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Chromosomal distribution and evolution of abundant retrotransposons in plants: gypsy elements in diploid and polyploid Brachiaria forage grasses

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Abstract

Like other eukaryotes, the nuclear genome of plants consists of DNA with a small proportion of low-copy DNA (genes and regulatory sequences) and very abundant DNA sequence motifs that are repeated thousands up to millions of times in the genomes including transposable elements (TEs) and satellite DNA. Retrotransposons, one class of TEs, are sequences that amplify via an RNA intermediate and reinsert into the genome, are often the major fraction of a genome. Here we put research on retrotransposons into the larger context of plant repetitive DNA and genome behaviour, showing features of genome evolution in a grass genus, *Brachiaria*, in relation to other plant species. We show the contrasting amplification of different retroelement fractions across the genome with characteristics for various families and domains. The genus *Brachiaria* includes both diploid and polyploid species, with similar chromosome types and chromosome basic numbers $x = 6, 7, 8$ and 9. The polyploids reproduce asexually and are apomictic, but there are also sexual species. Cytogenetic studies and flow cytometry indicate a large variation in DNA content (C-value), chromosome sizes, and genome organization. In order to evaluate the role of transposable elements in the genome and karyotype organization of species of *Brachiaria*, we searched for sequences similar to conserved regions of TEs in RNAseq reads library produced in *B. decumbens*. Of the 9,649 TE-like contigs, 4,454 corresponded to LTR-retrotransposons, and of these, 79.5% were similar to members of the *gypsy* superfamily. Sequences of conserved protein domains of *gypsy* were used to design primers for producing the probes. The probes were used in FISH against chromosomes of accesses of *B. decumbens, B. brizantha, B. ruziziensis* and *B. humidicola*. Probes showed hybridization signals predominantly in proximal regions, especially those for retrotransposons of the
clades CRM and Athila, while elements of Del and Tat exhibited dispersed signals, in addition to those proximal signals. These results show that the proximal region of Brachiaria chromosomes is a hotpot for retrotransposon insertion, particularly for the gypsy family. The combination of high-throughput sequencing and a chromosome-centric cytogenetic approach allows the abundance, organization and nature of transposable elements to be characterized in unprecedented detail. By their amplification and dispersal, retrotransposons can affect gene expression, they can lead to rapid diversification of chromosomes between species, and hence are useful for studies of genome evolution and speciation in the Brachiaria genus. Centromeric regions can be identified and mapped, and retrotransposon markers can also assisting breeders in the developing and exploiting interspecific hybrids.

**Key words:** centromeres, retrotransposons, FISH, in situ hybridization, metaviridae, grasses, genomics, genome organization, transposons, transposable elements, genetics, repetitive DNA, chromosomes

**Abbreviations**

PBS (Primer Binding Site)

PR (Protease)

RT (Reverse Transcriptase)

RT-Athila (Reverse Transcriptase of Athila lineage)

RT-CRM (Reverse Transcriptase of CRM lineage)

RT-Tat (Reverse Transcriptase of Tat lineage)

RNase H (Ribonuclease H)

INT (Integrase)
Introduction

The nuclear genome size of plants varies more than 2,000 fold, from 63.40 Mbp (1C = 0.0648 pg) in *Genlisea margaretae* (Greilhuber et al. 2006) to 259,000 Mbp (1C = with 264.9 pg) in the allohexaploid *Trillium hagae* (Zonneveld 2010; Pellicer et al. 2010). Sequencing and study of composition of genomes have shown that transposable elements (TEs) and responsible for much of the genome size variation in plants, in addition to those variations arising from differences in the levels of ploidy (Adams and Wendel 2005; Heslop-Harrison and Schmidt, 2012). Transposable elements represent up to 85% of the DNA (genome size) in cereals (Daron et al. 2014) or loblolly pine (Kamm et al. 1996; Neale et al. 2014), and TEs may increase their copy number leading to a rapid expansion in genome size between closely related species (Gregory 2005; Pearce et al. 1996). In *Arabis alpina*, Willing et al. (2015) show the genome expansion through amplification of a retrotransposon family compared to *Arabidopsis thaliana*. TEs can also involve genome contraction through a wide range of illegitimate recombination and deletion processes, which depends on a balance between insertion
Transposable elements are classified according to their mode of mobility into Class 1, retrotransposons that transpose via an RNA intermediate using a copy-and-past mechanisms; and Class 2, transposons that move via a DNA molecule using cut-and-paste events and may amplify before or during the transposition cycle (Hansen and Heslop-Harrison 2004). The LTR-retrotransposons (LTR-RTs) represent the majority of TEs identified in plant genomes (eg Bertioli et al. 2013). They are subdivided into two big super families: \textit{copia} (Pseudovirideae) and \textit{gypsy} (Metavirideae). The most striking difference between members of these two super families is the order of the integrase (INT) amino acid domain along the polyprotein gene encoded by the LTR retrotransposons (Hansen and Heslop-Harrison 2004; Wicker et al. 2007). \textit{Copia} are the best studied and understood retrotransposons in plant species, probably because they seem to be more conserved in plant evolution.

Plant LTR-RTs often accumulate in heterochromatin regions of chromosomes (Gao et al. 2008; Kejnovsky et al. 2012; Pearce et al. 1996), and are also found in proximal and non-recombining regions of sex chromosomes (VanBuren et al. 2015). An example of expansion of retrotransposons in centromeric heterochromatin is seen in a comparison of related species of \textit{Alstroemeria} (Kuipers et al. 1998). In \textit{Arabidopsis}, \textit{copia} elements are clustered at centromeres (Heslop-Harrison et al. 2003) and also occur along the chromosomes, while \textit{gypsy} elements are preferentially inserted in pericentromeric regions (Pereira 2004, who also considers evolutionary mechanisms and consequences for copy number and distribution of elements). In most monocots and dicots examined so far, centromere regions contain a specific lineage of \textit{gypsy} LTR-RTs called Centromeric Retrotransposons (CRs, called CRM in Maize), suggesting that santos et al. Distribution and evolution of retrotransposons in the forage grass \textit{Brachiaria}. Page 5.
these sequences play an important role in the function and evolution of centromeres (Zhong et al. 2002; Gao et al. 2015). The gypsy family of retroelements is also responsible for the genome expansion seen in Arabis alpina (Willing et al. 2015), while insertion and excision of DNA transposable element families such as hAT elements in Musa (Menzel et al. 2015) or MITEs in Brassica (Nouroz et al. 2015) leads to differences both in genome size and organization.

The activity of retrotransposons with LTRs can be modulated by DNA methylation and silencing processes, and is induced by a wide range of internal and external factors, including biotic and abiotic stresses (Takeda et al. 1998; Casacuberta and González 2013). As consequence, LTR-RTs may proliferate and induce mutations with biological effects depending of their insertion sites. Hybridization and allopolyploidization are also considered as potential genomic shocks involved in the accumulation of transposable elements and genome reorganization in plants (Parisod et al. 2010; Renny-Byfield and Wendel 2014; Zou et al. 2011). As well as amplification of genome sizes through transposable elements, most plant species have one or more rounds of whole-genome doubling events or polyploidy in their ancestry (see Heslop-Harrison 2012). These events may be followed by chromosomal loss and rearrangements, and there may be rapid genomic changes at the time of the polyploidization or hybridization events (Ma and Gustafson 2008; Gaeta et al. 2007).

The grass genus Brachiaria (Poaceae) is of African origin and comprises about 100 species (Renvoize et al. 1996), with many polyploids and hybrid species (Boldrini et al. 2009; Akiyama et al. 2010; Nielen et al. 2010) propagated sexually through seeds and vegetatively. The basic chromosome number is \( x=9 \), with species including \( 2n=2x=18 \) diploids and various ploidy to \( 2n=10x=90 \). Four of the species, B. Santos et al. Distribution and evolution of retrotransposons in the forage grass Brachiaria. Page 6.
ruziziensis, B decumbens, B. brizantha and B. humidicola, have great economic importance in production of forage and seeds in weak and acids soils (Nakamura et al. 2005). In general, hybrids between these species exhibit variable frequencies of univalent and multivalent pairing, asynchrony in cell divisions, and abnormal development of the microsporogenesis (Mendes-Bonato et al. 2002), suggesting that polyploidy and hybridization may cause a loss of “genomic homeostasis”. Together, these observations suggest the Brachiaria genus is a good model to use to compare the distribution and study the impact of TEs between diploid and polyploid species.

To understand the diversity of LTR-RTs in the organization of proximal chromosome regions in Brachiaria, we aimed to identify and characterize transcriptionally active gypsy LTR-RTs using RNAseq data generated from the diploid species B. decumbens. Probes related to four transcribed gypsy lineages were generated and used for in situ hybridization to chromosomes of diploids (B. decumbens, B. ruziziensis and B. brizantha) and polyploids (B. decumbens, B. humidicola, and B. brizantha) Brachiaria species. Probes of 5S rDNA and telomeres were used to identify chromosomes and their morphology. By showing different retroelement distributions, our data exemplify the genomic distribution of this important group of abundant sequences and contribute to the knowledge of genome structure, composition and evolution of diploid and polyploid Brachiaria species.

Materials and Methods

Biological samples
Samples of ten accessions belonging to four species of *Brachiaria* were used: i) B72 (2n = 4x = 36) and B183 (2n = 5x = 45) of *Brachiaria brizantha*, ii) R102 (2n = 2x = 18) of *B. ruziziensis*, iii) H16 (2n = 4x = 36), and H36, H38 and H112 (2n = 9x = 54) of *B. humidicola*, iv) D4 (2n = 2x = 18) of *B. decumbens*, and v) two interspecific hybrids with 2n = 4x = 36. Accessions were vegetatively propagated and are maintained in the Embrapa Gado de Corte (Brazilian Research Institute), Campo Grande, Mato Grosso do Sul State, Brazil.

**RNAseq analysis and design of FISH probes**

RNAseq data were produced from *B. decumbens* roots using Illumina HiSeq 2000 sequencing technology. RNA sequencing reads were assembled with Trinity (Grabherr et al. 2011). All contigs were first compared to the RepBase (Jurka et al. 2005) amino acids database (version 19.06; http://www.girinst.org/repbase/) using BLAST (BLASTx, E-value 1e-4) to classify them according to their similarities. The nucleotide contigs showing significant similarities to LTR-RTs coding regions were further compared to the reverse transcriptase (RT), integrase (INT) and capsid (GAG) amino-acid domains database downloaded from the Gypsy Database 2.0 (http://gydb.org/) (Llorens et al. 2011). The RT amino-acid domains from *B. decumbens* nucleotide contigs were extracted using GeneWise (https://www.ebi.ac.uk/Tools/psa/genewise/) with at least 150 amino-acid residues. Sequences were aligned using Muscle tools (Edgar 2004) and a Neighbor-joining tree was constructed using ClustalW and edited with FigTree (http://tree.bio.ed.ac.uk/software/figtree/).

Primers were designed on selected contigs with Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) and conserved regions were amplified by PCR. Sequences, including primers, regions and sizes are Santos et al. Distribution and evolution of retrotransposons in the forage grass *Brachiaria*. Page 8.
available in the Table 1. Inserts of the pTa794 clone containing the 5S rDNA sequence of *T. aestivum* (Gerlach and Dyer 1980) and the pLT11 clone containing the telomeric insert (TTTAGGG)_n from *A. thaliana* (Richards and Ausubel 1988) were also used in FISH. Probes were labeled with digoxigenin-11-dUTP or biotin-11-dUTP using the Invitrogen Bioprime CGH labelling kit.

**Fluorescent in situ hybridization**

Root tips were pretreated in 2 mM 8-hydroxyquinoline, 5% dimethylsulfoxide for 24 h at 9 °C and fixed in ethanol: acetic acid (3:1, v:v). Chromosome spreads were made as described by Schwarzacher and Heslop-Harrison (2000). Samples were digested in 2% cellulase Onozuka R-10 (Serva) and 20% pectinase (Sigma) for 5 h at 37 °C, squashed in a drop of 45% acetic acid, and coverslips removed in liquid nitrogen. Slides were sequentially treated with RNaseA (100 μg mL\(^{-1}\)) and pepsin (10 μg mL\(^{-1}\)), washed in 2× SSC, and dehydrated in an ethanol series. A hybridization mix (40 μL/slide) which consisted of 50% formamide, 2× SSC, 10% dextran sulphate, 25 ng salmon sperm DNA, 0.15% SDS, 400 ng of labeled probe and 0.25 mM EDTA, was denatured at 80 °C for 10 min, applied onto slide. Both were denatured at 70 ° for 7 min using a thermal cycler, followed by an overnight incubation at 37 °C in a humid chamber. Slides were washed in SSC buffer at 80% stringency, and detection was done with Alexa 594-antibiotin (red) and FITC-antidigoxigenin (green) in 5% BSA in SSC buffer. Post-detection washes were done in the SSC buffer followed by staining with DAPI (4 μg mL\(^{-1}\)/slide). Samples were mounted with antifade (Citifluor).

Preparations were analyzed with a Nikon E800 imaging epifluorescence microscope. Images were overlaid using Adobe Photoshop 6.0; image brightness, and contrast in each colour were optimized using only operations affecting the whole image.
results

The RNA sequences of *B. decumbens* were obtained using Illumina sequencing, and reads assembled into 126,601 unigenes, with an average size of 1kb (total 126,383,241 bp). 58% of unigenes (73,553; N50 1823) have a length larger than 500 bp. The functional annotation of transposable elements using BLASTx against the RepBase amino acid database showed that 9,649 unigenes (7.6%) have at least one match on these database of known proteins of TEs. Based on the BLASTx results, 4,454 TE unigenes (47.1%, Fig. 1) were classified into the Class I LTR-RTs group of retrotransposons. Among LTR-RT unigenes, *gypsy* super family members represented the majority (79.5%). The reverse transcriptase domains of identified LTR-RTs were extracted and used to draw a Neighbor-Joining tree with the reference RT domains from the Gypsy Database. Results showed that most of the lineages of *gypsy* and *copia* super families typically present in plants were also identified in the transcriptome of *B. decumbens* with the exception of the *Galadriel* lineage. Among the clades (Fig. 2), seven contigs were selected from four different *gypsy* lineages to design primers for PCR and FISH: *Del*, *CRM*, *Athila* and *Tat* (Table 1 and Fig. 2; Figs 3, 4, 5 for *in situ* hybridization results).

The universal probes of 5S rDNA (pTa794) of *T. aestivum* and TTAGGG\(_n\) (telomeric sequence, pLT11 clone of *A. thaliana*) were used to help identify chromosomes and detect possible variations in relation to hybridization site numbers. The 5S rDNA probe showed hybridization sites predominantly in proximal regions,
varying from four sites in *B. decumbens* 2x (Fig. 3C) to six in *B. brizantha* 5x (Fig. 4D).

The telomeric probe hybridized always at the chromosome ends, without evidence of ectopic telomeric sites (polyploid *B. humidicola* Fig. 4A and C; *B. brizantha* Fig. 5A).

FISH using *gypsy* probes showed differences in the chromosome distribution between lineages and between ploidy levels and species. The *Athila* probe, for instance, was located predominantly in the centromeric-pericentromeric regions of chromosomes of diploids *B. decumbens* (Fig. 3A, B), *B. ruziziensis* (Fig. 3E) and *B. brizantha* (Fig. 3I), but with few dispersed signals (Fig. 3A). In the polyploid *B. brizantha* (Fig. 5C), the *Athila* probe showed also centromeric-pericentromeric hybridization signals, but differences in location and abundance (strength of signal) was evident between chromosomes, with some minor sites (Fig. 5C).

The *Tat* probes showed relatively strong hybridization signals in the centromeric-pericentromeric regions, with dispersed signals in interstitial regions of diploid *B. decumbens* (Fig. 3C) and *B. ruziziensis* (Figs. 3D-E). Differences in the intensity and size of signals were seen between chromosomes within a genome (Fig. 3E). However, the polyploids showed differences in the signal location. *B. humidicola*, for instance, showed a more dispersed profile of hybridization, except for 14 chromosomes that exhibited accumulation of the *Tat* probe in centromeric-pericentromeric regions (Fig. 4A). In the polyploid *B. brizantha* (Fig. 5C-D), the signals produced using the reverse transcriptase as probe were weak (low intensity, most likely due to a weak probe), but they showed differential labeling of chromosomes (Fig. 5C). FISH using an INT probe of the same element showed stronger signals that were scattered and/or concentrated in about half of the chromosomes, while the other half of the signals were weak and dispersed (Fig. 5D).
FISH with CRM showed centromeric signals, with a few inconspicuous signals at pericentromeric-interstitial regions in diploids of *B. ruziziensis* (Fig. 3F, G) and *B. brizantha* (Fig. 3J). In the polyploids, the CRM probe hybridized in the proximal regions, but the signals were less intense. No dispersed signals were noted along chromosome arms of *B. humidicola* (Fig. 4C). In contrast, chromosomes of polyploid *B. brizantha* exhibited proximal signals, besides some interstitial dots in interstitial regions (Fig. 5A, and in the box below and to the right of the image). The Del probe showed dots scattered in all along chromosome regions of diploid *B. brizantha* (Fig. 3H, I), but with some few chromosomes with proximal signals. Likewise, in the polyploid *B. brizantha*, the Del probes showed signals as dots scattered along chromosomes, with marking in some proximal regions (Fig. 5B and inset box v and iv).

**Discussion**

Transposable element DNA families are relatively easily identified in genome sequences due to their repetitive nature and sequence signatures that facilitate recognition using bioinformatic tools (Lerat 2010; Janicki et al. 2011; Heitkam et al. 2014; Menzel et al. 2015). In plants, Class I transposable elements, particularly those of *gypsy* and *copia* LTR-RTs super-families, are the most frequent in relation to the other elements that occupy the plant genomes (Wicker et al. 2007). Because these elements are commonly expressed in plant genomes, we can identify them from a collection of RNAseq reads, using a model plant with no available sequenced genome. According to data obtained from RNAseq in *Prunus persica* (Rosaceae), for instance, the LTR-RTs represent 18.6%, (10% *gypsy* and 8.6% *copia*-like; Verde et al. 2013), while in DNA analysis of other monocots, retrotransposons (LTR-RTs) sequences can vary from 21.4% in *Brachypodium* (a small genome with most TEs located in the centromeric regions; Santos et al. Distribution and evolution of retrotransposons in the forage grass *brachytria*. Page 12.
Vogel et al. 2010) to 26% in rice, about 50% in sorghum and banana (including 25.7% copia-like and 11.6% gypsy-like; d’Hont et al. 2012), and more than 80% in wheat. Transposable elements and retrotransposon-related sequences are normally found in transcriptomes. Some of these sequences are ‘active’ and their insertion into the genome leads to variation that is detectable using IRAP (Inter-Retroelement Amplified Polymorphism) markers (Vicient et al. 2001; Alsayeid et al. 2015). However, few if any of the transcripts are likely to be translated (only from the small proportion of TE sequences with functional open reading frames) or reverse-transcribed, nor reinserted into the genome, in a single generation. Activation of transposable elements by stress conditions (including environmental, tissue culture or sexual hybridization) is well known, and new insertions can sometimes be detected following such events (Takeda et al. 1998; Parisod et al. 2010).

The assembly obtained from an RNAseq assembly of *B. decumbens* showed a predominance of expressed gypsy elements (79.5%), when compared with copia superfamily members, as well as members of other repetitive DNA families (Fig. 1). The main gypsy lineages in *B. decumbens* were of the Reina, Athila, Tat, Del and CRM, typical of many plant species with a range of families. Primers were designed for these four last lineages to amplify PCR products. Athila and Tat elements comprise two families of large gypsy LTR-RTs, with sizes ranging between 10 and 12 kb. These are recognized by a primer binding site (PBS) complementary to tRNA-Glu. Athila/Tat and form a large clade in the phylogeny proposed by Llorens et al. (2009) (see also Gypsy Database 2.0 http://gydb.org/index.php/Phylogeny:POL_LTR_retroelements), but both can be separated by differences in the homology and number of 3’-ORFs (see Chavanne et al. 1998). Probes of both groups of LTR retrotransposons were obtained from PCR from various conserved regions including RT from Athila-like, and RT, INT or RNaseH
from Tat-like, and in situ hybridization showed these were located preferentially in proximal chromosome regions, although in polyploids of B. decumbens and B. humidicola, the Tat probe was more scattered in interstitial regions than the Athila probe. Although both probes have been located in the proximal chromosome region, they are not typical centromeric retrotransposons, perhaps because they do not carry any chromodomain (Weber and Schmidt, 2009). The Bagy-1 LTR-RT, an element from the Del gypsy lineage that was initially reported from the barley genome, showed hybridization signals such as dots distributed in the terminal and interstitial chromosome regions, but with a greater concentration in the pericentromeric region. This dispersed distribution of Bagy-1 may be associated with the fact that this LTR-RT belongs to a group considered to be quite active in the grass genomes (Vicient et al. 2001). Notably, in the tetraploid accession of B. humidicola (Fig. 4A), the INT-Tat/Cyclops probe labels about half the chromosomes more strongly than the others, suggesting that the sequence has amplified in one of the ancestral genomes before they came together in the tetraploid.

Of all the LTR-RTs tested here, only the CRM-like elements belongs to chromoviruses, generally the most widespread clade of gypsy-like elements (Gorinšek et al. 2004). Centromere-specific chromoviruses are very common in both angiosperm and gymnosperm genomes, and are close to Reina, Tekay and Galadriel clades (Lorens et al. 2009). All the members of these clades present an integrase chromodomain at C-terminus (Gorinšek et al. 2004), so they can recognize and interact with modified centromeric histones, suggesting that these elements present an important role for the structure and the function of the centromere (Gao et al. 2008; Houben et al. 2007 for another centromeric retroelement in barley; Gao et al. 2015 in rice species).
The conserved regions of four representatives of *gypsy* lineages (*CRM, Athila, Del* and *Tat*) of *B. decumbens* showed a preference for integration into proximal chromosome regions, which is targeted to heterochromatin locations and suppression of recombination. This shows the non-random distribution of *CR* (centromeric retrotransposons) of *Brachiaria*. These retrotransposons lineages appeared also more concentrated in proximal chromosome regions of *Brachypodium distachyon*, with transposons-like distributed closer to the gene-rich regions (Vogel et al. 2010). In some cereals, such as barley, wheat and rice, centromeric and pericentromeric regions contain large occurrence of *gypsy* LTR-RTs (Cheng and Murata 2003; Nagaki et al. 2005). The accumulation of these elements into the proximal chromosome regions in these four species of *Brachiaria* is in agreement with the proposal of Bennetzen and Wang (2014) suggesting that TEs predominate in recombination-poor regions, which are genomic locations of low rate of unequal homologous recombination and gene conversion. These are also gene-depleted regions where retrotransposons insertion may have minimal effect.

Lisch and Bennetzen (2011) review the different rates of amplification, insertion, and removal of transposable elements, and show the association with epigenetic control. Together, it is clear that transposable elements have a major consequence on the complexity and organization of genomes in different plant species. Different transposable elements, as exemplified by *gypsy* retrotransposons in the *Brachiaria* species examined here, can have characteristic distributions which have different impacts on behavior of genomes and amplification of elements, and hence on the diversification of species.
The elements are not only important to understand for fundamental biological and evolutionary reasons, but also because of their abundance and consequences for gene expression. For breeding of crops, including the *Brachiaria* forage grasses, it is important to exploit the biodiversity within the whole genus by making crosses, synthetic hybrids and polyploids, and transposable elements can be used to provide markers, to define relationships between genomes in hybrids, and examine pairing and recombination at meiosis.

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Legends

**Figure 1.** Transposable element families in the transcriptome of *Brachiaria decumbens*, obtained from RNAseq. The proportions of major families of class I (retrotransposons, 59%) and class II (DNA, 41%) transposable elements are shown.

**Figure 2.** Cladogram showing the relationships between *Ty1-copia* and *Ty3-gypsy* retrotransposable element superfamilies identified in the transcriptome of *B. decumbens* within contigs/unigenes (black names) or as characteristic domains (red). Arrows indicate the *gypsy* LTR-RTs sequences used to design primers for *in situ* hybridization (Table 1).

**Figure 3.** *In situ* hybridization of *gypsy* retrotransposon probes to chromosomes (fluorescing blue with DAPI) of diploid species of *Brachiaria*. A) prometaphase of *B. decumbens* hybridized with RT-*Athila* probe (green). Signals were accumulated in centromeric-pericentromeric regions of all chromosomes, and no signals were detected in distal regions. B) metaphase of *B. decumbens* hybridized with RT-*Athila* probe (observe proximal signals in red) and with RT-*Tat* probe (green) hybridizing as dots along the chromosome arms, with some pericentromeric signals. C) prometaphase of *B. decumbens* hybridized with p*Ta*794 probe (red) and INT-*Tat/Cyclops* probe (green). The 5S rDNA shows four interstitial signals, while *Tat/Cyclops* probe exhibited signals spread from the centromeric region well into the interstitial regions. D) prometaphase of *B. ruziziensis* hybridized with INT-*Tat/Cyclops* probe (green), which showed also signals spread from the centromeric region, reaching interstitial regions. E) metaphase
of B. ruziziensis hybridized with RT-Athila probe (red), with signals accumulated in centromeric-pericentromeric regions of all chromosomes, and INT-Tat/Cyclops probe (green) with dot-like signals in pericentromeric regions and in interstitial-distal signals of few chromosomes. Note an interphase nucleus showing collocation of both probes. F) Metaphase of B. ruziziensis hybridized with RT-CRM probe (green). Observe only proximal signals, without interstitial-distal ones. G) Metaphase of B. ruziziensis hybridized with RT-CRM (red) and GAG-Circe (green) probes, showing collocation at centromeric region in all chromosomes. H) Metaphase of B. brizantha hybridized with RT-Del/bagy-1 (green), showing dot-like signals in proximal, interstitial and distal regions. However, note that there are differences of signals amount among chromosomes. I) Metaphase of B. brizantha hybridized with RT-Del/bagy-1 (green), showing dot-like signals in proximal, interstitial and distal regions, and RT-Athila probe (red) with centromeric-pericentromeric signals. J) Metaphase of B. brizantha hybridized with RT-CRM probe (green), showing only centromeric-pericentromeric signals, without distal ones. Bar = 5 μm.

Figure 4. In situ hybridization of gypsy retrotransposon probes to chromosomes in polyploids of B. humidicola (A, C) and B. brizantha (B, D): A) partial metaphase hybridized with telomeric probe (red), which shows dot-like signals distally positioned, and INT-Tat/Cyclops probe (green) along chromosomes with concentration of signals in centromeric-pericentromeric regions in almost half chromosomes. B) partial metaphase hybridized with RNAse-Tat probe (green). Signals appeared predominantly in centromeric-pericentromeric regions, with some signal extending into the interstitial regions of few chromosomes. C) chromosomes of B. humidicola hybridized with Santos et al. Distribution and evolution of retrotransposons in the forage grass Brachiaria. Page 28.
telomeric probe (red), which showed terminal signals, and with RT-CRM probe (green),
that showed proximal weak signals, and few interstitial dot-like signals. Strong green
signals are nonspecific. D) chromosomes of *B. brizantha* hybridized with pTa794 probe
(green), showing six interstitial-proximal signals, and INT-Tat-cyclop probe (red), with
signals predominantly centromeric-pericentromeric and few interstitial. Bar = 5 μm.

**Figure 5.** *In situ* hybridization of *gypsy* retrotransposon probes to chromosomes in
polyploids of *B. brizantha*: A) metaphase and nucleus hybridized with telomeric probe
(red), which shows dot-like signals terminally positioned, and RT-CRM probe (green)
with signals concentrated in centromeric-pericentromeric regions. In the box inset
below (i, ii and iii) telomeric dots and proximal CRM signals are presented. B) metap"