantha* (A.Rich.) R.D. Webster, *Urochloa decumbens* (Stapf) R.D. Webster and *Urochloa ruziziensis* (R. Germ. & C.M. Evrard) Crins (Ferreira et al., 2021).

Urochloa breeding programs developed since 1980 at important institutions have contributed to improvements in pasture systems. Despite these promising results, breeding programs for this genus are relatively recent, and some characteristics of the species, such as polyploidy and apomixis, pose additional challenges for breeders (Ferreira et al., 2021; Simeão et al., 2021). One strategy to explore the genetic variability of apomictic genotypes is to use them as pollen donors in crosses with sexual genotypes. In the '*brizantha*' agamic complex (*x* = 9), the artificial polyploidization of *U. ruziziensis* produced sexual tetraploid genotypes (2n = 4*x* = 36) (Ishigaki et al., 2009; Timbó et al., 2014a) and facilitated crossing with apomictic allotetraploids of *U. brizantha* and *U. decumbens* (Risso-Pascotto et al., 2005; Mendes-Bonato et al., 2007).

This procedure has enabled the availability of several cultivars worldwide (Ferreira et al., 2021). Several hybrids are continually being produced and evaluated by research centers and partner institutions to obtain new promising genotypes for current problems.

Effective identification of hybrids is one of the limiting factors in breeding because, as there is no emasculation before crossing, some plants of the progeny may be the result of self-fertilization (Valle & Pagliarini, 2009). In addition, the hybridization system often occurs via polycrosses with open pollination (Valle & Miles, 2001; Valle & Pagliarini, 2009), making it challenging to identify male parents and certify the hybridization. Therefore, the application of efficient procedures, such as molecular marker analysis, is essential for breeding programs and has been commonly used in *Paspalum* L. to confirm the hybrid condition in crosses with sexual and apomictic polyploid parents (Aguilera et al., 2011; Novo et al., 2017; Brugnoli et al., 2019; Ortiz et al., 2020).

Microsatellite or simple sequence repeats (SSR) are highly polymorphic markers with good reproducibility, relative abundance, and extensive genome coverage (Powell et al., 1996; Kalia et al., 2011). Several SSR markers have been described and evaluated in *Urochloa* (Jungmann et al., 2009a; Jungmann et al., 2009b; Vigna et al., 2011; Ferreira et al., 2016; Triviño et al., 2017), but have not yet been used for paternity analysis in the '*brizantha*' complex.

Moreover, information regarding the reproduction mode of hybrids is crucial for breeding. In this sense, the use of the p799/p780 marker, developed for the apospory-specific genome region (ASGR) (Akiyama et al., 2011), is efficient for diagnosing the mode of reproduction of several species and hybrids of *Urochloa* (Worthington et al., 2016; Worthington et al., 2019). This tool provides quick answers for different genotypes, helping breeders make decisions.

Considering the polyploidy and wide variation in the chromosome number of *Urochloa* genotypes (Penteado et al., 2000; Valle & Pagliarini, 2009), cytogenetics analyses are important at different breeding stages, including post-hybridization. Recently, cytogenetic analyses identified aneuploid hybrids from the crossing of *U. ruziziensis* (4*x*) with *U. decumbens* and *U. brizantha* (both 4*x*) (Paula et al., 2017; Moraes et al., 2019; Rocha et al., 2019).

In the present study, we tested SSR markers to confirm interspecific hybridization and paternity of *Urochloa* hybrids. Subsequently, we determined the mode of reproduction using the ASGR p779/p780 marker and performed cytogenetic analyses to determine the chromosome number, location of rDNA sites, and genome size of the hybrids and their respective parents.

Material and methods

Plant material

Analyses were conducted using nine genotypes (Table 1) provided by *Empresa Brasileira de Pesquisa Agropecuária* (Embrapa) *Gado de Leite*, Juiz de Fora, Minas Gerais State, Brazil. The selected hybrids exhibited interesting agronomic characteristics. They were obtained by polycrossing without prior emasculation and, therefore, with unknown male parents. Clones obtained by vegetative propagation of tillers were maintained in pots in a greenhouse under similar environmental conditions (temperature, humidity, photoperiod, irrigation, and fertilization).

Table 1. Genotypes of the evaluated *Urochloa* species and hybrids.

Identification	Genotype	Description
1M	*U. ruziziensis* 4*x*	female parent
7M	*U. ruziziensis* 4*x*	female parent
10M	*U. ruziziensis* 4*x*	female parent
1F/21	hybrid	1M x (?)*
7F/153	hybrid	7M x (?)*
10F/v5	hybrid	10M x (?)*
cv. Marandu	*U. brizantha* 4*x*	Pollen donor
genotype 2	*U. brizantha* 5*x*	Pollen donor
cv. Xaraés	*U. brizantha* 5*x*	Pollen donor

*unknown male parent

DNA extraction, polymerase chain reaction (PCR), and SSR analysis

DNA was extracted from young leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA integrity was assessed on a 1% agarose gel, and the samples were quantified using a NanoVue spectrophotometer.

Seventeen SSR markers (Table 2) described in the literature showing certified transfer to at least one species were tested (Jungmann et al., 2009a and b). For each reaction, 50 ng genomic DNA, 0.4 µM each primer, 1× Master mix (Cellco Biotec, São Carlos, Brazil), and water to complete the final volume of 25 µL were used. The thermocycler conditions were an initial denaturation at 95°C for 2 min.; followed by 35 amplification cycles of 95°C for 30 s, primer-specific annealing temperature (Table 2) for 50 s, and 72°C for 1 min. and 20 s; and an additional extension step at 72°C, for 7 min. and a hold step at 4°C. Amplification products were resolved by electrophoresis on a 3% agarose gel and, when necessary, on a 3% MetaPhor agarose gel, following the manufacturer's recommendations (Lonza, Rockland, ME, USA). Product size was determined by comparison with a 1 kb ladder (Invitrogen, Carlsbad, CA, USA). Owing to *Urochloa* polyploidy, the SSR bands were classified as absent or present, like a dominant marker.

Identification of the mode of reproduction with the p779/p780 marker

The p779/p780 marker, which amplifies ASGR-BBML, was used to identify the mode of reproduction (Akiyama et al., 2011). PCR was also performed using the ITS-F/ITS-R primer pair designed for the internal transcribed spacer (ITS) region of rDNA (Table 2), as a positive control. The amplification conditions were as described above, and the amplified products were visualized on a 1% agarose gel.

Nuclear DNA content

Samples of young leaf tissue (20–30 mg) of each genotype and *Pisum sativum* L. (internal reference standard, 2C value = 9.09 pg) were macerated in a Petri dish containing 1 mL ice-cold LB01 buffer (Doležel et al., 1989). The nuclear suspension was stained with 25 µL propidium iodide (1 mg mL^{-1}), and at least 10,000 nuclei per sample were quantified in a Facscalibur cytometer (BD Biosciences, San Jose, CA, USA). Histograms were obtained using CellQuest software and analyzed using WinMDI 2.8 software (http://www.cyto.purdue.edu/flowcyt/software/Winmdi.htm). The average genome size was estimated in pg, considering three replications per genotypes, and compared using the Scott-Knott test (Scott & Knott, 1974) at 5% probability using Sisvar software (Ferreira, 2011).

Chromosome preparations and fluorescent in situ hybridization (FISH)

Root tips were collected and pre-treated with a solution of cycloheximide (88.9 µM) and APM (amiprophos-methyl, 4 µM) for 2 hours at room temperature (RT). Subsequently, the material was fixed in methanol: acetic acid (3:1) solution for 24h at RT and stored in 90% ethanol at -20°C. The meristems were excised and submitted to enzymatic digestion in a mix comprising 0.7% Onozuka R10 cellulase, 0.7% cellulase, 1% pectolyase, and 1% cytohelicase for approximately 1h 40 min. at 37°C. Slides were prepared using cell dissociation and air-drying techniques (Fukui & Nakayama, 1996).

FISH was performed using probes for 35S rDNA (pTa71) and 5S rDNA sequences (RICRGAC1/RICRGAC2 primers; Table 2) labeled with biotin-16-dUTP or digoxigenin-12-dUTP, respectively, via nick translation and PCR. The chromosomes were denatured in 70% formamide (2× SSC) for 1 min. 10 s at 85°C, followed by dehydration in an alcohol series (70%, 90%, and 100%). The hybridization mix (50% formamide, 10% dextran sulfate, 2× SSC, 50–100 ng of probe) was denatured for 8 min at 95°C and immediately transferred to ice. Hybridization occurred for 40–48h in a humid chamber at 37°C. Probe detection was performed with rhodamine-conjugated anti-digoxigenin and Alexa fluor 488-conjugated streptavidin at 37°C for 1h after stringency washes in 2× SSC buffer at 42°C.

Chromosomes were counterstained with DAPI (4',6-diamidino-2-phenylindole) in a Vectashield Antifade mounting medium (1.5 µg mL^{-1}). At least ten metaphases were evaluated and captured for each genotype using a QImaging Retiga EXi CCD camera coupled with a BX 60 fluorescence microscope (Olympus, Tokyo, Japan). Images were processed using Adobe Photoshop version 23.5.2 software (https://www.adobe.com/br/products/photoshop.html).

Table 2. Primers used in polymerase chain reactions (PCR).

Primer	Primer (5'-3')	Annealing Temperature (°C)	Reference
BhUNICAMP003	CTGTCAGCATGGTTCACTAATAA	51	Jungmann et al., 2009a
	GCATCGAATAAAGCTCAGAAAG		
BhUNICAMP004	AGTTTGCTGCCTCTTTGATTC	51	Jungmann et al., 2009a
	GTGGCGCTACCTTCTTGTG		
BhUNICAMP005	CACTGCATCAACTACCCACCAC	52	Jungmann et al., 2009a
	GTCTGCTTTGCTTGCGTTACCT		
BhUNICAMP015	AGGGGAGAACTGTGGTGGTGTC	55	Jungmann et al., 2009a
	TCGAGTTTTGAGGGTGGGATTG		
BhUNICAMP018	TCCATATCCAACAAGGCAACAT	52	Jungmann et al., 2009a
	GCGCGAGCTGGTGAAGT		
BhUNICAMP020	CCGGCCGGTTCACTCGT	52	Jungmann et al., 2009a
	GGCACTCGGACTCCCATAACTC		
BbUNICAMP001	AGCGTGCTGATTCCGACCTT	52	Jungmann et al., 2009b
	GAAATGGCTATGCGAAATGTGT		
BbUNICAMP002	TCGCTCGTTTCATTCCTTTCAT	53	Jungmann et al., 2009b
	CGCACGCTTATACGACAACATT		
BbUNICAMP003	GCTACAGGCTACGTTGAGAAGA	52	Jungmann et al., 2009b
	ACAGGTGAGCAGTATGAGTCCA		
BbUNICAMP004	ACCCCGCTGCATACCCATAAT	52	Jungmann et al., 2009b
	TTTTTGCCCCATCACTCTCTTTG		
BbUNICAMP005	CGTTATGCGGCGACACT	48	Jungmann et al., 2009b
	AAGAAACTCCATGAAAAACAGA		
BbUNICAMP006	GGGGCCACTGACCAAAAC	52	Jungmann et al., 2009b
	GCGTAGCTACCCGAATCC		
BbUNICAMP009	AGGAGAGGGAGGAGAGGAGAAT	53	Jungmann et al., 2009b
	GCGAGTTGTTAATGAAGTGAGGA		
BbUNICAMP010	TCGTTCAGTTGAGTCGTCTTCT	52	Jungmann et al., 2009b
	GCCATTCCGCCATCATAG		
BbUNICAMP011	GCTCGGGAATACAGGCTTGAAA	53	Jungmann et al., 2009b
	GAAACGCGTCGGGGTGATT		
BbUNICAMP012	CGCGCATTACAGACTACAGA	48	Jungmann et al., 2009b
	TAGAGAAGGCAATTGATTAGATAG		
BhUNICAMP013	ATGCAGCGGAAACATCTC	52	Jungmann et al., 2009b
	ACTTCCTCCACTTCCACTCTG		
p779/p780	TATGTCACGACAAGAATATG	52	Akiyama et al., 2011
	TGTAACCATAACTCTCAGCT		
ITS-F/ITS-R	CGTGACCCTTAAACAAAACA	57	Torres González; Morton, 2005
	GGTCGTCTATGAGTCCTAAG		
RICRGAC1/RICRGAC2	GATCCCATCAGAACTCCAAG	60	Doleželová et al., 1998 (personal communications withFukui e Ohmido)
	CGGTGCTTTAGTGCTGGTATG		

Results

Paternity and mode of reproduction analyses

Seven of the seventeen SSR markers tested (Table 2) produced polymorphisms that were considered informative (Figure 1). The BhUNICAMP004 (Figure 1a) and BhUNICAMP015 (Figure 1b) primers produced polymorphic markers in *U. brizantha* genotype 2 and hybrid 7F/153 but were not present in the 7M maternal parent (*U. ruziziensis*), indicating the respective genotype as a pollen donor for this hybrid. The BhUNICAMP015 (Figure 1b) and BbUNICAMP001 (Figure 1c) primers formed polymorphic markers in the 10F/v5 hybrid and the Xaraés cultivar (*U. brizantha*), identifying it as the parent involved in the formation of this hybrid. The Marandu cultivar of *U. brizantha* was identified as the male parent of the 1F/21 hybrid based on the pattern of marks obtained using the BbUNICAMP013 primer (Figure 1d). The BhUNICAMP005, BhUNICAMP018, and BbUNICAMP004 primers, despite not producing polymorphisms among the possible

pollen donors, certified the hybrid condition of the 1F/21, 7F/153, and 10F/v5 genotypes because they produced polymorphic markers between the female and male parents that were present in the offspring (Figure 1e-g).

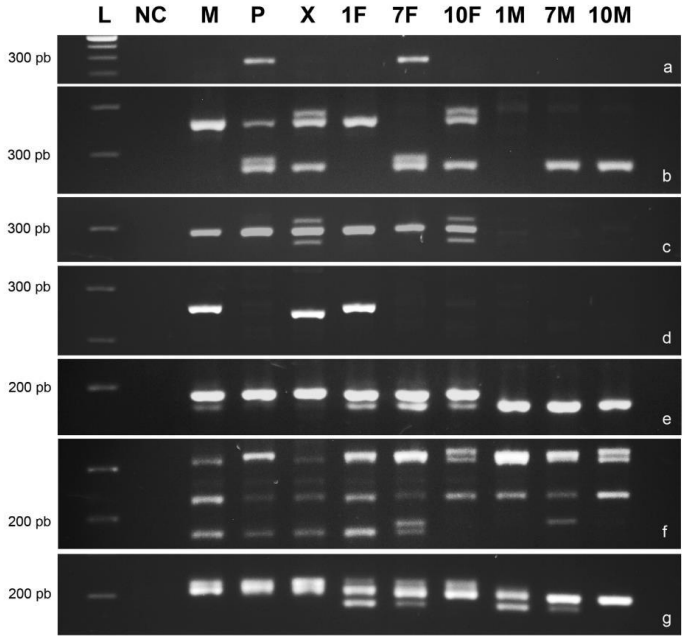

Figure 1. Polymorphic products amplified by polymerase chain reaction (PCR) with BhUNICAMP004 (a), BhUNICAMP015 (b), BbUNICAMP001 (c), BbUNICAMP013 (d), BhUNICAMP005 (e), BhUNICAMP018 (f), and BbUNICAMP004 (g) SSR markers in *Urochloa* genotypes. L, ladder; NC, negative control; M, *U. brizantha* cv. Marandu; P, *U. brizantha* genotype 2; X, *U. brizantha* cv. Xaraés; 1F, hybrid 1F/21; 7F, hybrid 7F/153; 10F, hybrid 10F/v5; 1M, *U. ruziziensis* 1M; 7M, *U. ruziziensis* 7M; 10M, *U. ruziziensis* 10M.

Eight markers (BhUNICAMP003, BbUNICAMP002, BbUNICAMP003, BbUNICAMP005, BbUNICAMP006, BbUNICAMP009, BbUNICAMP010, and BbUNICAMP012) showed good amplification but did not produce conclusive polymorphic markers. Two other markers (BhUNICAMP020 and BbUNICAMP011) did not show amplification or was nonspecific amplification.

The apomictic genotypes of *U. brizantha* presented a specific marker for the p779/780 apospory marker, which was not observed in the sexual parents of *U. ruziziensis* or the interspecific hybrids (Figure 2a). The ITS region of the rDNA was amplified in all genotypes, producing a fragment of approximately 550 bp (Figure 2b), confirming that the genomics DNAs were adequate for verifying the apospory marker.

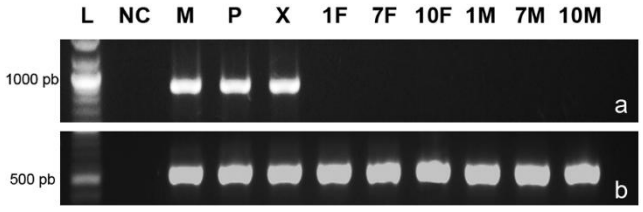

Figure 2. Polymerase chain reaction (PCR) amplified products of the p779/p780 marker (a) and internal transcribed spacer region (b) in *Urochloa* genotypes. L, ladder; NC, negative control; M, *U. brizantha* cv. Marandu; P, *U. brizantha* genotype 2; X, *U. brizantha* cv. Xaraés; 1F, hybrid 1F/21; 7F, hybrid 7F/153; 10F, hybrid 10F/v5; 1M, *U. ruziziensis* 1M; 7M, *U. ruziziensis* 7M; 10M, *U. ruziziensis* 10M.

Nuclear DNA content, chromosome number, and ribosomal DNA sites

The tetraploid genotypes of *U. ruziziensis* (2n = 4*x* = 36 chromosomes) were allocated to the same statistical group for the genome size (Table 3 and Figure 3). No statistical difference was detected in the monoploid genome size (C*x*) between genotype 2 (2n = 5*x* = 45) and cv. Marandu (2n = 4*x* = 36) (Table 3) in *U. brizantha*. The 1F/21 hybrid was identified as aneuploid (2n = 4*x* = 36+1) (Figure 4d) and was statistically different from the other hybrids with smaller genome sizes (Table 3 and Figure 3). Hybrids 7F/153 and 10F/v5 had statistically similar DNA content (Table 3) and 2n = 40 and 2n = 41 chromosomes, respectively (Figure 4e and f), confirming the interploid crosses.

The numbers of 35S and 5S rDNA sites varied between the hybrids and parents, but they were always in the terminal and interstitial positions in the chromosomes, respectively (Figure 4). *U. ruziziensis* (10M) and the three hybrids showed six, five, and three 35S rDNA sites and differed from the other genotypes. The 5S rDNA sites were the same for *U. ruziziensis* genotypes but varied between hybrids and between *U. brizantha* genotypes (Table 3 and Figure 4).

Table 3. Genome size, chromosome number, and number of ribosomal DNA sites of *Urochloa* genotypes.

Genotype	2C Value (pg)*	C*x* Value (pg)*	Ploidy	Chromosome number (2n)	rDNA sites	
					35s	5s
U. ruziziensis 1M	3.13[a]	0.78[a]	4*x*	36	4	8
U. ruziziensis 7M	3.07[a]	0.77[a]	4*x*	36	4	8
U. ruziziensis 10M	3.20[a]	0.80[a]	4*x*	36	6	8
Hybrid 1F/21	3.61[c]	0.90[c]	4*x*	37	5	7
Hybrid 7F/153	3.79[d]	-	interploid	40	3	6
Hybrid 10F/v5	3.88[d]	-	interploid	41	3	6
U. brizantha cv. Marandu	3.44[b]	0.86[b]	4*x*	36	4	6
U. brizantha genotype 2	4.27[e]	0.85[b]	5*x*	45	4	5
U. brizantha cv. Xaraés	4.45[f]	0.89[c]	5*x*	45	4	6

*Means followed by different letters are significantly different by Scott–Knott test, at 5% probability.

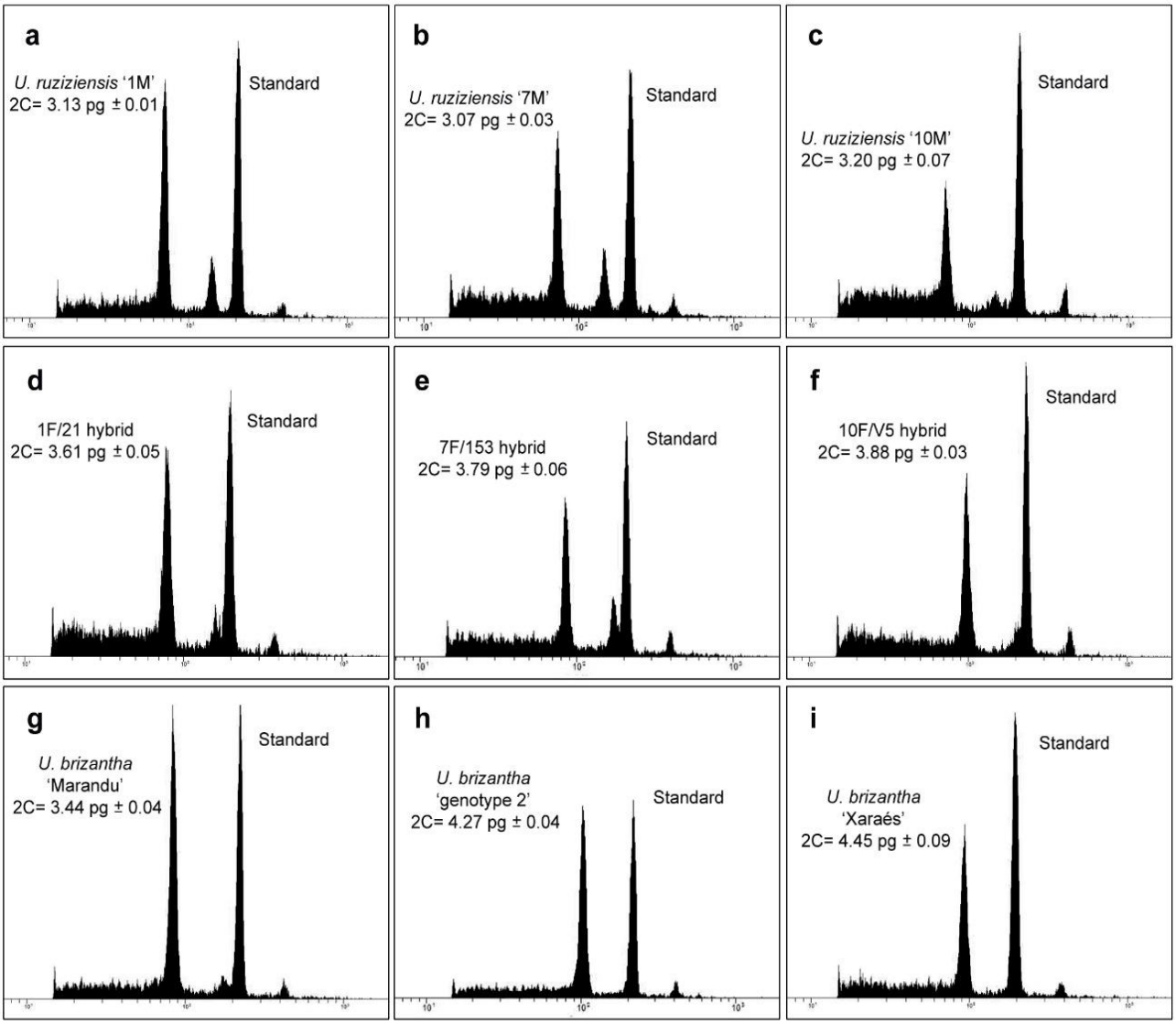

Figure 3. Flow cytometry histograms for quantification of nuclear DNA in picograms of *Urochloa* genotypes. Standard: Pisum sativum was used as an internal reference standard (2C = 9.09 pg)

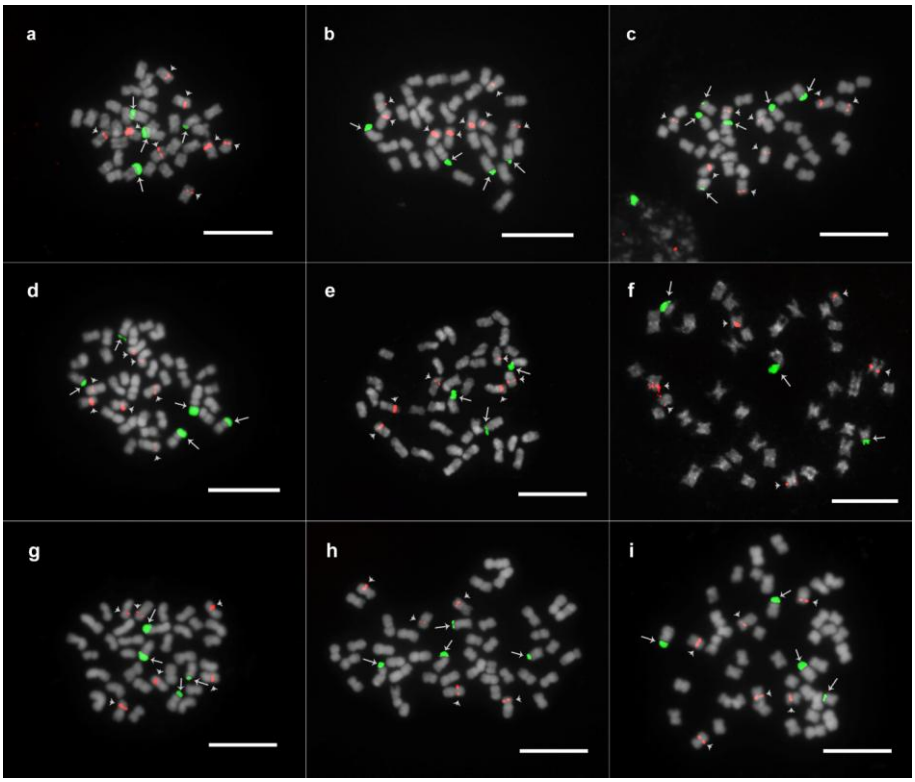

Figure 4. Location of 35S (arrow) and 5S (arrowhead) rDNA sites in mitotic metaphase chromosomes of parental and hybrids *Urochloa. U. ruziziensis*: a) 1M; b) 7M; c) 10M. Hybrids: d) 1F/21; e) 7F/153; f) 10F/v5. *U. brizantha*: g) cv. Marandu; h) genotype 2; i) cv. Xaraés; Bar: 10 µm.

Discussion

Interploid crosses and the new possibilities for breeding *Urochloa*

The SSR markers used in our study efficiently confirmed hybridization and identified the male parents involved in the crosses. In *Urochloa*, the identification of hybrids is usually based on contrasting phenotypic differences between parents and is corroborated by the intermediate characteristics observed in the hybrids (Valle & Miles, 2001). However, considering the greater plasticity of morphological characters and the limitation in the number of these markers, monitoring paternity has been a laborious and inaccurate task, especially among species of the '*brizantha*' complex, which are phylogenetically close (Triviño et al., 2017; Pessoa-Filho et al., 2017). Some authors have even considered *U. ruziziensis* and *U. decumbens* as synonyms of *Urochloa eminii* (Mez) Davidse (Plants of the World Online [POWO], 2023).

Urochloa Breeding via hybridization requires reliable methods for the effective identification of hybrids. Molecular markers are important tools because they enable direct access to the plant genotype without being influenced by environmental effects, early and rapid analysis of the material (Kordrostami & Rahimi, 2015). In grasses of *Paspalum* genus, random amplified polymorphism DNA (RAPD) and inter simple sequence repeat (ISSR) markers are commonly used to monitor crosses between apomictic and sexual genotypes and confirm the hybrid origin (Aguilera et al., 2011; Novo et al., 2017; Brugnoli et al., 2019; Ortiz et al., 2020). SSR markers could define unique genotypes and are excellent for hybrid identification and genotyping (Powell et al., 1996; Kalia et al., 2011). Several studies have used this marker for similar purposes in other economically important species, such as rice (Sundaram et al., 2008), citrus (Oliveira et al., 2002) and cotton (Selvakumar et al., 2010).

The chromosome number and estimated average DNA content of the parents and hybrids corroborated the results obtained with SSR markers and showed the occurrence of crosses between tetraploid and pentaploid parents in the formation of hybrids 7F/153 and 10F/v5. Our results suggested that the fusion of gametes with 18 chromosomes of *U. ruziziensis* (7M) + 22 chromosomes of *U. brizantha* genotype 2 resulted in the formation of hybrids 7F/153 (40 chromosomes) and 18 chromosomes of *U. ruziziensis* (10M) + 23 chromosomes of *U. brizantha* cv. Xaraés in 10F/v5 hybrid (41 chromosomes). This assumption is based on available information and was confirmed for the 7F/153 hybrid through genomic in situ hybridization (GISH) (Moraes et al., unpublished data). The application of GISH in other hybrids and parents can help to elucidate the genomic composition and inheritance of chromosomes involving species of the '*brizantha*' complex.

It is uncommon to use genotypes with odd ploidies as parents in *Urochloa* breeding. However, our results show that interesting genotypes with other ploidy levels can be used successfully in crosses of the '*brizantha'* complex. The formation of hybrids from interploid crosses has also been reported in previous studies on *Brassica* (Wen et al., 2012), wheat (Padmanaban et al., 2017), and strawberries (Luo et al., 2017). In the *Urochloa* genus, Hacker (1988) obtained a triploid intraspecific hybrid from a cross between the diploid and tetraploid cytotypes of *U. decumbens*. Nitthaisong et al. (2019) produced triploid hybrids between the diploid *U. ruziziensis* and *U. decumbens* and *U. brizantha*, which are both tetraploids.

Interploid hybrids obtained by crossing tetraploids and pentaploids had not yet been reported in the '*brizantha*' complex. These results bring new possibilities to *Urochloa* breeders, who can advance in exploring germplasm from the '*brizantha*' complex beyond the tetraploid level. In *Brassica*, interspecific interploid hybrids have been used as a bridge to transfer traits such as the yellow color of the seed to *Brassica napus* L. (Wen et al., 2012). Luo et al. (2017) identified strawberry hybrids with cold resistance from a cross between octaploid and dodecaploid genotypes. Padmanaban et al. (2017) reported the formation of pentaploid wheat hybrids (*Triticum aestivum* L. 6*x* x *T. durum* Desf. 4*x*), resulting in progenies with unique chromosomal constitutions and the potential to improve several important traits.

In addition to the interploid hybrids (7F/153 and 10F/v5), our analysis identified aneuploidy in the 1F/21 hybrid despite its origin involving parents with the same ploidy level. The detection of aneuploidy in this genus has been recurrent and has been reported in previous studies within the '*brizantha*' complex (Paula et al., 2017; Moraes et al., 2019; Rocha et al., 2019) and '*humidicola*' complex (Damasceno et al., 2023; Tomaszewska et al., 2023).

In some species, aneuploidy has been used as a strategy for genetic mapping and the formation of addition or substitution lines aimed at introgressing traits of interest (Humphreys et al., 2003; Molnár-Láng et al., 2015). The identification of aneuploids was recently reported in *Urochloa*. Therefore, cytogenetic and genomic studies are essential to evaluate and monitor the stability of hybrids, in addition to agronomic comparisons of performance and productivity with euploid hybrids. Evaluating parental meiosis to elucidate aspects of aneuploid gamete formation is also interesting. The parent of the 7F/153 hybrid (*U. brizantha* genotype 2) produces balanced and viable gametes, even with meiotic abnormalities (Moraes et al., unpublished data). In the aneuploid hybrids evaluated by Paula et al. (2017) and Moraes et al. (2019), *U. ruziziensis* was identified as the likely parent responsible for the observed trisomy and double trisomy.

Determining the sexual mode of reproduction of the three interspecific hybrids is important for breeding programs because they can be self-fertilized or backcrossed with parents. In turn, the apomictic progeny can be evaluated as potential cultivars (Valle & Miles, 2001). It is fundamental to evaluate the meiosis and pollen viability of hybrids to verify the fertility of these genotypes for future crossings. Agronomic traits must be evaluated, and potential improvements determined, which can be increased in the *Urochloa* germplasm. Finally, new progenies must be evaluated for their modes of reproduction and cytogenetics, as certain chromosomal instabilities are expected over generations in sexual genotypes with aneuploidy and interploidy. Apomictic plants tend to circumvent possible problems resulting from aneuploidy; however, because of their pseudogamy, viable pollen grains are necessary for forming viable seeds (Miles & Valle, 1996).

Like SSR markers, the p779/p780 marker is an important tool for *Urochloa* breeding, with its efficiency certified in a diverse panel of species and hybrids (Worthington et al., 2016; 2019). Together, these markers enable the evaluation of many genotypes in a shorter period at a reduced cost, which is considered advantageous for breeding programs.

Parental chromosome number and ribosomal DNA sites

Previous studies had already described karyotypic data on species, cultivars, and hybrids with reports on the chromosome number and/or rDNA sites (Bernini & Marin-Morales, 2001; Akiyama et al., 2010; Nielen et al., 2010; Nani et al., 2016; Paula et al., 2017). However, the interploidy findings in this study and the recurrent identification of aneuploid hybrids led to the need to investigate comparative cytogenetic characteristics at the family level (parental and respective hybrids).

The chromosome number (2n = 36 chromosomes) of female parents (1M, 7M, and 10M) corresponds to synthetic tetraploid *U. ruziziensis* (Ishigaki et al., 2009; Timbó et al., 2014a). Several studies have reported the occurrence of a polyploid series with diploid, tetraploid, pentaploid, and hexaploid cytotypes for *U. brizantha* (Penteado et al., 2000; Mendes-Bonato et al., 2002; Risso-Pascotto et al., 2003; Tomaszewska et al., 2023).

Chromosome counts (2n = 36) and genome size were reassessed in this study for cv. Marandu ratified the information previously obtained for tetraploids of *U. brizantha* (Bernini & Marin-Morales, 2001; Akiyama et al., 2010; Nielen et al., 2010; Timbó et al., 2014b; Nani et al., 2016; Nitthaisong et al., 2016). Genotype 2 and cv. Xaraés had a chromosome number compatible with the pentaploid level (Akiyama et al., 2010). Although tetraploidy is predominant in *Urochloa*, pentaploidy is the second most frequent ploidy (18.5%) identified in *U. brizantha* in a sample of 222 accessions (Penteado et al., 2000). The nuclear genome sizes estimated for both pentaploid genotypes corroborated a previous description of another pentaploid cytotype of *U. brizantha* (Moraes et al., 2021).

FISH using both rDNA probes revealed variations in the evaluated species compared to previous descriptions. The *U. ruziziensis* genotypes evaluated in our study showed two additional 5S rDNA sites compared to the study by Paula et al. (2017), but were compatible with the duplication of the four 5S rDNA sites of diploid plants (Akiyama et al., 2010; Nani et al., 2016). The 10M genotype presented a pair of 35S rDNA sites in addition to the other two parents and another previously evaluated tetraploid (Paula et al., 2017). The six 35S rDNA sites also extrapolated the generally expected duplication results of the two 35S rDNA sites identified in the diploids of this species (Akiyama et al., 2010; Nani et al., 2016).

For the Marandu cultivar, the number of rDNA sites aligned with previous reports (Akiyama et al., 2010; Nielen et al., 2010). Akiyama et al. (2010) reported variation in the number of rDNA sites between different tetraploid cytotypes of *U. brizantha*. For pentaploids, the number of rDNA sites differed from previous descriptions, which identified five sites for both rDNAs in cv. Xaraés (Akiyama et al., 2010), or five 35S rDNA sites and eight 5S rDNA sites in another pentaploid cytotype (Moraes et al., 2021).

The variation of rDNA sites has been related to transposable elements near or dispersed in the rDNA loci, increasing the possibility of chromosomal rearrangements (Raskina et al., 2008). Furthermore, the repetitive character of rDNA loci and their organization in the genome may provide frequent opportunities for ectopic recombination events, leading to variations in locus size, number, and sites distribution (Goffová & Fajkus, 2021).

The allopolyploidy proposed for the tetraploid and pentaploid cytotypes of *U. brizantha* (Nielen et al., 2010; Paula et al., 2017; Moraes et al., 2021) may also be related to the frequent reports of variations in rDNA sites. With the apomictic mode of reproduction, characteristic of the polyploid cytotypes of *U. brizantha*, apomixis can act by fixing the formed karyotypic variations. Macháčková et al. (2022) sequenced the ITS region of the sexual and apomictic taxa of *Taraxacum* and identified few variants in the sexual taxa, while the apomictic taxa presented a significant number of variants. Additionally, the number of 35S rDNA sites in the hybrids differed from what was expected, considering the number of sites in the parents, demonstrating the dynamic inheritance of these sequences.

The positioning pattern of the 35S and 5S rDNA sites presented by the genotypes corroborates previous reports involving species of the '*brizantha*' complex (Akiyama et al., 2010; Nielen et al., 2010; Nani et al., 2016; Paula et al., 2017; Moraes et al., 2021). The preferential positioning of 35S rDNA at the terminal region of chromosomes is a trend in angiosperms, while 5S rDNA sites have a more variable distribution, with frequent reports in interstitial regions (Roa & Guerra, 2012, 2015; Garcia et al., 2017). The genotypes evaluated in the present study showed a higher number of 5S rDNA loci than 35S rDNA loci, which differed from the relationship identified by Garcia et al. (2017) for most plants. A similar situation was observed in *Taraxacum*, where most taxa had one 35S rDNA locus for two 5S rDNA loci per monoploid complement (Macháčková et al., 2022).

Some studies have also indicated a tendency for polyploids to reduce the number of sites per monoploid complement with increasing ploidy levels (Roa & Guerra, 2012; Garcia et al., 2017). Our results indicate that *U. brizantha* may present this pattern, as pentaploid genotypes exhibited the same number of 35S and 5S rDNA sites, the latter including a reduction compared to the tetraploid cv. Marandu. Considering the number of 35S rDNA sites per monoploid complement, *U. brizantha* cv. Marandu has 1 × 35S, while pentaploids have 0.8 × 35S; that is, they have less than one 35S rDNA site per monoploid complement. Future studies on cytotypes of *U. brizantha* with other levels of ploidy, such as diploid and hexaploid genotypes, and even other tetraploid and/or pentaploid genotypes, may clarify aspects related to variations in rDNA sites and karyotypic patterns.

Conclusion

SSR markers efficiently determined the pollen donors of the three crosses and identified *U. brizantha* cv. Marandu, *U. brizantha* genotype 2 and *U. brizantha* cv. Xaraés as the male parents of hybrids 1F/21, 7F/153,

and 10F/v5. The three hybrids exhibited a sexual mode of reproduction. Hybrid 1F/21 showed aneuploidy, with 2n = 4*x* = 36+1 chromosomes, whereas hybrids 7F/153 and 10F/v5 were interploids, resulting from crosses between tetraploids and pentaploids, and had 2n = 40 and 2n = 41 chromosomes, respectively. The number of rDNA sites of the parents and hybrids indicates dynamic behavior and inheritance, while the position of the sites on the chromosomes is conserved within the '*brizantha*' complex.

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