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Original Paper

Construction of AFLP-based cosegregation groups of tetraploid Plicatula species and identification of markers linked to apomixis

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Abstract

Most species of Plicatula are important native forages. This work aimed to build framework cosegregation groups of the apomictic tetraploid race of *Paspalum guenoarum* cv. Rojas and localize the locus controlling apomixis in the species. An interspecific population derived from crossing a completely sexual tetraploid plant of *P. plicatulum* and an apomictic tetraploid individual of *P. guenoarum* cv. Rojas was used. Both, disomic and tetrasomic inheritance were detected in both parental genotypes. In *P. guenoarum*, ten cosegregation groups were built, including 50 markers expanding for 583 cM. The estimated genome coverage was 63.95%. The apomixis locus was located in the linkage group M8, together with seven other loci (four paternal and three biparental markers). The group extended for 59 cM. The four paternal markers showed strong linkage to apomixis, and two of them mapped at 4 and 7 cM at both sides of the locus. Five female linkage groups were constructed with markers segregating from *P. plicatulum*. One of them (F3) being homologous to the male group carrying apomixis. The linkage groups presented here constitute the first genetic frame for species of Plicatula group. Moreover, molecular markers linked to apomixis in *P. guenoarum* can assist fundamental research and breeding programs.

Key words: apomixis, apospory, cv. Rojas, genetic mapping, Plicatula group.

Resumen

La mayoría de las especies de Plicatula son importantes forrajeras nativas. El objetivo de este trabajo fue construir grupos de cosegregación marco de la raza tetraploide apomíctica de *Paspalum guenoarum* cv. Rojas y localizar el locus que controla la apomixis en la especie. Se empleó una población interespecífica, derivada del cruzamiento entre una planta tetraploide completamente sexual de *P. plicatulum* y un individuo tetraploide apomíctico de *P. guenoarum* cv. Rojas. En ambos parentales se observó tanto herencia disómica como tetrasómica. En *P. guenoarum* se construyeron diez grupos de cosegregación, incluyendo 50 marcadores distribuidos en 583 cM. La cobertura estimada del genoma fue de 63,95 %. El locus de la apomixis se localizó en el grupo de ligamiento M8, junto a otros siete loci (cuatro marcadores paternos y tres biparentales), distribuidos en 59 cM. Los cuatro marcadores paternos mostraron fuerte ligamiento a la apomixis, y dos de ellos mapearon a 4 y 7 cM a ambos lados del locus. Se construyeron cinco grupos de ligamiento femeninos con marcadores segregantes de *P. plicatulum*, uno de ellos (F3) homólogo al grupo masculino que porta la apomixis. Los grupos de ligamiento que aquí se presentan constituyen el primer marco genético para especies del grupo Plicatula. Además, los marcadores moleculares ligados a la apomixis en *P. guenoarum* serán útiles a la investigación básica y a los programas de mejoramiento.

Palabras clave: apomixis, aposporia, cv. Rojas, mapeo genético, grupo Plicatula.

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Introduction

Plicatula is one of the taxonomic informal groups within the subgenus *Paspalum sensu stricto* (Chase 1929). It includes species morphologically related to *P. plicatulum* Michx., which is characterized by spikelets with transversely wrinkled lemma and shining dark-brown anthecium (Quarin *et al*. 1997). This group contains about 30 species, most of which are tetraploid and apomictic, though several of them have sexual diploid counterparts (Aguilera *et al*. 2011). Most species of Plicatula are important native forages and some of them like *P. guenoarum* Arechav. (Ramírez 1954; Urbani & Quarin 2002; Urbani *et al*. 2002; Evers & Burson 2004), *P. plicatulum* (Oram 1990) and *P. atratum* Swallen (Kretschmer *et al*. 1994; Quarin *et al*. 1997; Urbani & Quarin 1997) have become cultivated pastures in subtropical regions of the Americas. Nevertheless, because of the apomictic mode of reproduction, all released cultivars were selected from natural ecotypes without any genetic improvement through breeding.

"Apomixis" in angiosperms means asexual (agamic) reproduction by seeds, *i.e.*, "agamospermy" ("seed apomixis") (Nogler 1984). This mode of reproduction leads to the generation of maternal offspring which normally are genetically exact replicas of the mother plant (Nogler 1984). Apomixis is a heritable trait that guarantees clonal propagation of any genetic combination through seeds without loss of heterosis (Spillane *et al*. 2004). Moreover, apomixis eliminates the restrictions imposed by meiosis on the fertility of wide hybrids. Therefore, genetically distant progenitors can be crossed allowing full exploitation of the available germplasm (Spillane *et al*. 2004). Breeding programs based on this technology are currently being conducted in naturally apomictic forage grasses of *Paspalum* (Acuña *et al*. 2011; Novo *et al*. 2017, 2020), as well as in other apomictic grasses (Miles 2007). In most *Paspalum* species apomixis is of aposporous type, characterized by the formation of non-reduced embryo-sacs from somatic cells of the nucellus, the parthenogenetic development of the embryo and the endosperm formation after the fertilization of the non-reduced polar nuclei by a sperm cell (pseudogamy) (Ortiz *et al*. 2013).

The generation of two autotetraploid completely sexual plants (4*c*-4*x* and 7*d*-4*x*) of *P. plicatulum* by colchicine treatment of diploid seeds (Sartor *et al*. 2009), allowed to perform intra and interspecific crosses between species of the Plicatula group at the tetraploid level. Crosses between 4PT (a clone from 4*c*-4*x*) with a natural apomictic tetraploid individual of *P. guenoarum* cv. Rojas GR19 yielded a population of 182 inter-specific hybrids that segregate for the mode of reproduction in a 1:1.6 ratio of apomictic vs. sexual hybrids (Aguilera *et al*. 2015). This outcome indicated that the inheritance of apomixis in the Plicatula group fit with the general model proposed in other *Paspalum* species, in which apomixis is transmitted as a monogenic dominant factor with distorted segregation ratios (Martínez *et al*. 2001; Pupilli *et al*. 2001; Ortiz *et al*. 2013). Following these first analyses, several other inter-specific segregating populations were obtained from crosses between 4PT and other species of this group (Novo *et al*. 2013, 2016, 2017, 2019, 2020). All these works open the possibility for generating improved cultivars of Plicatula species by combining hybridization and apomixis. For this goal, the availability of molecular markers linked to the trait is a fundamental tool for diminishing the cost and time of the breeding programs. Genetic maps of the apomixis-controlling genomic region (ACR) were reported for several species of *Paspalum* including *P. notatum* Flüggé, *P. simplex* Morong, *P. malacophyllum* Doell. and *P. procurrens* Quarin (Pupilli *et al*. 2001, 2004; Martínez *et al*. 2003; Stein *et al*. 2004, 2007; Hojsgaard *et al*. 2011). These analyses concluded that ACR is located in a conserved chromosome segment characterized by strong repression of recombination, various extent of synteny with the subtelomeric part of rice chromosome 12 long-arm and structural features of heterochromatin, i.e. repetitive elements, gene degeneration and gene expression deregulation (Calderini *et al*. 2006; Podio *et al*. 2012; Siena *et al*. 2016).

The objectives of this work were *i*) to assess the general (disomic or polysomic) inheritance type of tetraploid races of *P. plicatulum* and *P. guenoarum*; *ii*) to build cosegregation groups of *P. guenoarum* by using single dose AFLP markers; and *iii*) to identify molecular markers linked to apomixis locus.

Material and Methods

Plant material

A tetraploid F_1 mapping family of 91 hybrids (55 sexual and 36 apomicts) was used (Fig. 1). These plants are part of a larger population segregating for the mode of reproduction obtained

by crossing a completely sexual tetraploid (2*n* = 4*x* = 40) genotype of *P. plicatulum* (4PT) and one individual (GR19) of the tetraploid (2*n* = 4*x* = 40) apomictic cultivar (cv.) Rojas of *P. guenoarum* (Aguilera *et al*. 2015). The plant 4PT is a vegetatively propagated clone of 4*c*-4*x,* an experimentally-generated completely sexual tetraploid individual of *P. plicatulum* (Sartor *et al*. 2009). The mode of reproduction (apomixis/sexuality) of each F_1 hybrid was determined by using the flow cytometric analysis of individual seeds by Aguilera *et al*. (2015).

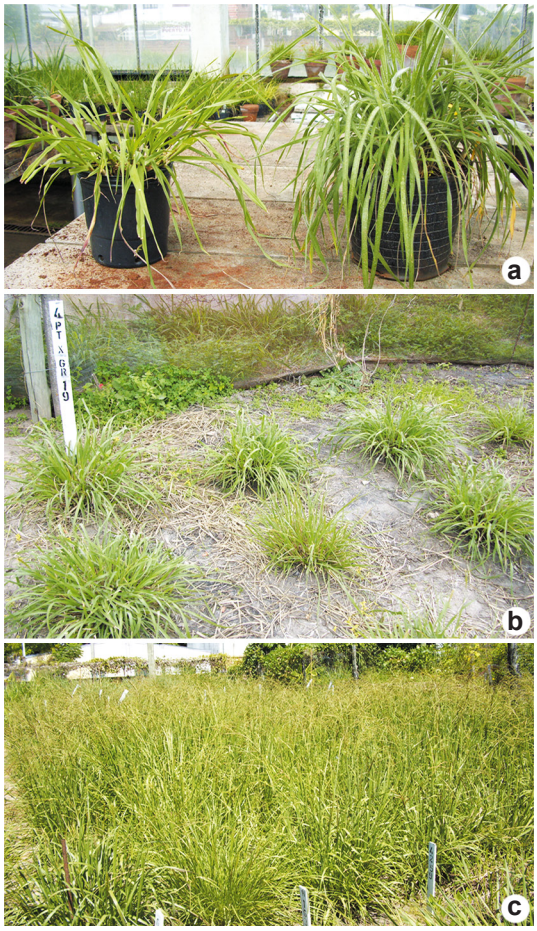

Figure 1 – a-c. Parental genotypes and the F_1 mapping population – a. the apomictic tetraploid pollen donor parent GR19 on the left, and the completely sexual tetraploid pistillate parent 4PT, on the right, cultivated at the greenhouse; b. some of the F_1 individuals growing at the field a few weeks after transplanting; c. the F_1 mapping population during the flowering period. Note the variablity in the phenotypic semblance of the parents (a) and in the F_1 individuals (b), and the general appearance of F_1 individuals with interesting forage traits (c).

AFLP markers procedure and BSA

Genomic DNA was isolated from 0.4 g of fresh leaves of the parental genotypes and F_1 hybrids, following the method of Dellaporta *et al*. (1983) with the modifications of Ortiz *et al*. (1997). Sample quality controls were performed by measuring absorbance at 260 and 280 nm and electrophoresis at 25 Volts in 1% TAE 1X agarose gels. DNA samples were stored at a final concentration of 0.3 μg/μl at -20 °C. Amplified fragment-length polymorphism (AFLP) markers were generated according to Vos *et al*. (1995) with the modifications described as follows. Genomic DNA (1 μg) was simultaneously digested with 5 units (U) of each *Eco*RI (Promega, USA) and *Mse*I (New England BioLabs, USA) restriction enzimes, 1X OPA buffer, 5 mM of 1,4-dithiothreithol (DTT, ICN Lab.), 50 ng/μl of bovine serum albumin solution (BSA, New England BioLabs), and sterile distilled water in a final volume of 25 μl, at 37 °C for 4 h, and at 72 °C for 10 minutes for stopping digestion. For *Eco*RI and *Mse*I adapters generation, 10 μl of single strand *Eco*RI forward (5'-CTC GTG ACT GCG TAC C-3') and reverse (5'-AAT TGG TAC GCA GTC-3') and 20 μl of single strand *Mse*I forward (5'-GAC GAT GAG TCC TGA G-3') and reverse (5'-TAC TCA GGA CTC ATC-3') oligonucleotides (IDT, USA) were mixed separately, incubated at 96 °C for 10 min, and at room temperature for 30 min. Both double strand adapters were diluted with distilled water at 50 pM/μl. Then *Eco*RI adapters were diluted (1:10) to a final concentration of 5 pM/μl. After digestion, 7.5 μl of adapter ligation solution (10 mM ATP, 5X OPA buffer, 250 mM DTT, 10 μg/μl BSA), 5 pM *Mse*I adapter, 0.5 pM *Eco*RI adapter, 15U T4 DNA ligase (Bioneer) and 0.425 μl of distilled water for a final volume of 10 μl were added to each digestion sample. The ligation mixture was incubated overnight at room temperature. Pre-amplification reactions were performed with *Eco*RI (E) and *Mse*I (M) primers designed by University of British Columbia, Canada (UBC) carrying one selective base (E01 5'-GAC TGC GTA CCA ATT CA-3' and M01 5'-GAT GAG TCC TGA GTA AA-3', respectively). Then selective amplifications were carried out using E and M primer combinations from UBC with three selective bases (sequences available at <https://wheat.pw.usda.gov/ggpages/keygeneAFLPs.html>). A screening for informative

primer combinations was carried out by performing bulked segregant analysis (BSA) (Michelmore *et al*. 1991). Bulks were prepared by combining 2.5 μg of DNA (300 ng/μl) from each 10 sexual (sexual bulk) and each 10 apomictic (apomict bulk) hybrids. For selective amplification, DNA from both parental genotypes and sexual and apomictic bulks were assayed with 48 E/M primer combinations (E31M31-M37, E31M39, E31M42, E32M31-M37, E32M39, E32M42, E33M31-M33, E33M37-M38, E34M32-M35, E34M37-M39, E34M42, E35M32-M35, E35M37, E35M39, E35M42, E36M32-M35, E36M37-M39, E38M32, E38M38-M39). Thereafter, primer pairs showing polymorphisms between parental genotypes and/or detecting polymorphisms between bulks were assayed over the 91 F_1 individuals of the mapping family. Amplification reactions were performed using a Bio-Rad thermocycler (MyCycler Thermal cycler #170-9701). For the pre-amplification step, PCR reactions were performed for 20 cycles with the following cycle profile: a 30 s DNA denaturation step at 94 °C, a 60 s annealing step at 56 °C, and a 60 s extension step at 72 °C (Vos *et al*. 1995), including 2 μl of the ligation mix, 0.2 mM of dNTPs solution, 1.2 ng/μl of each E01 and M01 primer, 1U of GoTaq DNA polymerase and 1X Taq buffer (all final concentrations) (Promega), and distilled water to a final volume of 25 μl (Ortiz *et al*. 2001). Pre-amplification products were diluted (1:10) and used as templates for selective amplifications. Selective amplification cycles were carried out at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s 1 time, then 12 cycles (cycle 2–13) in which the annealing temperature was reduced by 0.7 °C in each cycle, and 23 cycles (cycle 14–36) of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s (Vos *et al*. 1995), including 2 μl of the preamplification, 0.2 mM of dNTPs solution, 1.2 ng/μl of each E and M primer, 1U of GoTaq DNA polymerase and 1X Taq buffer (all final concentrations) (Promega), and UP water to a final volume of 10 μl (Ortiz *et al*. 2001). After amplification, 2 μl of loading dye (95% formamide, 10 mM NaOH, 0.05% bromophenol blue and 0.05% xylene cyanol) were added to the PCR products, denatured at 95 °C for 5 min and immediately placed on ice. Polyacrylamide gels were prepared according to Sambrook *et al*. (1989) with the following modifications: for preparing 6% gels an acrylamide: bisacrylamide (19:1, 40% w/v) stock solution, 7M urea, 0.5X TBE buffer, and distilled water. From this solution, 50 ml were mixed with 391.66 μl of 10% ammonium persulfate (APS) solution, and 39.16 μl of N,N,N',N'-tetrametiletilenodiamina (temed) for about 50 ml of 6% polyacrylamide gel. The gel was pre-electrophoresed at constant power of 60 W, at 50 °C for 2 h. Then, 5.5 μl of the denatured samples and 2.5 μl of the molecular weight marker (Biodynamics, Argentina) were loaded onto denaturing 6% polyacrylamide gels and electrophoresis was carried out at by applying a constant power of 60 W at a temperature of 50 °C for 1 h 20 min using a Scie-Plas electrophoresis cell connected to a PowerPac/3000 power supply (Espinoza *et al*. 2006). Amplification products were visualized by using silver nitrate following the Silver Staining-System protocol of Promega (Promega, USA) and digitized using an HP Scanjet 4670 scanner (Hewlett-Packard).

Data recording and segregation analyses

All polymorphic AFLP bands were scored as present (relevant allele), or absent (non-detected allele) in 4PT, GR19, sexual and apomictic bulks and each F_1 individual. Our tetraploid F_1 mapping family was considered as a pseudo-testcross population derived from tetraploid heterozygous non-inbred parents, in which loci with different types of segregation can be observed (Ritter *et al*. 1990). A 1:1 (presence:absence) segregation ratio was expected for polymorphic markers segregating from one parent present in single-dose (single-dose amplification fragments, SDAFs, Aaaa), regardless the type of inheritance disomic or polysomic of the species. Polymorphic markers in double-dose (double-dose amplification fragments, DDAFs, AAaa) are expected to segregate in 3:1/1:0 or 5:1, in cases of disomic or polysomic inheritance, respectively (Wu *et al*. 1992). Moreover, segregating markers from both parental genotypes (biparental single-dose fragments, BSDFs, Aaaa x Aaaa), will segregate with an expected ratio of 3:1 (presence:absence), either for disomic or polysomic inheritance (Ritter *et al*. 1990). The apomixis trait was considered as controlled by a single-allele of a dominant locus segregating from the male parent with a slight distorted segregation ratio 1:1.6 (apomixis:sexuality) (Aguilera *et al*. 2015). In all cases, a Chi-square (χ^2) test was applied to determine the goodness of fit (at $P < 0.01$) between the observed and the expected number of genotypes for each class of segregation ratio. Polymorphic fragments were named by a four-digit number designating the specific *Eco*RI and *Mse*I primers,

respectively used in the selective amplification, followed by a final number to differentiate fragments derived from the same amplification.

Determination of inheritance type and construction of cosegregation groups

Markers with segregation ratios of DDAFs were used for determining the type of inheritance prevailing in the parental individuals. Segregation of DDAFs that fit with a 3:1 ratio was considered as the result of disomic inheritance, *i.e.* as derived from an allopolyploid individual. DDAFs with segregation ratios that fit with a 5:1 value were assumed as derived from polysomic inheritance, *i.e.* as originated from an autopolyploid individual (Wu *et al*. 1992). The mapping strategy was based on the detection of SDAFs proposed by Wu *et al*. (1992) and the linkage analysis was carried out using the computer software JoinMap v.3.0 (Van Ooijen & Voorrips 2001). Genotypes were codified according to a cross pollinated population type (CP) with linkage phases unknown (Van Ooijen & Voorrips 2001). In order to examine the information derived from both parents, segregation data from 4PT and GR19 were analyzed independently. Accordingly, two different data files were created, each containing the genotypic information of the 91 F_1 individuals for specific SDAFs (1:1), plus all BSDFs (3:1) (Ritter *et al*. 1990). In addition, SDAFs segregating from GR19 that fit to the 1:1.6 ratio were included in the paternal data set. Map units in centimorgan (cM) were derived from the Kosambi (1944) mapping function. The cosegregation (linkage) groups were determined by two-points linkage analysis varying the LOD score values between 6.0–2.0 and a recombination frequency θ = 0.45 [the maximum detectable recombination fraction (MaxR) for coupling markers in a population of 91 individuals from an autotetraploid species (Wu *et al*. 1992)]. Within each cosegregation group, marker positions were determined at a threshold LOD = 0.50. Mapped BSDFs were used to identify the putative female and male homologous cosegregation groups (Maliepaard *et al*. 1998) and, when possible built a join map between male and female linkage groups. Female and male linkage groups were named as F or M respectively, followed by a number indicative of each group. To calculate the genome coverage in the male parent, the observed and expected genome lengths were compared. The observed genome length (Go) was calculated adding the length of all linkage groups, while the expected genome length (Ge) was estimated by multiplying the length of each linkage group by the factor f = (m+1)/(m-1), where m is the number of loci in each linkage group of the map. Then, the total expected genome length was estimated as the sum of Ge from all linkage groups. Finally, the genome coverage was calculated as the ratio of (Go / Ge) x 100 (Chakravarti *et al*. 1991).

Results and Discussion

Screening of AFLP markers and segregation analysis

In order to speed the identification of informative markers screening of primer combinations was coupled with a bulked segregant analysis (BSA, Michelmore *et al*. 1991). Briefly, a total of 48 E/M primer pairs combinations were assayed over 4PT, GR19, a sexual bulk and an apomictic bulk. This initial search allowed generating more than 3,200 total AFLP markers of which about 650 were polymorphic between the parents (present in 4PT and absent in GR19, or viceversa) (Fig. 2). The mean percentage of polymorphism between parents was 20.32%. Of this group of polymorphic markers, 250 (38.46%) segregated from the female parent 4PT, and 400 bands (61.54%) were from the male parent (GR19). Based on these results, 17 primer combinations producing clear amplification patterns, detecting polymorphism among 4PT and GR19, and showing specific bands of the apomictic parent and bulk, were selected for assaying in the mapping population.

Analysis of 17 informative AFLP primer combinations (E31M32, E31M33, E31M35, E31M42, E32M33, E32M39, E33M32, E33M33, E34M32, E34M42, E35M32, E35M33, E35M34, E35M42, E36M32, E36M33, and E36M38) in the mapping family produced 348 fragments, including maternal, paternal and biparental markers, of which 226 were polymorphic between parents. On average, 20.47 segregating markers (5.00 maternal, 8.29 paternal, and 7.17 biparental) per primer pair were obtained. A total of 85 maternal, 141 paternal and 120 biparental markers were obtained. Since the mapping population used in this work was a tetraploid F_1 family derived from non-inbred and highly heterozygous parents, different allelic configurations were expected (Ritter *et al*. 1990). Accordingly, the segregation ratio of each marker in the mapping family was analyzed to detect SDAFs and DDAFs segregating from 4PT and GR19, as well as, BSDFs segregating from both parents. The total number of markers scored in each class

of segregation ratio is shown in Table 1. In 4PT, three different types of segregating markers were scored. One group segregated as SDAFs (1:1), other group showed presence/absence values congruent with DDAFs for both disomic (3:1) and polisomic (5:1/3:1 or 5:1) and a third group showed distorted ratios that did not fit to any of the expected values (Tab. 1). In the male parent (GR19), four categories were discriminated, SDAFs that exclusively fit to 1:1 ratio, DDAFs segregating in 5:1, 3:1 ratios or both, distorted markers that did not fit with any expected value, and distorted markers that agree with 1:1.6 ratio (Tab. 1). The latest group was included in the analysis because the 1:1.6 ratio was observed for the segregation of apomixis vs sexuality in a 4PT x GR19 F_1 family (Aguilera *et al*. 2015). Finally, out of the 122 bands segregating from both parents, almost half of them were classified as BSDFs, fitting to a 3:1 ratio (Tab. 1). These results indicate that most polymorphic bands segregated as SDAFs or BSDFs revealing, as expected, a high heterozygosity in both (4PT and GR19) parental genotypes. Interestingly, a number of DDAF segregating exclusively in 5:1, as well as, in 5:1 or 3:1 ratio from each parental plant was detected. A segregation ratio of 5:1 in a tetraploid cytotype can only by explained by polysomic inheritance (Wu *et al*. 1992), *i.e.*, considering

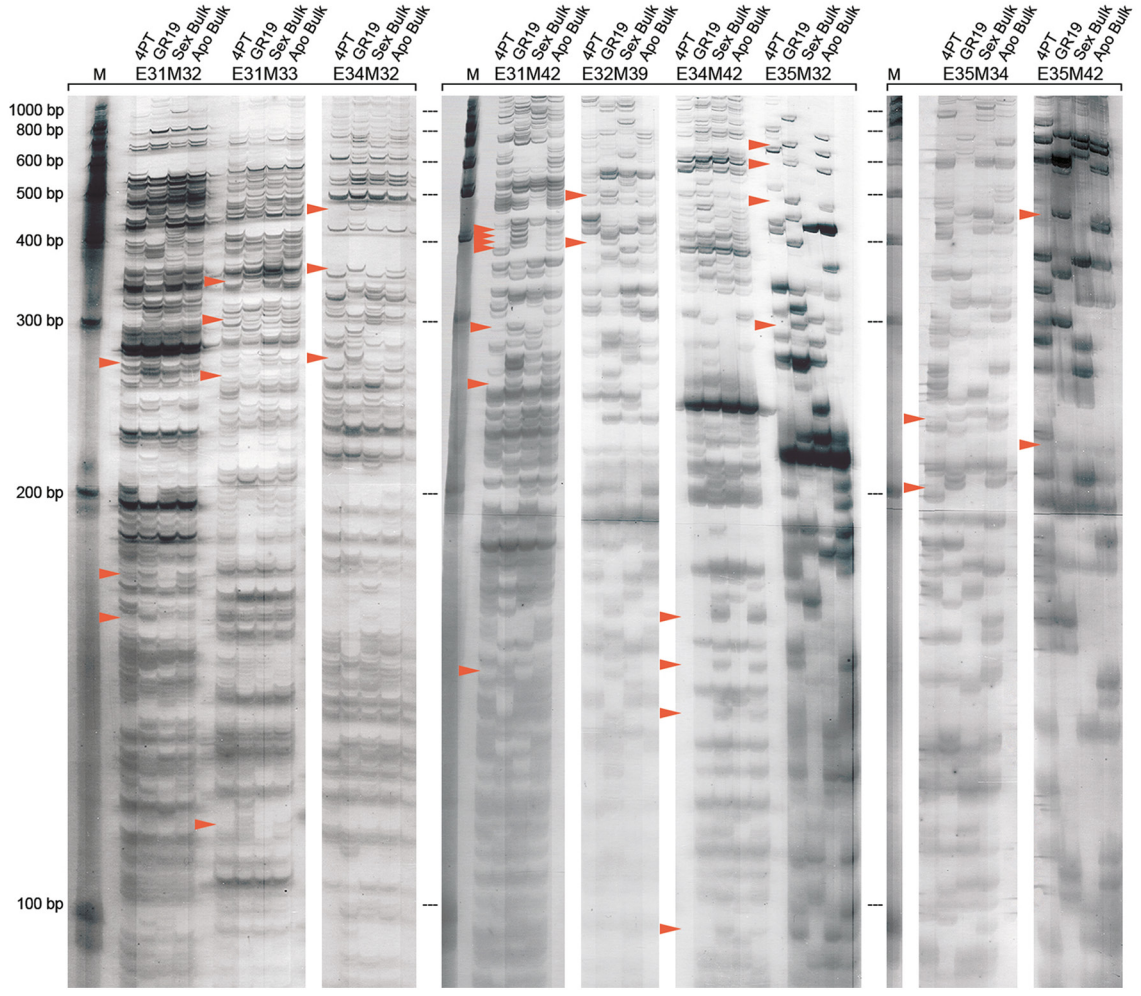

Figure 2 – BSA carried out by assaying different *Eco*RI (E) and *Mse*I (M) selective primer combinations. Silver stained polyacrylamide gels visualizing amplification patterns for the parental genotypes (4PT and GR19) and sexual and apomictic bulks (Sex and Apo Bulk), using nine E-M primer combinations. M = molecular weight marker. Arrow heads indicate molecular markers putatively linked to apomixis locus. Square brackets indicate primer combinations assayed simultaneously in a same polyacrylamide gel.

Table 1 – Number of markers scored in each class of segregation ratio. SDAF = single-dose amplification fragments; DDAF = double-dose amplification fragments; BSDF = biparental single-dose amplification fragments; BMDF = biparental multiple-dose amplification fragments; a = distorted, *i.e.* markers that not fit to any of the expected segregation ratios at $P < 0.01$; b = markers that fit simultaneously to both segregation ratios at $P < 0.01$.

Type of marker	Allelic configuration and expected presence: absence segregation ratios						Total
	SDAF		DDAF		Dist.[a]		
	1:1 (%)	5:1 (%)	5:1/3:1[b] (%)	3:1 (%)	1:1.6 (%)	Other	
Maternal	44 (51.77)	3 (3.53)	14 (16.47)	10 (11.76)	-	14 (16.47)	85
Paternal	70 (49.30)	4 (2.82)	11 (7.75)	15 (10.56)	25 (17.60)	17 (11.97)	142
Biparental	BSDF		BMDF		Dist.[a]		
	3:1 (%)		(11:1/7:1) + (35:1/15:1) (%)				
	59 (48.37)		49 (40.16)		14 (11.47)		122

the random assortment of the four homologs. In addition, markers segregating exclusively in a 3:1 ratio must be derived from disomic inheritance (Wu *et al*. 1992), *i.e.* pairing between only two chromosomes of the four. Besides, those markers that showed segregation values not different from 5:1 to 3:1 ratio might correspond to any of the two classes. Overall, these results suggest that both polysomic and disomic inheritances are present in both 4PT and GR19 genotypes. This outcome is in agreement with previous observation of chromosome pairing at meiosis that suggested an autotetraploid origin of the species (Burson & Bennett 1971; Aguilera *et al*. 2011). Accordingly, bi- to multivalent chromosome associations are expected and thus some genome regions could present disomic or polysomic inheritance, as was also detected in other species of *Paspalum* (Stein *et al*. 2004). Moreover, it was shown recently that the quadrivalent formation is these species are rare, being bivalents the more frequent associations (Novo *et al*. 2019).

Construction of linkage groups and mapping apomixis locus

The linkage analysis in tetraploid *P. guenoarum* cv. Rojas GR19 was based on a total of 154 markers, including 70 paternal SDAFs (1:1 and 1:1.6 ratios), 24 distorted paternal SDAFs that fit exclusively to 1:1.6, the apomictic trait, and 59 BSDFs segregating from both parents (Tab. 1). Cosegregation groups were determined by two-points linkage analysis at LOD score values between 6.0–2.0 and recombination frequency $\theta = 0.45$. Using these parameters, 48 markers were distributed in 10 linkage groups (LGs M1 to 10) with three or more markers in each one. For most of the cosegregation groups sufficient linkage information allowed group markers into genetic maps. Solely for group M10 insufficient linkage information prevented from including all markers on the map at one step. So, initially a group of three markers was fixed. Then, two additional markers were added maintaining the setting parameters (LOD = 6.0–2.0, $\theta = 0.45$). No weak nor suspect linkages were detected across the 10 linkage groups, therefore, a set of well-supported cosegregation groups of GR19, including 50 loci (28 SDAFs, 21 BSDFs and apomixis) were built (Fig. 3). The mean distance between markers was 14.57 cM (1 to 34 cM), and the total recombination length span over 583 cM. The mean length of linkage groups was 58.30 cM (7–102 cM) and the mean number of markers per group was five (3–10 markers). One-hundred and two markers remained unlinked. The estimation

of the genome size resulted in 911.71 cM, and thus the genome coverage by the recombination map of GR19 was about of 63.95%. Although the coverage of this first analysis is still reduced, the number of linkage groups defined in the analysis is coincident with the basic chromosome number of the species (n = 10). Thus, some groups could represent genomic regions well scattered over the whole genome. Interesting, out of 17 AFLP primer combinations assayed over the mapping population, four (E31M32, E31M42, E34M32, and E34M42) generated markers cosegregating with the apomixis locus (Fig. 3). The cosegregation group carrying the trait (M8) spanned on about 59 cM, and was defined by seven AFLP loci, including four paternal SDAFs and three biparental BSDFs. All SDAFs mapping in this group showed a distorted segregation that fit to 1:1.6 ratio. This outcome indicated that the inheritance of apomixis in the Plicatula group fit with the model proposed for other *Paspalum* species, in which the apomictic donor (*i.e. P. guenoarum*) carry a simplex (Aaaa) dominant factor responsible for the trait, and the sexual female parent (*i.e. P. plicatulum*) is a null or homozygous recessive for apomixis locus (aaaa) (Martínez *et al*. 2001; Pupilli *et al*. 2001; Ortiz *et al*. 2013). In addition, segregation distortion affecting the transmission of apomixis was also evidenced, although it appears to be less obvious that informed by other species (Zilli *et al*. 2015). Apomixis locus resulted tightly linked to markers M344220 (LOD = 18.67, θ = 0.04, 4 cM) (Figs. 3; 4), M314229 (LOD = 13.52, θ = 0.09, 7 cM), and loosed linked to markers M343235 (LOD = 10.65, θ = 0.11, 13 cM), and M313247 (LOD = 8.15, θ = 0.17, 15 cM) (Fig. 3). Therefore, although a close association between markers and apomixis was found, some extend of recombination around apomixis locus was observed in *P. guenoarum*. Similarly, recombinant molecular markers at both side of apomixis locus were reported in *Brachiaria* spp. (Pessino *et al*. 1997, 1998). Molecular markers linked to apomixis were also detected in other species of *Paspalum* (Pupilli *et al*. 2001; Martínez *et al*. 2003*;* Stein *et al*. 2004, 2007; Hojsgaard *et al*. 2011), buffelgrass [*Cenchrus ciliaris* L., syn. *Pennisetum ciliare* (L.) Link] (Jessup *et al*. 2003), Guineagrass [*Megathyrsus maximus* (Jacq.) B. K. Simon & S. W. L. Jacobs, syn. *Panicum maximum* Jacq.] (Ebina *et al*. 2005) and Kentucky bluegrass (*Poa pratensis* L.) (Porceddu *et al*. 2002). Particularly, sequencing of BAC (bacterial artificial chromosomes) clones bearing molecular markers completely linked to apospory in *P. simplex*, and chromosomal walking from markers cosegregating with the trait in *P. notatum* were performed to reveal genes included in the ACR. Those analyses revealed several putative protein-coding regions and a large number of highly repetitive sequences (Ortiz *et al*. 2017). However, although genetic studies suggest that apomixis in *Paspalum* spp. is controlled by a single genomic region, the inherent characteristics

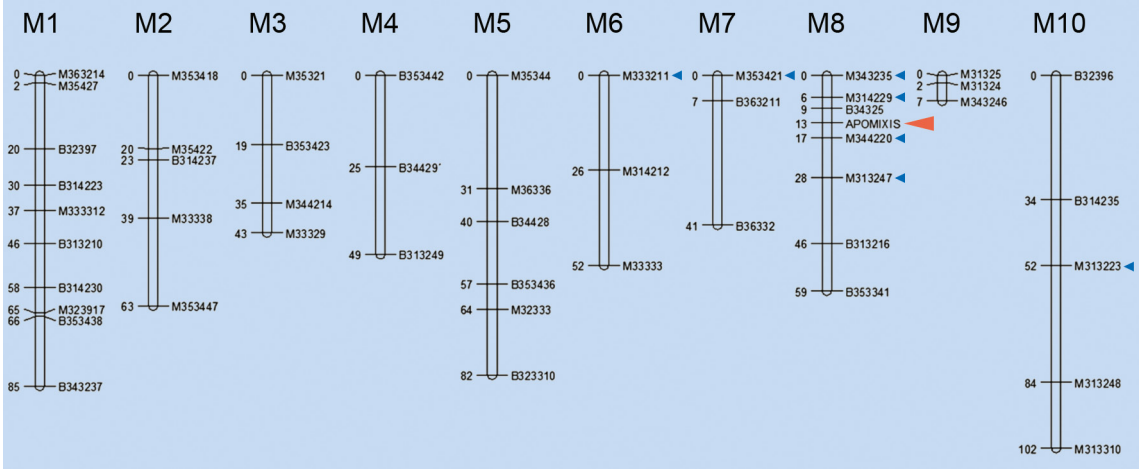

Figure 3 – Genetic linkage groups of apomictic tetraploid (2n = 4x = 40) *Paspalum guenoarum* cv. Rojas. Male linkage groups are indicated as M1 to M10. Distances in cM (Kosambi) and markers' names are indicated on the left and the right of each group, respectively. One biggest arrowhead (orange) indicates the apomixis locus on linkage group M8. Minor arrow heads (blue) point AFLP markers that fit to 1:1.6 ratio.

of the ACR hinder the identification of trait's key determinants through positional mapping strategies (Ortiz *et al*. 2020). More recently, a floral reference transcriptome of apomictic and sexual *P. notatum* was produced in order to investigate putative apomixis candidate genes previously identified, as well as reveal molecular routs involved in this asexual reproductive mode (Ortiz *et al*. 2017).

On the other hand, segregating data corresponding to the sexual tetraploid female parent *P. plicatulum* 4PT encompassed a total of 103 markers, including 44 maternal SDAFs and 59 BSDFs. Likewise, in GR19, the linkage groups were defined at LOD = 6.0–2.0 and $\theta = 0.45$. Using these parameters only five linkage groups with three or more markers could be identified (LGs F1 to 5). Only 28 markers, including 15 maternal SDAFs and 13 BSDFs, were included in linkage groups. The total genetic distance covered in this case was only 307 cM (Fig. 5). Finally, in order to determine putative homologous linkage groups among GR19 and 4PT, markers segregating from both parents as BSDFs (3:1) were used as "allelic bridges" (Maliepaard *et al*. 1998). A total of 21

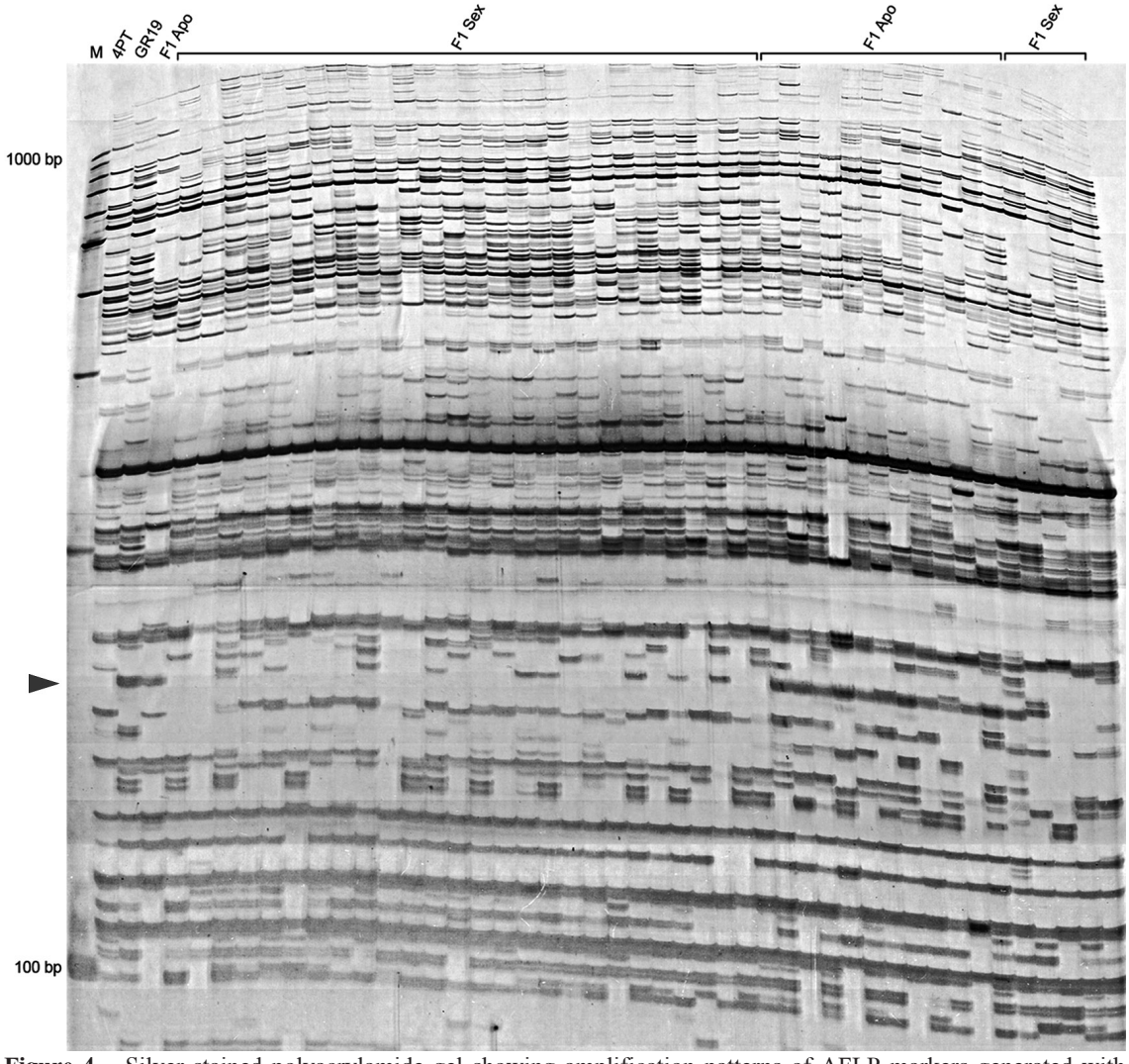

Figure 4 – Silver stained polyacrylamide gel showing amplification patterns of AFLP markers generated with E34M42 primer pair on parental genotypes (4PT and GR19) and sexual and apomictic F_1 hybrids. M = molecular weight marker. F_1 Apo: apomictic F_1 individuals. F_1 Sex: sexual F_1 individuals. Arrow indicates a band present in GR19 and in apomictic descendants, but absent in 4PT and sexual hybrids. The AFLP maker mapped at 4 cM from apomixis locus.

and 13 BSDFs were mapped to eight paternal (M1-M5, M7-M8, M10) and five maternal (F1-F5) cosegregation groups, respectively (Figs. 3, 5). Linkage group's homology was assumed when the same BSDFs were shared by a male and a female linkage group. Following this criterion 12 BSDFs mapped simultaneously on four paternal (M1, M4, M5, M8) and four maternal (F1, F3, F4, F5) groups were identified. The corresponding male/female homologous linkage groups were: M4-F1-M1-F5, M5-F4 and M8-F3 (Fig. 6a). Interestingly, three biparental markers of the female group F3 (B34325, B353341 and B313216) were shared with the linkage group carrying the apomixis locus (M8) (Fig. 6a). The female group F3 is characterized by six markers (three SDAFs and three biparental) over 63 cM. This association marks the *P. plicatulum* linkage group related to apomixis locus and could be of interest for further investigations. In most cases, the relative order of the markers was well conserved, supporting the homology relationships. Then join (maternal and paternal) maps were constructed with the aid of program JoinMap 3.0 combining linkage information of groups carrying at least two common markers. This analysis allowed to build four combined cosgegregation groups (M4 + F1, M1+F5, M5+F4 and M8+F3) (Fig. 6b). The information on homology relationships between *P. guenoarum* GR19 and 4PT is significant for breeding programs. Recently new interspecific

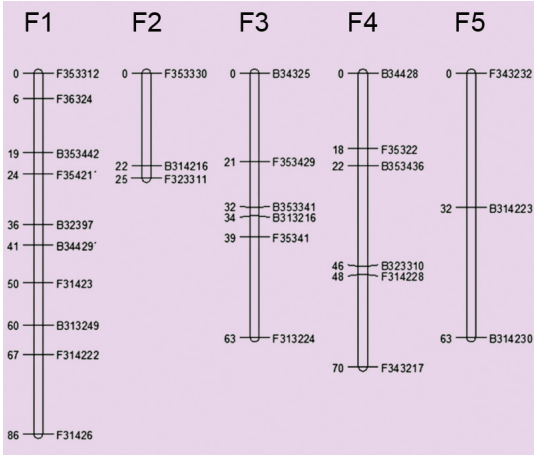

Figure 5 – Five female linkage (cosegregation) groups of sexual tetraploid ($2n = 4x = 40$) *Paspalum plicatulum* genotype 4PT indicated as F1 to F5. Distances in cM (Kosambi) and markers' names are indicated on the left and the right of each group, respectively.

families have been created by crossing 4PT with diverse apomictic *P. guenoarum* genotypes and other Plicatula species as pollen donors (Novo *et al*. 2017). Moreover, several apomictic hybrids derived from these crosses have been selected based on valuable agronomic characteristics, i.e. frost tolerance, persistence and cattle preference with the final objective of developing superior apomictic lines (Novo *et al*. 2017). In this sense, the determination of homology relationships between maternal and paternal groups would help to saturate the linkage groups of sexual and apomictic strains and identify markers linked to interesting agronomic traits segregating from both parents.

Although a high level of polymorphisms was detected between parents of the mapping cross, the amount of SDAFs segregating from 4PT was not sufficient to achieve a significant coverage of the maternal genome. Probably, several markers corresponded to non-segregating loci (*i.e.* AAAA or AAAa) that have been arisen as result of the autopoliploidization. In contrast, in the male parent the set of SDAFs detected enabled the construction of 10 linkage groups which cover about two-thirds of the paternal genome. In both cases, and considering the constrains associated with the mapping in polyploids, additional cosegregation groups should be detected by incorporating new SDAFs for covering the chromosome complement of both genotypes.

The present study adds information about the genetic structure of Plicatula species and the apomixis locus in this group of grasses. Information on the type of inheritance based on molecular data revealed that both disomic and polysomic inheritances can be expected in *P. plicatulum* and *P. guenoarum*. The preliminary linkage groups of *P. guenoarum* can be used as a genetic framework for basic and applied studies in species of the Plicatula group, several of which are currently included in breeding schemes. In particular, the identification of molecular markers linked to apomixis locus will help the discrimination of hybrid progenies by their mode of reproduction at early stages of development and thus, reducing the time and cost of conducing undesirable (sexual) individuals to maturity in breeding programs. Moreover, the putative female homologous of the linkage group carrying the trait was also identified. The results presented here would be useful for fundamental

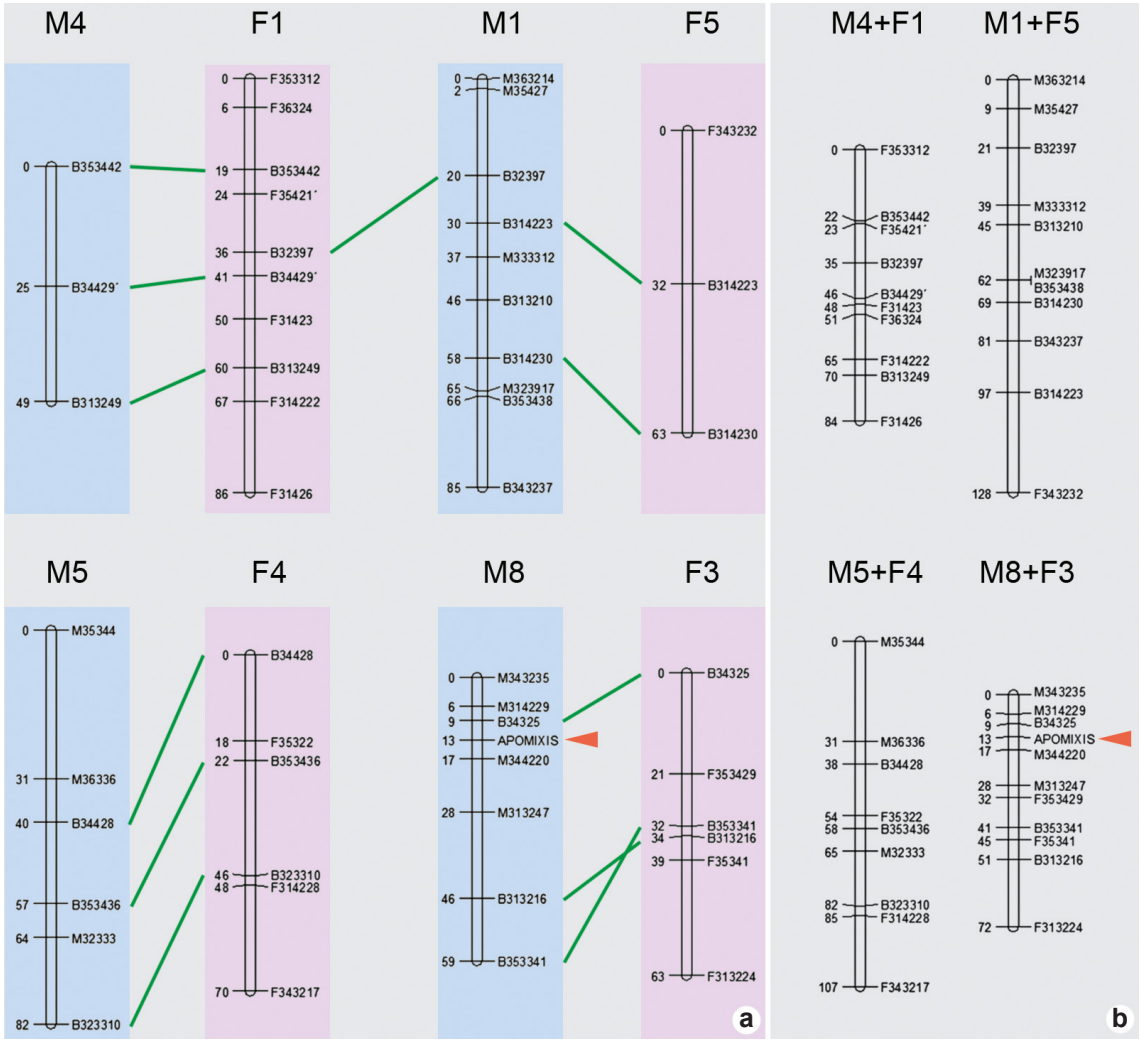

Figure 6 – a-b. Identification of putative homologous male (GR19) and female (4PT) linkage groups – a. homologous groups sharing common markers (M4 and F1, M1 and F1, M1 and F5, M5 and F4, and M8 and F3). Lines connect the same biparental marker in the different male and female linkage groups (allelic bridges); b. join maps of male and female homologous groups M4 and F1, M1 and F5 (above), M5 and F4, and M8 and F3 (below). Arrowheads indicate the aposporous apomixis locus on linkage group M8 and on the M8 and F3 join map.

genetic studies concerning apomixis, as well as, for breeding programs involving tetraploid cytotypes of these and other species of the Plicatula group of *Paspalum*.

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