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RESEARCH

Performance of the molecular marker p779/p780 for classifying mode of reproduction and its association with agronomic traits in a guineagrass breeding program

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Abstract Apomixis in guineagrass (*Megathyrsus maximus*) breeding offers significant advantages, including the fixation of heterosis in the F_1 generation, simplification of seed production, and enhancement of pasture uniformity in cultivars. Nevertheless, the segregation of mode of reproduction—apomictic versus sexual—within breeding populations necessitates the development of efficient and large-scale screening methods for the identification and selection of apomictic individuals. The objectives of this study were: (1) to evaluate the accuracy of the molecular marker p779/p780 in classifying the mode of reproduction of genotypes across diverse genetic backgrounds within a guineagrass breeding program; (2) to assess the correlation between the marker and key agronomic traits; and (3) to implement marker-assisted selection (MAS) in a biparental population. To this end, the mode of reproduction of genotypes from the Embrapa guineagrass breeding program was determined using both conventional methodologies and the p779/p780 molecular marker. A logistic regression model was employed to assess the marker's ability to classify the mode of reproduction. Additionally, correlation analyses between the marker and agronomic traits, as well as MAS application, were conducted in a biparental population. The p779/p780 marker demonstrated high classification accuracy (>0.90) across multiple genetic backgrounds within the breeding program, indicating its robustness and reliability. However, despite its overall effectiveness, the marker was not found to be perfectly associated with the mode of reproduction in segregating populations. Inheritance of the marker followed

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a monogenic pattern (1 sexual:1 apomictic), and no significant correlations were observed between the marker and key agronomic traits. The implementation of MAS for mode of reproduction at early selection stages can enhance the efficiency of guineagrass breeding programs by enabling a high selection accuracy of apomictic genotypes without compromising selection gains for other agronomic traits.

Keywords *Megathyrsus maximus* · *Panicum maximum* · Apomixis · Molecular markers · Marker-assisted selection

Introduction

Guineagrass (*Panicum maximum* Jacq., currently classified as *Megathyrsus maximus* (Jacq.) B. K. Simon & S. W. L. Jacobs) is an autotetraploid forage species of high agronomic importance, particularly in tropical regions, where it plays a central role in livestock production systems (Muir & Jank 2004). In Brazil—recognized as the world's second-largest beef producer and the leading beef exporter—approximately three million hectares of guineagrass have been planted annually over the past five years, according to seed market data (Ministry of Agriculture, Livestock and Food Supply of Brazil 2023). The success and expansion of this species are largely attributable to advances in genetic improvement, which have led to the development and dissemination of high-yielding cultivars with improved tolerance to both biotic and abiotic stresses (Jank et al. 2014).

The species primarily reproduces via apomixis, a form of asexual reproduction by seeds (Warmke 1954; Nogler 1984). Apomixis in guineagrass is considered facultative, as sexual reproduction processes are occasionally observed, though these occur at relatively low frequencies (Savidan 1982). Microsporogenesis is typically initiated in apomictic plants, resulting in male gametes that are produced entirely through sexual mechanisms. A small number of fully sexual and diploid plants, originally collected from the species' center of origin in East Africa, have undergone chromosomal duplication. These plants enabled successful crosses with apomictic accessions at the tetraploid level, thereby facilitating their utilization in breeding programs (Savidan 1981).

Apomixis is a trait of significant importance in both production systems and breeding programs (Miles 2007). In production systems, it ensures enhanced genetic stability of the cultivar, greater ease of pasture establishment, and increased uniformity within the pasture. These factors contribute to more effective management of animal grazing. In breeding programs, crosses between sexual female parents and apomictic pollen parents generate progenies that segregate 1:1 for sexual and apomictic plants, due to the monogenic inheritance of the mode of reproduction (Savidan 1980). This allows for the rapid fixation and propagation of superior apomictic hybrids as early as the first generation following the cross, thereby streamlining the process of cultivar development.

Due to the critical role of apomixis, its evaluation in hybrid candidates for cultivar development is essential. The primary techniques for assessing apomixis are progeny testing and embryo sac analysis (Leblanc and Mazzucato 2001). While these methods are effective in determining the mode of reproduction, they have notable limitations. These include long periods of time and space requirements in the field to obtain and evaluate the progenies in the progeny test, as well as the need for plants with flowers, skilled labor, and the use of potentially toxic chemicals during embryo sac analysis. As a result, these techniques are typically low-throughput, which complicates the assessment process, particularly in the early stages of selection.

Early identification of apomictic individuals during the selection process significantly enhances the efficiency of guineagrass breeding programs, as it enables the allocation of resources toward the evaluation of promising cultivar candidates. Marker-assisted selection (MAS) represents a promising approach for large-scale screening of mode of reproduction, particularly in the initial stages of selection. Despite this potential, no molecular markers have yet been validated for effective and routine use in guineagrass breeding programs for the identification of apomictic genotypes. Several research efforts have aimed to identify markers linked to apomixis (Ebina et al. 2005; Bluma-Marques et al. 2014; Deo et al. 2020). Although these studies have reported markers showing strong linkage to genomic regions associated with apomixis, their broader adoption has been limited. This limitation is likely attributable to challenges in reproducibility and a lack of robustness when applied

across the diverse genetic backgrounds commonly encountered in breeding populations.

Studies aimed at identifying molecular markers linked to apomixis have been conducted across several grass genera, including *Pennisetum* and *Cenchrus* (Conner et al. 2008; Akiyama et al. 2011), *Poa* (Albertini et al. 2001), *Paspalum* (Calderini et al. 2011), and *Urochloa/Brachiaria* (Worthington et al. 2016, 2019). Among the markers identified, the primer pair p779/p780 has emerged as particularly significant. This primer pair amplifies a genomic fragment located within the ASGR-BBML-like region—an apomixis-specific genomic region (ASGR)—which is thought to play a central role in controlling parthenogenesis across multiple species (Conner et al. 2015).

The p779/p780 marker has demonstrated conservation across several grass species and has proven effective in classifying mode of reproduction in multispecies panels from the genera *Pennisetum*, *Cenchrus*, and *Urochloa*, as well as in selected guineagrass accessions (Worthington et al. 2019). In an F_1 mapping population of *Urochloa* developed by the International Center for Tropical Agriculture (CIAT), the marker exhibited perfect linkage with the ASGR and demonstrated high predictive accuracy for mode of reproduction, highlighting its potential utility in marker-assisted selection within the genus (Worthington et al. 2019). As a result, CIAT has incorporated the p779/p780 marker into its breeding programs for *Urochloa* and guineagrass. However, despite its application, no studies have yet validated the predictive capacity of this marker for determining the mode of reproduction in segregating populations of guineagrass.

The potential association between molecular markers linked to apomixis and other agronomic traits under selection is a critical consideration in the context of marker-assisted selection (MAS). Such associations, whether arising from pleiotropic effects or genetic linkage (Lynch and Walsh 1998), can substantially influence selection outcomes. Therefore, understanding these relationships is essential to ensure the efficiency and reliability of MAS in plant breeding programs. In *Paspalum simplex*, for instance, a SCAR-type marker strongly associated with apomixis was reported to have no significant association with morphological or agronomic traits (Brugnoli et al. 2019). Under these conditions, the use of the marker for selecting apomictic genotypes would not interfere with the selection for other traits, supporting its utility in MAS. However, this remains the only published study that specifically investigated potential associations between apomixis-linked markers and traits of agronomic importance. Additional research is therefore warranted to elucidate the genetic linkage between apomixis-controlling regions and economically important traits in other forage species.

The application of MAS for apomixis in guineagrass holds considerable promise, particularly in the early stages of breeding programs, where large population sizes necessitate efficient high-throughput screening strategies. Marker-based classification of mode of reproduction can be integrated into both biparental (Deo et al. 2020) and multiparental (Worthington and Miles 2015) breeding schemes, thereby increasing the overall efficiency of selection processes. In this context, the objectives of the present study were: (1) to assess the accuracy of the p779/p780 molecular marker for classifying mode of reproduction across different genetic backgrounds in a guineagrass breeding program; (2) to estimate the correlation between the marker and key agronomic traits; and (3) to implement marker-assisted selection in a biparental population.

Material and methods

The mode of reproduction of 187 genotypes from various sources within the Embrapa Beef Cattle guineagrass breeding program was evaluated simultaneously using conventional techniques and the molecular marker p779/p780. The genotypes included 11 apomictic cultivars, 10 elite sexual hybrids used as female parents in crosses (Hybrids-1), 53 hybrids from intermediate selection stages and different crosses (Hybrids-2), and 113 hybrids from a biparental population (Hybrids-3) (Table 1). The apomictic cultivars represent both older and more recent genotypes released by Embrapa and other Brazilian companies. Hybrids-1 are sexual genotypes derived from crosses and selected for various agronomic traits within the breeding program. Hybrids-2 are genotypes that underwent two stages of selection: initial individual plant selection for traits such as disease resistance, regrowth capacity, agronomic merit, and leafiness, followed by selection based on experimental

Table 1 Information on the genotypes used for validating the p779/p780 marker in the guineagrass breeding program at Embrapa Beef Cattle

Group	Breeding information			Analysis for mode of reproduction		
	No. of Genotypes	Origin	Selection status	Embryo sacs clearing	Progeny test	p779/p780 marker
Cultivars	11	Germplasm bank	Released cultivars or accessions used as male parents in crosses	11	0	11
Hybrids-1	10	Diverse F1 crosses	Selected elite hybrids used as female parents in crosses	4	10	10
Hybrids-2	53	Diverse F1 crosses	Hybrids selected from a clonal trial	50	3	53
Hybrids-3	113	F1 cross	Unselected hybrids from an F1 cross	113	5	113
Total	187	–	–	178	18	187

plot evaluations for traits like forage production, leaf percentage, nutritive value, disease resistance, and regrowth capacity. These hybrids were not specifically selected for their mode of reproduction. Hybrids-3 represent a sample of genotypes from a biparental population that did not undergo any selection process.

Determination of the mode of reproduction

Conventional analyses: embryo sacs

Altogether, 178 genotypes were evaluated using the embryo sac analysis method (Table 1). For this analysis (Fig. 1A), 40 to 60 pistillate flowers of each genotype were collected at the anthesis stage, dissected, and fixed in an FAA solution in the proportion 40:14:3:3 (Formalin 40%: Distilled water: Ethyl alcohol 95%: Glacial acetic acid) for 24 h at room temperature (Raposo et al. 2019). After this period, the fixative was replaced with 70% (v/v) ethyl alcohol, and the flowers were stored at 4 °C. The pistils were extracted from the flowers using a stereoscopic microscope and tweezers, then stored in 70% ethyl alcohol and clarified according to the protocol proposed by Young et al. (1979). The clarified pistils were stored in methyl salicylate under refrigeration at 4 °C. For the morphological analysis of the embryo sacs, slides were prepared with 30 pistils per genotype, which were kept humidified in methyl salicylate.

After preparing the slides, evaluations to determine the mode of reproduction were carried out using a microscope with differential interference contrast (DIC) (Fig. 1B). Plants that presented only reduced embryo sacs of the *Polygonum* type were classified as sexual plants, that is, presenting an egg cell, a central cell with two polar nuclei and antipodal cells at the chalazal pole; plants that presented at least one unreduced embryo sac of the *Panicum* type, that is, containing an egg cell, a single polar nucleus and absence of antipodal cells or with multiple embryo sacs (Nakagawa 1990) were classified as apomictic. Sterile, atrophied, undefined and empty embryo sacs were identified, but disregarded in the analyses.

Conventional analyses: progeny test

Eighteen genotypes were evaluated by the progeny test (Table 1) in the field at Embrapa Beef Cattle (20° 25′ longitude, 54° 40′ latitude and altitude 565 m). Seeds from Hybrids-1 (10 sexual parents) in crosses with apomictic parents were collected and used to produce 60 seedlings, which were evaluated in six plots of ten plants each in the summer of 2018 (December to May). To evaluate the other eight genotypes (from Hybrids-2 or Hybrids-3), seeds of each genotype were used to obtain 54 seedlings, which were evaluated in 12 m^2 plots in the summer of 2022 (January to May).

Mother plants that gave rise to progenies with uneven morphology for traits such as plant height, leaf width and aspect (decumbent, brittle at the tips or erect), disease resistance and flowering were classified as sexual, while mother plants that gave rise to progenies with uniform morphology were classified as apomictic (Fig. 2). Nine genotypes evaluated by the progeny test were also evaluated by the embryo sac method.

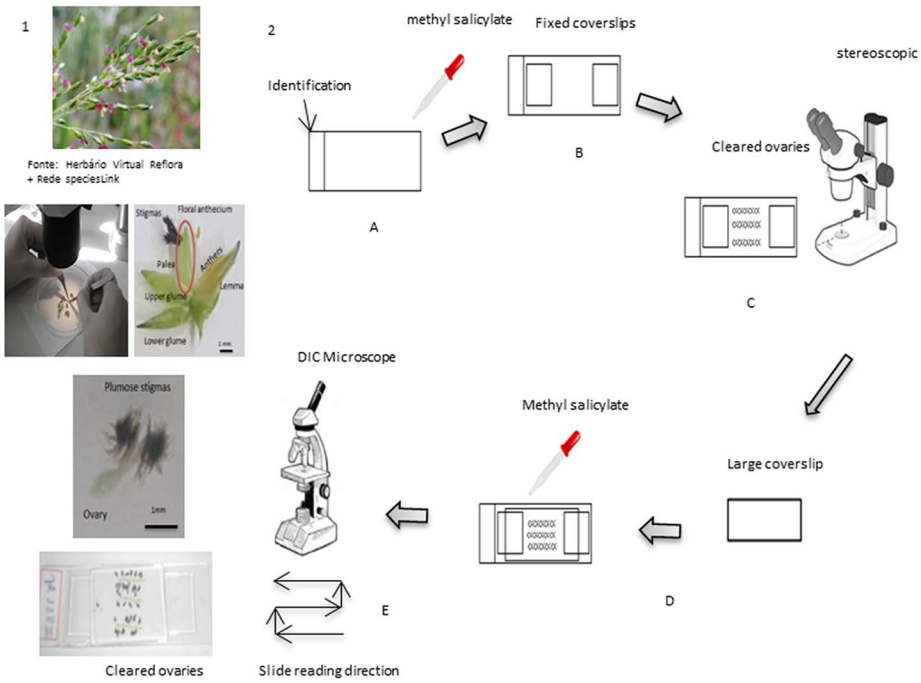

Figure 1- A Diagram illustrating the methodology used to study the mode of reproduction in guineagrass at the Plant Cytogenetics Laboratory of Embrapa Beef Cattle. 1) Inflorescences at anthesis showing yellow anthers and purple stigmas; dissected hermaphroditic flower; ovary with purple stigmas; microscope slide containing clarified ovaries prepared for reproductive mode analysis. 2) Schematic representation of the slide assembly procedure used for the analysis of clarified ovaries.

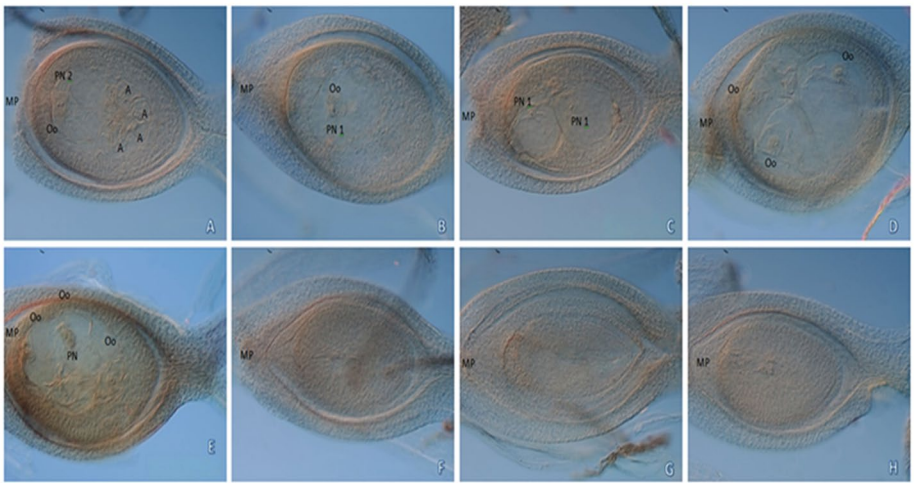

Figure 1- B - Cleares ovules showing the embryo sacs morphology in different individuals of guineagrass. A) embryo sac reduced present in sexual plants; B) embryo sac unreduced presente in apomictic plants; C) 2 embryo sacs unreduced present in apomictic plants ; D) and E) multiple embryo sacs present in apomictic plants ; F) sterile embryo sac; G) emtpty embryo sac; H) atrophied embryo. Note in C, D and E the aposporous embryo sacs are in the same ovule. A = antipodes; Oo = egg apparatus; MP = micropyle; PN = polar nucleus.

Fig. 1 **A** Diagram illustrating the methodology used to study the mode of reproduction in guineagrass at the Plant Cytogenetics Laboratory of Embrapa Beef Cattle. (1) Inflorescences at anthesis showing yellow anthers and purple stigmas; dissected hermaphroditic flower; ovary with purple stigmas; microscope slide containing clarified ovaries prepared for reproductive mode analysis. (2) Schematic representation of the slide assembly procedure used for the analysis of clarified ovaries. **B** Cleares ovules showing the embryo sacs morphology in different individuals of guineagrass. **A** embryo sac reduced present in sexual plants; **B** embryo sac unreduced presented in apomictic plants; **C** 2 embryo sacs unreduced present in apomictic plants; **D**, **E** multiple embryo sacs present in apomictic plants; **F** sterile embryo sac; **G** empty embryo sac; **H** atrophied embryo. Note in **C**, **D** and **E** the aposporous embryo sacs are in the same ovule. *A* antipodes, *Oo* egg apparatus, *MP* micropyle, *PN* ploar nucleus

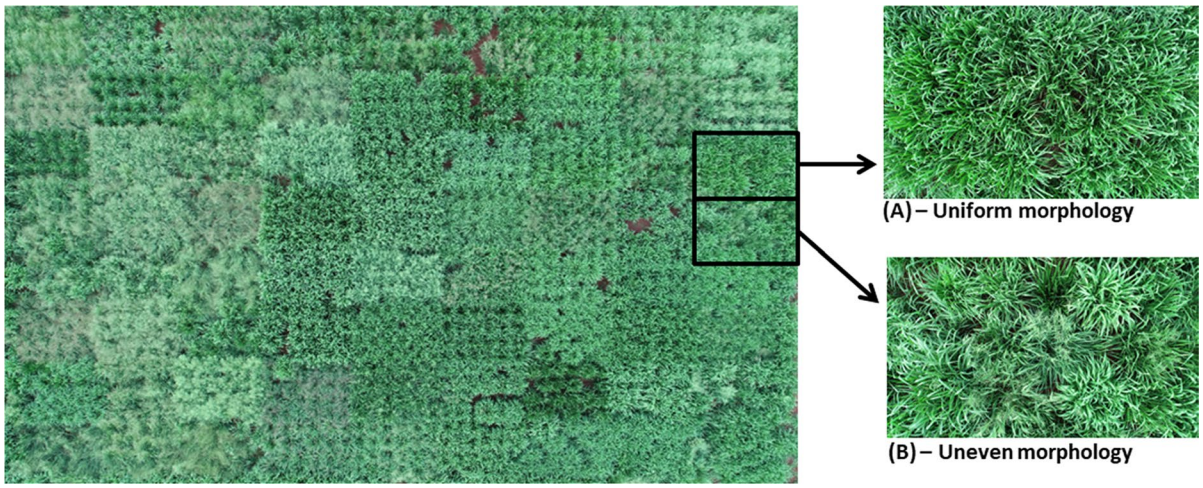

Fig. 2 Progeny test field of guineagrass plants at Embrapa Beef Cattle, Brazil. **A** Progeny from an apomictic plant; **B** progeny from a sexual plant

Analyses through the molecular marker p779/p780

Analyses with the marker p779/p780 were performed for all genotypes (187) used in this study (Table 1). Genomic DNA was extracted from young leaves using the protocol described by Bonato et al. (2002), with adaptations. After extraction, the DNA was quantified using a spectrophotometer at 260 nm, with its purity assessed by the reading ratio at wavelengths of 260/280 nm. The quality and concentration of the DNA were verified in a 0.8% agarose gel, using phage λ DNA as the standard, applying 30 to 300 ng.

PCR analyses were performed using the pair of primers p779/p780 (5′ TATGTCACGACAAGAATA TG; 3′ TGTAACCATAACTCTCAGCT), in a final volume of 15 µL, containing 2 ng/µL of genomic DNA; 1X PCR buffer; 1.5 mM $MgCl_2$; 0.125 mM dNTPs; 0.5 µM forward and reverse primers; 0.5 U Taq DNA polymerase (Invitrogen, Taq DNA Polymerase, Brazil), and sterile Milli-Q water (Worthington et al. 2016). Amplifications were performed in Veriti model thermocyclers (Applied Biosystems), with initial cycles of 5 min at 94 °C; 35 cycles of 30 s at 94 °C; 30 s at 57 °C; 1 min at 72 °C; and ending with a cycle of 72 °C for 10 min. After PCR amplification, the products were subjected to electrophoresis in a 1.5% agarose gel (1.5 g of agarose; 100 mL 1X TBE-93 mM Tris, 89 mM boric acid, and 2 mM EDTA), and visualized under UV light on a Gel Doc XR + Bio Rad system, and subsequently analyzed. To detect the mode of reproduction, the amplification product was analyzed, where the presence of the band indicated that the individual was apomictic and its absence indicated that the individual was sexual (Fig. 3). Plants from the breeding program with a known mode of reproduction were used as positive and negative controls.

To verify the occurrence of potential analytical errors, all cases in which the conventional and marker-based analyses produced discordant results for the mode of reproduction were reanalyzed using both methodologies. The final results, including those obtained from the reanalyses, are presented in Table 1 of the supplementary material.

The hypothesis of a 1:1 segregation ratio between sexual and apomictic individuals for both the mode of reproduction and the molecular marker p779/p780 in the Hybrids-2 and Hybrids-3 populations was tested using the chi-square (χ^2) test, calculated using the following formula:

$$\chi 2 = \sum \frac{(FO - FE)^2}{FE}$$

where *FO* and *FE* denote the observed and expected frequencies, respectively, for each genotypic class (sexual or apomictic). The calculated χ^2 values were compared against the critical value from the chi-square distribution table for 1 degree of freedom at the 5% significance level ($\chi^2_{0.05,1} = 3.84$).

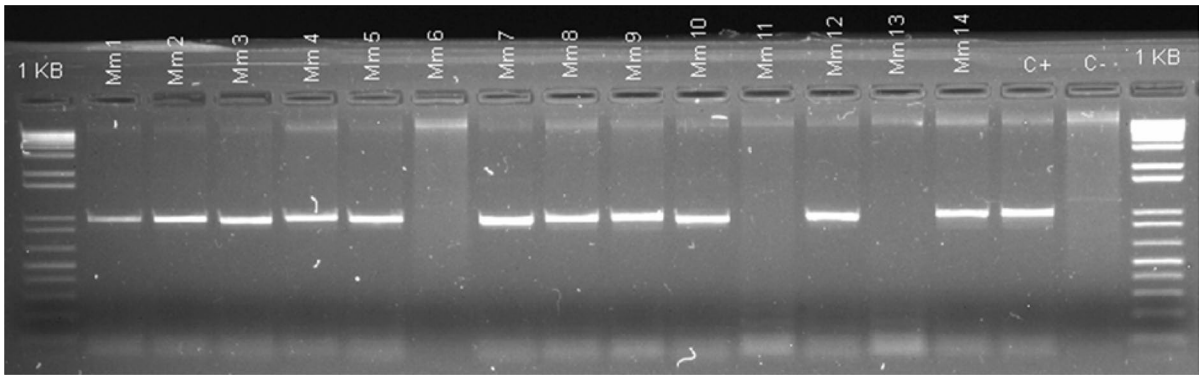

Fig. 3 Electrophoresis agarose gel at 1.5% of PCR product using the molecular marker p779/p780 in a sample of guinea-agrass individuals of the S12 × Miyagui population (Mm1, Mm2, … Mm14). C+ and C− are the positive (apomictic) and the negative (sexual) controls. 1KB is the type of DNA ladder consisting of 13 fragments from 250 to 10,000 base pairs. The presence of the band with about 900 pb indicates that the individual is apomictic and its absence indicates that it is sexual

Analyses of the ability to classify the mode of reproduction with the molecular marker p779/p780

All genotypes (n = 187) that were simultaneously evaluated using the p779/p780 molecular marker and conventional methods—embryo sac clearing and/or progeny testing—were included in the analysis to assess the predictive performance of the marker. The results obtained from conventional analyses were considered the reference standard for determining the mode of reproduction and served as the benchmark for comparison. To estimate statistical parameters and evaluate the classification ability of the p779/p780 marker, a binomial logistic regression model was employed, as defined by the following function (James et al. 2013):

$$p(X) = \frac{e^{\beta_0+\beta_1 X}}{1 + e^{\beta_0+\beta_1 X}},$$

where $p(X)$ is the probability of the occurrence of the event (i.e., classification as apomictic), β_0 and β_1 are parameters estimated via the maximum likelihood method, and X is the predictor variable, representing the marker class (0 = sexual, 1 = apomictic).

The bootstrap method was used to evaluate the classification capacity of the regression model. In each sampling, the data set was divided into a training set to estimate parameters of the model (80% of the observations—149 genotypes) and a validation set (20% of the observations—38 genotypes). The results of the model returned a probability that varies between 0 and 1. To classify the mode of reproduction, a cutoff point of 0.80 was applied, i.e. when the probability was above 0.8, the most likely mode of reproduction was apomixis; when the probability was below 0.8, the most likely mode of reproduction was sexuality. One thousand resamples were performed to estimate the classification ability of the marker. In each resampling, a confusion matrix was obtained, which allowed us to evaluate the quality of the model in classifying the mode of reproduction.

With this matrix, the number of true positives (TP), true negatives (TN), false positives (FP) and false negatives (FN) were computed. Thus, it was possible to obtain the following performance parameters of the model:

$$accuracy = \frac{TP + TN}{TP + TN + FP + FN};$$

$$error\ rate = 1 - accuracy;$$

$$false\ positive\ rate = \frac{FP}{TN + FN};$$

$$true\ positive\ rate = \frac{TP}{TP + FP};$$

$$false\ negative\ rate = \frac{FN}{TP + FP};$$

$$true\ negative\ rate = \frac{TN}{TN + FN}.$$

The mean results of the parameters were obtained and their confidence intervals were constructed using the t test at 95% probability based on the values of 1000 resamples. The analyses were carried out using the '*caret*' package (Kuhn 2008), and basic functions of the R software (R Core Team 2022).

Estimation of the genetic correlation between p779/780 marker and agronomic traits

To estimate the correlation between agronomic traits and the p779/p780 marker, agronomic evaluations were conducted on the Hybrids-3 population. This biparental population was derived from a cross between a sexual genotype and the apomictic cultivar 'Miyagui'. The sexual parent was developed through successive cycles of selection by the Embrapa Beef Cattle breeding program, while the apomictic parent is a commercial cultivar widely adopted by Brazilian ranchers.

Agronomic evaluations of the hybrids were conducted under field conditions at Embrapa Beef Cattle. An augmented block design with five blocks was employed, in which the hybrids were treated as regular (non-replicated) treatments, while the controls—cultivars 'Mombaça', 'Miyagui', and the sexual parent—were included as common (replicated) treatments. Each plot consisted of a single plant, with spacing of 1.5 × 1.5 m between plants.

The evaluations were carried out during four harvests, one during the 2019 dry season (1st cut 24/10/2019) and three during the rainy season in 2019/2020 (2nd-06/12/2019, 3rd-10 /01/2020, 4th-13/02/2020). Before each harvest, the canopy height of the plant was evaluated by measuring from the soil to the mean height of the canopy (CH, cm). Leaf dry matter production (LDMY), stem + sheath dry matter production (SDMY) and dead material production (DMP) were calculated and converted into g plant^{-1}. Total dry matter production (DMY) corresponded to the sum of LDMY, SDMY and DMP. The percentage of leaves (LP) in relation to stems was calculated as $LP = \left[\frac{LDMY}{LDMY+STMY}\right] x100$. The forage density, equivalent to the leaf volumetric density (LVD), was calculated as $LVD = \frac{LDMY}{CH}$, in g/cm. The clump area was estimated as $\pi * \left(\frac{D}{2}\right)^2$, in which D is the diameter of the clumps (cm), measured with a ruler positioned horizontally in relation to the soil.

Regrowth density (RD) was evaluated by visually scoring regrown tillers seven days after each harvest using a visual scale ranging from 0 to 100%. Regrowth speed (RS) was obtained by calculating the mean of the growth of two leaves (GL cm, one at the edge and one at the center) of the clumps between seven and nine days after each harvest (DAH), using the following expression: $RS = \frac{GL}{DAH}$.

Genetic-statistical analyzes for agronomic traits were carried out considering the following mixed linear model:

$$y_{ijk} = \mu + g_i + b_k + h_j + gb_{ik} + gh_{ij} + bh_{kj} + \varepsilon_{ijk}$$

In which y_{ijk} is the phenotypic value of the genotype i, in the block k, in the harvest j; μ is the intercept of the model; g_i is the random effect of the genotype i; b_j is the random effect of the block k; h_j is the fixed effect of the harvest j; gb_{ik} is the random effect of the interaction between the genotype i and the block k; gh_{ij} is the random effect between the genotype i and the harvest j; bh_{kj} is the random effect of the interaction between the block k and the harvest j; and ε_{ijk} residual effect, where $\varepsilon_{ijk} \sim N(0, \mathbf{R})$, in which $\mathbf{R}$ is the matrix of residual variances and covariances. Variance components were estimated using the restricted maximum likelihood (REML) method. Subsequently, the best linear unbiased predictors (BLUP) were calculated, which represent the genotypic predicted values of the hybrids in the population. The heritability coefficient was calculated using the following formula (Cullis et al. 2006).

$$H^2_{Cullis} = 1 - \frac{\bar{v}^{Blups}_{\Delta\ldots}}{2\sigma\hat{}^2_g}$$

In which $\bar{v}^{Blups}_{\Delta\ldots}$ is the mean variance of the difference between pairs of BLUPs and $\sigma\hat{}^2_g$ is the genotypic variance. The predicted genotypic means for the traits were obtained for each hybrid using the equation: $\underline{X}_{PREDm_i} = \mu + Blup_i$, in which μ is the intercept of the model, and $Blup_i$ is the random effect corresponding to each hybrid i in the population. For these analyses, the ASReml-R 4.2 package was used (Butler et al. 2023).

Based on the predicted genotypic means, Pearson correlations and their significances were calculated between all traits, including the p779/p780 marker. Subsequently, the magnitudes of the correlations were presented using a heatmap graph. All analyses were performed using basic R software packages.

Marker-assisted selection using p779/p780 in a biparental population

Marker-assisted selection (MAS) using the p779/p780 molecular marker was implemented in the Hybrids-3 population. To assess the effectiveness of MAS based on this marker, the following selection strategies were applied:

1. marker-assisted selection for the mode of reproduction aiming at selection of apomictic hybrids;
2. marker-assisted selection for the mode of reproduction aiming at selection of sexual hybrids;
3. Selection based solely on a selection index derived from the predicted genotypic means of agronomic traits. To construct the selection index, the traits leaf dry matter yield (LDMY), leaf proportion (LP), regrowth density (RD), regrowth speed (RS), and leaf volumetric density (LVD) were standardized to a mean of 0 and a standard deviation of 1. The selection index was calculated using the following formula: $Z_k = \sum_1^t r_k^t$, in which Z_k represents the selection index value for the hybrid k, and r_k^t represents a genotypic mean of the individual k for the trait t. The selection index was ranked in descending order and used to select hybrids considering a selection intensity of 20%, that is, the 26 hybrids with the highest values for the index were selected;
4. selection combining the index and marker-assisted selection, aiming to obtain apomictic hybrids; and,
5. selection combining the index and marker-assisted selection, aiming to obtain sexual hybrids.

The unselected population served as the baseline for evaluating the selection strategies employed. After selection, the mean of the selected hybrids was calculated. To compare the effectiveness of different selection strategies, the confidence intervals of the means were determined using a t-test with 95% confidence.

Results

Evaluation of the mode of reproduction in guineagrass genotypes

A total of 5141 pistils from 178 genotypes were analyzed using the embryo sac method, yielding an average of 29 pistils per genotype (see Tables 1 and 2 in supplementary material). Among these genotypes, 79 were classified as sexual and 99 as apomictic. In the sexual genotypes, 63% of embryo sacs were classified as Polygonum type, while 37% were excluded from analysis due to being undefined, empty, atrophied, or sterile. For the apomictic genotypes, 18%, 14%, and 45% of embryo sacs were categorized as Polygonum, Panicum, and multiple types, respectively; the remaining 23% of embryo sacs were excluded from the analysis for similar reasons (undefined, empty, atrophied, or sterile). These results highlight a high rate of invalid embryo sacs in sexual plants and demonstrate a predominance of multiple embryo sacs in apomictic genotypes. Furthermore, a range of meiotic embryo sac occurrence (0–73%) was observed

Table 2 Mode of reproduction of genotypes from different sources of the guineagrass breeding program evaluated by conventional techniques and by the p779/p780 marker

Techniques	Phenotype class	Sources of genotypes				Total
		Cultivars	Hybrids-1	Hybrids-2	Hybrids-3	
Embryo sac/progeny test	Sexual	0	10	22	53	85
	Apomictic	11	0	31	60	102
	Sex:Apo ratio	–	–	0.7:1.0	0.9:1.0	–
p779/p780 marker	Sexual	0	10	22	51	83
	Apomictic	11	0	31	62	104
	Sex:Apo ratio	–	–	0.7:1.0	0.8:1.0	–

in apomictic genotypes, suggesting varying levels of facultative apomixis.

Analyses using the progeny test showed that 11 genotypes were sexual and seven were apomictic, i.e. they generated morphologically uneven and uniform plant progenies, respectively. Among the genotypes analyzed by this technique, nine were also analyzed by the embryo sac method, with a 100% coincidence in the results between both conventional techniques.

Considering the genotypes evaluated using both conventional methods, 85 were classified as sexual and 102 as apomictic (Table 2). As expected, all cultivars were classified as apomictic, while the elite sexual parents (Hybrids-1) were all classified as sexual. The ratio of sexual to apomictic genotypes in the Hybrids-2 and Hybrids-3 populations were 0.7:1.0 and 0.9:1.0, respectively. The chi-square test for mode of reproduction in Hybrids-3 was not significant ($\chi^2 = 0.43$, $p > 0.05$), indicating no statistical differences between the observed and expected segregation ratios. This suggests that the segregation of the mode of reproduction in this population follows a 1 sexual:1 apomictic ratio, further supporting the hypothesis of monogenic control. Interestingly, the same hypothesis was not rejected for Hybrids-2 ($\chi^2 = 1.5$, $p > 0.05$), even after two stages of selection for agronomic traits.

The analysis using the p779/p780 marker, conducted on all genotypes in this study, identified 83 sexual hybrids (absence of the marker band) and 104 apomictic hybrids (presence of the marker band). The ratio of sexual to apomictic hybrids in both the Hybrids-2 and Hybrids-3 populations closely matched the results obtained through conventional techniques, indicating strong concordance between conventional and marker analyses.

Performance of the p779/p780 marker to classify the mode of reproduction

The logistic regression coefficient β presented a highly significant value (4.62, $p < 0.001$) using the maximum likelihood method (result not shown). This indicates that the presence of the band for the p779/p780 marker was strongly associated with the occurrence of apomictic mode of reproduction, while the absence of the band was related to sexual reproduction.

The performance of the model using the p779/p780 marker as a variable to classify the mode of reproduction in the guineagrass breeding program is presented in Table 3. The mean predictive accuracy of the model was high, at 90.9%, while the error rate was low, at 9.1%. The false positive rate (FPR) and false negative rate (FNR) were 12.8% and 6.8%, respectively, indicating a higher FPR. The true positive and true negative rates were 90.5% and 91.6%, respectively. The confidence intervals for the parameters were narrow, suggesting high precision in the estimates for the data set used.

Although the marker demonstrated high performance in predicting the mode of reproduction for the genotypes in the breeding program, it misclassified seventeen genotypes (Table 1, supplementary material). Of these misclassified genotypes, 10 were false positives (i.e., they presented the marker band but were sexual) and seven were false negatives (i.e., the marker band was absent but the genotypes were apomictic). The majority of misclassified genotypes (15) were from Hybrids-3, with only 2 misclassified genotypes from Hybrids-2.

Genetic correlation between the p779/p780 marker and agronomic traits in the F1 population

Significant genetic variability was observed for all agronomic traits. The heritability of the traits ranged from moderate (0.41) to high (0.82), with most traits exhibiting heritability values greater than 0.5, indicating that the majority of the observed variation was due to genetic factors (data not shown).

A heatmap of correlations between traits revealed three distinct groups (Fig. 4). The first group consisted solely of the p779/p780 marker, which showed no significant correlation with any agronomic trait.

Table 3 Measures of performance of the model with the marker p779/p780 to classify the mode of reproduction (MR) in guineagrass

Measure	Value	CI[b]
Accuracy (%)	90.9	90.7–91.3
Error rate (%)	9.1	8.7–9.3
False positive rate (%)	12.8	12.3–13.4
False negative rate (%)	6.8	6.5–7.1
True positive rate (%)	90.5	90.1–90.8
True negative rate (%)	91.6	91.2–92.0

[a]Mean over 1000 resampling

[b]95% confidence intervals based on a t-test

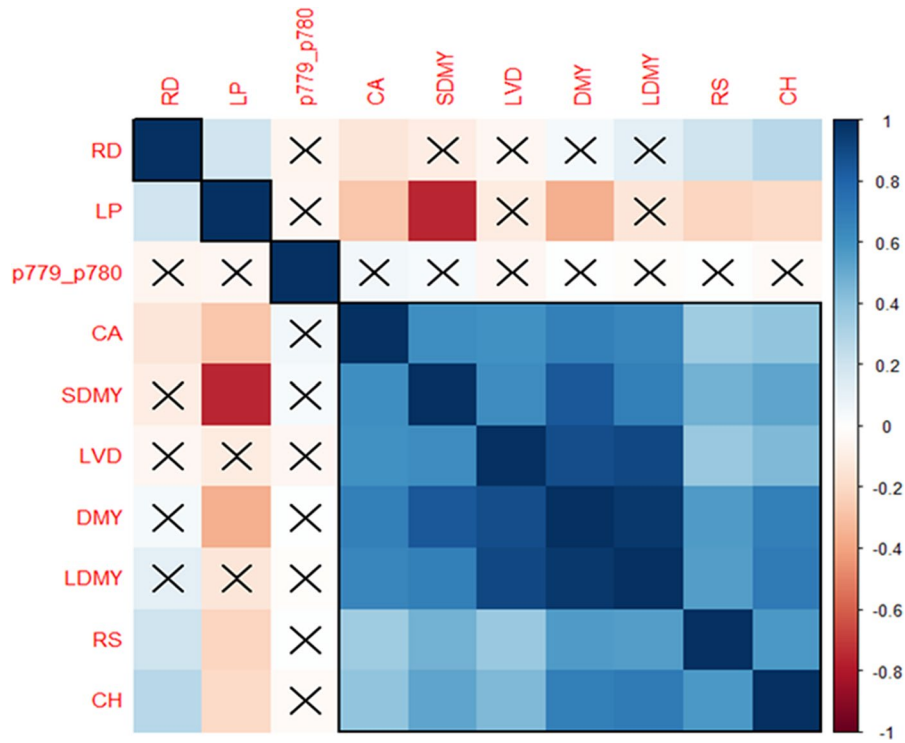

Fig. 4 Heatmap of correlation between the marker p779/p780 and diverse agronomic traits in an F1 guineagrass population. X: absence of significance with 95% of probability. *DMY* dry matter yield, *LDMY* leaf dry matter yield, *SDMY* stem dry matter yield, *CH* canopy height, *LP* leaf percentage, *LVD* leaf volumetric density, *RD* regrowth density, *RS* regrowth speed, *CA* clump area

The second group included traits related to forage production (DMY, LDMY, SDMY), canopy height, clump area, regrowth speed, and forage density, all of which were positively correlated. The third group comprised leaf percentage and regrowth density, which also showed a positive correlation. These results are noteworthy as they demonstrate that the marker was not associated with key agronomic traits for guineagrass breeding, while further reinforcing previously established correlations between related traits in the species.

Direct and indirect selection responses by using MAS with p779/p780 marker

Selection strategies 1 and 2 utilized the molecular marker exclusively as a selection criterion, as detailed in Table 4. Using this method in the biparental population, 62 hybrids were classified as apomictic and 51 as sexuals. Among the selected hybrids, nine were false positives (misclassified as apomictic) and six were false negatives (misclassified as sexual), as their classifications were not validated by conventional methods.

The indirect selection responses on agronomic traits after applying MAS were assessed by comparing the means and corresponding confidence intervals of the selected hybrids for each strategy with the population mean. No significant differences were observed in the agronomic trait means between the selected hybrids and unselected population, which was expected given the lack of correlation between the molecular marker and agronomic traits. Thus, the use of the p779/p780 marker effectively selected genotypes with the desirable mode of reproduction without affecting agronomic traits.

Strategy 3, which employed selection based solely on a selection index for key agronomic traits, was implemented to represent the conventional selection approach that does not incorporate marker-assisted selection (MAS) for mode of reproduction. Under this strategy, nearly all evaluated traits exhibited significant increases in their mean values relative to the overall population mean. Notably, the indirect selection response concerning mode of reproduction yielded a nearly balanced distribution of sexual (n = 14) and apomictic (n = 12) hybrids. This outcome suggests that selection based exclusively on agronomic performance does not favor either mode of

Table 4 Averages of the unselected and of the selected genotypes after applying different selection strategies with and without the p779/p780 in a guineagrass biparental population

Selection strategy[a]	No. genotypes	Traits								
		MR	Agronomic[b]							
			DMY	LDMY	SDMY	CH	LP	LVD	RD	RS	CA
Unselected	113	mix	719 [702–736]	528 [517–540]	171 [164–177]	83 [81–84]	78 [77–78]	7.1 [6.8–7.4]	61 [59–64]	5.4 [5.3–5.5]	0.20 [0.19–0.20]
MAS-1 (1)	62	apo	711 [688–734]	523 [507–538]	169 [161–178]	82 [80–83]	78 [77–78]	7.0 [6.6–7.4]	60 [57–64]	5.4 [5.2–5.5]	0.20 [0.19–0.20]
MAS-0 (2)	51	sex	729 [706–752]	536 [520–551]	172 [163–181]	83 [82–85]	78 [77–78]	7.3 [6.8–7.7]	62 [59–66]	5.4 [5.3–5.6]	0.20 [0.19–0.20]
Index-IS (3)	26	mix	820 [784–855]	619 [595–643]	179 [165–192]	88 [85–90]	79 [78–80]	8.1 [7.5–8.8]	65 [60–70]	5.8 [5.6–6.0]	0.21 [0.19–0.22]
MAS-1 + IS (4)	12	apo	850 [798–903]	647 [612–682]	184 [164–204]	88 [84–92]	79 [78–80]	8.4 [7.4–9.4]	64 [56–71]	5.8 [5.4–6.1]	0.21 [0.19–0.23]
MAS-0 + IS (5)	14	sex	793 [744–842]	595 [562–628]	175 [156–193]	88 [84–91]	79 [78–80]	7.9 [7.0–8.8]	67 [59–74]	5.8 [5.5–6.1]	0.20 [0.19–0.22]

[a]MAS-1 and MAS-0 refers to the marker-assisted selection with p779/p780 for apomictic and sexual hybrids, respectively; and Index-IS refers to the selection based on the selection index only

[b]*DMY* total dry matter yield (g plant^{-1}), *LDMY* leaf dry matter yield (g plant^{-1}), *SDMY* stems dry matter yield (g plant^{-1}), *CH* canopy height (cm), *LP* leaf percentage, *RD* regrowth density (%), *LDMD* leaf density (g/cm), *RS* regrowth speed (cm/day), *CA* clump area (cm^2), *MR* mode of reproduction

reproduction, thereby resulting in an approximately equal representation of sexual and apomictic genotypes among the selected individuals.

In strategies 4 and 5, where the marker was combined with the selection index, the selection process identified only apomictic hybrids (12) in strategy 4 and sexual hybrids (14) in strategy 5. When comparing with mean trait values of the selected hybrids in strategy 3, no significant effect of the marker was observed on the means of the top selected hybrids in strategies 4 and 5. Among the hybrids selected for apomictic mode of reproduction, three were false positives, while two false negatives were identified among the hybrids selected for sexual mode of reproduction. These results were anticipated, given the presence of false positive rate (FPR) and false negative rate (FNR) by using the marker p779/p780.

Discussion

The findings of this study provide a clear insight into the potential of the p779/p780 molecular marker as a tool for improving guineagrass breeding programs. The significant association of the marker with the mode of reproduction suggests its reliability in distinguishing between sexual and apomictic genotypes. Moreover, its lack of association with critical agronomic traits such as forage production further supports its value, as it does not interfere with the selection of important traits. This characteristic establishes the marker as the most promising candidate for application in guineagrass breeding programs, as it enables the selection of the preferred mode of reproduction without adversely affecting key agronomic trait performance.

The p779/p780 marker is strongly associated with parthenogenesis within the apospory-specific genomic region (ASGR) in several *Cenchrus/Pennisetum* species, including *Brachiaria/Urochloa* and guineagrass. Its utility in distinguishing between sexual and apomictic genotypes has been well documented in tropical forage species, with previous studies demonstrating consistent and reliable performance (Conner et al. 2015; Worthington et al. 2016, 2019). The findings of the present study further validate the effectiveness of the p779/p780 marker in guineagrass, confirming its high classification accuracy (>0.9) for identifying apomictic individuals. However, the presence of misclassified genotypes—both false positives and false negatives—indicates that the marker is not perfectly linked to the gene(s) governing the mode of reproduction in guineagrass. This observation suggests the possibility of recombination events between the marker and the target gene(s), or alternatively, the existence of secondary loci influencing apomixis in this species. These findings highlight the complexity of the genetic control of apomixis in guineagrass and underscore the need for further investigation to fully elucidate the underlying mechanisms.

The significant association with the mode of reproduction in Embrapa's breeding program reinforces the marker's potential as an effective diagnostic tool in guineagrass. Notably, the marker was found to be robust across various genotypes (accessions, hybrids), crosses, and different breeding stages, demonstrating its wide applicability. In addition, the p779/p780 marker outperforms other markers identified in previous studies, such as those reported by Bluma-Marques et al. (2014), which exhibited lower selective efficiency in different crosses. This makes the p779/p780 marker the most efficient tool for detecting the mode of reproduction in guineagrass, thereby contributing significantly to the advancement of marker-assisted selection (MAS) strategies in this species.

Genetic correlations between traits may arise due to pleiotropy or linkage disequilibrium among genes (Lynch and Walsh 1998). In the present study, the p779/p780 marker showed no significant correlation with any agronomically relevant traits. This suggests that the genomic region controlling apomixis in guineagrass neither harbors pleiotropic genes affecting both mode of reproduction and agronomic traits, nor is in linkage disequilibrium with loci associated with those traits. A similar pattern was observed in *Paspalum simplex*, where no significant associations were found between the mode of reproduction (linked to a SCAR marker) and morphological or agronomic traits (Brugnoli et al. 2019). However, a contrasting result was reported by Deo et al. (2020) in a study with guineagrass, which identified QTLs in linkage group 2—the same region as the apomixis locus—associated with both mode of reproduction and agronomic or nutritive value traits. While this could theoretically support a genetic correlation, the study did not present direct correlation estimates between the marker and the traits. Thus, the findings of the present work provide evidence that, in this dataset, the p779/

p780 marker functions independently of agronomic performance, reinforcing its potential as a selective tool for mode of reproduction without introducing confounding selection pressures on key traits.

Another line of evidence supporting the independence between agronomic traits and mode of reproduction is provided by the analysis of Hybrids-2. Despite undergoing two selection phases targeting distinct agronomic traits, the ratio of sexual to apomictic hybrids was statistically non-significant. This stability suggests that the selection process did not influence the mode of reproduction. Further empirical evidence from the breeding program corroborates this finding, confirming that no significant relationship exists between mode of reproduction and key agronomic traits. Collectively, these results suggest that apomixis can be effectively utilized as a genotype fixation strategy, with minimal impact on the selection of other important agronomic traits in the breeding of the species.

Regarding selection based solely on the p779/780 marker in the biparental population, the results indicated a response to selection exclusively for the mode of reproduction trait (Strategies 1 and 2). In contrast, selection based solely on the selection index (Strategy 3) resulted in a response to selection only for agronomic traits. In this case, the selected hybrids exhibited a mix of sexual and apomictic hybrids, maintaining the expected genetic model ratio of 1:1 for mode of reproduction, as observed in previous studies (Savidan 1981; Ebina et al. 2005; Bluma-Marques et al. 2014; Deo et al. 2020). This outcome aligns with practices currently adopted in the breeding program. These results can be explained by the lack of significant correlation between the marker and the agronomic traits, as demonstrated in earlier findings.

The selection of candidates for apomictic cultivars or sexual parents must take into account multiple traits, including the mode of reproduction. A key advantage of combining molecular marker selection with the genotypic index (Strategies 4 and 5) is that only hybrids possessing all the traits of interest will progress to the next stage of selection. For example, if the goal is to select only hybrids suitable for cultivar development (apomictic) in our biparental population, only 12 hybrids would advance to the next phase, compared to 26 hybrids selected using the index alone. This approach enables an approximate 50% reduction in evaluation costs or, alternatively, allows for the inclusion of twice as many desirable genotypes (apomictic or sexual) in subsequent selection phases, thereby enhancing the probability of identifying superior genotypes.

Although the p779/p780 marker demonstrated high accuracy, the continuous search for additional molecular markers remains essential. As observed in the biparental population, the presence of false positives highlights the need for conventional techniques to verify the mode of reproduction in later selection phases. The ideal scenario would be for the classification model to achieve 100% accuracy, eliminating the need for confirmatory assessments. Given that apomixis is governed by multiple processes—including apomeiosis, parthenogenesis, and pseudogamy (Kaushal et al. 2019)—it is plausible that additional genomic regions contribute to its regulation. Further investigation is warranted to elucidate the complete genetic architecture underlying apomixis in guineagrass.

Although the limited number of pistils evaluated in the present study precluded quantification of the extent of facultative apomixis in the apomictic hybrids, its occurrence was nevertheless confirmed. Embryo sacs originating from sexual processes were observed in the apomictic hybrids. A more detailed understanding of the inheritance of facultative apomixis could improve the efficiency of selective processes in guineagrass, enabling the development of strategies that produce apomictic hybrids with a low rate of sexuality. However, this aspect was not the focus of the current study.

Applications in guineagrass breeding

The application of the p779/p780 marker offers substantial advantages in guineagrass breeding programs, including reduced time, costs, and labor, while expanding the scale of hybrid evaluation. One of the main applications of this marker is in the early selection of apomictic hybrid candidates for cultivar development from biparental crosses. In this strategy, seeds from a specific cross are germinated, and seedlings are used to collect DNA for marker screening. After performing marker-assisted selection for the mode of reproduction, only hybrids exhibiting the desired band are advanced to the next selection phases. As demonstrated in this study, this approach allows for the evaluation of twice as many apomictic

hybrids with the same resources compared to strategies that do not employ marker-assisted selection, thereby enhancing the probability of success in the breeding program. Notably, the p779/p780 marker offers the advantage that selection for mode of reproduction does not negatively impact agronomic traits, as shown by the results of the selection strategies (1 and 2) presented in Table 4.

The p779/p780 marker can also be effectively applied to facilitate recurrent selection strategies, as proposed by Worthington and Miles (2015), aimed at exploiting heterosis in apomictic species. In this strategy, two populations (A and B), derived from distinct heterotic groups, are created by crossing sexual and apomictic plants within the same heterotic group. Notably, the mode of reproduction will segregate in a 1:1 ratio (sexual: apomictic) within each population, following the theoretical model of monogenic control for the trait. After sowing the seeds from these crosses, a representative sample of plants from each population is evaluated for mode of reproduction at the seedling stage using the p779/p780 marker. For example, 200 plants may be considered, although this number could vary based on the program's evaluation capacity. At this stage, the sexual plants (100) from each population (A and B) are selected and prepared for random crosses with two apomictic plants from a different population. This results in interpopulational hybrids: sexual A × apomictic B and sexual B × apomictic A. Following this, after sowing 20 interpopulational hybrids from each cross, a total of 8000 hybrids are obtained. These hybrids undergo analysis for mode of reproduction using the marker. According to the expected segregation, 4000 of these will be apomictic. These apomictic hybrids are then evaluated for agronomic traits across multiple environments in experiments with replications. After these evaluations, the best-performing interpopulational apomictic hybrids (A × B and B × A) are selected for advancement in the cultivar development process. The apomictic and sexual parents of these hybrids in each population are recombined (A sexual × A apomictic or B sexual × B apomictic) to form new base populations A and B, which are improved and segregate for mode of reproduction.

It is crucial to emphasize that, in this last scheme, the logistics required to evaluate the mode of reproduction trait can only be effectively managed through the use of molecular markers, due to the need for early, large-scale assessment of the mode of reproduction.

Final considerations

The p779/p780 marker demonstrated monogenic inheritance and is strongly associated with the mode of reproduction in guineagrass, making it a valuable tool for various breeding strategies. Additionally, the marker is not associated with the main agronomic traits, such as forage production, regrowth, and plant structure traits. These characteristics make the p779/p780 marker an excellent choice for use in guineagrass breeding programs.

However, it is important to highlight that, despite its high accuracy, the presence of false positive and false negative results indicates the need for further studies to determine whether markers perfectly linked to the apomixis locus exist, or if other secondary loci are controlling the trait. Additionally, it is essential to retrain the logistic model to incorporate new evaluations using both conventional techniques and the p779/p780 marker. This retraining is particularly crucial when incorporating data from hybrids derived from parents not previously used in model training, as they may represent new genetic patterns.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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