A parthenogenesis gene candidate and evidence for segmental allopolyploidy in apomictic

*Brachiaria decumbens*

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Running title

Parthenogenesis gene candidate in apomictic *Brachiaria*

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Abstract

Apomixis, asexual reproduction through seed, enables breeders to identify and faithfully propagate superior heterozygous genotypes by seed without the disadvantages of vegetative propagation or the expense and complexity of hybrid seed production. The availability of new tools such as genotyping-by-sequencing and bioinformatics pipelines for species lacking reference genomes now makes the construction of dense maps possible in apomictic species, despite complications including polyploidy, multisomic inheritance, self-incompatibility, and high levels of heterozygosity. In this study we developed saturated linkage maps for the maternal and paternal genomes of an interspecific *Brachiaria ruziziensis* (R. Germ. and C.M. Evrard) x *B. decumbens* Stapf. F₁ mapping population in order to identify markers linked to apomixis. High resolution molecular karyotyping and comparative genomics with (*Setaria italica* (L.) P. Beauv) provided conclusive evidence for segmental allopolyploidy in *B. decumbens*, with strong preferential pairing of homologs across the genome and multisomic segregation relatively more common in chromosome 8. The apospory specific genetic region (ASGR) was mapped to a region of reduced recombination on *B. decumbens* chromosome 5. The *Pennisetum squamulatum* (L.) R.Br. *PsASGR-BABY BOOM-like (psASGR-BBML)* specific primer pair p779/p780 was in perfect linkage with the ASGR in the F₁ mapping population and diagnostic for reproductive mode in a diversity panel of known sexual and apomict *Brachiaria* (Trin.) Griseb. and *P. maximum* Jacq. germplasm accessions and cultivars. These findings indicate that *ASGR-BBML* gene sequences are highly conserved across the Paniceae and add further support for the postulation of the *ASGR-BBML* as candidate genes for the apomictic function of parthenogenesis.
Apomixis, asexual reproduction through seed, results in progeny that are genetically identical to the female parent (Asker and Jerling 1992). Apomictic reproduction is found naturally in many economically important forage grass genera and is highly desirable in the sense that superior heterozygous genotypes can be propagated faithfully through seed over many generations without the expense and difficulty of hybrid seed production from inbred parental lines or vegetative propagation. Apospory, a common form of apomixis found in Paniceae grass genera including *Brachiaria* (Trin.) Griseb. (syn. *Urochloa*), *Cenchrus L./Pennisetum* Rich., *Panicum L.* (syn. *Megathrysus*), and *Paspalum* L. involves two sequential processes. An unreduced embryo sac first develops from an adjacent somatic nucellar cell (apomeiosis), which then develops into a viable embryo without fertilization (parthenogenesis) (Barcaccia and Albertini 2013; Hand and Koltunow 2014). Recently, the *ASGR-BBML* gene family have been postulated as candidate genes for parthenogenesis in the genus *Cenchrus/Pennisetum* (Conner et al. 2015). However, the role of the *ASGR-BBML* has yet to be established in other apomictic species and many questions remain regarding the role of apomixis in genomic stability and polyploid evolution. To date these questions have largely been addressed through cytogenetic studies (e.g. Mendes-Bonato et al. 2002) or population genetics (e.g. Akiyama et al. 2011; Lovell et al. 2013). The development of dense genetic maps in apomictic species would complement these studies and facilitate molecular breeding in tropical forages, but factors including lack of investment, self-incompatibility, multisomic inheritance, and high levels of heterozygosity have thus far prevented the construction of saturated linkage maps in polyploid apomicts.
Brachiaria forage grasses are widely sown across the global, and especially American tropics, where they have drastically increased the efficiency of cattle production, particularly in areas with marginal soils (Miles et al. 2004). Brachiaria grasses are most economically important in Brazil, where they are planted on 99 Mha, accounting for 85 percent of sown pasture land (Jank et al. 2014). In addition to extensive pasture systems in Latin America, Brachiaria is also planted in intensive smallholder systems in Africa and Asia (Hare et al. 2013; Khan et al. 2014; Maass et al. 2015). The three most important commercial species, Brachiaria brizantha (A. Rich.) Stapf (palisadegrass), B. decumbens Stapf (signalgrass), and B. humidicola (Rendle) Schweick (koroniviagrass) exist primarily as apomicts with varying levels of polyploidy, though diploid sexual genotypes are also found in nature (Valle and Savidan 1996). Brachiaria brizantha and B. decumbens form an agamic complex with a diploid sexual species of lesser commercial importance, B. ruziziensis Germain & Evrard (ruzigrass) (2n = 2x = 18) (Lutts et al. 1991). Microsporogenesis occurs normally in apomictic plants; therefore, apomictic genotypes can be crossed to sexual plants as pollen donors to generate progeny segregating for reproductive mode. Thus, the development of a synthetic autotetraploid sexual (2n = 4x =36) B. ruziziensis genotype through colchicine doubling (Swenne et al. 1981) facilitated recombination between sexual plants and tetraploid apomictic Brachiaria pollen donors and enabled the establishment of breeding programs at the International Center for Tropical Agriculture (CIAT) and Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) [Brazilian Enterprise for Agricultural Research] in the late 1980s (Miles 2007).

Efficient and reliable discrimination between apomictic and sexual offspring is of paramount importance to Brachiaria breeding programs because only apomict genotypes can be released as true-breeding cultivars (Miles 2007). Apospory is usually inherited as a single
dominant Mendelian factor denoted as the "apospory-specific genomic region" (ASGR) (Ozias-Akins and van Dijk 2007). Thus, the progeny generated from a cross between a sexual female parent and an apomictic pollen donor are expected to segregate for reproductive mode on a 1:1 basis. Embryo sac analysis and progeny tests can be used to assess individuals in segregating populations for reproductive mode, but both are time consuming and expensive. In contrast, molecular markers tightly linked to apomixis can be used to inexpensively and rapidly assess the reproductive mode of thousands of segregating progeny at the seedling stage (Worthington and Miles 2015).

Marker assisted selection (MAS) for apomixis has been used routinely in the CIAT Brachiaria breeding program since 2009, providing considerable savings in time and money used to establish sexual hybrids unsuitable for further use in the breeding program (Worthington and Miles 2015). The marker used to screen new hybrids is a Sequenced Characterized Amplified Region (SCAR) marker named ‘N14’ developed from a Random Amplified Polymorphic DNA (RAPD) marker linked to apomixis in B. decumbens CIAT 606 (cv. Basilisk) and B. brizantha CIAT 26646 (cv. La libertad) (Pedraza Garcia 1995). N14 is diagnostic for apomixis when CIAT 606 is used as the pollen parent, but performs inconsistently in populations derived from crosses with some apomict accessions of B. decumbens and B. brizantha and does not amplify in the more distantly related commercial species B. humidicola (unpublished data).

Parallel mapping efforts at EMPRAPA with Amplified Fragment Length Polymorphism (AFLP) markers and Restriction Fragment Length Polymorphism (RFLP) probes developed in maize and rice showed that apomixis in B. brizantha CIAT 6294 (cv. Marundu) was linked to a total of nine markers. Four linked markers were syntenic with maize chromosome 5 and two markers were syntenic with rice chromosome 2, which in turn is also mostly syntenic with maize chromosome
5 (Pessino et al. 1997, 1998). Another recent study identified a RAPD marker linked to apomixis in *B. humidicola* (Zorzatto et al. 2010). However, CIAT researchers were unable to generate bands linked to apomixis in segregating *B. humidicola* crosses using the published primers.

Apomixis research is relatively more advanced in the closely related Paniceae genus and *Cenchrus/Pennisetum*. In *Pennisetum squamulatum* (L.) R. Br. (syn. *Cenchrus squamulatus*), the ASGR has been mapped to a physically large hemizygous region of reduced recombination (Ozias-Akins et al. 1998). Sequence analysis of bacterial artificial chromosome (BAC) clones in *P. squamulatum* revealed the presence of multiple copies of the *PsASGR-BABY BOOM-like* (*PsASGR-BBML*) gene in the ASGR region (Conner et al. 2008). The *ASGR-BBML* genes have been posited as candidate genes for parthenogenesis in *Pennisetum* and *Cenchrus* based on multiple lines of evidence including similarity to *BABY BOOM* (*BBM*) genes associated with somatic embryogenesis in *Brassica* and *Arabidopsis* (Boutilier 2002), failed parthenogenesis in a recombinant *C. ciliaris* plant lacking *CcASGR-BBML* genes (Conner et al. 2013), reduced parthenogenetic embryo development in apomictic F$_1$ RNAi transgenic plants with reduced *PsASGR-BBML* expression (Conner et al. 2015), and induced parthenogenesis and production of haploid offspring in transgenic sexual pearl millet plants expressing *PsASGR-BBML* (Conner et al. 2015). The primer pair p779/p780 was developed from sequences in the 4$^{th}$ and 7$^{th}$ exons of *ASGR-BBM-like2* and amplifies a region including three introns of 95 bp, 266 bp, and 154 bp (Akiyama et al. 2011). This primer pair was linked to the ASGR in F$_1$ populations developed with *P. squamulatum* and *C. ciliaris* as apomictic pollen parents and validated in a diversity panel of apomictic and sexual *Pennisetum* and *Cenchrus* species, where it amplified in all apomictic species but no sexual species (Akiyama et al. 2011). The presence of *ASGR-BBML*
genes in other aposporous Paniceae genera and potential of p779/p780 as a diagnostic marker for apomixis in tropical forage grass breeding programs has yet to be tested.

Genetic framework maps have been constructed with AFLP and RFLP markers in several aposporous Paniceae genera including Cenchrus (Jessup et al. 2003), Panicum (Ebina et al. 2005), Paspalum (Stein et al. 2007), and Brachiaria (Thaikua et al. 2016). However, these maps are generally poorly saturated, with fewer linkage groups than expected for a pseudo-testcross map with a separate linkage group for each chromosome or an excess of very sparse and/or short linkage groups. Some efforts have been made to assess homology among linkage groups and assess the prevalence of preferential pairing in polyploid species (Jessup et al. 2003; Stein et al. 2007). While tetrasomic inheritance was proposed for Paspalum notatum Flügge (Stein et al. 2007), preferential (disomic) chromosome pairing was also identified in certain chromosomal segments including the apospory linkage group (Stein et al. 2004). Jessup et al. (2002) also found evidence for disomic inheritance of the apospory linkage group in Cenchrus ciliaris L. However, the ratio of repulsion- versus coupling-phase linkages in the maternal and paternal genetic maps suggests that C. ciliaris is a segmental allopolyploid with substantial variation in the relative prevalence of multisomic and disomic pairing among parental genotypes (Jessup et al. 2003). Evidence regarding ploidy and chromosomal pairing in Brachiaria is limited to cytogenetic studies. Bivalent chromosome associations were predominant in a panel of B. brizantha accessions and in B. decumbens CIAT 606, however, a low frequency of multivalent associations in all tested polyploids suggested that they may be segmental allopolyploids composed of partially homologous sub-genomes (Mendes-Bonato 2002; Mendes-Bonato et al. 2002).
Recent advances such as genotyping-by-sequencing (GBS) and bioinformatics pipelines for species lacking reference genomes make the construction of dense maps possible in polyploid apomict species despite their complicated genomics. Genotyping-by-sequencing is a high-throughput genotyping platform which uses a reduced representation library strategy to reduce the genome to a subset of restriction enzyme recognition sites and integrate single nucleotide polymorphism (SNP) discovery and genotype calling into a single step (Elshire et al. 2011). Genotyping-by-sequencing has been used to generate saturated genetic maps in highly heterozygous clonally propagated specialty crops with few molecular resources such as red raspberry (Rubus idaeus subsp. idaeus L.) (Ward et al. 2013) and is much more cost effective for developing high-density linkage maps than fixed SNP arrays in specialty crops. This is especially true for multisomic polyploids, where only the fraction of markers with simplex segregation patterns can be mapped using a pseudo-testcross strategy in software designed for diploid species (Van Ooijen 2011). Recently GBS was also used to construct a saturated genetic linkage map in an autotetraploid alfalfa (Medicago sativa L.) F<sub>1</sub> mapping population (Li et al. 2014) using a pseudo-testcross strategy, demonstrating the effectiveness of this approach in multisomic polyploid species.

The principal objective of this study was to develop the first saturated linkage maps of polyploid apomict species using GBS in an interspecific B. ruziziensis x B. decumbens (2n = 4x = 36) F<sub>1</sub> mapping population (n = 169) segregating for reproductive mode. These saturated maps were then used to assess synteny with foxtail millet (Setaria italica (L.) P. Beauv), a diploid sexual species in the tribe Paniceae, and evaluate meiotic interactions among homologs and homeologs by molecular karyotyping. Lastly, the genetic mapping population and a diversity panel of Brachiaria and Panicum accessions with known reproductive mode were used to
identify flanking markers linked to the ASGR in *B. decumbens* and test for conservation of
*ASGR-BBML* genes across the Paniceae using the primer pair p779/p780.

**Methods**

**Evaluation of reproductive mode**

Parents and F₁ progeny of the BRX 44-02 (*B. ruziziensis*) x CIAT 606 (*B. decumbens*) mapping population were classified as apomictic or sexual by cytoembryological observation of benzyl benzoate:dibutyl phthalate cleared pistils using differential interference contrast (DIC) microscopy following Crane and Carman (1987) with minor modifications. At least 30 pistils with normally developed embryo sacs were evaluated for each of the 167 F₁ progeny assessed for reproductive mode. Progeny with only Polygonum type embryo sac development were scored as sexual, while progeny with any pistils that had enlarged vacuolated nucellar cells or further Panicum type embryo sac development were scored as apomictic.

**GBS and SNP genotype calling**

Genotyping-by-sequencing libraries were prepared following Heffelfinger *et al.* (2014). The methylation-sensitive restriction enzyme *HincII* (R0103, New England Biolabs), which recognizes a degenerate six base pair sequence, was used for digestion. Libraries were constructed for the 169 F₁ progenies and the two parents and were sequenced as 75 bp paired-end reads on the Illumina HiSeq 2500 in rapid run mode by the Yale Center for Genome Analysis (http://medicine.yale.edu/keck/ycga/index.aspx) following the manufacturer’s protocol. Depths of coverage for each sample are provided in Table S1. *De novo* SNP discovery and genotype calling was conducted using the Tassel 3.0 Universal Network Enabled Analysis Kit (UNEAK)
pipeline (Lu et al. 2013). A greater number of reads are required to make accurate genotypic
calls in tetrasomic polyploid populations than diploid populations. Thus, strict genotype calling
thresholds were employed following the recommendations of Li et al. (2014) in order to reliably
distinguish between homozygotes (AAAA) and triplex heterozygotes (AAAB).

**PCR based marker reactions**

The parents and progeny of the mapping population were also evaluated with the SCAR marker
N14, ASGR-BBML specific primers p779/p780, and six Kompetitive Allele Specific PCR
(KASP) assays (K42517, K62444, K76831, K100912, K171196, and K207542) designed based
on ASGR-linked GBS markers from the CIAT 606 parental map (Table S2, Table S3). The four
most tightly linked KASP markers, N14, and p779/p780 were also used to evaluate a *Brachiaria*
diversity panel composed of *B. brizantha*, *B. decumbens*, *B. ruziziensis*, and *B. humidicola*
accessions with a mixture of apomictic, sexual, and unknown reproductive mode from the CIAT
genetic resources program forages collection and four interspecific apomictic hybrid cultivars.
Five *C. ciliaris* accessions and 10 *P. maximum* accessions with mixed reproductive mode were
also evaluated with N14 and p779/p780.

**Linkage Map Construction**

Separated genetic linkage maps of BRX 44-02 and CIAT 606 were constructed in JoinMap 4.1
following the two-way pseudo-testcross strategy (Van Ooijen 2011). Markers that were
heterozygous in only one parent, had less than 20% missing data, and had a segregation ratio of
heterozygote to homozygote progeny of less than 2:1 were classified as single-dose allele (SDA)
markers and used in map construction. Markers that were heterozygous in BRX 44-02 and fit the
expected 5:1 segregation ratio for double-dose allele (DDA) markers were used to identify
homologs and in the maternal haplotype map and to generate joint linkage maps for *B. ruizienisis* chromosomes 1-9 in TetraploidMap (Hackett *et al.* 2007). Areas of segregation distortion were identified based on deviation of SDA markers from expected allelic ratios according to the $\chi^2$ test following Li *et al.* (2014).

**Synteny analysis and molecular karyotyping**

Extended tag pair sequences of SDA and DDA markers were queried against the foxtail millet genome ([http://www.phytozome.net/foxtailmillet.php](http://www.phytozome.net/foxtailmillet.php)). Markers that aligned to a unique position in the foxtail millet genome ($P < 1e-4$) were used to assign each linkage group to a chromosome and identify homologues. High resolution molecular karyotyping was then used to detect meiotic associations between chromosomal regions with differing degrees of homology and homeology across the paternal CIAT 606 genome following (Mason *et al.* 2014). Chromosome segregation and recombination events in CIAT 606 were also manually inspected by visualization of changes in allele presence or absence along the parental haplotype map in the progeny.

**Data availability**

File S1 contains a list with detailed information for all the supplementary tables and figures. More detailed information about the materials and methods used in this study can be found in File S2. Table S1 provides information on depth of coverage for parents and progeny. KASP primer sequences are given in Table S2. Table S3 contains the genotype scores of p779/p780, N14, KASP assays, and the GBS derived SDA and DDA markers evaluated in the BRX 44-02 x CIAT 606 population. UNEAK sequences of the GBS derived SDA and DDA markers with variant alleles designated as 'query' and 'hit' according to Lu *et al.* (2013) can be found in Table S4. Breeders and scientists interested in applying p779/p780 in their own programs can access
information including primer sequences, PCR conditions, and genetic resources at the Integrated Breeding Program diagnostic marker site ([https://www.integratedbreeding.net/298/breeding-services/predictive-markers?marker=58](https://www.integratedbreeding.net/298/breeding-services/predictive-markers?marker=58)).

**RESULTS**

*Analysis of Reproductive Mode*

The 167 F₁ phenotyped progeny segregated for reproductive mode at a 1:1 ratio ($\chi^2 = 0.006, P = 0.94$) ([Table 1, Table S5](#)), supporting the hypothesis that apospory is inherited as a single dominant genetic factor in *Brachiaria*. A small portion of pistils with abnormal embryo sacs were found in both apomictic and sexual plants ([Table S5, Figure S1J](#)). Progeny classified as sexual had only Polygonum type embryo sacs in all normally developed pistils, while progeny classified as apomictic had normally developed pistils with only Panicum type embryo sacs, only Polygonum type embryo sacs, or with Panicum and Polygonum type embryo sacs together. The average proportion of Panicum type embryo sacs observed in progeny classified as apomicts was 0.57 and ranged from 0.07-1.00 ([Table S5, Figure S2](#)). Apomictic progeny had significantly more embryo sacs per pistil than sexually reproducing progeny ($P < 0.01$). Sexual progeny usually had a single embryo sac per pistil ([Table 1, Table S5](#)), although four sexual plants had a low portion of “twin” embryo sac development. In contrast, plants scored as apomicts often had multiple embryo sacs per pistils ([Table 1, Table S5, Figure S1F-I](#)).

*Genotyping by Sequencing*

After quality filtering and processing with the UNEAK pipeline, a total of 87.4 million of the original 484.1 million sequencing reads ([Table S1](#)) were assigned to 147,496 tag pair sites. After markers with a missing genotype score in either parent were removed, a total of 10,479
polymorphic GBS markers were identified with an average of 44% missing genotype calls per marker in the F$_1$ progeny. Of these markers, 3912 had less than 20% missing genotype calls among the F$_1$ progeny. Within the dataset with a maximum threshold of 20% missing data per marker, a total of 1916 markers (49%) were classified as SDAs. Of the SDA markers, 706 markers were heterozygous in BRX 44-02 and 1210 markers were heterozygous in CIAT 606. A further 281 (8%) of markers in the dataset fit a 5:1 segregation ratio ($\chi^2$, $P < 0.05$) and were classified as DDAs. Two hundred and sixty one of the DDA markers were heterozygous in BRX 4402, while only 20 of the markers were heterozygous in CIAT 606. The ratio of SDA to DDA markers in BRX 44-02 was 3:1, while the SDA:DDA ratio was 61:1 in CIAT 606 (Table S6).

Genetic linkage maps and synteny with foxtail millet

The 706 GBS SNP markers heterozygous in BRX 44-02 were placed in 34 linkage groups, with between three and 71 markers per linkage group and two ungrouped markers (Table 2, Table S7, Figure 1A). The total length of the BRX 44-02 haplotype map was 1985 cM with an average marker density of one per 2.8 cM. The 1210 GBS SNP markers heterozygous in CIAT 606 and markers N14 and p779/p780 were assigned to 36 linkage groups in JoinMap 4.1 (Table 2, Table S7, Figure 1B). The number of markers per linkage group ranged from 14 to 72. The total map length was 2693 cM, with an average of one marker every 2.2 cM.

Two hundred and twelve (30%) and 356 (29%) of the GBS SNP markers heterozygous in BRX 44-02 and CIAT 606 respectively mapped to unique positions on the foxtail millet reference genome at a cutoff E-value of $<1 \times 10^{-5}$ (Figure 2, Table S7). However, the distribution of markers with unique positions on the foxtail millet physical map was uneven across chromosomes. The number of markers mapped to each chromosome of foxtail millet ranged from 33 (chromosome 8)
to 90 (chromosome 3) (Table S7). Using this information we were able identify the four homologous linkage groups chromosomes from the CIAT 606 genetic map corresponding to each of the nine base chromosomes of *B. decumbens* and assign them names based on synteny with diploid foxtail millet (2n = 2x = 18) (Figure 2B).

The BRX 44-02 maternal genetic map had several poorly saturated linkage groups and only 26 of 34 linkage groups with more than two markers that mapped to unique physical positions in a single foxtail millet chromosome (Figure 2A). Therefore, shared linkages with DDA markers were used to complement synteny data and assist in the identification of homologous linkage groups (Table S8). While most *B. ruziziensis* chromosomes had four linkage groups corresponding to each of the four haplotypes (homologous chromosomes), chromosomes 2 and 9 had only three corresponding linkage groups (Table 2, Table S7). One of the two unlinked markers mapped to a unique physical position on foxtail millet chromosome 2 and was linked to DDA marker which also had shared linkage with SDAs mapped to *B. ruziziensis* linkage groups 2 a-c, indicating that it likely is the lone marker from the fourth homolog of *B. ruziziensis* chromosome 2 (Table S8, Table S9; Figure S3). Combined linkage maps for each *B. ruziziensis* chromosome, generated with 50 markers selected from each homologous linkage group and DDAs in linkage with those SDA haplotypes, ranged in length from 83-116 cM (Table S8; Figure S3). Synteny with foxtail millet was conserved in the joint map, indicating that map ordering was correct. (Figure S4).

The only observed reciprocal translocation between *Brachiaria* and foxtail millet was between the proximal tip of chromosome 3 (0-2 Mbp) and the distal tip of chromosome 7 (33-36 Mbp) (Table S7, Table S9, Figure S4, Figure S5). Inversions between the foxtail millet physical map and *B. ruziziensis* and *B. decumbens* genetic maps were relatively more common. A consistent inversion was observed between the proximal arm of foxtail millet chromosome 1 and the CIAT
606 haplotype maps (1a-d) and BRX 44-02 chromosome 1 joint map. Other inversions between the proximal arm of foxtail millet chromosomes 3, 5, and 6 and the BRX 44-02 joint genetic maps of those chromosomes (Table S9, Figure S4) were supported by parallel inversions in at least two of the four CIAT 606 haplotype maps for each chromosome, though the marker density in the remaining haplotype maps was not sufficient to verify whether the inversion was present in all homologs of *B. decumbens* (Table S7, Figure S5).

**Preferential pairing**

A strong peak in segregating allele read frequency (ratio of reads for the segregating allele to total reads) was observed around 0.25 for GBS SDA markers included in the BRX 44-02 maternal haplotype map, as expected for SDA markers in an autotetraploid parent (AAAB) (Figure 3A). In contrast, two peaks at 0.25 and 0.5 were observed for segregating allele read frequency in GBS SDA markers in the CIAT 606 genetic map (Figure 3B). The peak at 0.5 is stronger than the peak at 0.25, indicating that some SNPs are present in all four haplotypes of each chromosome, but the majority of SNPs are found in only one of two differentiated sub-genomes of *B. decumbens*. A third peak in segregating allele read frequency around 0.33 is likely the result of the overlapping tails of the normal curves around peaks at 0.25 and 0.5.

High-resolution molecular karyotyping was used to test each marker pair for significant linkage and segregation, enabling the identification of two sets of preferentially pairing homologs for each *B. decumbens* chromosome (Figure 4, Table S10). *Brachiaria decumbens* haplotypes were named such that ‘a’ and ‘b’ are a set of preferentially pairing homologs and ‘c’ and ‘d’ form the second set of homologs. There is no evidence that the haplotypes named ‘a’ and ‘b’ for any given chromosome belong same sub-genome as haplotypes ‘a’ and ‘b’ in the other
chromosomes. Visual inspection of allelic inheritance in the 169 F₁ progeny revealed that 90-99% of segregation patterns matched expectations for homologous pairing in all chromosomes except chromosome 8 (Table 3).

Non-homologous pairing was relatively more common in *B. decumbens* chromosome 8, with the four haplotypes pairing with their primary homologs only 75% of the time (Table 3). In addition to segregation between the primary homologs pairs, Figure 4C shows significant segregation between linkage groups ‘a’ and ‘d’ as well as between linkage groups ‘b’ and ‘c’ in *B. decumbens* chromosome 8. The segregating allele read ratios are all significantly higher (*P* < 0.05) for haplotypes ‘a’ and ‘b’ than ‘c’ and ‘d’ in every *B. decumbens* chromosome except 8, indicating that more SNPs are present in all four haplotypes and the sub-genomes may be less differentiated in chromosome 8 compared to the rest of the genome (Table 2). Progeny with unbalanced gametes (evidenced by the presence of segregating alleles in three or one of the four haplotypes in each chromosome, instead of two of four as expected) were also observed in five out of nine *B. decumbens* chromosomes. The incidence of unbalanced gametes was higher in progeny with lower primary homolog pairing rates, peaking at 7% in chromosome 8 (Table 3).

The higher incidence of multisomic pairing in *B. decumbens* chromosome 8 is also demonstrated by the distribution of DDA markers. Of the 20 GBS markers heterozygous in CIAT 606 that fit the 5:1 segregation ratio of heterozygotes to homozygotes expected for DDAs, four were linked in coupling and repulsion with the four haplotypes of chromosome 8 (Table S8). Only one other DDA marker was linked to coupling and repulsion with the four haplotypes of chromosome 3. The remaining markers were either in linkage with only two haplotypes from chromosomes 1, 3, 5, 6, or 7 or duplicated in two sets of chromosomes. Interestingly, seven DDA
markers were in linkage with SDA markers from chromosomes 1 and 4, indicating that these SNPs are duplicated in more than one chromosome.

**Segregation distortion**

In the BRX 44-02 maternal linkage map only 21 (3%) of the 704 mapped markers were distorted (P < 0.01) (Table S7). No genetic regions met the criteria for distortion (at least three distorted markers skewed in the same direction in a region where the smoothed LOESS curve was over LOD = 3.0) (Figure S6A). However, marker saturation in many maternal haplotypes was poor and regions of segregation distortion could remain undetected. Of the 1212 mapped markers in the CIAT 606 paternal linkage map, 82 (7%) were distorted (Table S7). A major distortion region was identified in haplotypes ‘a’ and ‘b’ of CIAT 606 chromosome 9, with an overabundance of heterozygote progeny in the markers mapped to the center of linkage group 9a and an excess of homozygote progeny in the corresponding position of linkage group 9b (Figure S6B).

**Genetic mapping of the ASGR**

The ASGR was mapped to position 42.5 cM of CIAT 606 linkage group 5c, a region syntenous with foxtail millet chromosome 5 (Table S7, Figure 5). A total of six markers, including the GBS markers TP145804 and TP265637, the GBS-derived KASP markers K42517 and K62444, the SCAR marker N14, and the **ASGR-BBML** marker p779/p780, were in perfect linkage with the ASGR. The ASGR was flanked at 7.7 cM proximal by TP176128 and at 0.6 distal by TP262274, K76831, and K207542. Seventeen of 34 markers mapped to linkage group 5c aligned to unique positions on foxtail millet chromosome 5 physical map (Table S7, Figure 5). The only marker in perfect linkage with the ASGR that mapped to a unique physical position on foxtail millet chromosome 5 was TP2655637, which aligns to 21.9 Mbp, slightly distal of the centromeric
A second marker in perfect linkage with the ASGR, TP145804, mapped to position 41.3 Mbp on foxtail millet chromosome 3, which is also distal to the centromere (Table S7). The distal flanking markers K76831, K207542, and TP262274 map to positions 24.1, 26.4, and 27.8 Mbp on the distal arm of foxtail millet chromosome 5. The proximal arm of *B. decumbens* chromosome 5 is inverted relative to foxtail millet, and the most closely linked proximal marker to the ASGR which maps to a unique position on the foxtail millet physical map, TP150336, maps to position 5.7 Mbp (Table S7, Figure 5). No markers on the four homologs *B. decumbens* chromosome 5 were mapped to physical positions in the region 12.5-21.9 Mbp on foxtail millet chromosome 5 (Figure S5).

The ASGR-BBML-specific marker p779/p780, SCAR marker N14, and four KASP markers (K42517, K62444, K76831, and K207542) were subsequently validated in a diversity panel (n = 162) comprising four apomictic interspecific *Brachiaria* hybrid cultivars and accessions of *B. brizantha* (n = 82), *B. decumbens* (n = 13), *B. ruziziensis* (n = 11), and *B. humidicola* (n = 52) with a mixture of apomictic, sexual, and unknown reproductive mode from the CIAT genetic resources program forages collection. Only p779/p780 was broadly predictive for apomixis in a wide range of *Brachiaria* species and *P. maximum* (Figure 6, Table S11). Markers N14, K42517, K76831, and K207542 all failed to amplify in *B. humidicola*. The KASP marker K62444 amplified well in most *B. humidicola* accessions, but misclassified 30 of 38 accessions with known reproductive mode. In contrast, the primer pair p779/p780 produced bands in all *B. humidicola* samples except CIAT 26146, the only identified natural polyploid sexual accession (Jungmann *et al*. 2010).

N14 and the four KASP markers were better predictors of apomixis in the *B. ruziziensis/B. brizantha/B. decumbens* agamic complex, but all misclassified at least some accessions with known reproductive mode. Seven, 13, 26, 30, and 31 of the 91 hybrid cultivars and accessions in
the *B. ruziziensis/B. brizantha/B. decumbens* agamic complex classified for reproductive mode were misclassified with N14, K207542, K62444, K76831, and K42517 respectively (Table S11). Eight, 14, 27, 31, and 32 of the 91 hybrid cultivars and accessions in the *B. ruziziensis/B. brizantha/B. decumbens* agamic complex classified for reproductive mode were misclassified with N14, K207542, K62444, K76831, and K42517 respectively (Table S10). In comparison *B. decumbens* CIAT 26186 and *B. brizantha* CIAT 26179 were the only apomictic germplasm accessions in the *B. ruziziensis/B. brizantha/B. decumbens* agamic complex potentially misclassified as sexual by p779/p780. CIAT 26186 was classified as an apomictic tetraploid by EMBRAPA (Penteado *et al.* 2000; Valle *et al.* 2008), but was previously classified as sexually reproducing in unpublished preliminary reproductive mode trials at CIAT. CIAT 26179 was likewise classified as an apomictic tetraploid by EMBRAPA (Valle 1990; Penteado *et al.* 2000). This accession has not been phenotyped for reproductive mode at CIAT, but is labeled as a *B. ruziziensis* accession in the CIAT database (http://isa.ciat.cgiar.org/urg/foragecollection.do) and fits the taxonomic descriptors for *B. ruziziensis*, a diploid sexual species (Renvoize *et al.* 1996). Thus, research is warranted to determine whether the accessions labeled as CIAT 26186 (EBC D017, BRA-004588) and CIAT 26179 (EBC B295) in CIAT and EMBRAPA Beef Cattle are actually different genotypes. However, the possibility that these are rare ASGR recombinants (Conner *et al.* 2013) cannot be excluded and should be investigated further. The p779/p780 primer pair it amplified bands in five *C. ciliaris* accessions and five *P. maximum* accessions from the CIAT genetic resources collection, while failing to produce bands in five putatively sexual progeny of the synthetic autotetraploid *P. maximum* Tift SPM92 (Figure 6, Table S10). The ten *C. ciliaris* and *P. maximum* accessions which produced bands when evaluated with p779/p780 were not phenotyped for reproductive mode; however, sexuality is rare in these species.
Discussion

Genotyping-by-Sequencing and Genetic linkage maps

The higher read depth needed for reliable genotype calling and requirement that only SDA markers be used for mapping in complex tetraploid genomes results in a lower number of total mapped GBS markers compared to diploid, inbred species (Li et al. 2014). Still, with 1916 total markers distributed across the parental linkage maps, the maps developed in this study have over three times the marker density of the best currently available genetic maps in tetraploid apomictic species (Jessup et al. 2003; Stein et al. 2007; Thaikua et al. 2016). The total number of sequence reads and average sequence reads per progeny obtained in this study using the GBS protocol of Heffelfinger et al. (2014) with the HincII restriction enzyme were comparable to those obtained by Li et al. (2014) in their study of tetraploid alfalfa using the Elshire et al. (2011) protocol with ApeKI (R0643L; NEB). Over twice as many polymorphic SNPs were identified in that study; however, the mean percentage of missing genotype calls was much lower in this study and a very similar total number of polymorphic markers with less than 20% missing data were obtained in the two studies. Overall, both protocols work well and demonstrate the utility of GBS as an approach for the generation of dense genetic maps in complex polyploid species.

Synteny was highly conserved between B. decumbens, B. ruziziensis, and foxtail millet (Setaria italica), the closest relative of Brachiaria with a publically available reference genome (Zhang et al. 2012). The conservation of marker order and genome structure compared to the foxtail millet physical map provided evidence for the validity of the genetic maps and enabled us to assign linkage groups to chromosomes and identify homologs. The only major structural rearrangements identified between Brachiaria and foxtail millet were a reciprocal translocation
between the proximal and distal tips of chromosomes 3 and 7, and inversions on chromosomes 1, 3, 5, and 6. This marked conservation of genome structure indicates that breeders and geneticists working on *Brachiaria* and other forage genera in the Paniceae would likely benefit from applying the wealth of genomic tools being developed in foxtail millet (Muthamilarasan and Prasad 2015) to their own species.

While the CIAT 606 paternal linkage map had 36 linkage groups, as expected for a tetraploid with a base chromosome number of nine, the BRX 44-02 map had only 34 linkage groups. The lower marker density in the BRX 44-02 maternal haplotype map is not surprising given the synthetic autotetraploid origin of this accession and the high proportion of heterozygous markers fitting DDA segregation ratios. The incorporation of DDA markers with SDA markers from homologous haplotype groups into joint linkage groups resolved some questions about the relative positions of the markers on the individual SDA linkage groups (Hackett *et al*. 2007). However, the utility of this approach would be greatly increased by the development advanced mapping software for autoployploids capable of ordering more than 50 markers per linkage group.

**Preferential pairing and segmental allopolyploidy**

The concept of segmental allopolyploidy was first introduced by Stebbins (1947, 1950) as an intermediate form of polyploidy between allopolyploidy and autoployploidy, where plants composed of partially homologous parental genomes (AAA’A’) display a mixture of disomic and multisomic inheritance. Preferential pairing among homologous chromosomes is expected to predominate, with occasional homeologous pairing through the formation of multivalents and/or bivalents with switched pairing partners (A with A’). Segmental allopolyploidy is generally considered a transient state in the evolutionary trajectory of new polyploids toward diploidization.
and stable allopolyploidy, with exclusive bivalent pairing of primary homologs, or autopolyploidy, with sets of fully homologous chromosomes pairing at random (Sybenga 1996). Previous cytological evidence for segmental allopolyploidy in apomictic *Brachiaria* species was supported by high-resolution molecular karyotyping. Strong preferential pairing among primary homologs was demonstrated by highly significant segregation among alleles mapped to two pairs of homologous SDA linkage groups in each *B. decumbens* chromosome. The presence of two overlapping peaks at 0.25 and 0.5 for segregating allele read frequency in GBS-SDA from the paternal genetic linkage map and the scarcity of markers heterozygous in CIAT 606 that fit the expected segregation ratio for DDAs (5:1) provide secondary supporting evidence for the presence of two related but differentiated sub-genomes tetraploid *B. decumbens*.

Successful homologous chromosome pairing and normal bivalent formation in meiosis are highly dependent on sequence similarity. Therefore, meiotic instability and resulting defects, including unbalanced gametes and aneuploidy, are common in F1 plants derived from wide hybridization events. These problems can generally be resolved by meiotic restitution upon polyploidization through meiotic non-reduction as summarized by De Storme and Mason (2014). However, aneuploid gametes can still be produced by allopolyploid meiosis if non-homologous pairing and intergenomic recombination compromise the meiotic stability of the parental chromosomal complements (Comai 2005). Therefore, genetic mechanisms such as the *pairing homeologous* 1 locus in hexaploid wheat (Sears 1976; Prieto *et al.* 2004) have evolved to prevent homeologous pairing in wheat and promote strict disomic inheritance.

Alternatively, Stebbins (1950) proposed that segmental allopolyploids may stabilize by functional autopolyploidization over many successive generations of recombination between homeologous chromosomes. Stebbins also proposed that a stable (secondary) segmental
allopolyploid might evolve with some sets of fully homologous chromosomes and other chromosome sets further differentiated with exclusively preferential pairing, though Sybenga (1996) argued that no polyploids of this type have been found in nature and segmental allopolyploidy does not occur in established natural polyploids. The evidence for segmental allopolyploidy in *B. decumbens* presented in this study add to a growing body of literature refuting this claim and demonstrating the existence of naturally occurring segmental allopolyploids in apomict grasses (Mendes-Bonato *et al.* 2002; Jessup *et al.* 2003) and sexually reproducing species (Boff and Schifino-Wittmann 2003; Shinohara *et al.* 2010). The prevalence of segmental allopolyploidy in apomictic grass species suggests that apomixis may effectively arrest the processes of diploidization and/or functional autopolyploidization, enabling genotypes to remain in a neopolyploid state indefinitely.

Additionally, the high-resolution molecular karyotyping approach (Mason *et al.* 2014) used in this study enabled us to evaluate the prevalence of preferential pairing at each individual chromosome. Visual inspection of allelic inheritance in the F<sub>1</sub> progeny indicated that homologs paired preferentially in at least 90% of instances across the genome except in chromosome 8, where significant segregation among markers mapped to secondary homeologs was observed and segregation patterns in the mapping population progeny suggest that non-homologous pairing occurs in approximately 25% of meiotic episodes. Thus *B. decumbens* is the best example to date of a stable (secondary) segmental allopolyploid with most chromosome sets exhibiting strong preferential pairing and chromosome eight evolving toward functional autopolyploidization.

The presence of meiotic abnormalities is common in both neopolyploids and in the microsporocytes of polyploid apomicts (De Storme and Mason 2014). The most commonly observed meiotic abnormalities observed in polyploid *Brachiaria* were related to irregular
chromosome segregation (e.g. precocious migration of univalent to poles in metaphases, laggard chromosomes during anaphases, and formation of micronuclei in microspores), chromosome stickiness, and impaired cytokinesis (Mendes-Bonato et al. 2001; Risso-Pascotto et al. 2006; Ricci, Souza-Kaneshima, Felismino, et al. 2011; Ricci, Souza-Kaneshima, Pagliarini, et al. 2011). In this study, we also found evidence of progeny with unbalanced gametes in five out of nine B. decumbens chromosomes, peaking at 7% in chromosome 8. The correlation between non-homologous pairing and formation of unbalanced gametes observed in this study suggests that apomicts with strong preferential pairing across the genome may produce more viable male gametes. Reduced fertility is considered an important constraint to the success of newly formed polyploids (Ramsey and Schemske 2002). Thus, apomixis may have evolved in unstable segmental allopolyplloid grasses as a means to promote fertility despite irregular chromosome segregation caused by non-homologous pairing in meiosis.

Implications for seed yield and producibility

Although polyploid apomicts are able to forego meiosis during megasporogenesis, the high prevalence of unreduced gamete formation observed in these species is not without consequence for commercial tropical forage seed production. Apomicts in the Paniceae are pseudogamic (Barcaccia and Albertini 2013), meaning that the secondary nuclei of apomictic embryo-sacs must be fertilized with viable pollen gametes for normal endosperm development and seed production. Seed yield potential is an important trait that determines whether a new variety can be profitably produced and distributed to farmers, and failed seed set is persistent limitation in Brachiaria, where caryopsis formation rarely exceeds 30% (Hopkinson et al. 1996). Abnormal tetrad frequency appears to be significantly correlated with non-viability of pollen grains (Souza et al. 2015). The implications of the relationship between the incidence of meiotic abnormalities and
reduced pollen viability are particularly troubling for bred hybrid genotypes. Because there are no known natural allotetraploid sexuals in the B. ruziziensis/B. brizantha/B. decumbens agamic complex, synthetic autotetraploid genotypes (AAAA) have been used as the source of sexuality in inter- and intraspecific crosses with segmental allopolyploid (BB’B’) pollen donors (Swenne et al. 1981; Simioni and Valle 2011; Souza et al. 2015). Therefore, pairing affinity is likely reduced in progeny (AABB’), which receive male gametes with low homology (BB’). Indeed, meiotic abnormalities appear to be more common in progeny of both inter- and intra-specific crosses than natural apomictic accessions (Risso-Pascotto et al. 2005; Fuzinatto et al. 2007, 2012; Mendes-Bonato et al. 2007; Felismino et al. 2010; Souza et al. 2015). Many natural apomictic accessions, including CIAT 606 (cv. Basilisk), produce adequate seed and are successful as cultivars despite low pollen fertility (Mendes-Bonato et al. 2001). Thus, the production of viable pollen may not be as critical in apomictic species as in sexual species, where seed development is dependent on successful pollination of both the embryo and the endosperm (Souza et al. 2015). Still, at least one hybrid Brachiaria cultivar with excellent forage traits, good stress resistance, and ample flowering failed in the marketplace due to poor seed yield caused by low seed set (Hare et al. 2007), indicating that further research on the relationship between meiotic abnormalities and seed yield in tropical forages is warranted.

Inheritance of apomixis and conservation of the ASGR in the Paniceae

Apomixis was inherited as a single dominant Mendelian factor in the BRX 44-02 x CIAT 606 mapping population. Segregation distortion has been associated with apomixis in other apomictic polyploids including P. notatum (Martínez et al. 2001), Tripsacum dactyloides (Grimanelli et al. 1998), and interspecific Pennisetum glaucum x P. squamulatum hybrids (Roche et al. 2000). However, the only distorted region identified in the present study was on linkage
groups ‘a’ and ‘b’ on *B. decumbens* chromosome 9, indicating that there may be a lethal or sub-lethal detrimental factor located on CIAT 606 linkage group 9b.

The ASGR was mapped to position 42.5 cM of CIAT 606 linkage group 5c, a region syntenous with foxtail millet chromosome 5. The number of markers in perfect linkage with the ASGR, large physical distance between ASGR-flanking markers in tight genetic linkage, and proximity of linked markers to the foxtail millet chromosome 5 centromere all indicate that the ASGR is located in a region of suppressed recombination, as has been demonstrated in *P. squamulatum* (Ozias-Akins et al. 1998). Tight clustering of markers in perfect linkage with the ASGR has also been observed in other Paniceae mapping populations (Jessup et al. 2002; Martínez et al. 2003; Ebina et al. 2005; Stein et al. 2007; Thaikua et al. 2016), though the lack of available information about physical distances between markers in previous studies made it difficult to assess whether recombination was indeed suppressed.

Interestingly, the ASGR was previously linked, in *B. brizantha* CIAT 6294, to RFLP probes designed from rice chromosome 2 and maize chromosome 5 (Pessino et al. 1997, 1998). Foxtail millet chromosome 5, on the other hand, is mostly syntenic with rice chromosome 1 and maize chromosome 3 (Zhang et al. 2012). The ASGR has also been linked to markers from sorghum chromosome 4, which has homology to maize chromosomes 2 and 10, in *C. ciliaris* (Jessup et al. 2002); and rice chromosome 12 in *Paspalum* (Pupilli et al. 2004). Most recently, the ASGR-carrier chromosome of *P. squamulatum* was found to be collinear with foxtail millet chromosome 2 and sorghum chromosome 2 by *in silico* transcript mapping and fluorescence *in situ* hybridization (FISH) (Sapkota et al. 2016).
Jessup et al. (2002) suggested that the implication of different ASGR-carrier chromosomes in these studies may indicate the independent evolution of apomixis in multiple grass species. However, subsequent comparative genomics with ASGR-linked BACs in Cenchrus and Pennisetum species showed that apomixis more likely evolved as a single event and was spread to other species through hybridization or phylogenetic diversification (Ozias-Akins et al. 2003; Akiyama et al. 2011). The perfect linkage of the ASGR-BBML specific primers p779/p780 with the ASGR in this mapping population and its excellent diagnostic ability for reproductive mode in the diversity panel indicates conservation of the ASGR in Cenchrus/Pennisetum and the more distantly related genera Brachiaria and Panicum. These findings also support the hypothesis of a common origin for aposporous apomixis in the Paniceae tribe. While the ASGR appears to be highly conserved across the Paniceae, the ASGR-carrier chromosome has undergone significant rearrangement and translocation during hybridization. In all apomictic Pennisetum species the ASGR is located in the telomeric region of the carrier chromosome, while it is inverted and located in an interstitial region in Cenchrus (Goel et al. 2006). In B. decumbens the ASGR is located adjacent to the centromere of chromosome 5, though previously identified linkages of the B. brizantha ASGR with markers from maize chromosome 5 and the lack of transferability of SNPs linked to apomixis in the B. ruziziensis/B. brizantha/B. decumbens agamic complex to B. humidicola indicate that the ASGR may have been translocated to different chromosomal backgrounds even within Brachiaria.

**Conclusions**

The development of dense molecular maps in tetraploid Brachiaria species has provided useful new information about the genomic organization and evolution of polyploid apomicts. The diagnostic ability of the primers p779/p780 for reproductive mode in the F₁ mapping
population and diversity panel of known sexual and apomict *Brachiaria* germplasm accessions and cultivars indicates that ASGR-BBML gene sequences are strongly conserved across the Paniceae and adds further support for the postulation of the ASGR-BBML as candidate genes for the apomictic function of parthenogenesis. Molecular karyotyping also provided conclusive evidence for segmental allopolyploidy in *B. decumbens*, suggesting that apomixis may have evolved as a means of meiotic restitution to ‘fix’ inherently unstable states of segmental allopolyploidy that would otherwise be lost to natural selection. Future studies focused on comparative genomics of ASGR sequences from diverse Paniceae genera should yield interesting information about the evolution of apomixis and may aid in the identification or validation of candidate genes related to aposporous initial formation.

In addition to providing new evidence about the evolution of apospory in the Paniceae and its role in segmental allopolyploidy, the development of saturated genetic linkage maps in polyploid apomict species has immediate applications for tropical forage breeding. The high density linkage maps developed in this study can be used to map QTL for agronomically important traits in *Brachiaria*, including traits related to pollen fertility and seed yield, and should permit anchoring of sequence scaffolds in the *B. ruziziensis* diploid reference genome. Furthermore, the perfect linkage of p779/p780 with the ASGR in the BRX 44-02 x CIAT 606 F$_1$ mapping population and its excellent predictive ability for reproductive mode in a large diversity panel with multiple species of *Brachiaria* suggest that this is a useful genetic marker for apomixis that can be applied for marker assisted selection in a wide range of breeding programs. Further research should be conducted to confirm whether p779/p780 is diagnostic for apomixis in populations of *B. humidicola* and *P. maximum* segregating for reproductive mode.
Acknowledgements

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Authors Contributions: M.W. provided overall conceptual guidance for the project, conducted molecular mapping, analyzed and interpreted the data, and drafted the manuscript. C.H. conducted bioinformatics and synteny analyses. D.B. phenotyped the mapping population and assisted in manuscript preparation. C.Q. and Y.P.Z. performed library preparation, conducted molecular marker analyses, and assisted in analysis and interpretation of data. J.G.P. assisted in molecular karyotyping analysis and figure preparation. J.V. performed the draft assembly of the *B. ruziziensis*
diploid genome. J.M. developed the mapping population. S.D. and J.T. provided conceptual
support for the project.

**Figure Captions**

**Figure 1** The 34 linkage groups of the BRX 44-02 maternal linkage map (A) and the 36 linkage
groups of the CIAT 606 paternal haplotype map (B). Homologous linkage groups (a-d) were
identified and assigned to chromosomes (1-9) based on synteny with foxtail millet (*Setaria
italica*) and shared linkage with double dose allele markers. Marker position are expressed in
centiMorgans (cM).

**Figure 2** Alignment of markers mapped to the *Brachiaria ruziziensis* BRX 44-02 (A) and *B.
decumbens* CIAT 606 (B) genetic linkage maps with unique physical positions on the foxtail
millet (*Setaria italica*) reference genome at a cutoff of E-value < 1 x 10^{-5}. Markers mapped to
haplotypes a, b, c, and d of each chromosome are represented with red, blue, green, and violet
dots.

**Figure 3** Relative depth of coverage (read frequency) of the segregating allele in heterozygous
loci in *Brachiaria ruziziensis* BRX 44-02 (A) and *B. decumbens* CIAT 606 (B).

**Figure 4** Linkage and segregation of markers in (A) the entire CIAT 606 paternal haplotype
map, (B) CIAT 606 chromosome 1 homologs a-d, and (C) CIAT 606 chromosome 8 homologs
a-d. Single nucleotide polymorphism markers are arranged by their genetic position (not drawn
to scale). Regions with statistically significant linkage are indicated in red, orange, and yellow,
while genetic regions with significant segregation are indicated with shades of blue.
Figure 5 Comparison of CIAT 606 linkage group 5c with the S. italica chromosome 5 physical map. Genetic positions are expressed in centiMorgans (cM) and each unit of the physical map represents 5 x 10^3 bp. The apospory-specific genomic region (ASGR) is highlighted with a red box.

Figure 6 PCR amplification products from the ASGR-BBML primer pair p779/p780 with genomic DNA of Cenchrus ciliaris 36100, Brachiaria decumbens CIAT 606, B. ruziziensis BRX 44-02, B. humidicola CIAT 16888, B. humidicola CIAT 26146, B. brizantha CIAT 6294/6297, Panicum maximum CIAT 26924, and P. maximum PI 570664. Of these materials, BRX 44-02, CIAT 26146, and PI 570664 are putatively sexual. AP = apomictic, SX = sexual, ND = not determined.
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Table 1 Segregation ratio for reproductive mode of in F₁ progeny of the BRX 44-02 x CIAT 606 mapping population, range of proportion Polygonum and Panicum type embryo sacs observed in the progeny of each phenotypic class, and average number of embryo sacs per pistil observed in progeny classified as sexual and apomictic.

<table>
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<th>Reproductive mode</th>
<th>Number of progeny</th>
<th>Proportion of Polygonum type embryo sacs</th>
<th>Proportion of Panicum type embryo sacs</th>
<th>Embryo sacs per pistil</th>
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<td>Sexual</td>
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<td>1.00</td>
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<tr>
<td>Apomictic</td>
<td>83</td>
<td>0-0.93</td>
<td>0.07-1</td>
<td>2.11</td>
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<td>Total</td>
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### Table 2

Number of single dose allele markers, length, and mean ratio of segregating allele reads to total reads in the heterozygous parent in each of the 70 linkage groups in the BRX 44-02 and CIAT 606 haplotype maps.

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<th>CIAT 606</th>
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<td>Length (cM)</td>
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</tr>
<tr>
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<td>Total</td>
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</table>
Table 3 Percentage of *Brachiaria decumbens* chromosomes segregating with primary homologs based on complementary allelic inheritance patterns (presence of segregating alleles in one homolog and absence in the other with or without recombination) between homologs in the 169 F\(_1\) hybrid progeny of the BRX 44-02 x CIAT 606 mapping population and percentage of progeny with unbalanced gametes.

<table>
<thead>
<tr>
<th>Chromosome</th>
<th>Segregation with primary homolog (%)</th>
<th>Unbalanced gametes(^a) (%)</th>
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<tbody>
<tr>
<td>1</td>
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<tr>
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<td>8</td>
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<td>7</td>
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<tr>
<td>9</td>
<td>99</td>
<td>0</td>
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<tr>
<td>Overall</td>
<td>93</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\) Progeny with the segregating alleles present in three or one of the four haplotypes in each chromosome (instead of two of four haplotypes as expected) were classified as unbalanced gametes.
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