South African Journal of Botany 88 (2013) 118–123

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Obtaining sexual genotypes for breeding in buffel grass

M. Quiroga, K. Grunberg, A. Ribotta, E. López Colomba, E. Carloni, E. Tommasino, C. Luna, S. Griffa *

Instituto de Fisiología y Recursos Genéticos Vegetales (IFRGV), Centro de Investigaciones Agropecuarias (CIAP) ex IFFIVE, Instituto Nacional de Tecnología Agropecuaria (INTA), Camino 60 cuadras Km 5.5, Córdoba, Argentina

ARTICLE INFO

Article history:
Received 11 July 2012
Received in revised form 21 November 2012
Accepted 2 April 2013
Available online 25 July 2013

Edited by DI Thompson

Keywords:
Cenchrus ciliaris
Hibridization
Facultative apomixis
Forage breeding

ABSTRACT

Buffel grass (*Cenchrus ciliaris* L. syn. *Pennisetum ciliare* (L.) Link) is a species that is highly tolerant to drought and is used primarily as forage in drier regions throughout the subtropics and tropics. It reproduces mainly by apomixis and the acquisition of obligate sexual genotypes or facultative apomicts with high levels of sexuality is required for performing crosses and plant improvement. The aim of this study was to obtain sexual genotypes from controlled crosses using obligate apomictic cultivars and a sexual line. Twelve putative hybrid F1 plants were selected morphologically and two of them were identified as sexual genotypes by PCR using specific primers for reproductive mechanism. Cytoembryological analysis showed 65.5 and 71.3% meiotic embryo sacs in these plants and their hybrid nature was corroborated by AFLP. Both highly sexual genotypes could be used as female parents in crosses for obtaining improved cultivars of buffel grass.

1. Introduction

Cattle production in northwestern Argentina (NOA) is essentially pastoral, and subtropical and tropical perennial grasses are the main cultivated forage resources. This region extends from Cordoba, central Argentina, to the west and northwest of the country, and is characterized by low, seasonal rainfall (average 350 mm), high temperatures and high solar radiation sunlight in summer, as well as soils with varying textures and degrees of salinity.

In Argentina, buffel grass (*Cenchrus ciliaris* L.), among subtropical pastures, has demonstrated an excellent performance, and is adapted to soil and harsh climatic conditions prevailing in the NOA. This is a mainly obligate apomictic species (Snyder et al., 1955), and the use of obligate sexual or apomictic genotypes with high levels of sexuality is the only alternative for conventional crosses (Burson et al., 2012; Hussey et al., 1993). However, Bashaw (1962) identified a source of sexuality, an off-type plant, product of a mutation that showed poor forage aptitude (Griffa et al., 2005), significant susceptibility to salt stress (Griffa, 2010; Lanza Castelli et al., 2010) and little genetic contribution for apomictic F1s of agronomic interest (Griffa et al., 2005).

Using this source of sexuality, we have obtained several apomictic F1s, one of them, named Lucero INTA-PEMAN, with superior forage characteristics compared to its parents, while the remaining hybrids obtained showed marked phenotypic similarity to the female parent (Griffa, 2002; Griffa et al., 2005). These results suggest that the possibility of obtaining promising (F1) genotype frequency by hybridization will depend largely on favorable combinations of polygenes for quantitative characteristic additive effects, and/or the occurrence of heterosis in component characters of biomass production (Griffa et al., 2006). New and improved sexual genotypes from hybridization of the introduced sexual source, with cultivars widespread in the country and genetically divergent from female parental lines (Griffa, 2010), would lead to the availability of new female parents (Valle and do Savidan, 1998) in buffel grass for breeding purposes.

Progenies obtained from controlled crosses of sexual lines and apomictic cultivars can be sexual or apomictic hybrids. The reproductive method may be determined by using specific molecular markers (PCR) that were found linked to the apomixis genomic region (ASRG) in buffel grass (Gustine et al., 1997; Jessup, 2005; Jessup et al., 2002; Lubbers et al., 1994; Ozias-Akins et al., 1993), and by embryo sac analysis (Sartor et al., 2005, 2009). However, the sexuality source is self-compatible (Bashaw, 1962; Hanselka et al., 2004), and therefore it is important to determine whether the sexual progenies obtained are hybrids. For that purpose, available molecular techniques, such as RAPD and AFLP, are widely used (de Benedetti et al., 2000; Griffa et al., 2006; Oropeza and García, 1997). The aim of this study was to obtain and characterize new and improved sexual genotypes to be used as female parents in conventional crosses in buffel grass.

2. Material and methods

2.1. Plant material

Plant material consisted of 30 plants of the introduced sexual source, arranged in 3 rows of 10 plants each, and field grown. The apomictic

* Corresponding author at: INTA–IFRGV (Instituto de Fisiología y Recursos Genéticos Vegetales), CIAP (Centro de Investigaciones Agropecuarias) ex IFFIVE, Camino 60 Cuadras Km 5.5 Córdoba - X5020ICA, Argentina. Tel.: +54 9 351 4974343; fax: +54 351 4974330.
E-mail address: sgriffa@yahoo.es (S. Griffa).

0254-6299/$ – see front matter
http://dx.doi.org/10.1016/j.sajb.2013.04.016

cultivars used were Biloela, Nunbank, Molopo, Americana, Nueces, Messina, Boorara, Toowomba, and *Cenchrus* sp. The sexual line was represented by plants grown from seeds provided by the Germplasm Bank of Texas A&M University College Station, TX (USA), and male parents (apomictic cultivars) were field grown in the Experimental Area IFRGV-INTA (Córdoba, Argentina).

2.1.1. Controlled crosses

Thirty female plants were cloned and field grown. Because buffel grass exhibits protogyny (Fisher et al., 1954; Snyder et al., 1955) there was no need for emasculation to produce controlled hybrids (Shafer et al., 2000). Individual panicles completely emerged from the flag leaf sheath were daily placed into acetate tubes prior to stigma exsertion. Hybridizations were made during the summer months by means of the crossing technique proposed by Sherwood et al. (1994) with modifications (Griffa, 2010). Pollen of the apomictic cultivars was collected each morning between 8 and 10 am and the inflorescences were pollinated, tagged, marked and bagged until seed maturation. Seed was harvested, dried naturally and stored cold until the following spring. Seeds collected from each pollinated panicle were planted in separate pots and the seedlings were transplanted to greenhouse and subsequently planted in the field, spaced 1 m × 1 m between them in the Experimental Area of the Institute of Plant Physiology and Genetic Resources (IFRGV) (INTA). Putative hybrid F1 plants were placed in rows, maintaining the identification of those that derived from a single pollinated panicle.

2.1.2. Preliminary evaluation and selection of putative F1 hybrid plants

Preliminary evaluation of putative F1 hybrid plants was performed by the character plant height, a component that in forage production has a direct influence on dry matter production (Daher et al., 2004), has high coefficient of genetic determination (CGD = 89%) (Griffa, 2002) and is stable in different environments. It also has a high discriminating power among cultivars and is easily measured (Griffa et al., 2011). Plant weight and height are widely used in genetic improvement of *C. ciliaris* (Griffa, 2002; Griffa et al., 2011). Those putative F1 hybrid plants that were different from either both parents or from the female parent were selected for further morphological characterization.

2.1.3. Morphological evaluation of putative F1 hybrid plants

Previously selected plants were propagated vegetatively and three plants per clone were obtained. The selected clones were evaluated in the field, using a completely randomized design with three replications. For that purpose, morpho-agronomic characters (Table 1) with capacity to discriminate genotypes in buffel grass were used, most of which had coefficient of genetic determination between 50 and 89% (Griffa, 2002). Seven vegetative and reproductive characters were measured in 18 main tillers with panicles at harvest (six tillers per individual cloned/three cloned individuals/plant selected). The dimensions of the flag leaf were evaluated in 18 other main tillers with panicles of approximately 2 cm, emerging from the flag leaf sheath. At the end of the growing season, in April, (six months after being planted) the adult plants were cut to 10 cm of soil and weighed to record total fresh weight (Table 1).

2.1.4. Statistical analysis

An ANOVA was performed with the morphological characters; the means were further compared using the multiple comparisons Di Rienzo, Guzman, and Casanoves test (DGC) (Di Rienzo et al., 2002) with a confidence level of 5%. Multivariate analysis of clusters was conducted to observe the phenotypic divergence among the materials and between the materials and their respective parent through the measured characters. In all cases, statistical analyses were made using InfoStat software (Di Rienzo et al., 2011).

Table 1
Morphological characters evaluated, mode of data collection, and ontogenetic stage at data collection in *Cenchrus ciliaris*.

Morphological character	Ontogenic stage
-*Plant height (PLH)* (measured in m from ground level to the apex of the panicle of a main tiller). -*Length of panicle (LP)* (measured in cm from the base to the apex of floral peduncle of a main tiller). -*Internodal length (INTL)* (distance in cm between 3rd and 4th node). -*Tiller diameter (TD)* (measured in mm on a main tiller immediately below the 3rd node) -*Number of nodes per tiller (NUD)* (measured on a main tiller) -*Number of vegetative branches per tiller (VB)* (number of secondary axes of a main tiller without panicles) -*Number of reproductive branches per tiller (RB)* (number of secondary axes of a main tiller with panicles)	Harvest
-*Flag leaf lamina length (FLL)* (measured in cm from ligule to the distal tip of lamina) -*Flag leaf lamina width (FLW)* (measured in cm at 2 cm from the base of lamina) -*Flag leaf sheath length (FSL)* (measured in cm from the node to the base of ligule) -*Total leaf length (TLL)* (length of lamina plus length of sheath of flag leaf in cm)	Panicle emergence
-*Fresh weight (FW)* (weight of plant in kg, measured immediately after the cut)	Post-harvest

2.2. Reproductive mechanism identification

2.2.1. PCR technique

Because materials obtained from F1 hybrids may be apomictic or sexual genotypes, the selected F1s were evaluated by PCR with the following specific markers linked to the apomixis sequence genomic region (ASGR) (Jessup, 2005): PCAB10, Q8H and UGT197 (Table 2).

Reagents and the PCR program adjusted in buffel grass (Griffa, 2010) were the following: Mastermix final volume of 20 μl, 10× buffer with 15 mM $MgCl_2$, 25 mM $MgCl_2$, 1 mM dNTP, 5 mM of each primer (forward and reverse), GoTaq DNA polymerase from Promega (5 U/μl) and 25 ng/μl of sample DNA. The thermal cycler program was as follows: 94 °C for 3 min and 10 cycles of 94 °C for 30 s, 64 °C for 30 s (−1 °C/cycle) and 72 °C for 45 s, followed by 36 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and then storage at 4 °C. PCR products were visualized in 2% agarose gel.

2.2.2. Clearing embryo sacs

Selected putative F1 hybrid plants, found sexual by PCR, were additionally analyzed cytoembryologically. Immature inflorescences, in partial emergence of the flag leaf and without exertion of stigmas and anthesis, were fixed in FAA (18 parts Ethanol 70%:1 part Formaldehyde 37%:1 part glacial acetic acid) for 24 h. Pistils were dissected out of the florets and cleared using the method of Young et al. (1979). One hundred mature ovules per genotype were directly mounted for

Table 2
Markers linked to apomixis, forward and reverse sequences and weight of the amplified fragment in base pairs (bp).

Marker	5′————————> 3′	bp
PCAB10	F: TTCGAAATCGCATAGGTGAG R:GAGCCTTTCTTTATTTACCCAGTG	211
Q8H	F: GAGCTTGNCCAATCGGGAAA R: ATGGTGATGGATCTTTTGGAC	800–850
UGT197	F: GGATGAATAAAACGGTGTTGGGAG R: GAACAACCGCACAAGTGAGAGAA	850

observation under bright field microscopy using DIC at 20×. Ovules containing one reduced embryo sac with egg apparatus, binucleated central cell, and antipodals in the chalazal region were classified as sexual. Ovules with multiple or single embryo sacs with egg apparatus, central cell and no antipodals were classified as apomictics.

2.2.3. Determination of F1 hybrid plants selected by AFLP

In the selected putative F1 hybrid plants, which were previously found sexual, AFLP was used to determine whether their origin was sexual or by self-pollination. Genomic DNA was isolated from young leaves of an individual plant by using the protocol described by de Gustine et al. (1996) with minor modifications (Griffa et al., 2006). About 700 ng of genomic DNA was first digested with EcoRI and secondly with MseI. The restricted genomic DNA fragments were ligated to EcoRI and MseI adapters and constituted the template for further amplifications. Pre-amplification primers had one selective nucleotide. Pre-amplification products were diluted (1:3) with nuclease-free H_2O and used as templates for selective amplification.

Ten combinations of the EcoRI and MseI AFLP primers supplied by the manufacturer (containing three selective nucleotides) were used for selective amplification. The combinations were the following: EcoRI + ACC-MseI + ACC; EcoRI + ACG-MseI + CAC; EcoRI + AAC-MseI + CAG; EcoRI + ACG-MseI + CAG; EcoRI + AAC-MseI + CTA; Eco RI + ACC-Mse I + CTG; Eco RI + ACG-Mse I + CTA; Eco RI + ACG -Mse I + CTG; Eco RI + AAC-Mse I + CAC and Eco RI + ACG Mse I + CAA. An Eppendorf thermocycler was used for both pre-amplification and selective amplification. Following amplification, PCR products were mixed with 2 μl of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol), denatured at 95 °C for 5 min and immediately placed on ice. Five microliter of the denatured samples were loaded onto denaturing 6% polyacrylamide gels and electrophoresis was performed, applying a constant power of 60 W at a temperature of 50 °C for 2 h in a BIO-RAD Sequi-Gen electrophoresis cell connected to a PowerPac/3000 power supply. Amplification products were visualized with $AgNO_3$ according to Benzouza et al. (2006).

2.2.4. Data analysis

The presence or absence of amplified fragments was scored as 1 or 0, respectively. The data were analyzed using the Infogen software (Balzarini and Di Rienzo, 2012). Genetic similarities based on Jaccard's coefficient were calculated between putative hybrid sexual progeny and their parents. A phenogram using only polymorphic bands was constructed according to the unweighted pair-group method using arithmetic averages (UPGMA; Sneath and Sokal, 1973).

3. Results and discussion

3.1. Morphological evaluation of putative F1 hybrid plants

Of the seven hundred putative F1 hybrid progeny that were planted, only 12 were selected as off-type, and the remaining progeny showed marked phenotypic similarity with the female parent, according to Griffa et al. (2005).

Five selected off-type plants were from the crossing of the sexual line and cv. Messina (collection No. 153) (Table 3), and 7 were the product of the sexual line with cv. Boorara (collection No. 136) (Table 4). The ANOVA results showed that the F1 progeny from the crossing between the sexual line and cv. Messina had a PLH lower than the male parent and similar to its sexual parent, except for F1 plants x153-3-4-8 and x153-3-9-1, which were intermediate between both parents. These would be advantageous for new material, as their smaller size would be beneficial in the use of photosynthates, reduced transpiration and increased water use efficiency. Three F1 plants had smaller LP than both parents. F1 plants x153-3-4-7 and x153-1-9-1 had higher values of LP and were similar to the sexual line. A greater LP would probably increase the number of spikelets available for crosses and, according to Griffa et al. (2012), it would also increase seed yield in these materials. A high value of LP would also be a desirable heritable trait in progenies obtained from crosses using these genotypes as parents. For INTL, the F1 plant x153-3-4-3 and Messina showed the lowest length values of all genotypes. TD and NUD were similar to the female parent and had a lower average value than Messina. There were no differences among the materials in VB; for RB, F1 x153-3-4-3 was similar to both parents, and the remaining F1 plants presented higher average values, thus increasing the number of inflorescences available to be used as female sources in crosses. Moreover, seed production can be increased in the progeny. In leaf dimensions, blade length, blade width and total length (FLL and FLW, TLL), except x153-1-9-1, F1 plants had lower values than both parents, indicating increased nutritional quality by lower accumulation of fibrous support structures (Agnusdei et al., 2009; Avila, 2009; Avila et al., 2010; Di Marco, 2010). In FSL, F1 progeny was similar to both parents, except x153-1-9-1 which had the greatest length. It should also be noted that this plant showed the greatest total fresh weight (FW) of all genotypes (Table 2), but without significant differences.

As regards the morphological evaluation of the putative F1 hybrid plants obtained from the cross of the sexual line and cv. Boorara (Table 3), PLH was lower than Boorara and similar to the sexual parent, except for plants x136-1-7-22 and x136-3-9-3, which had similar average PLH to Boorara. Reduced PLH plant height is a highly heritable trait (Griffa, 2002) and desirable in a forage grass. In LP the parent Boorara showed the highest average values, and the F1 plants had

Table 3
Morpho-agronomic evaluation of putative hybrids, produced by crossing sexual line (female parent) x cv. Messina (male parental). Different letters indicate significant differences ($p < 0.05$).

	Genotype	PLH	LP	INTL	TD	NUD	VB	RB	FLL	FLW	FSL	TLL	FW
Parents	Sexual line	1.20 a	11.30 b	12.48 b	3.29 a	7.33 a	48.3 a	2.17 a	28.01 c	0.79 b	11.27 b	38.48 c	0.77 a
		(0.22)	(0.73)	(1.53)	(0.60)	(1.72)	(1.47)	(0.75)	(3.77)	(0.14)	(0.68)	(3.98)	(0.17)
	Messina	1.53 c	13.57 c	10.22 a	4.07 b	11.44 b	6.00 a	1.33 a	31.17 c	0.66 b	9.74 b	40.92 c	1.15 a
		(0.08)	(1.07)	(1.62)	(0.43)	(0.01)	(2.00)	(1.41)	(3.94)	(0.15)	(0.81)	(3.91)	(0.03)
Offspring	x153-3-4-3	1.18 a	9.33 a	9.67 a	2.74 a	8.33 a	6.33 a	2.33a	14.67 a	0.37 a	9.83 b	24.50 b	0.38 a
		(0.03)	(0.58)	(1.15)	(1.15)	(0.77)	(1.15)	(2.52)	(5.86)	(0.06)	(1.15)	(7.00)	(0.00)
	x153-3-4-7	1.26 a	10.67 b	12.67 b	3.25 a	7.00 a	6.00 a	4.67 b	10.00 a	0.30 a	7.17 a	17.17 a	0.68 a
		(0.03)	(0.58)	(0.29)	(0.97)	(0.00)	(1.00)	(2.52)	(3.61)	(0.00)	(1.53)	(2.52)	(0.00)
	x153-3-4-8	1.31 b	9.67 a	12.67 b	3.01 a	7.67 a	6.33 a	12.00 b	12.00 a	0.40 a	10.33 b	23.25 b	0.86 a
		(0.08)	(0.58)	(2.02)	(0.59)	(0.58)	(1.15)	(2.52)	(2.83)	(0.00)	(3.62)	(7.42)	(0.00)
	x153-3-4-13	1.26 a	9.00 a	12.33 b	3.36 a	8.00 a	6.33 a	4.33 b	16.13 a	0.40 a	9.00 b	25.13 b	0.72 a
		(0.03)	(0.00)	(1.15)	(0.73)	(0.00)	(0.58)	(0.58)	(5.01)	(0.10)	(0.87)	(4.30)	(0.00)
	x153-1-9-1	1.40 b	12.00 b	13.33 b	3.57 a	8.00 a	6.33 a	4.67 b	22.50 b	0.50 a	15.00 c	36.50 c	1.40 a
		(0.10)	(0.00)	(0.58)	(0.39)	(1.73)	(1.15)	(1.53)	(3.54)	(0.10)	(2.00)	(4.95)	(0.00)

Table 4
Morpho-agronomic evaluation of putative hybrids, produced by crossing sexual line (female parent) x cv. Boorara (male parental). Different letters indicate significant differences ($p < 0.05$).

	Genotype	PLH	LP	INTL	TD	NUD	VB	RB	FLL	FLW	FSL	TLL	FW
Parents	Sexual line	1.20 a	11.30 a	12.48 a	3.29 a	7.33 a	48.3 a	2.17 a	28.01 b	0.79 b	11.27 b	38.48 b	0.77 a
		(0.12)	(0.61)	(1.37)	(0.45)	(1.72)	(1.22)	(0.75)	(4.00)	(0.11)	(0.84)	(3.98)	(0.17)
	Boorara	1.60 b	13.25 b	11.00 a	4.43 b	11.83 b	7.33 a	1.50 a	35.18 c	1.03 c	13.48 c	48.66 c	2.05 b
		(0.05)	(0.52)	(1.10)	(0.57)	(0.41)	(2.80)	(1.79)	(4.96)	(0.17)	(0.57)	(5.00)	(0.64)
Offspring	x136-1-7-11	1.33 a	10.33 a	13.00 a	3.96 a	8.67 a	6.00 a	4.33 a	13.00 a	0.30 a	8.67 a	21.50 a	0.78 a
		(0.06)	(1.53)	(1.00)	(0.20)	(0.58)	(1.00)	(0.58)	(5.29)	(0.06)	(0.58)	(4.95)	(0.00)
	x136-1-7-2	1.35 a	11.17 a	11.17 a	4.41 b	8.00 a	5.33 a	4.33 a	15.00 a	0.50 a	8.00 a	23.00 a	0.70 a
		(0.05)	(2.75)	(1.04)	(0.73)	(1.00)	(1.15)	(1.53)	(5.00)	(0.10)	(1.00)	(4.58)	(0.00)
	x136-1-7-22	1.38 b	11.43 c	11.67 a	3.58 a	8.33 a	6.33 a	4.00 a	13.50 a	0.60 a	9.17 a	22.67 a	0.72 a
		(0.11)	(0.93)	(0.58)	(0.03)	(0.58)	(0.58)	(1.00)	(3.50)	(0.26)	(0.76)	(4.50)	(0.00)
	x136-3-9-1	1.41 a	10.5 b	11.83 a	3.56 a	8.33 a	5.67 a	3.67 a	21.25 b	0.43 a	10.50 b	31.50 b	1.12 a
		(0.09)	(0.50)	(0.35)	(0.39)	(0.58)	(0.58)	(0.58)	(7.29)	(0.06)	(1.89)	(7.07)	(0.00)
	x136-1-7-19	1.41 a	10.00 a	14.00 b	3.58a	8.00 a	6.00 a	3.67 a	23.67 b	0.43 a	9.17 a	32.83 b	0.90 a
		(0.02)	(1.00)	(1.00)	(0.44)	(1.00)	(0.00)	(1.15)	(1.89)	(0.23)	1.61)	(0.58)	(0.00)
	x136-1-7-12	1.30 a	9.50 a	12.33 a	3.66 a	7.67 a	5.67 a	4.00 a	16.17 a	0.30 a	8.33 a	24.50 a	0.94 a
		(0.00)	(0.00)	(0.76)	(0.53)	(0.58)	(0.58)	(1.00)	(1.26)	(0.00)	(0.29)	(1.50)	(0.00)
	x136-3-9-3	1.53 b	12.00 a	10.83 a	3.74 a	8.00 a	6.33 a	3.00 a	27.63 b	0.50 a	11.33 b	38.97 b	0.83 a
		(0.06)	(1.32)	(1.04)	(0.29)	(1.00)	(0.58)	(2.00)	(2.68)	(0.10)	(1.53)	(3.33)	(0.00)

lower values than Boorara and similar to those of the sexual line, except x136-1-7-22 and x136-3-9-3, which had intermediate LP values between both parents. In INTL, all F1 progeny were similar to both parents, except x136-1-7-19 which had the greatest length. For TD, the F1 plants had narrower tillers, similar to the sexual line, an advantageous trait for increased quality (Ribotta et al., 2005; Griffa et al., 2011), and F1 plant x136-1-7-2 exhibited wider stems, as Boorara. In NUD, all progeny were similar to the female parent and only Boorara differed in a higher number of nodes per tiller. There was no difference among genotypes for VB and RB (Table 3). Most F1 plants showed shorter leaves (TLL) than both parents, a trait that has been associated with high nutritional quality (Agnusdei et al., 2009; Avila, 2009; Avila et al., 2010). Three F1 plants had similar TLL values to that of female parent, but smaller than TLL values of the male parent. For FLW all F1 progeny had lower values than both parents. In FSL, F1 plants had lower values than both parents, except for two plants that were similar to the sexual line (x136-3-9-1 and x136-3-9-3), but shorter than the male parent. Four F1 progenies presented the lowest TLL, whereas three F1 plants showed lower values than Boorara and similar to those of the female parent. For FW all F1 progeny were similar to the sexual line and Boorara had the greatest value.

Cluster analysis of all the morphological characters (Fig. 1) showed well-defined clusters. The first cluster was composed of the parental Boorara (cv. 136) and the second cluster included the parental Messina (cv. 153) together with the genotype x136-3-9-3. The sexual line (sex1) was separated from the others, and a third cluster gathered eight F1 plants. Plants x153-3-4-3 and x153-1-9-1 were also separated, being the most different among all the selected progenies. The progeny were quite distant from the parents, particularly the female parent, and might therefore be considered putative hybrids.

3.2. Identification of the reproductive mechanism

3.2.1. PCR technique

By means of the PCAB10 marker (Fig. 2), of all the progeny selected, four F1 plants (x66-2-1-2, x153-1-9-1, x136-1-7-11 and x136-1-7-19) did not amplify the fragment linked to apomixis. Later, these four offspring were tested with primers UGT197 (Fig. 3) and Q8H (Fig. 4), confirming the sexual reproductive system of only two of them: x153-1-9-1 and x136-1-7-11. Although the three markers are at the same distance from apolocus (50.4 cM) (Jessup, 2005),we could not obtain the same result with PCAB10, possibly due to the instability of the 3′ extreme of the latter, which makes it nonspecific.

3.2.2. Embryo sac analysis

One hundred mature ovules were observed per genotype of the putative F1 hybrid plants x153-1-9-1 and x136-1-7-11, and were directly mounted for observation under bright-field microscopy using DIC at 20×. We observed 71.3% and 65.5% of sexual sacs for both plants, respectively. These high levels of sexuality would indicate that both plants can be used as new female parents in buffel grass.

3.3. Determination of hybrid F1 plants by AFLP

Putative hybrid F1 plants x153-1-9-1 and x136-7-11, cultivars Boorara and Messina (male parent, each) and the sexual line were analyzed by AFLP. A total of 392 bands were amplified, of which 30.10% were polymorphic and 69.9% were monomorphic; UPGMA analysis allowed differentiation of all genotypes analyzed in this study (Fig. 5).

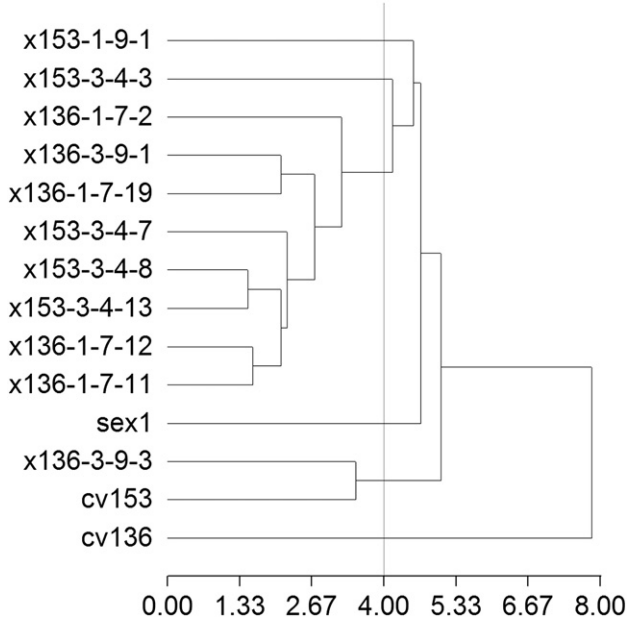

Fig. 1. Dendrogram resulting from cluster analysis on the proximity of the putative F1 hybrid genotypes of buffel grass, and their male parents, the cvs. Messina and Boorara (No. of entries 153 and 136, respectively) and the female parent, the sexual line introduced (sex1) with all morphological characters measured.

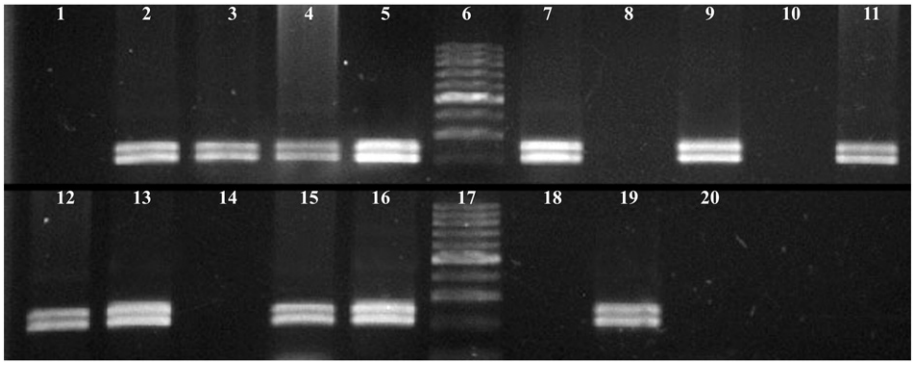

Fig. 2. Electrophoresis gel with apomixis marker PCAB10. Column 1 to 5 and 7 to 16: F1 plants; 6 and 17: molecular weight marker; 18: female parent: sexual line (negative control) (absent band); 19: male parent (control +) (present band); 20: reagent control.

The phenogram showed a phenetic correlation of 0.99, indicating that there was no distortion between the phenogram and the similarity matrix. The number of bands detected was an average of 39.2 for the primer combinations tested, ranging from 14 bands amplified for the combination EcoRI + ACC-MseI + ACG up to 94 bands amplified with the combination: EcoRI + AAC-MseI + CAC.

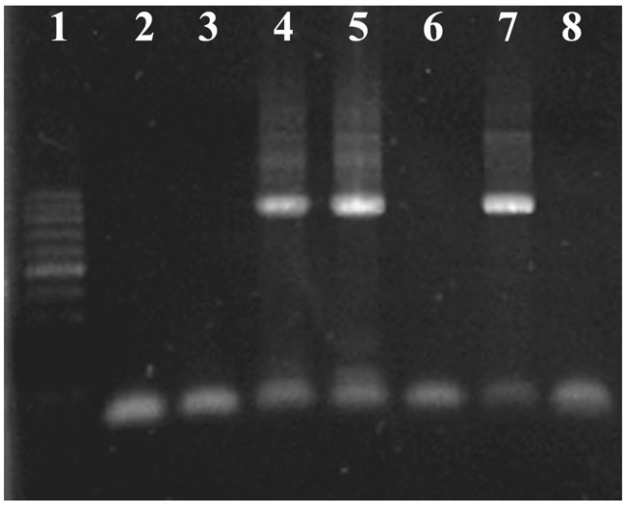

Fig. 3. Electrophoresis gel with apomixis marker UGT197. Column 1: molecular weight marker; 2 to 5: 4 F1 plants found sexual by PCAB10 primer; 6: female parent: sexual line (negative control) (absent band); 7: male parent (control +) (present band); 8: reagent control.

The phenogram (Fig. 5) showed that the genetic distance between the genotypes ranged from a maximum value of 0.45 (distance between the sexual line and the remaining genotypes) to a minimum value of 0.24 (between the half-sib F1 plants, x153-1-9-1 and x136-1-7-11). Shorter distances were observed between the sexual line and F1 plants in a previous study (Griffa et al., 2006). The male parents grouped together with a Jaccard's coefficient equal to 0.33. Espinoza et al. (2006) reported that the greatest distance found among 41 accessions of *Paspalum notatum* of different geographical origins, ploidy and reproductive methods was only 0.36. The F1 plants were not grouped with any of their parents and the high genetic distance from the female parent indicates that they are hybrids and that they are not generated by self-pollination. Interestingly, sexual F1 plants x153-1-9-1 and x136-1-7-11 exhibited 2 and 8 unique bands, respectively, which could be used as specific markers of these materials. Furthermore, both plants showed 2 and 3 bands shared with their respective parental, which are not present in the sexual parent. The presence of some bands from the apomictic parents in these sexual F1 plants also suggests their hybrid nature.

In summary, the technique of crosses between the sexual line and apomictic cultivars of buffel grass allowed us to obtain putative hybrid F1 plants, from which12 off-type plants were selected based on the evaluation of morphological characters. By means of PCR, using markers linked to apomixis and cytoembryological analysis of cleared embryo sacs, two of these 12 plants selected, showed high percentages

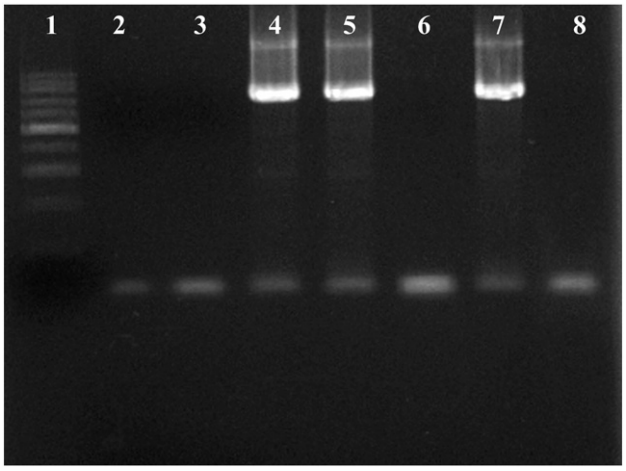

Fig. 4. Electrophoresis gel with apomixis marker Q8H. Column 1: molecular weight marker; 2 to 5: 4 F1 plants found sexual by PCAB10 primer; 6: female parent: sexual line (negative control) (absent band); 7: male parent (control +) (present band); 8: reagent control.

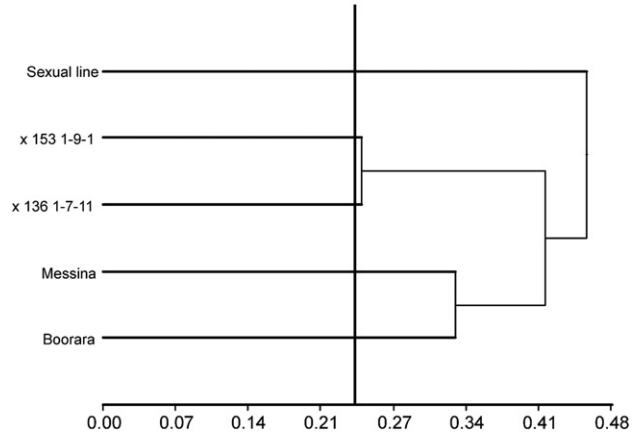

Fig. 5. Genetic relationship among apomictic cultivars (male parents), sexual line (female parent) and two F1 sexual genotypes (x153-1-9-1 y x136-1-7-11) in *Cenchrus ciliaris* L. Jaccard's coefficient was used to estimate the genetic distance. Phenogram was constructed by the UPGMA method.

of sexuality. Both plants were identified as hybrids using AFLP. Therefore, these new genotypes with high levels of sexuality, x136-1-7-11 with some promising traits for higher quality forage, and x153-1-9-1 for biomass yield, could be incorporated into the breeding program of buffel grass as new female parents to obtain improved apomictic cultivars.

Acknowledgments

The authors thank the Agencia Nacional de Promoción de Ciencia y Tecnología (ANPCyT project PICT Nº507) and INTA (Project Nº261821) for their support for this research.

References

Agnusdei, M.G., Nenning, F.R., Di Marco, O.N., Aello, M.S., 2009. Variaciones de calidad nutritiva durante el crecimiento vegetativo de gramíneas megatérmicas de diferente porte y longitud foliar (*Chloris gayana* y *Digitaria decumbens*). Revista Argentina de Producción Animal 29, 13–25.

Avila, R.E., 2009. Contenido y digestibilidad in vitro de la fibra de láminas de *Chloris gayana* Kunth. en relación a la edad y tamaño foliar. Tesis para optar al título de Magíster Scientie en Producción Animal Facultad de Ciencias Agrarias, Unidad Integrada Balcarce. Universidad Nacional de Mar del Plata, Argentina (90 pp.).

Avila, R.E., Di Marco, O.N., Agnusdei, M.G., Mayoral, C., 2010. Digestibilidad de la fibra y materia seca de dos gramíneas megatérmicas (*Chloris gayana* y *Cenchrus ciliaris*) de diferente porte: Relación con la edad y largo foliar. Revista Argentina de Producción Animal 30 (1), 1–13.

Balzarini, M.G., Di Rienzo, J.A., 2012. InfoGen Versión 2012. FCA, Universidad Nacional de Córdoba, Argentina (URL http://www.info-gen.com.ar).

Bashaw, E.C., 1962. Apomixis and sexuality in buffel grass. Crop Science 2, 412–415.

Benbouza, H., Jacquemin, J.M., Baudoin, J.P., Mergeai, G., 2006. Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. Biotechnology, Agronomy, Society and Environment 10 (2), 77–81.

Burson, B.L., Actkinson, J.M., Hussey, M.A., Jessup, R.W., 2012. Ploidy determination of buffel grass accessions in the USDA National Plant Germplasm System collection by flow cytometry. South African Journal of Botany 79, 91–95.

Daher, R.F., Vander Pereira, A., Gonzaga Pereira, M., da Silva Lédo, F.J., Teixeira do Amaral Junior, A., Anda Rocabado, J.M., Fortes Ferreira, C., Dessaune Tardin, F., 2004. Análise de trilha de caracteres forrageiros do capim-elefante (*Pennisetum purpureum* Schum.). Ciência Rural 34 (5), 1531–1535.

De Benedetti, L., Burchi, G., Mercuri, A., Pecchioni, N., Faccioli, P., Schiva, T., 2000. Random amplified polymorphic DNA (RAPD) analysis for the verification of hybridity in interspecific crosses of Alstroemeria. Plant Breeding 119, 443–445.

Di Marco, O., 2010. Manejo de Pasturas. Calidad Nutritiva. Visión Rural 83, 11–14.

Di Rienzo, J.A., Guzmán, A.W., Casanoves, F., 2002. A multiple comparisons method based on the distribution of the root node distance of a binary tree. Journal of Agricultural, Biological and Environment Statistics 7 (2), 1–14.

Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M., Robledo, C.W., 2011. InfoStat Versión 2011. Grupo InfoStat, FCA Universidad Nacional de Córdoba, Argentina.

Espinoza, F., Daurelio, L., Pessino, S., Valle, E., Quarín, C., 2006. Genetic characterization of *Paspalum notatum* accessions by AFLP markers. Plant Systematics and Evolution 258, 147–159.

Fisher, W., Bashaw, E., Holt, E., 1954. Evidence for apomixis in *Pennisetum ciliare* and *Cenchrus setigerus*. Agronomy Journal 46, 401–404.

Griffa, S.M., 2002. Caracterización de una estirpe sexual y cultivares apomícticos de Buffelgrass (*Cenchrus ciliaris* L.). Tesis de Magister Universidad Nacional de Córdoba, Argentina (163 pp.).

Griffa, S., 2010. Caracterización bioquímica y molecular de germoplasma, evaluación de la tolerancia a la salinidad y obtención de híbridos en buffel grass. Tesis Doctoral Universidad Nacional de Córdoba, Córdoba, Argentina (137 pp.).

Griffa, S., Ribotta, A., Muñoz, N., López Colomba, E., Ojeda, M., Luna, C., Grunberg, K., Díaz, D., Biderbost, E., 2005. Análisis de la herencia de caracteres de interés agronómico en *Cenchrus ciliaris* L. XXXIV Congreso Argentino de Genética. Trelew, Chubut, Argentina, p. 150.

Griffa, S., Díaz, D., Ribotta, A., Lanza Castelli, S., Muñoz, N., López Colomba, E., Luna, C., Grunberg, K., Biderbost, E., 2006. Molecular genetic discrimination of Buffel grass genotypes and F1 hybrids for breeding purposes using amplified fragment length polymorphism analyses. Grass and Forage Science 61, 454–458.

Griffa, S., Ribotta, A., Luna, C., Bollati, G., López Colomba, E., Tommasino, E., Carloni, E., Quiroga, M., Grunberg, K., 2011. Evaluación morfológica del cultivar de buffelgrass "Lucero INTA-PEMAN" en condiciones de sequía. Revista de Investigaciones Agropecuarias RIA 37 (1), 87–91.

Griffa, S., Quiroga, M., Ribotta, A., López Colomba, E., Carloni, E., Tommasino, E., Luna, C., Grunberg, K., 2012. Relationship between seed yield and its component characters in *Cenchrus* spp. Electronic Journal of Plant Breeding 3 (1), 701–706.

Gustine, D., Sherwood, R., Huff, D.R., 1996. Isozyme, protein and RAPD markers within a half-sib family of Buffel grass segregation for apospory. Crop Science 36, 723–727.

Gustine, D., Sherwood, R., Huff, D.R., 1997. Apospory-linked molecular markers in Buffel grass. Crop Science 37, 947–951.

Hanselka, W.C., Hussey, M.A., Ibarra, F.F., 2004. Buffelgrass. In: Moser, L.E. (Ed.), Warm-season (C4) Grasses: Agronomy Monograph, 45. ASA, CSSA, and SSSA, Madison, WI, pp. 477–502.

Hussey, M.A., Bashaw, E.C., Hignight, K.W., Wipff, J., Hatch, S.L., 1993. Fertilisation of unreduced female gametes: a technique for genetic enhancement within the *Cenchrus–Pennisetum* agamic complex. In: Baker, M.J., et al. (Ed.), Proceedings of the International Grassland Congress, 17th, Palmerston North, New Zealand. 8–21 Feb, pp. 404–405.

Jessup, R.W., 2005. Molecular tools for marker-assisted breeding of Buffelgrass. Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy. Graduate Studies of Texas A&M University, USA (65 pp.).

Jessup, R.W., Burson, B.L., Burow, G.B., Wang, Y.W., Chang, C., Li, Z., Paterson, A.H., Hussey, M.A., 2002. Disomic inheritance, suppressed recombination, and allelic interactions govern apospory in buffelgrass as revealed by genome mapping. Crop Science 42, 1688–1694.

Lanza Castelli, S., Grunberg, K., Muñoz, N., Griffa, S., López Colomba, E., Ribotta, A., Biderbost, E., Luna, C., 2010. Oxidative damage and antioxidant defenses as potential indicators of salt-tolerant *Cenchrus ciliaris* L. genotypes. Flora 205, 622–626.

Lubbers, E.L., Arthur, L., Hanna, W.W., 1994. Molecular markers shared by diverse apomictic *Pennisetum* species. Theoretical and Applied Genetics 89, 636–642.

Oropeza, M., García, E., 1997. Utilización de marcadores moleculares para la identificación de variedades de caña de azúcar. Phyton 61, 81–85.

Ozias-Akins, P., Lubbers, E., Hanna, W., McNay, J.W., 1993. Transmission of the apomictic mode of reproduction in *Pennisetum*: co-inheritance of the trait and molecular markers. Theoretical and Applied Genetics 85, 632–638.

Ribotta, A., Griffa, S., López Colomba, E., Grunberg, K., Biderbost, E., 2005. Determinación del contenido proteico en materiales en proceso de selección de *Cenchrus ciliaris* L., *Chloris gayana* K. y *Panicum coloratum* L. Pastos y Forrajes 28 (3), 241–246.

Sartor, M.E., Espinoza, F., Quarín, C.L., 2005. Análisis del sistema reproductivo en plantas autotetraploides inducidos de *Paspalum plicatulum*. Comunicaciones Científicas y Tecnológicas. Universidad Nacional del Nordeste, Argentina (Resumen A-008, 4 pp.).

Sartor, M., Quarin, C., Espinoza, F., 2009. Mode of reproduction of colchicine-induced *Paspalum plicatulum* tetraploids. Crop Science 49 (4), 1270–1276.

Shafer, G., Burson, B., Hussey, M., 2000. Stigma receptivity and seed set in protogynous buffelgrass. Crop Science 40, 391–397.

Sherwood, R.T., Berg, C.C., Young, B.A., 1994. Inheritance of apomixis in Buffel grass. Crop Science 34, 1490–1494.

Sneath, P.H.A., Sokal, R.R., 1973. Numerical taxonomy: the principles and practice of numerical classification. W. H. Freeman, San Francisco, CA, USA.

Snyder, L.A., Hernandez, A.R., Warmke, H.E., 1955. The mechanism of apomixis in *Pennisetum ciliare*. Botanical Gazette 116, 109–221.

Valle, C.B., do Savidan, Y.H., 1998. Genética, Citogenética y Biología reproductiva de Brachiaria. In: Miles, J.W., Maass, B.L., do Valle, C.B. (Eds.), Brachiaria: Biología, Agronomía y Mejoramiento. Gado do Corte, Brasil, pp. 163–180.

Young, B., Sherwood, R., Bashaw, E., 1979. Cleared-pistil and thick-sectioning techniques for detecting aposporous apomixis in grasses. Canadian Journal of Botany 57, 1668–1672.