Differentiation of apomictic and sexual genotypes of *Brachiaria* spp., using molecular markers

Diferenciación de genotipos apomícticos y sexuales de *Brachiaria* spp., con marcadores moleculares

Jaime Poblete-Vargas[1], Ernestina Valadez-Moctezuma[2*], Gabino García de los Santos[1], Carlos Martínez-Flores[3], Armando Peralta-Martínez[4]

[1]Programa de Semillas, Colegio de Postgraduados Campus Montecillo. Km 36.5 Carretera México- Texcoco, CP. 56230, Montecillo, Texcoco, Estado de México, México.
[2]Laboratorio de Biología Molecular, Departamento de Fitotecnia. Universidad Autónoma Chapingo. Carretera México-Texcoco Km 38.5 Chapingo, CP. 56230, Estado de México, México.
[3]Programa Global de Maíz. Centro Internacional de Mejoramiento de Maíz y Trigo. Carretera México-Veracruz. Km. 45, Colonia El Batán, CP. 56237, Texcoco, Estado de México, México. .
[4]Agroproductos de Iguala, S.A. de C.V. Hidalgo No. 23, Col. Centro, CP. 40400, Iguala, Guerrero, México.
*Corresponding author: evaladez@chapingo.mx

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ABSTRACT. Some species of *Brachiaria* are cultivated in the tropics because of their high productivity and drought resistance; their apomictic hybrids are of interest because of the almost null segregation and fixation of the hybrid vigor in seeds. In this research, Amplified Fragment Length Polymorphism (AFLP) and Cleaved Amplified Polymorphic Sequence (CAPS) markers were used to differentiate apomictic tetraploid and sexual diploid *Brachiaria* parents and their progeny. Polymorphism detected with AFLP was 91.3% and with CAPS 79.7%. Phenograms differentiated each genotype and the clustering of parents and their progeny was by their degree of genetic relatedness. AFLP did not separate the apomictic genotypes from the sexual ones, but CAPS markers did so through the C15-8 marker. Some apomictic genotypes of the progeny inherited it from their *B. ruziziensis* sexual mother, which may be related to some segregating character of the parental line.
Key words: AFLP; CAPS; DNA; forage; polymorphism.

RESUMEN. Algunas especies de *Brachiaria* se cultivan en los trópicos por su alta productividad y resistencia a sequía; sus híbridos apomicticos, son de interés por la casi nula segregación y fijación del vigor híbrido. En este trabajo se utilizaron marcadores Amplified Fragment Length Polymorphism (AFLPs) y Cleaved Amplified Polymorphic Sequences (CAPS) para diferenciar progenitores apomícticos tetraploides y sexuales diploides de *Brachiaria* y su progenie. El polimorfismo detectado con AFLPs fue del 91.3% y con CAPS del 79.7%. Los fenogramas diferenciaron a cada genotipo y agrupación de progenitores con la progenie por su grado de parentesco genético. Los AFLPs no separaron a los genotipos apomícticos de los sexuales, pero los CAPS si lo lograron mediante el marcador C15-8; algunos genotipos apomícticos de la progenie, lo heredaron de la madre sexual *B. ruziziensis*, lo que puede estar relacionado con algún carácter segregante de la línea progenitora.
Palabras clave:

INTRODUCTION

Livestock production is a key sector in the economies of Latin American countries, occupying a large area of land with productive potential (FAO 2009). Among the forage plants used, the genus *Brachiaria*, which is characterized by having cultivated forage pastures, stands out (Do Valle and

Miles *et al*. 2001). The success of these pastures is due to the fact that many species have a wide natural variation in agronomic traits, such as biomass production, nutrient quality, drought tolerance, tolerance to flooded soils and high seed yields (Miles *et al*. 2004). The qualities of these forage species have been the reason for developing hybrids with resistance to cuckoo spit, the frothed-up sap caused by the spittlebug (*Aeneolamia postica*), which limits the passage of water and nutrients in the plant (De la Cruz-Llanas *et al*. 2005), and to foliar fungi such as *Rhizotocnia*; hybrids have also been developed that are adapted to acid soils with high levels of aluminum (Rao *et al*. 2006) and that have higher production of high digestibility forage (Lascano 2002). Most commercially cultivated *Brachiaria* species are tetraploid apomicts; only *B. ruzizienzis* reproduces sexually and its recombination with apomictic genotypes can generate hybrids with desirable characteristics (Miles *et al*. 2004).

Apomixis is present in some *Brachiaria* species, which implies that the progeny are genetically identical to the mother plant, so they do not present segregation, a phenomenon that is often of importance in plant breeding (Grimanelli *et al*. 2001). This character is considered relevant in agriculture, due to the possibility of transforming crops of worldwide importance from sexual reproduction to apomictic clones (Bicknell and Koltunow 2004). Apomixis has great agricultural interest, as its fixation would allow maintaining the genetic stability of hybrids and varieties for an indefinite period, while the progeny of sexual reproduction maintain their genetic variation (Koltunow *et al*. 1995). *B. decumbens*, *B. brizantha* and *B. ruziziensis* are considered the most important species in this regard, since they belong to the same agomictic complex, with the first two being apomictic and the last one sexual (Do Valle and Miles 2001, Risso-Pascotto *et al*. 2005).

Traditionally in *Brachiaria*, morphological descriptors and embryo sac analysis with pistil thinning techniques are used to differentiate progeny and determine whether F_1 individuals are apomictic or sexual (Savidan 2000), while genetic-molecular marker techniques have been used successfully since the 1990s, with the advantage that they provide results in a short time from any plant tissue and any plant age; they are efficient because they are not affected by the environment. In most cases, the markers complement the information from the morphological markers (Azofeifa-Delgado 2006). In particular, the Amplified Fragment Length Polymorphism (AFLP) and Cleaved Amplified Polymorphic Sequence (CAPS) techniques are used to sample the entire genome (Zorzatto *et al*. 2010). Therefore, the objective of the present study was to apply the AFLP and CAPS techniques to differentiate interspecific and sexual apomictic genotypes of *Brachiaria* spp, selected as apomictic in the field.

MATERIALS AND METHODS

Plant material

We used young seedling leaves from 30 hybrid genotypes from three artificial hybrid tetraploid (2n + 2n= 4n = 36) male (♂) apomictic *Brachiaria brizantha* x *B. ruziziensis* parents; five tetraploid female (♀) *Braploiaria ruziziensis* (4x = 36) parents and 22 hybrids of the progeny resulting from 10 crosses selected in the field with the apomixis character (Table 1). The parent ♂ 18 (BR/NO 1873) is registered in Mexico as cultivar Mulato I, characterized by having high genetic uniformity; parent ♂ 71 (BR/NO 1371) is facultative, only produces viable pollen and is identified as self-compatible (CIAT 2002); it was used as a pollinator because it is a cuckoo spit-resistant apomict. Parent ♂ 94 (AIG 2094) is a seed-producing, drought-resistant hybrid. The progeny tests for identifying the reproductive mode of the mother plants and the 22 apomictic hybrids were carried out in an experimental lot in Tuxpan, Iguala county, Guerrero state, Mexico, located at 18° 21' NL, 99° 29' WL.

DNA extraction

From 1 g of lyophilized tissue obtained from the mixture of the genotypes listed in Table 1, the DNA was obtained with the cetyltrimethylammo-

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Table 1. Genetic material and keys used in molecular characterization.

No.	Genotype		Key*	Origen**
1	Parent ♂ 4 X	Apomictic	71	*Brachiaria brizantha x B. ruziziensis*
2	Parent ♂ 4 X	Apomictic	94	*Brachiaria brizantha x B. ruziziensis*
3	Parent ♂ 4 X	Apomictic	18	*Brachiaria brizantha x B. ruziziensis*
4	Parent ♀ 2 X	Sexual	29	*Brachiaria ruziziensis*
5	Parent ♀ 2 X	Sexual	110	*Brachiaria ruziziensis*
6	Parent ♀ 2 X	Sexual	41	*Brachiaria ruziziensis*
7	Parent ♀ 2 X	Sexual	100	*Brachiaria ruziziensis*
8	Parent ♀ 2 X	Sexual	143 A	*Brachiaria ruziziensis*
9	Progeny	Apomictic	4118-6	41 x 18
10	Progeny	Apomictic	4118-10	41 x 18
11	Progeny	Apomictic	4118-12	41 x 18
12	Progeny	Apomictic	2918-14	29 x 18
13	Progeny	Apomictic	2918-18	29 x 18
14	Progeny	Apomictic	10018-1	100 x 18
15	Progeny	Apomictic	10018-6	100 x 18
16	Progeny	Apomictic	10018-16	100 x 18
17	Progeny	Apomictic	143A 18-3	143A x 18
18	Progeny	Apomictic	143A 18-23	143A x 18
19	Progeny	Apomictic	4171-9	41 x 71
20	Progeny	Apomictic	4171-20	41 x 71
21	Progeny	Apomictic	10071-4	100 x 71
22	Progeny	Apomictic	10071-8	100 x 71
23	Progeny	Apomictic	4194-19	41 x 94
24	Progeny	Apomictic	4194-24	41 x 94
25	Progeny	Apomictic	143A 94-3	143A x 94
26	Progeny	Apomictic	143A 94-4	143A x 94
27	Progeny	Apomictic	143A 94-11	143A x 94
28	Progeny	Apomictic	11094-1	110 x 94
29	Progeny	Apomictic	11094-7	110 x 94
30	Progeny	Apomictic	11094-16	110 x 94

*In the hybrids (9-30) the number after the hyphen corresponds to the number of plants resulting from the cross with apomictic characteristics considered in the study. ** In the hybrids (9-30) the numbers correspond to the parents involved in the crossing.

nium bromide (CTAB) method (Dellaporta *et al.* 1983, Zhang and Stewart 2000).

Molecular evaluation

DNA quantification was performed with spectrophotometry at 260 nm and its quality was estimated on 1% agarose gels with 1X TAE buffer (Sambrook *et al.* 1989).

AFLP

Digestion, adapter ligation, pre-amplification reactions and selective amplification were performed as indicated by GIBCO-BRL Life Technologies AFLP™. For the selective amplification, the following primer combinations were evaluated: E-AAC + M-CAC, E-AAC + M-CTG, E-ACC + M-CAC, E-ACC + M-CTG, E-AGG + M-CTC, E-AGG + M-CTA, E-ACT + M-CTC y E-ACT + M-CTA and those which produced clear differences between parents and progeny were selected (Table 2). The fragments obtained in the selective amplification were separated on denaturing 6% polyacrylamide gels (19:1), 7.5 M urea and 1X TBE buffer in a BioRAD® vertical sequencing chamber and an E-C Apparatus Corporation® model 3000PW power source. The reagents required for electrophoresis and staining were prepared according to Sambrook *et al.* (1989). Prior to running the polymerase chain reaction (PCR) products, the gel was pre-run for 30 min at 55 °C. Subsequently, 10 μL of the samples previously mixed and heated with a run buffer at 65 °C were loaded and kept cold until their run. Electrophoresis was run at 80 watts (1000 volts) for 3 h; the gel was then stained in 0.2% silver nitrate solution for 30 min and developed with sodium carbonate solution, made with 30 g of sodium car-

Table 2. Primers, number of fragments and level of polymorphism detected with the Amplified Fragment Length Polymorphism (AFLP) and Cleaved Amplified Polymorphic Sequences (CAPS) techniques in parents and progeny of the genus *Brachiaria*.

No.	Primers	Total fragments	Polymorphic fragments	% polymorphism
AFLP				
1	E AAC + M CAC	54	47	87.04
2	E ACC + M CAC	43	40	93.02
3	E ACT + M CTC	29	28	96.55
4	E ACT + M CTA	35	32	91.42
	Total	161	147	91.30
CAPS				
1	C-06 5'-GAA CGG ACT C-3'	27	23	85.18
2	C-08 5'-TGG ACC GGT G-3'	15	12	80.00
3	C-15 5'-GAC GGA TCA G-3'	23	17	73.91
4	C-19 5'-GTT GCC AGC C-3'	19	15	78.95
	Total	84	67	79.76

bonate (Na_2CO_3) per L of distilled water, which was maintained at 6 °C; before use, 3 mL of 37% formaldehyde and 400 μL of sodium thiosulfate (10 mg mL^{-1}) were added. When the first bands were developed, after approximately 4 min, the solution was discarded and replaced with fresh solution to continue the development for another 15 min. Finally, the gels were photodocumented for analysis.

CAPS

It was performed with a modification, which consisted in digesting the DNA with the *Eco RI* enzyme prior to PCR, while in the original technique the amplified fragments are digested (Konieczny and Ausubel 1993). The modification provided reliable, informative and reproducible results between experiments, because the conformational change in the molecule used in PCR (fragments) exhibits a less complicated topography. PCR amplifications were performed on an Applied Biosystems® 9700 thermocycler according to the methodology of Williams *et al.* (1990). The selected RAPD primers were C-06, C-08, C-15 and C-19, of 25 primers previously evaluated by the ROTH Company, Germany (Table 2). The thermocycling consisted of one minute at 94 °C for pre-denaturation, 38 cycles [94 °C, 20 s; 40 °C, 15 sec; 72 °C, 60 s] and a 5-min final extension cycle at 72 °C. The products were separated in acrylamide (29: 1) double-layer gels where the run-off gel was prepared at 16%. The run buffer was 1X Tris-Glycine and it was run for 4 h with 40 V/cm in a C.B.S. Scientific CO® model MVG-216-33 vertical chamber, with an E-C Apparatus Corporation® model EC-105 power source. The gels were stained with 0.2% silver nitrate ($AgNO_3$), visualized and documented with a white light transilluminator (Sambrook *et al.* 1989).

Statistical analysis

A binary matrix was constructed with the products amplified in each technique. Genetic similarities were calculated with Nei and Li/Dice distance (Nei and Li 1979) and the method for clustering was the Unweighted Pair Group Method with Arithmetic mean (UPGMA) with a 500-replicate bootstrap analysis. The program used was FreeTree v 0.9.1.50 and the trees were drawn with the TreeView program (Win32) 1.6.6. A simple correspondence factor analysis was also carried out with the molecular data obtained with both techniques, to determine the percent contribution of the markers to the differentiation of the genotypes with respect to apomictic or sexual character.

RESULTS AND DISCUSSION

The number of markers obtained was acceptable and informative; 147 corresponded to AFLP, of which 91.3% were polymorphic, while 87 were obtained with CAPS with 79.7% polymorphism. Both types of markers differentiated each of the genotypes studied (Table 2). In the consen-

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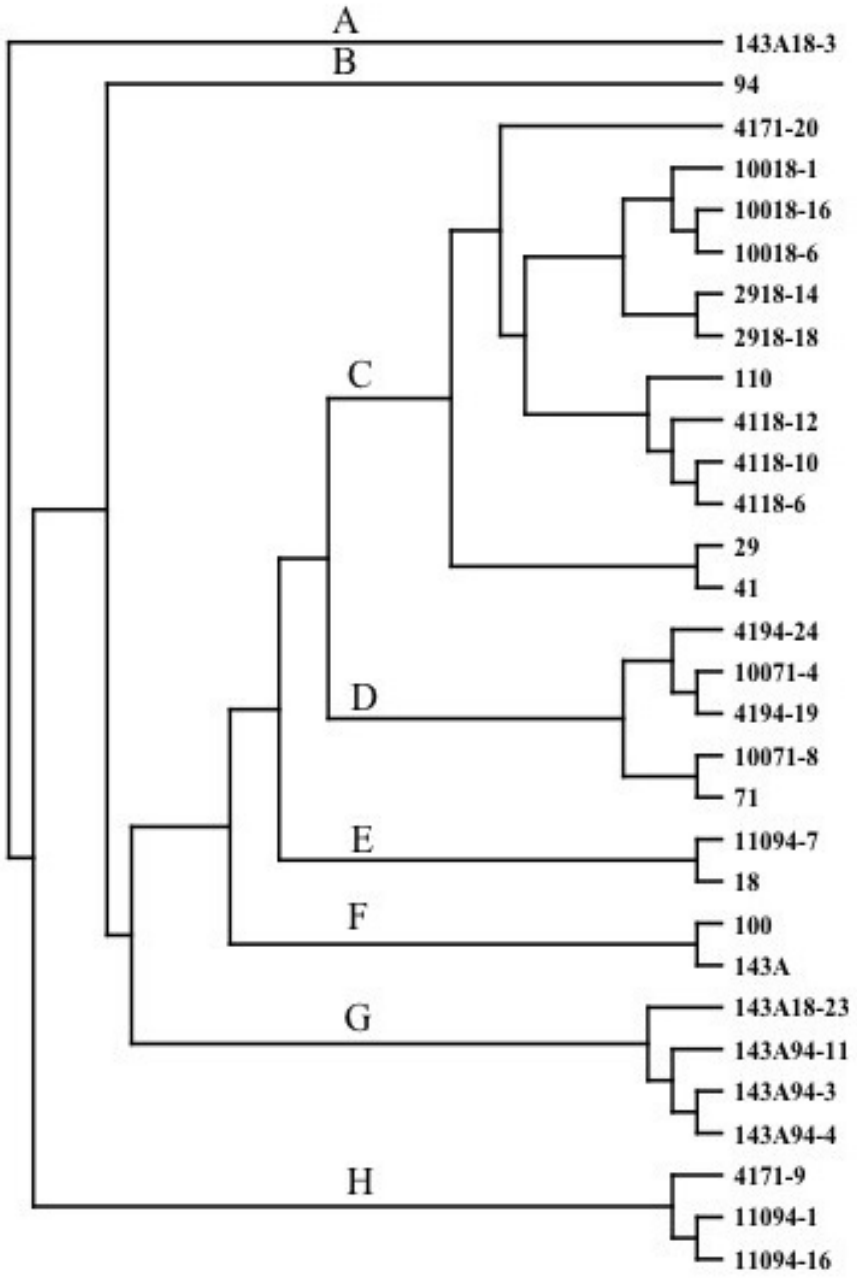

Figure 1. Consensus phenogram of apomictic and sexual genotypes of *Brachiaria* spp., constructed with Amplified Fragments Length Polymorphism (AFLP) genetic markers. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering and random sampling with 500 replicates (bootstrapping).

sus phenogram, constructed with the AFLP markers (Figure 1), three main groups are distinguished: group A comprised by the hybrid 143A-18-3; group B defined by the ♂ 94 and distant from the other two males; and group H with genotypes 4171-9, 11094-1 and 11094-16. Group B was related to the rest of the genotypes, which were clustered based on close genetic similarities. Groups C and G show

groupings of full siblings. The C group concentrates genotypes with greater influence of ♂ 18 [10018-1, 10018-16 and 10018-6] and [4118-12, 4118-10 and 4118-6], which is a dominant apomict, and ♀ 110, 29 and 41; this group also includes the genotype 4171-20, which must share characters with the parent ♀ 41 and the indicated half-siblings. Group G contains full siblings originating from ♀ 143A and ♂ 94 [143A94-11, 143A94-3 and 143A94-4, in addition to genotype 143A18-23, which apparently shares more information with parent ♀ 143A than with ♂ 18. On the other hand, the genotype 143A18-3 is found as an independent group (group A) when it was expected to be grouped with its genetic similarities. The discrepancy in this behavior may be due to mutations in the genome that generate polymorphisms in the profiles obtained. These alterations are common in plants of asexual/vegetative reproduction and it is relatively easy to detect them with molecular markers. Group D contains ♂ 71 and some of its progeny, group E has ♂ 18, and finally group F contains ♀ 100 and 143A. The fact that all the females were distributed throughout the phenogram suggests the genetic contribution of *B. ruziziensis* used as a female parent. The AFLP markers were able to differentiate all the genotypes and to show the close genomic relationship between some of them; however, they were insufficient to mark the locus or loci responsible for the apomixis (Hand and Koltunow 2014).

Figure 2 shows the grouping formed with CAPS data; ♂ 18 remained independent of the two main groupings, group I and group II, suggesting less genetic similarity according to these markers. Group I includes the parent ♂ 94, the genotypes with greater genetic influence of this parent, as well as some genotypes related to the parents ♂ 18 and ♂ 71. Group II comprises the females 29, 110 and 41, as well as the hybrids that shared more characters with them, but also ♂ 71, probably due to its facultative character. The other two females, 100 and 143A, were grouped independently. As in the phenogram with AFLP data, the groupings with CAPS were also made up of subgroups of full siblings; for example, group I included 4: [10018-16, 10018-6]; [143A94-3, 143A94-4]; [11094-16, 11094-1, 11094-7] and [2918-18, 2818-14], while group II comprised 2 subgroups, [4118-10 and 4118-12] and [4194-19, 4194-24], mainly. There are several matches between the two phenograms; there were groupings of full siblings, as well as females 100 and 143A or 29 and 41, which stayed together but in separate groups, which explains their close genetic resemblance. On the other hand, in both phenograms it was possible to separate the females from the males; however, in the case of CAPS, ♂ 71 was not separated from the group of females 29, 110 and 41, perhaps due to their particular qualities. The distribution behavior observed in both phenograms can be useful, especially when considering the alternative of selecting any of the females used in the present study due to their degree of genetic similarity, or ♂ 18 and 94 that showed less relatedness between them in both marker systems. Another observation was that the progeny was mainly influenced by the genetic characters of the parents ♂ 18 and ♂ 94, and to a lesser degree by ♂ 71.

The search for characters associated with apomixis or sexuality with techniques that detect anonymous markers such as those used in this study has proved difficult, unless sufficient molecular data are considered; this probability increases if precise information is available for the two loci that are assumed to control apomixis in plants (Noyes and Rieseberg 2000). DNA markers associated with apomixis (AFLPs and SCARs) have been reported for the genera *Paspalum* (Labombarda *et al*. 2002), *Pennisetum* (Ozias-Akins *et al*. 1998), *Hieriacium* (Catanach *et al*. 2006) and *Trichloris crinite* (Cavagnaro *et al*. 2006). In this study, the CAPS markers detected a DNA fragment called C15-8 that differentiated the sexual parents (♀) from the apomictic ones (♂) and that could be associated with the sexuality of *B. ruziziensis*; this may indicate the absence of this distinctive character in the apomictic genotypes used as males. This fragment was inherited from hybrids such as 9, 10, 12, 14, 15, 16, 18, 19, 22, 26 and 27 (Figure 3) that were selected at the field level as apomicts,

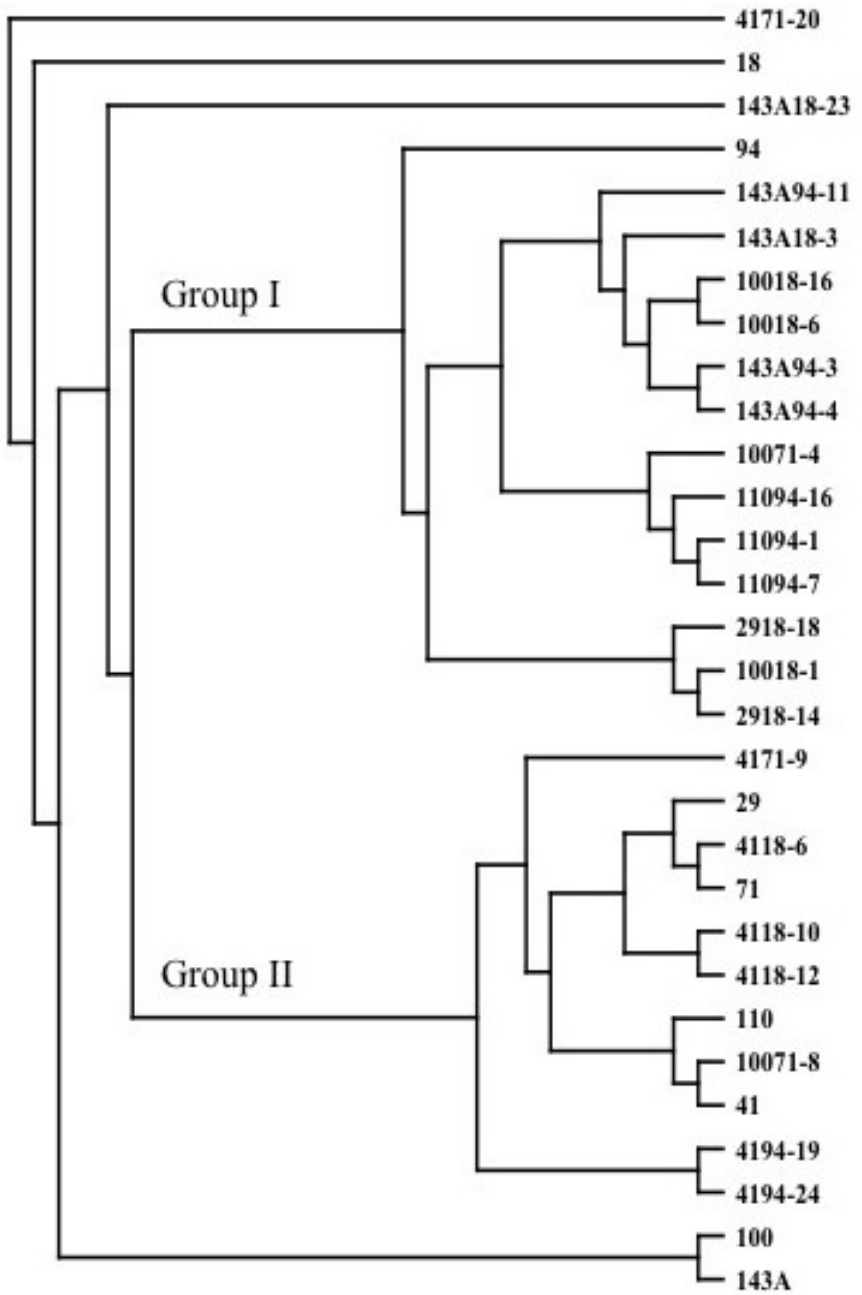

Figure 2. Consensus phenogram of apomictic and sexual genotypes of *Brachiaria* spp., constructed with Cleaved Amplified Polymorphic Sequences (CAPS) genetic markers, Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering and random sampling with 500 replicates (bootstrapping).

which may indicate that the *B. ruziziensis* line used as female is not the same as that of the tetraploid males. This observation can also be supported by separating the females in the two phenograms obtained. An alternative way of determining the effect of this marker is to observe the behavior at field level with respect to sexuality in different environments, to confirm it in a traditional way. The informa-

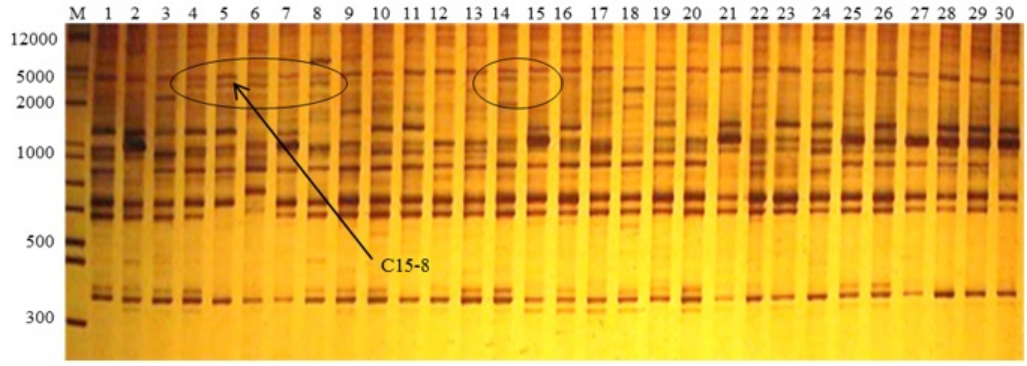

Figure 3. Cleaved Amplified Polymorphic Sequences (CAPS) profiles of 30 genotypes of Brachiaria spp., obtained with the ROTH C-15 primer. Lanes 1-3 correspond to 4X males, 4-8 to 2X females and 9-30 to hybrids. The ellipses indicate a distinctive DNA fragment in the females (lanes 4-8) that were inherited by some hybrids and named C15-8. Lane M indicates the molecular weight marker ϕX174 DNA /HaeIII.

Table 3. Eigenvalues of genotypes of the genus *Brachiaria*, product of simple correspondence factor analysis.

Principal Component	Eigenvalue	Explained variance in (%)	Cumulative variance in (%)
AFLP			
1	0.074	13.55	13.55
2	0.0627	11.47	25.02
3	0.0602	11.02	36.03
4	0.0396	7.25	43.29
5	0.0382	7.00	50.28
6	0.0338	6.18	56.47
CAPS			
1	0.0654	12.86	12.86
2	0.0628	12.36	25.22
3	0.0441	8.67	33.89
4	0.0366	7.2	41.08
5	0.0346	6.81	47.9
6	0.0324	6.37	54.27

tive capacity of this CAPS technique has also been shown by authors such as Möhring *et al.* (2005) who detected self-incompatibility characters in *Brassica* species. The relative ease of differentiating sexual genotypes (*Brachiaria ruziziensis*) from interspecific apomicts (*B. brizantha* and *B. ruziziensis*) in the present study could be favored by the genomic contrast of the species types involved in the crosses.

The simple correspondence factor analyses (Table 3) performed for the AFLP and CAPS data allowed weighting the value contributed by each component (amplicon) (Dore and Ojasso 2001). With both techniques, the first six principal components explain 56.47% for AFLP and 54.27% for CAPS, which suggests the effectiveness of the markers used to differentiate each of the genomes. The AFLP and CAPS techniques were appropriate to estimate the genetic relationship of parents and hybrids of the genus *Brachiaria*. The genetic contribution of male parents 18 and 94 was shown in the progeny, compared to that provided by parent ♂ 71. The CAPS technique was able to clearly differentiate the diploid genotypes of *B. ruziziensis* (female) from the tetraploid genotypes of *B. brizantha* X *B. ruziziensis* used as males. The CAPS factorial analysis allowed correlating the DNA fragment called C15-8 as a distinctive character of the female sexual parents, unlike that of the male apomictic parents.

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