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Molecular markers linked to apomixis in *Panicum maximum* Jacq.

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***Panicum maximum* Jacq. is an important forage grass of African origin largely used in the tropics. The genetic breeding of this species is based on the hybridization of sexual and apomictic genotypes and selection of apomictic F_1 hybrids. The objective of this work was to identify molecular markers linked to apomixis in *P. maximum* to determine easily and at an early stage, the reproductive mode of F_1 hybrids, so to assist the breeding program. A bulked segregant analysis was performed using 184 random amplified polymorphic DNA (RAPD) primers in an F_1 population of *P. maximum* segregating for reproductive mode. Four RAPD markers linked to apomixis were identified and mapped in this population. These markers showed good selection efficiency, ranging from 77.3 to 88%, with 81.3% when analyzed together. Two of these markers were easily transferred to another F_1 population of *P. maximum*. In this population, the selection efficiency was also high for both markers; 84.8 and 90%, when analyzed together. These markers may be used in the assisted selection for reproductive mode in both F_1 progenies of *P. maximum* studied here and in other populations to which they can be transferred.**

Key words: Guinea grass, apospory, reproduction mode, random amplified polymorphic DNA (RAPD), selection efficiency.

INTRODUCTION

Pastures play an important role in the national economy of most countries in the tropics since livestock production depends largely on both quantity and quality of forage crops established for ruminants. Only in Brazil, approximately 171 million hectares are planted to pastures (ABIEC, 2013).The main important cultivated pastures are grasses of African origin, which in general, show great adaptation to the Brazilian climate and soils. Among these grasses, *Panicum maximum* Jacq. stands out due to its good forage quality,high yield, wide

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adaptability and ease of establishment (Jank et al., 2008).

Panicum maximum, has both facultative apomixis and obligate sexual modes of reproduction (Savidan, 1980, 1982). Apomixis in *P. maximum* has been characterized as the result of apospory, embryo sac development from a somatic cell, followed by parthenogenesis and development of an embryo from the unfertilized egg cell (Savidan et al., 1989). Sexuality in this species was first discovered by Combes and Pernes (1970) in diploid accessions from East Africa.

For genetic breeding of apomictic species, it is necessary to identify sexual plants to use as maternal genitors in the crosses with apomictic plants, donors of pollen. In general, sexual genotypes in nature are diploid being necessary to undergo chromosome duplication before or after the crosses (Dall'Agnol and Schifino-Wittmann, 2005).

Crosses between sexual tetraploid genotypes of *P. maximum,* obtained from colchicine treatment, and apomictic genotypes have been done since 1971 in the Ivory Coast (Savidan, 1980). The hybrid progenies obtained from these crosses include sexual and apomictic hybrids in the Mendelian proportion of 1:1, demonstrating that apomixis in *P. maximum* is dominant over sexuality and determined by a single gene, the sexual tetraploids being defined as aaaa and the apomictic genotypes as Aaaa (Savidan, 1981).

A large and representative germplasm collection of *P. maximum* was introduced in 1982 through a cooperation-agreement between the Brazilian Agricultural Research Corporation (EMBRAPA) and the French Institute de Recherche pour le Développement (IRD - former ORSTOM - Office de la Recherche Scientifique et Technique d'Outre-Mer) (Jank et al., 2011). Embrapa Beef Cattle Center received from ORSTOM 426 apomictic accessions and 417 sexual genotypes (Jank et al., 2008).

The main thrust of the initial cultivar development efforts in Brazil were focused on the selection of useful commercial genotypes directly from the collection of apomictic accessions and resulted in the release of the cultivars Tanzânia, Mombaça and Massai (Jank et al., 2008).

A hybridization program was also initiated aiming to combine agronomic characteristics of interest and thus increase pasture productivity. After the hybridization, in order to continue the breeding program, the mode of reproduction of these progenies must be identified. Currently, the method used is the characterization of the anatomy of clarified ovaries analyzed under differential interference contrast microscopy. This is a difficult and laborious method, because it involves the harvest of inflorescences in anthesis in the adult plant, and the determination of the anatomical structures can only be made after specific training (Jank, 1995). Therefore, the development of alternative methods to access easier and more precociously the mode of reproduction in *P. maximum* have become extremely important.

A method that stands out for its speed and accuracy is the use of molecular markers that co-segregate with apomixis and can be used precociously in the earlier stages of plant development, with DNA samples extracted from seeds or seedlings (Bhat et al., 2005). This technique showed to be efficient in the apomictic species *Brachiaria* sp. (Pessino et al., 1997), *Pennisetum squamulatum* (Ozias-Akins et al., 1998), *Poa pratensis* (Barcaccia et al., 1998), *Paspalum simplex* (Labombarda et al., 2002; Gualtieri et al., 2006), *P. maximum* (Ebina et al., 2005), *Cenchrus ciliaris* (Gualtieri et al., 2006; Yadav et al., 2012), *Hypericum perforatum* (Barcaccia et al., 2007; Schallau et al., 2010), and *Brachiaria humidicola* (Zorzatto et al., 2010), among others.

The main objective of the present work was to identify molecular markers linked to apomixis in *P. maximum* to determine, easily and in early stages, the reproductive mode of F_1 hybrids, so to assist the breeding program.

MATERIALS AND METHODS

The experiment was conducted in the Laboratory of Plant Biotechnology of Embrapa Beef Cattle, located in Campo Grande, Mato Grosso do Sul State, Brazil.

Plant material

Two F_1 populations, called A and DE, were previously characterized for mode of reproduction by the technique of anatomy of clarified ovaries observed under differential interference contrast microscopy. Population A consisted of 40 apomictic hybrids and 35 sexual hybrids obtained from crossing *P. maximum* S10 (tetraploid sexual genotype) and cv. Tanzânia (tetraploid apomictic accession ORSTOM T58). Population DE consisted of 12 apomictic and 21 sexual hybrids obtained from crossing S12 (tetraploid sexual genotype) and cv. Tanzânia. A Chi-square (χ^2) test was performed on these progenies to verify whether the genetic segregation between apomictic x sexual hybrids fit the expected Mendelian model. Thirty-five accessions from the *P. maximum* germplasm collection from Embrapa were also analyzed (13 sexual genotypes and 22 apomictic accessions), to verify the presence or absence of the linked markers.

Molecular analysis

The DNA was extracted from young leaves by the Bonato et al. (2002) method. DNA purity and concentrations were estimated using a NanoDrop1000 (Thermo) spectrophotometer and on 0.8% agarose gel stained with ethidium bromide (5 $\mu g.mL^{-1}$).

A bulked segregant analysis (Michelmore et al., 1991) was carried out to identify molecular markers linked to apomixis using only population A. Two bulks were prepared, an apomictic bulk (AB) and a sexual bulk (SB), each one containing equimolar quantities of DNA from 10 hybrids characterized by differential interference contrast microscopy. A total of 184 RAPD primers were used for amplification of the bulks (AB and SB) and parents (S10 and

Table 1. Chi-square test (χ^2) for population A segregation and the markers segregation (P<0.05).

Parameter	Observed proportion		Chi- square test (χ^2)	
	Mode of reproduction			
	Apomitic	Sexual	Segregation	χ^2
Anatomical evaluation	40	35	1:1	0.33
Marker				
PM_A01	43	32	1:1	1.61
PM_01	31	41	1:1	2.25
PM_16	36	39	1:1	0.12
PM_U07	27	40	1:1	3.10

Tanzânia). Then, the primers potentially linked to apomixis were applied to the entire population A.

The polymerase chain reactions (PCR) were performed with a final volume of 25 µL, using, 1xTaq DNA polymerase buffer (Invitrogen); 1.5 mM $MgCl_2$ (Invitrogen); 0,2 mM dNTPs (Invitrogen); 0.4 µM primer (Operon Thecnologies); 1.0 U de Taq DNA polimerase (Invitrogen), 30 ng de DNA and H_2O to complete the volume. The thermal conditions consisted of 5 min at 94°C, 40 cycles of 1 min at 94°C, 1 min at 35°C, 2 min at 72°C followed by 7 min at 72°C. The amplification products were separated on a 2% agarose gel, stained with ethidium bromide and photo documented using L.Pix Image systems (Loccus Biotechnology).

Data analysis

The amplification of the potential markers was analyzed as binary, with 1 for presence and 0 for absence of the marker. The binary data was used to confirm the segregation 1:1 of the markers by the chi-square test (χ^2) with 5% probability ($p \geq 0.05$). The linkage analysis was conducted with the GQMOL 1.0.0 computer software (Cruz and Schuster, 2007) using the Kosambi function and a map was constructed. The LOD score used was 3 with a maximum distance of 30 cm. The selection efficiency (SE) of the markers linked to the apomixis locus (Apo locus) was calculated based on the comparison between the phenotypic and the genotypic (markers) according to Silva et al. (2007).

The molecular markers linked to apomixis in population A were also tested on population DE and in some apomictic accessions and sexual genotypes from the germplasm collection, to analyze the transferability of these markers.

RESULTS

The χ^2 test of the apomictic and sexual hybrids from both A (Table 1) and DE (Table 2) populations were not significantly different from the 1:1 ratio, according to the expected Mendelian model. Of the 184 RAPD primers tested on the segregant bulks from population A, 16 did not amplify (8.7%). Of the remaining 168 primers, only 14 (8.3%) amplified polymorphic markers between the bulks. Four of these primers (OP-01, OP-16, OP-A01, and OP-U07) amplified markers that resulted in a good fit considering 1:1 segregation ratio (Table 1).

The linkage analysis revealed that these markers co-segregated with the Apo locus in this population, and that the PM_A01 marker amplified with primer OP-A01, showed the smallest distance, and the marker PM_U07 amplified with primer OP-U07, showed the largest distance (Figure 1).

The selection efficiencies (SE) for PM_A01, PM_01, PM_16 and PM_U07 were 88, 80, 81.3 and 77.3%, respectively, and the SE of the four markers together was 81.3%. After the confirmation of linkage between the four markers and the Apo locus, the amplifications with these four primers were done on population DE. In this case, three markers presented a good fit in the 1:1 segregation ratio and one did not, PM_16 marker (Table 2). However, only two markers co-segregated with the Apo locus by the linkage analysis (PM_A01 and PM_01). For PM_U07 marker the LOD score was lower than 3.0. The two markers were mapped 7.66 and 18.49 cM, respectively (Figure 1).

The SE was also calculated for these two markers in population DE. The PM_A01 marker presented the highest value (90%) and the PM_01 marker presented 84.8%. Together, the markers showed 90% selection efficiency.

As for the accessions from the germplasm collection, four sexual plants amplified none of the markers, being potential genotypes for use in future crosses, where the hybrids will inherit the markers only from the apomictic parent. Eighteen (18) of the 22 apomictic accessions amplified at least one of the markers, which shows that a high percentage of apomictic accessions from the germplasm collection presents the markers.

DISCUSSION

Earlier studies in *P. maximum* proposed a simplex genotype for tetraploid apomicts (Aaaa) and a homozygous recessive genotype for sexual plants, whether diploid (aa) or tetraploid (aaaa) (Savidan, 1981). The

Table 2. Chi-square test (χ^2) for population **DE** segregation and the markers segregation (P<0.05).

Parameter	Observed proportion		Chi- square test (χ^2)	
	Mode of reproduction			
	Apomitic	Sexual	Segregation	χ^2
Anatomical evaluation	12	21	1:1	2.44
Marker				
PM_A01	15	18	1:1	3.66
PM_01	11	22	1:1	1.61
PM_16	10	23	1:1	5.12
PM_U07	15	18	1:1	0.27

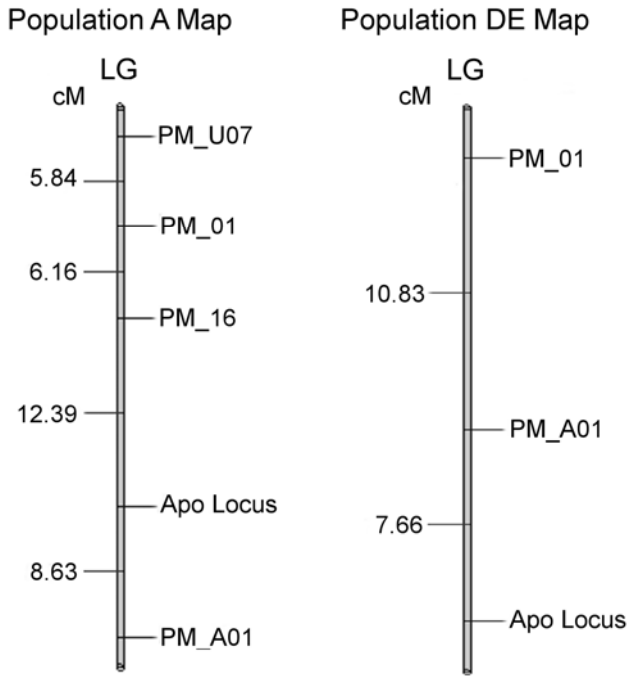

Figure 1. Linkage maps of the two populations of *P. maximum.* On the left is the linkage map of population A and on the right is the linkage map of population DE. On each linkage map the marker name is shown on the right and the estimated map distance is shown on the left.

results presented here for both reproductive modes and molecular markers corroborated this hypothesis, allowing the use of the bulked segregant analysis strategy and RAPD technique to prospect markers linked to apomixis in this species. Four RAPD markers were identified and mapped in population A and two of these were transferable to population DE, without the need to develop Sequenced Characterized Amplified Region Marker (SCARs).

The fact that the other two markers have not been transferred may be due to the small size of population DE. According to Sanglard and Melo (2011), the segregation distortion is most likely an effect caused by the low number of genotypes in the population. One of the four markers which was not transferred had a segregation distortion. Of the others three markers that co-segregated with the apomixis trait in population DE, only two showed LOD score equal or higher than 3.0, which shows that there is a possibility for a thousand times greater of the markers to be linked with the locus of

interest than an independent segregation. Once more, the small size of the DE population may have interfered in the results by not containing enough samples of meiotic events (Cruz and Schuster, 2007).

The identification of the region containing the apomictic locus in *P. maximum* was also attempted previously by Ebina et al. (2005). In their study, they used AFLP and RAPD markers to generate a linkage map and to identify molecular markers tightly linked to apomixis. Only AFLP markers linked to the apomixes trait, none of the RAPD markers linked, however, different RAPD primers were used than in our study.

The SE of the markers (individually or together) in both populations of *P. maximum* studied in this work may be considered high, since they can be assessed in the early stages of plant development, using small amounts of any tissue, and in a large number of individuals. This result demonstrated the potential of the use of these markers for assisted selection of reproductive mode in *P. maximum*.

The availability of a molecular marker linked to the Apo locus in *P. maximum* has immediate implications in the breeding of this commercially very important grass for it provides an efficient tool for rapid, early and reliable screening of the progenies for mode of reproduction without having to wait until flowering and to execute the time-consuming extraction, clarification and examination of ovaries.

In conclusion, this study identified molecular markers linked to apomixis in *P. maximum*. These markers can be used for phenotyping the reproductive mode of F1 hybrids. Each apomictic hybrid derived from crosses between sexual x apomictic plants is potentially a unique cultivar or a hopeful candidate regardless of the heterozigous or homozygous genetic background of the parents. The sexual hybrids can be used in new cycles of crossing and selection.

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