FABÍOLA CARVALHO SANTOS

CARACTERIZAÇÃO CITOGENÉTICA E GENÔMICA DO GÊNERO *BRACHIARIA* UTILIZANDO DNAS REPETITIVOS

Londrina
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Tese apresentada ao Programa de Pós–Graduação em Genética e Biologia Molecular, da Universidade Estadual de Londrina, como requisito parcial para a obtenção do título de Doutora.

Orientador: Dr. André Luís Laforga Vanzela.

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Aos meus pais Dorval e Sônia e aos meus irmãos Ivo e Flávia com a graça de DEUS

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RESUMO

O gênero *Brachiaria* pertence à tribo Paniceae e compreende cerca de 100 espécies. A maioria das espécies é poliploide, sendo preferencialmente tetraploides ($2n = 4x = 36$) e apomíticas facultativas, tais como *B. decumbens* e *B. brizantha*. Apesar de existirem ecótipos apomíticos de outras espécies, *Brachiaria ruziziensis* é a única de reprodução sexual e diploide ($2n = 2x = 18$), utilizada em cruzamentos para seleção de novas cultivares. A localização de marcas cromossômicas com DNAs repetitivos e não repetitivos constitui uma importante ferramenta para o esclarecimento da organização dos genomas. As técnicas citomoleculares permitem localizar desde regiões de DNA repetitivo até sequências específicas de DNA nos cromossomos e núcleos. O principal foco genético dos estudos em *Brachiaria* é elucidar o tipo de ploidia e a origem dos poliploides no gênero. Para isso, é necessário buscar genomas e complementos monoploides que ofereçam informações acerca das combinações que deram origem a esses poliploides. O objetivo foi contrastar informações obtidas dos cariótipos convencionais, conteúdo C de DNA e a ocorrência de sequências de LTR-RTs gypsy em *Brachiaria*. Nosso maior avanço obtido neste trabalho envolveu o reconhecimento de elementos de transposição a partir de uma coleção de sequências de ESTs de *B. decumbens*. As sondas produzidas servirão como base para comparações com mapas moleculares e melhorar a definição dos mecanismos de diferenciação dos genomas e cariótipos no gênero.

Palavras-chave: Hibridização *in situ*. Citometria de fluxo. Gramíneas forrageiras. Nível de ploidia. Poaceae. Retrotranposons. rDNA. Elementos transponíveis.

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ABSTRACT

The genus *Brachiaria* belongs the tribe Paniceae and contains about 100 species. Most species in this genus is polyploidy, preferably tetraploid (2n = 4x = 36) and apomictic, such as *B. decumbens* and *B. Brizantha*. Although there are most apomictic ecotypes the other species, *Brachiaria ruziziensis* is always found the sexual and diploid (2n = 2x = 18), and the one used in crosses for selection of new improved cultivars with higher genetic variability. The use of cytomolecular techniques citomoleculares allows find from regions of repetitive DNA to specific DNA sequences in chromosomes and nuclei. The main focus of genetic studies in *Brachiaria* is to elucidate the type of ploidy and origins of polyploidy in the genus. For this it is necessary to search for genomes and monoploids complements that provide information about the combinations the origins these polyploid. In order to understand the karyotypes and genomes of *Brachiaria*, this work proposed to identify and localize LTR-RTs gypsy elements, based on a collection of sequences of ESTs of *B. decumbens*. The probes will enhance the construction of the molecular maps and improve the definition of the mechanisms of differentiation of genomes and karyotypes in the genus.

Keywords: Flourescent *in situ* hybridization. Flow cytometry. Grasses forage. Ploidy level. Poaceae. Retrotransposons. rDNA. Transposable elements.

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Introdução

Aspectos reprodutivos e citogenéticos de Brachiaria

O gênero *Brachiaria* (com algumas espécies com sinonímia *Urochloa* (Shirasuna, 2010)) pertencente a família Poaceae, é de origem africana e compreende cerca de 100 espécies (Renvoize et al., 1996). Essas têm grande importância na atividade pecuária por viabilizar a produção de forrageiras em solos ácidos e pobres. O Brasil possui aproximadamente 170 milh.es de hectares de pastagens tropicais (IBGE, 2007), dos quais mais de 120 milhões de hectares dessas pastagens são cultivadas. 45 milhões dessas têm predominância do gênero *Brachiaria* (Sano et al., 1999, Macedo 2005). A produção de sementes de forrageiras tropicais no Brasil foi intensificada a partir dos anos 70 e, desde então, o país destaca-se como maior produtor, consumidor e exportador mundial, com o mercado movimentando cerca de R$ 1,2 bilhão por ano. A produtividade brasileira anual gira em torno de 100 mil toneladas de sementes puras viáveis, sendo que 20% são destinadas à exportação (Embrapa, 2011).

A produção de *Brachiaria* possui alta incidência de apomixia, que é a formação de sementes sem que haja a fecundação. Isto acarreta a formação de clones da planta mãe, dificultando assim a ampliação da variabilidade e o desenvolvimento de novas cultivares, já que as características paternas não são herdadas (Karia, 2006). Contudo, a apomixia pode ser vantajosa para os programas de melhoramento quando o objetivo é a fixação de caracteres agronômicos favoráveis em plantas elite, como por exemplo o vigor híbrido (Hanna e Bachaw, 1987; Grossnikaus et al., 2003; Spilane et al., 2004). Em plantas com reprodução sexual, o saco embrionário é do tipo Polygonum, com oito núcleos polares, mas em plantas apomíticas, o saco embrionário é do tipo Panicum com apenas quatro células (uma célula ovo, duas sinérgides e um núcleo polar). O gênero

Brachiaria possui apomixia facultativa, permitindo assim produzir proles sexuais e apomíticas em algumas espécies (Valle, 1990; Valle e Savidan, 1996).

O conhecimento do nível de ploidia e do modo de reprodução em *Brachiaria* é importante para determinar estratégias de cruzamentos e de condução dos programas de melhoramento. Esses têm como objetivo obter híbridos com características desejáveis de dois ou mais genitores agronomicamente promissores, tais como tolerância a estresses bióticos e abióticos, alta produtividade, bom valor nutritivo e, principalmente, resistência à cigarrinhas-das-pastagens (Valle et al., 2009).

A maioria das espécies do gênero *Brachiaria* é poliploide, tetraploides (2*n* = 4*x* = 36) e apomíticas facultativas, tais como *B. decumbens* e *B. brizantha* (Basappa et al., 1987; Mendes-Bonato et al., 2006a; Utsunomiya et al., 2005; Risso-Pascotto et al., 2006). O número cromossômico básico ainda é alvo de muitas especulações. Foi relatado para o gênero *x* = 7 e 9, com predominância de *x* = 9 (Darlington e Wylie, 1995; Dujardin, 1979; Basappa et al., 1987; Morrone e Zuloaga, 1992; Valle e Savidan, 1996). Risso-Pascotto et al. (2006) relataram a ocorrência de *x* = 6 para acessos de *B. dictyoneura* com 2*n* = 24 e *B. humidicola* com 2*n* = 42, além disso, Boldrini et al. (2009) observaram na meiose presença de seis univalentes em *B. humidicola* propondo também o número básico *x* = 6.

Em Poaceae, os númerous básicos de cromossomos encontrados variam de *x* = 2 a 18, e os números somáticos variam de 2*n* = 4 a 2n = 265 (De Wet, 1987). Em Panicoidea, subfamília a qual o gênero *Brachiaria* pretence, foram relatados números básicos *x*= 5, 6, 7, 8, 9, 10 e 12. Existem dados na literatura sobre *B. decumbens* e *B. brizantha* (Hochst.), que relatam que o número cromossômico básico *x* = 9 é encontrado naturalmente nos acessos 2*x* para a *B. ruziziensis* nos acessos 2x, 4x e 5x para a *B. decumbens* (Goulart, 2008; Penteado et al., 2000) e nos acessos 2x, 4x, 5x e 6x para a *B.*

brizantha (Penteado et al., 2000). Esses números são resultantes provavelmente de eventos de poliploidização (Stebbins, 1985), associados à perda e/ou ganho de cromosomos.

Brachiaria ruziziensis é de repodução sexual e diploide ($2n = 2x = 18$), o que possibilita a realização de cruzamentos, inter e intraespecíficos quando duplicada e consequentemente ampliação da variabilidade para seleção de novas cultivares. O sucesso dos cruzamentos interespecíficos para obtenção de cultivares promissoras só foi obtido após a *B. ruziziensis* sexual ser tetraploidizada viabilizando o cruzamento com acessos tetraploides de *B. decumbens* e *B. brizantha* (Gobbe et al., 1981, Ishigaki et al., 2009; Pinheiro, et al., 2000; Swenne, et. al., 1981; Timbó et al., 2012).

As cultivares produzidas e disponíveis no mercado são, em geral, originadas de poucos acessos de *B. brizantha, B. decumbens* e *B. humidicola*, o que provoca um estreitamento na base genética dos materiais direcionados ao melhoramento (Keller-Grein et al., 1996). Atualmente, estão registradas no Ministério da Agricultura, Pecuária e Abastecimento (MAPA) mais quinze cultivares do gênero *Brachiaria* aptos para produção, e comercialização (BRASIL, 2011). Duas delas, *B. brizantha* cv. Marandú e *B.decumbens* cv. Basilisk, respondem por mais de 80% do volume de sementes de forrageiras tropicais comercializadas no Brasil (BRASIL, 2007). Em relação à grande extensão territorial de nosso país, isso demonstra que há pouca variabilidade genética disponível podendo trazer prejuízos enormes para o setor agropecuário.

Híbridos interespecíficos exibem, em geral, anormalidades meiótica, que afetam em graus variados a fertilidade e comprometem a formação de sementes viáveis (Valle e Pagliarini, 2009). Algumas irregularidades meióticas têm sido frequentemente descritas nos acessos poliplóides em análises citológicas de espécies de *Brachiaria*, como ascensão precoce de cromossomos para os pólos nas metáfases, cromossomos

retardatários nas anáfases, levando à formação de micronúcleos nas telófases e tétrades. Outras anormalidades, como aderência cromossômicas, também foram observadas em alguns acessos (Mendes Bonato et al., 2002, Risso-Pascotto et al., 2003, 2006). Tais irregularidades meióticas comprometem a viabilidade dos grãos de pólen (Mendes-Bonato et al., 2004; Risso-Pascotto et al., 2004), pois elas podem levar à esterilidade completa do gameta masculino, sendo essa, a provável causa da baixa produção de sementes em alguns acessos. Mesmo que alguns híbridos tenham qualidade de forragem, resistência à pragas e doenças, esses não poderão ser liberados comercialmente, e a escassez de sementes desses híbridos comprometem sua adoção para implantação/renovação de pastos e de produção de sementes.

Algumas evidências apontam a hibridação natural como a fonte das irregularidades meióticas (Basappa et al*.*, 1987), como reportado em *B. decumbens, B. ruziziensis*, *B. brizantha* var. *brizantha* e *B. humidicola* (Boldrini et al., 2010). Entretanto, as informações ainda são superficiais e especulativas. A associação de informações morfológicas, citológicas e moleculares possibilitou estabelecer relações de parentesco, e forneceu subsídios aos taxonomistas para detectar características intermediárias entre *B. brizantha, B. decumbens* e *B. ruziziensis* (Tohme et al., 1996).

A identificação morfológica das espécies de *Brachiaria* ainda é confusa, e isso traz uma série dificuldades para os programas de melhoramento (Shelton, 2007). Embora o gênero tenha sido reconhecido no século XIX, a classificação botânica de *Brachiaria* não é considerada consistente em razão da dificuldade de definição clara de características morfológicas. Uma dificuldade foi a classificação errônea de *B. ruziziensis* e *B. brizantha*, e a delimitação de cv. 'Basilisk', amplamente difundido como *B. decumbens* e não como *B. brizantha* (Maass 1996; Renvoize et al., 1996). *Brachiaria humidicola* e *B. dictyoneura*, têm características vegetativas bastante

similares (Renvoize et al., 1996), o que gera confusões por parte dos agrônomos.

Aproximadamente 40% das espécies de *Brachiaria* é conhecida sob o ponto de vista citogenético e, para a maioria delas, as informações são limitadas às contagens cromossômicas (Bernini e Marin-Morales, 2001; Utsunomiya et al., 2005; Risso-Pascotto et al., 2009), comportamento meiótico (Mendes-Bonato et al. 2006a; Boldrini et al., 2011). Informações sobre mapeamento físico com sondas de DNAr (Nielen et. al., 2010) e citometria de fluxo (Ishigaki et al., 2010; Timbó et al., 2012,2014) foram disponibilizadas recentemente, contudo, nenhuma dessas contribuições possibilitou o reconhecimento de complementos monoploides ou das fontes das inúmeras irregularidades meioticas já reportadas em híbridos de *Brachiaria* (Mendes-Bonato et al., 2006b, 2007).

Bernini & Marin-Morales (2001) caracterizaram o cariótipo de 12 acessos e cinco espécies de *Brachiaria*. Estes autores relataram variações no número cromossômico em *B. decumbens* ($2n$ = 18 e 36) e em *B. humidicola* ($2n$ = 36, 42 e 54). *Brachiaria jubata* e *B. brizantha* apresentaram $2n$ = 36 e *B. ruziziensis,* $2n$ = 18. Nestas análises, com exceção de um acesso de *B. humidicola* (x = 7), o número básico predominante foi 9. Os cariótipos foram classificados como simétricos com tendência à assimetria em direção aos poliplóides, além da presença de satélite no par de cromossomos 8. Anteriormente, Valle et al. (1987) havia identificado os nove cromossomos por meio de mapas paquitênicos, distribuição e tamanho dos cromômeros e medições cromossômicas. Esses dados mostraram que a maioria dos cromossomos são submetacêntricos, com a região organizadora do nucléolo (RON) no par 7. Nielen (2010) encontrou o satélite no mesmo par de cromossomos que Bernini e Marin-Morales (2001) no acesso analisado de um diplóide sexual de *B. brizantha*, sugerindo então possíveis alterações estruturais durante a evolução desses acessos.

Análises citogenéticas convencionais permitem comparar populações e espécies quanto ao número, tamanho e forma dos cromossomos, além do comportamento meiótico. Contudo, técnicas mais sofisticadas de citogenética molecular fornecem maiores detalhes sobre a ocorrência de rearranjos estruturais, como microdeleções, inversões e translocações, correlacionadas às divergências genéticas. Neste sentido, o uso associado de diferentes ferramentas citogenéticas torna mais eficaz os estudos de caracterização cromossômica e evolução cariotípica, resolução de problemas taxonômicos e biossistemáticos (Sharma e Sem, 2002).

Citometria de fluxo

A determinação da ploidia por contagem de cromossomos é muito utilizada quando se trata de pequeno número de materiais. Entretanto, em programa de melhoramento, quando a quantidade de plantas é grande a técnica é laboriosa. A citometria de fluxo vem sendo utilizada com mais eficácia na quantificação de DNA nuclear em um número grande de amostras plantas.

Os valores C e Cx, ou seja, o conteúdo de DNA de um complemento haploide de cromossomos não replicados, fornecem uma comparação mais útil nos casos de ampla amostragem (Bennett et al., 2000, 2005; Schifino-Wittmann, 2001). Esta técnica tem como princípio a análise da intensidade de fluorescência do núcleo após o DNA ser corado com fluorocromos específicos, como o brometo de etídeo, iodeto de propídeo, DAPI ou outros.

Essa técnica posssibilita avaliar um grande número de indivíduos com rapidez, além de permitir a discriminação das progênies de híbridos interespecíficos geradas por fecundação ou apomixia, sendo assim adequado para o gênero *Brachiaria*. A maior dificuldade para os melhoristas está justamente na identificação morfológica desses

híbridos. A citometria de fluxo tem sido aplicada com sucesso em estudos de hibridizações em milho alotetraploides (Hammatt et al., 1991) salgueiro chorão (Thibault, 1998) e híbridos monoploides de *Limonium* (Morgan et al., 1995). Em espécies de *Hieracium*, por exemplo, o híbrido originado de duas espécies (*H. brachiatum* e *H. leptophyton*) exibiu um conteúdo C de DNA intermediário aos dos genitores (Brautigam e Brautigam, 1996). Sabharwal e Dolezel (1993) conseguiram identificar híbridos interespecíficos no gênero *Brassica*, os quais eram compostos por genomas de diferentes tamanhos (A, B e C).

A quantificação do valor C de DNA é importante também para projetos de análises de sequenciamento (Hardie et al., 2002), relações filogenéticas (Moscone et al., 2003; Suda et al., 2003), sistemática e taxonomia vegetal (Volgmayr e Greilhuber, 1998; Misset e Gourret, 1996; Lysák e Doležel, 1998). Para Bennett (1972) a determinação do tamanho do genoma é importante também por que o conteúdo de DNA influencia em caracteres fenotípicos, fenológicos, comportamento ecológico. Contudo, essa técnica vem sendo empregada no melhoramento de plantas para monitorar a estabilidade do nível de ploidia (Bonos et al., 2002; Casler et al., 2006; Gore et al., 2011), certificar indivíduos duplicados (Pinheiro, 2000; Latado et al., 2007; Campos et al., 2009), identificar haploides e duplohaploides em culturas de anteras e ovários, inferência de novos níveis de ploidia em resultados de cruzamentos (Keller-Grein et al., 1996, Hirsch et al., 2001), caracterizar germoplasmas (Bartos et al., 2005; Pillay et al,. 2006), detectar indivíduos aneuploides (Roux et al., 2003; Ozaki et al., 2004) e apomíticos (Matzk et al., 2000; Kelley et al., 2009), ciclo celular (Pozarowski, 2004) e na identificação de híbridos interespecíficos (Keller et al., 1996; Buitendijk et al., 1997; Bare et al., 1998; Thibault, 1998). Estudos de quantificação do valor C de DNA em

gramíneas foram publicados nos gêneros *Pennisetum* (Martel et al., 1997), *Brachiaria* (Penteado et al., 2000; Timbó, 2012) e *Agrostis* (Bonos et al., 2002).

Em espécies de *Brachiaria,* estudos de citometria de fluxo vêm sendo realizados para a determinação do nível de ploidia de diferentes genótipos como subsídio aos programas de melhoramento. Como há um volume muito grande de experimentos no campo, essa análise pode subsidiar na certificação de genótipos e avaliação das progênies de híbridos interespecíficos. Letterielo et al. (1999), por exemplo, associaram os resultados de contagens cromossômicas e de determinação do valor C de DNA para certificação da ploidia em acessos de *Brachiaria brizantha* e mencionaram, pela primeira vez, a ocorrência de acessos pentaplóides com $2n = 5x = 45$ nesta espécie. Penteado et al. (2000) determinaram o nível de ploidia de uma coleção de germoplasma de 435 acessos, pertencentes a 13 espécies de *Brachiaria*. Neste caso, a citometria mostrou-se muito eficaz na determinação de vários níveis de ploidia. Nos estudos de Ishigaki et al. (2009) e Timbó et al. (2014) foram relatados que o conteúdo de DNA em *B. ruziziensis* pode variar de cerca de 1,41 pg para as genomas diploides a 2,77 pg para genomas tetraploides.

Diferenças nos valores de conteúdo de DNA em picogramas, quando são comparadas entre amostras com os mesmos níveis de ploidia, podem ser explicadas de diferentes maneiras: i) pela plasticidade dos genomas decorrentes de variações interespecíficas (Bennett e Leitch, 1995, 2005) e intraespecíficas (Murray, 2005; Ozkan et al., 2010; Smarda e Bures, 2006; Smarda et al., 2008) ou ii) por variações decorrentes da distribuição geográfica (Dolezel et al., 2007). Contudo, outros autores como Greilhuber (2005), Greilhuber e Obermayer (1998a,b), Baranyi e Greilhuber (1999) e Bennett et al. (2003), comparam as variações de acordo com diferentes metodologias empregadas e concluíram que tais diferenças poderiam surgir em decorrência dos

procedimentos técnicos, além daqueles dos próprios genomas. Os efeitos extragenômicos poderiam estar relacionados com a presença de metábolitos secundários (Noirrot et al., 2003; Walker et al., 2006), ou coleta do material em diferentes posições geográficas, preparação das amostras, padrão de referência em picogramas, tampão de extração dos núcleos, interferência de inibidores endógenos de coloração, Greilhuber (2005).

Outra hipótese para a explicação das variações no tamanho do genoma é a da amplificação/redução na quantidade de elementos de tranposição, tamanho dos cromossomos e presença de heterocromatina. Flavell et al. (1980), sugeriram que sequências de DNA repetitivo podem influenciar na determinação do tamanho do genoma. Bennetezen et al. (2005), estudando presença de retrotranposons em *Arabidopsis,* sugeriram que as diferenças na quantificação do DNA são causadas pelos mecanismos de regulação e gerações de deleções entre espécies, causadas por recombinação ilegítima.

De acordo com Bernini e Marin-Morales (2001) os cromossomos em *Brachiaria* são muito parecidos em forma e tamanho, o que dificulta a discriminação das espécies baseadas em informações cariotípicas convencionais. Como argumentado anteriormente, a citometria de fluxo possibilita resultados mais refinados, permitindo em alguns casos uma comparação mais segura. Um exemplo de eficácia da citometria de fluxo foi o estudo de Morgans-Richard (2004), o qual conseguiu discriminar dois genomas pela diferença de 22% nos valores C de DNA em *Hieracium pilosella* e *Hieracium praealtum,* cujos cariótipos são praticamente indistinguíveis.

Como parte dos programas de melhoramento genético da *Brachiaria*, a avaliação sistemática dos genótipos deve ser feita em diferentes gerações para identificar materiais duplicados e potenciais candidatos ou elites presentes na coleção de trabalho

além de contaminação dos mesmos. Dentre as avaliações, a citogenética juntamente com a técnica de citometria de fluxo podem fornecer informações mais rápidas para discriminação de genótipos, certificação de ploidias, certificação de progênies de híbridos interespecíficos, análise das anormalidades meióticas, determinação de número cromossômico e de similaridades cariotípicas, entre outros. Estas informações podem auxiliar a tomada de decisão dos melhoristas e contribuir para reduzir o tempo na escolha dos genótipos mais estáveis e compatíveis.

Hibridização in situ com fluorocromos (FISH)

A FISH emprega sondas de DNA para a localização física de sequências específicas nos núcleos e cromossomos. Excelentes estudos foram realizados empregando a FISH (Fukui et al. 2001; Heslop-Harrison et al. 2000; Macas et al. 2011; Suzuki et al. 2012; Wang et al. 2013), e permitiram não somente o reconhecimento dos pares cromossômicos, mas também estabelecimento dos níveis de manutenção ou quebra de sintenias, bem como identificar as áreas de maior expressão gênica ao longo dos braços cromossômicos. A produção de sondas, a qual consideramos o ponto mais crítico na área da citogenética molecular, vem sendo resolvida com maior sucesso após a disseminação e o barateamento do sequenciamento genômico. Sondas podem ser obtidas a partir da caracterização de DNAs repetitivos, como retrotranposons e DNAs satélites, gerados após a montagem de *reads* de saída de sequenciamento de nova geração. A partir desses *contigs* é possível desenhar *primers* para produção de sondas diretamente por PCR, com posterior hibridizações *in situ* e construção de mapas físicos (Heslop-Harrison 2000; Yang et al. 2012; Alipour et al., 2013; Kolano et al,. 2013).

É fato que a maior fração do genoma dos eucariontes superiores é formada por DNAs repetitivos, que variam desde dinucleotídeos a mais de 10.000 pares de bases.

Esses elementos repetitivos podem ser divididos em dois grupos: sequências em *tandem*, como por exemplo, as repetições teloméricas, DNAr e os DNAs satélites e sequências dispersas: compreendendo principalmente a maioria dos elementos transponíveis (Schmidt e Heslop-Harrison, 1998; Heslop-Harrison, 2012).

Sequências repetitivas podem ser organizadas em categorias: i) repetições que ocorrem em tandem e formam grandes blocos incluindo familias de satélites com mais de 100 pb, ii) sequências de minisatelites com 10 a 100 pb, iii) sequências simples repetidas, com 2 a 10 pb, além de outras famílias gênicas (ribossômicas, e elementos de transposição) (Heslop-Harrison, 2000). Essas categorias podem apresentar diferentes repetições e variações, e que podem ser empregadas como marcas específicas para espécies ou cromossomos (Willard e Waye, 1987; Heslop-Harrison, 2000; Friesen et al., 2001; Dechyeva et al., 2003).

Nos cromossomos, a composição, a estrutura, a origem, o aumento e a diminuição dessas seqüências têm sido os principais parâmetros no estudo da evolução dos cariótipos e, por conseqüência, da variabilidade genética (Sharma e Sem, 2002). O grande número de cópias e a tendência à formação de blocos fazem dos DNAs repetitivos alvos ideais para experimentos de hibridização *in situ*, pois são mais fáceis de serem localizados. As sequências de DNAr 45S e 5S, por exemplo, são bastante conservadas, permitindo que sondas originalmente isoladas de diferentes espécies, como trigo (Gerlach e Bedbrook, 1979; Gerlach e Dyer, 1980), possam ser utilizadas na localização desses segmentos em diferentes espécies vegetais. Além disso, a característica conservada dessas sequências e a sua presença em um ou mais sítios cromossômicos fizeram do DNAr 45S e 5S importantes marcas cromossômicas para estudos citogenéticos (Heslop-Harrison, 2000).

A FISH foi recentemente empregada no gênero *Brachiaria*, sendo necessários

estudaram mais profundos em busca da delimitação dos possíveis genomas monoploides. Nielen et al. (2010) estudaram a morfologia dos cromossomos e foi o primeiro a utilizar a hibridização *in situ* (FISH) com genes ribossômicos 5S e 45S em diploides sexuais e um tetraploide apomítico de *B. brizantha*. Esses autores encontraram indícios de similaridades morfológicas entre os acessos tetraploides estudados previamente por Bernini e Marin-Morales (2001). Há indícios de possíveis alterações estruturais na evolução cariotípica deste gênero, contudo a precariedade e a baixa definição no reconhecimento dos centrômeros deixa uma série de dúvidas sobre a organização dos cariótipos e a origem dos poliploides. Akiyama et al. (2010), utilizaram sondas de DNAr 5S e 25S de rDNA em *B. brizantha* e cv. Mulato (*B. brizantha x B. ruziziensis*) e encontraram uma correlação da sonda 25S com o nível de ploidia

Os elementos transponíveis (TEs) são componentes importantes do genoma e constituem grande parte do material genético de diversos eucariotos (Feschotte et al., 2002). Esses elementos são classificados em duas classes. A classe I é composta por elementos cuja transposição é intermediada via um RNA (copia e cola), e englobam os não LTRs (LINEs e SINEs) e os LTR compostos pelos Ty3-*gypsy* e Ty1-*copia*, além do BEL e DIRS. A classe II engloba elementos que a transposição se movimentam por corta e cola mediado pela tranposonsase (tranposons). Os retrotransposons utilizam um RNA intermediário e a enzima transcriptase reversa para produzir uma nova molécula de DNA. Por ação da integrase, o DNA recém sintetizado é integrado ao genoma em posições *cis*, formando blocos, ou *trans*, com caráter disperso (Kazazian, 2004). Este tipo de mecanismo pode levar ao acúmulo de muitas cópias, aumentando significativamente o tamanho dos genomas, como por exemplo, em espécies de *Poaceae* (Langdon et al., 2000). A distribuição desses elementos nos genomas tem sido

analisada em um grande número de plantas utilizando a hibridização *in situ* (Friesen et al., 2001).

Apesar da origem e do papel biológico dessas sequências móveis variarem de acordo com a natureza das sequências e dos genomas, elas possuem um importante papel na geração de variabilidade genética e plasticidade genômica (Flavell et al., 1977; Evgenev, 1997). Os elementos transponíveis, por exemplo, movimentam-se nos genomas e causam mutações resultando ou não em mudanças fenotípicas. Tais mutações muitas vezes são favoráveis, contrbuindo para o aumento da diversidade genética existente na natureza (Charlesworth et al., 1994).

Os TEs constituem grande parte do material genético de diversos eucariotos, alcançando 45% do genoma humano e variando entre 50-80% do genoma de algumas gramíneas (Feschotte et al., 2002). Esses elementos são classificados de acordo com a similaridade da sequência e o modo de transposição (Charlesworth et al., 1994; Kubis et al., 1998). Em *Brachypodium* os TEs ocupam 21,4% do genoma, comparados com 26% do arroz, 54% em sorgo e mais que 80% em trigo (Bennetzen, 1997; International-Brachypodium-Initiative, 2010).

Os elementos de transposição, além de gerar marcas cromossômicas em blocos ou dispersas quando analisados por FISH (Yuyama et al., 2012), são muito comuns em centrômeros de diversas espécies, sobretudo alguns da superfamília *gypsy*-CRM. Esses elementos poderiam oferecer informações adicionais sobre a organização dos centrômeros, o que é uma das demandas nos estudos cromossômicos em *Brachiaria*. Retrotransposons têm sido encontrados em regiões centroméricas em plantas, como por exemplo, em *Arabidopsis* (Tsukahara et al., 2012) e *Triticum* (LI et al., 2013). Peterson-Burch et al. (2004) observaram em um estudo com *Arabidopsis*, que linhagens como *TAT* e *Athila* foram encontradas em regioes pericentromericas. Como mencionado

anteriormente, os poliploides em *Brachiaria* exibem uma série de irregularidades meióticas, incluindo a eliminação de cromossomos e complementos ao longo da meiose (Mendes-Bonato et al., 2002), e isso poderia estar relacionado ao funcionamento do centrômero. Este exemplo mostra claramente a demanda em estudar mais profundamente a organização e localização de DNAs repetitivos de *Brachiaria* por técnicas citomoleculares visto que a identificação de genomas mais próximos nas espécies do gênero poderá auxiliar na definição de cruzamentos mais promissores que podem levar a uma redução nas anormalidades meióticas aumentando assim a viabilidade polínica e consequentemente genitores mais férteis.

Estudos tentaram elucidar a hipótese do tipo de ploidia no gênero *Brachiaria*. Mendes-Bonato et al. (2002), por exemplo, estudou o comportamento meiótico em espécies de *Brachiaria* e encontrou uma frequência baixa de multivalentes, a qual considerou um indício de alopoliploidia segmental. Nielen et al. (2010) também sugeriram a alopoliploidia, corroborando com a hipótese de outros autores (Mendes-Bonato et al., 2002; Mendes et al., 2006; Boldrini et al., 2009; Akiyama et al., 2010). Trabalhos realizados por Akiyama et al. (2008 e 2010) conseguiram distinguir cromossomos utilizando a FISH com DNAr 5S e 45S em diferentes espécies de *Brachiaria*. Além disso, Akiyama et al. (2010) indicaram a presença de diferentes genomas nas espécies de *Brachiaria*.

Alguns autores relataram variações intraespecíficas e interespecíficas no conteúdo C (Ishigaki et al., 2009; Ishigaki et al., 2010; Penteado et al.,, 2000; Timbó et al., 2014). Uma das explicações sobre as variações no tamanho dos genomas é relativa às variações na quantidade/qualidade e ativação/silenciamento de DNAs repetitivos. Em *Oryza* e *Arabidopsis*, por exemplo, há diferenças no conteúdo C de DNA que são causadas por mecanismos diferenciais de regulação e acúmulo de retrotransposons, associados ou não

a alterações estruturais cromossômicas (Bennetzen et al., 2005, 2007; Gregory, 2003). Esses elementos genômicos são alvos interessantes do ponto de citogenético já que pode ocupar grandes frações dos genomas variando, por exemplo, de 21,4% em *Brachypodium* até 80% em *Triticum* (International-Brachypodium-Initiative 2010, Charles et al., 2008).

Faltam informações sobre marcas cromossômicas ou genômicas que possibilitem o reconhecimento e a seleção mais precisa de genomas e matrizes ótimas para as futuras hibridações nos programas de melhoramento, onde espécies mais próximas poderão ser indicadas para formação de híbridos interespecíficos viáveis. Portanto, há a necessidade de uma revisão mais robusta sobre a constituição cariotípica frente ao que está descrito na literatura, haja vista que a demanda atual das pesquisas depende do reconhecimento correto dos pares cromossômicos e dos complementos monoploides nos híbridos. Com o uso de técnicas citogenéticas mais sofisticadas, podem ser fornecidos detalhes sobre o reconhecimento dos pares cromossômicos e com isso, permitir maior clareza sobre a ocorrência de rearranjos estruturais, tais como microdeleções, inversões e translocações, correlacionadas às divergências genéticas nos diploides e poliploides. Assim, esperamos gerar informações que contribuíram para a resolução de problemas genéticos, taxonômicos e agronômicos para este grupo de plantas.

Objetivo Geral

Com o objetivo de gerar informações mais detalhadas sobre os cariótipos, conteúdo C de DNA e os genomas das espécies de *Brachiaria,* pretendemos identificar, caracterizar e determinar ocorrência e a distribuição de marcas genoma-específicos utilizando características cromossômicas e genômicas obtidos por citogenética convencional, molecular e citometria de fluxo. Famílias de DNA repetitivos foram

caracterizados de dados de RNAseq de *B. decumbens*, sobretudo diferentes famílias de proteínas RT da superfamília *Ty3/gyspsy*. Diante disso será possível interferir sobre as relações inter e intraespecíficas, detectar possíveis variações que ocorreram na diferenciação dos cariótipos e na organização dos genomas das espécies de *Brachiaria*, do ponto de vista do conteúdo de DNA e ocorrência de LTR-RTs.

7. Objetivos específicos

- Caracterizar os cariótipos e conteúdos C de DNA de *Brachiaria* incluindo espécies diplóides e poliplóides (*B. decumbens, B. brizantha, B. ruziziensis, B. humidicola* e *cultivares Llanero*) utilizando as seguintes ferramentas citogenéticas: i) coloração convencional, ii) citometria de fluxo, iii) hibridização *in situ* com as sondas de diferentes famílias protéicas de membros da superfamília *Ty3/gyspsy.*
- Utilizar os resultados obtidos pela caracterização cariotípica das espécies para identificar fisicamente os locais preferenciais de ocorrência de elementos gypsy-like. Estes, por sua vez, serão empregados em uma análise comparativa entre poliplóides e diplóides do gênero *Brachiaria*, a fim de auxiliar no entendimentoo dos os processos evolutivos envolvidos na diferenciação cromossômica e genômicas.
- Utilizar todas as informações geradas para construir idiogramas mais seguros que auxiliam na identifica de espécies base para o melhoramento, a fim de buscar os prováveis genomas que compõem os poliploides. O ganho desse estudo seria sugerir genótipos alvo para produção de híbridos com meiose mais regular e assim oferecer subsídios para os programas de melhoramento.

Referências

Akiyama Y. *et al.* (2008). Estimation of genome size and physical mapping of ribosomal DNA in diploid and tetraploid guineagrass (Panicum maximum Jacq.). **Grassland Science**, v.54, n.2, p.89–97.

Akiyama Y. *et al.* (2010). Morphological diversity of chromosomes bearing ribosomal DNA loci in *Brachiaria* species. **Japanese Society of Grassland Science**, v.56, n.4, p. 240-242.

Alipour A. *et al.* (2013). Structural characterization of copia-type retrotransposons leads to insights into the marker development in a biofuel crop, *Jatropha curcas*. **Biotech nology for Biofuels, *v.*6,** n.1, p.129.

Bare P *et al.* (1998). Relationship between parental chromosomic contribution and nuclear DNA content in the coffee interspecific hybrid C. pseudozanguebariae · C. liberica var. 'dewevrei'. **Theoretical and Applied Genetics,** v.96, n.2, p. 301–305.

Bartos J. *et al*. (2005). Nuclear genome size and genomic distribution of ribosomal DNA in *Musa* and *Ensete* (Musaceae): taxonomic implications. **Cytogenetic and Genome Research**, v. 109, n. 1/3, p. 50-57.

Basappa G.P, Muniyamma M., Chinnappa C.C. (1987). An investigation of chromosome numbers in the genus *Brachiaria* (Poaceae:Paniceae) in relation to morphology and taxonomy. **Canadian Journal Botany,** v.65, n.11, p. 2297-2309.

Baranyi M e Greilhuber J. (1999). Genome size in Allium: in quest of reproducible data. **Annals of Botany,** v.83, n.6, p. 687–695.

Bennett M.D. (1972). Nuclear DNA content and minimum generation time in herbaceous plants. Proceedings of the Royal Society of London, Series BÐBiological Sciences, n.181, n.63, p. 109-135.

Bennett M.D. e Leitch I.J. (1995). Nuclear DNA amounts in angiosperms. **Annals of Botany**, v.76, p. 113–176.

Bennett M.D. e Leitch, I.J. (2000). Nuclear DNA amounts in angiosperms and their modern uses - 807 new estimates. **Annals Botany**, Bristol, v.86, n. 4, p.859-909.

Bennett M.D e Leitch I.J. (2003). Plant DNA C-values database (release 6.0), http://www.rbgkew.org.uk/cval/homepage.html (accessed 20 Oct. 2014).

Bennett M.D. e Leitch I.J. (2005). Nuclear DNA amounts in angiosperms - progress, problems and prospects. **Annals Botany,** v. 95, n.1, p. 45-90.

Bernini C. e Marin-Morales M.A. (2001). Karyotype analysis in *Brachiaria* (Poaceae) species, **Cytobio**s, v.104, n.407, p.157-171.

Bennetzen J.L., Kellogg E.A. (1997). Do plants have a one-way ticket to genomic obesity? **Plant Cell,** v.9, n.9, p.1509–1514.

Bennetzen J.L. *et al.* (2005). Mechanisms of recent genome size variation in flowering plants, **Annals of Botany**, v.95, n.1 p.127–132.

Bennetzen J.L. (2007). Patterns in grass genome evolution, **Current Opinion in Plant Biology**, v.10, n.2 p.176–181.

Boldrini K.R., Pagliarini M.S., Valle C.B. (2009). Meiotic behavior of a nonaploid accession endorses x = 6 for *Brachiaria humidicola* (Poaceae). **Genetics and Molecular Research,** v.8, n.3, p.1444-1450.

Boldrini K.R., Pagliarini M.S., Valle C.B. (2010). Evidence of natural hybridization in *Brachiaria humidicola* (Rendle) Schweick. (Poaceae: Panicoideae: Paniceae). **Journal of Genetics,** v.89, n.1, p.91-94.

Boldrini K.R *et al.* (2011). Meiotic behavior in nonaploid accessions of *Brachiaria humidicola* (Poaceae) and implications for breeding. **Genetics and Molecular Research,** v.10, n.1, p.169-176.

Bonos S.A., Plumley, K.A., Meyer, W.A. (2002). Ploidy determination in Agrostis using flow cytometry and morphological traits. **Crop Science,** v.42, n.1, p. 192–196.

Buitendijk J.H., Boon E.J., Ramanna M.S. (1997). Nuclear DNA Content in Twelve Species of AlstroemeriaL. and some of their hybrids. **Annals of Botany**, v.79 n.4, p.343–353.

Bräutigam S., Bräutigam E. (1996). Determination of the ploidy level in the genu *Hieracium* Subgenus *Pilosella* (Hill) S.F. Gray by flow cytometric DNA analysis. **Folia Geobot Phyotax,** v.31**,** n.3, p. 315–321.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. SNPC – Registro Nacional de Cultivares. Disponível em: . Acesso em: 23 jul. 2007. BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. SNPC – Registro Nacional de Cultivares. Disponível em: . Acesso em: 27 jan. 2011.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. SNPC – Registro Nacional de Cultivares. Disponível em: . Acesso em: 23 jul. 2007. BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. SNPC – Registro Nacional de Cultivares. Disponível em: . Acesso em: 27 jan. 2011.

Campos J.M.S. *et al*. (2009). *In vitro* induction of hexaploid plants from triploid hybrids of *Pennisetum purpureum* and *Pennisetum glaucum*. **Plant Breeding,** v. 128, n. 1, p. 101-104.

Casler M.D., Vogel K.P., Beal A.C. (2006). Registration of WS4U and WS8U switchgrass germplasms. **Crop Science**, v. 46, n.2, p.998–999.

Charlesworth B., Sniegowski P., Stephan W. (1994). The evolutionary dynamics of repetitive DNA in eukaryotes. **Nature**, v. 371, n.15, p. 215-220.

Charles M. *et al.* (2008). Dynamics and differential proliferation of transposable elements during the evolution of the B and A genomes of wheat**. Genetic**, **v.**180**,** n.2, p.1071-1086.

Darlingtion C.D. e Wylie A.P. (1995). Chromosome atlas of flowering plants. 2nd edn. London: Geroge Allan & Unwin.

De Wet J.M.J. (1987). Hybridization and polyploidy in the Poaceae. In T. R. Soderstrom, K. W. Hilu, C. S. Campbell, and M. A. Barkworkth [eds.], Grass systematics and evolution. Smithsonian Institution Press, Washington, DC, p. 188-194.

Dechyeva D., Gindullis F., Schmidt T. (2003). Divergence of satellite DNA and interspersion of dispersed repeats in the genome of the wild beet *Beat procumbens*. **Chromosome Research**, v.102, n.4, p. 521-530.

Dujardin M. (1979). Additional chromosome numbers and meiotic behavior in tropical African grasses from western Zaire. **Canadian Journal of Botany,** v.57, n.8, p.864-876.

Doležel J., Greilhuber J. (2007). Suda J.Estimation of nuclear DNA content in plants using flow cytometry. **Nature Protocols,** n.2, p.2233–2244.

EMBRAPA. Banco de notícias Embrapa Gado de Corte - 18 de julho de 2011. Disponível em: **<http://www.cnpgc.embrapa.br/index.php?pagina=bancodenoticias/2011/18072011_ curso.html>**. Acessado em: 08 de set. de 2011.

Evgen'ev M.B. *et al*. (1997). *Penelope*, a new family of transposable elements and its possible role in hybrid dysgenesis in *Drosophila virilis*. **Proceedings of the National Academy of Sci USA,** v.94, n.1, p.196–201.

Feschotte C., Jiang N., Wessler S.R. (2002). Plant transposable elements: where genetics meets genomics. **Nature Reviews Genetics,** v.3, p.329–341.

Fukui K.I *et al.* (2001). Physical arrangement of retrotransposon-related repeats in centromeric regions of wheat. **Plant & Cell Physiology**, v.42, n.2, p.189-196.

Friesen N., Brandes A., Heslop-Harrison, J.S. (2001). Diversity, origin and distribution of retrotransposons (*gypsy* and *copia*) in conifers**. Molecular Biology and Evolution**, v.18**,** n.7, p.1176–1188.

Flavell R.B., Rimpau J. (1977). Smith D.B .Repeated sequence DNA relationships in four cereal genomes. **Chromosoma (Berl***)*, v. 63, n.1, p. 205-222.

Flavell R.B. (1980). The molecular characterization and organization of plant chromosomal DNA sequences. **Annual Review of Plant Physiology**, v.31, p.569-596.

Gerlach W. L., Bedbrook, J. R. (1979). Cloning and characterization of ribosomal RNA genes from wheat and barley. **Nucleic Acids Research** v.7, n.7, p.1869-1885.

Gerlach W.L., Dyer T.A. (1980). Sequence organization of the repeated units in the nucleus of wheat which contain 5S-rRNA genes. **Nucleic Acids Research,** v.8, n.21, p.4851-4865.

Gregory T.R. (2003). *Is small indel bias a determinant of genome size.* **Trends in Genetics,** *v.*19, n.9, p.485*–488.*

Gobbe J., Swenn A., Louant B.P. (1981). Diploides naturals et autotétraploides induits chez *Brachiaria ruziziensis* Germain et Evrard: critères d'identification. **Agron Tro** n.36, p.339–346.

Goulart J.C. (2008). Citogenética e anatomia foliar de acessos e progênies de Brachiaria. Lavras: UFLA 71p (Dissertação mestrado).

Gore M.A. *et al.* (2011). Complex ploidy level variation in guayule breeding programs. **Crop Science,** v.51, n.1, p.210–216.

Greilhuber J e Obermayer R. **(**1998a**)** Genome size variation and maturity group in the soybean, Glycine max. In: Lelley T, ed. Current topics in plant cytogenetics related to plant improvement. **Proceedings of the International Symposium on Current Topics in Plant Cytogenetics Related to Plant Improvement**, Tulln, Austria, 21–22 Feb. 1997. Tulln: Institute for Agrobiotechnology, 290–296.

Greilhuber J. e Obermayer R. (1998b). Genome size variation in Cajanus cajan (Fabaceae): a reconsideration. **Plant Systematics and Evolution** n.212, n.1/2, p.135–141.

Greilhuber J. (2005). Intraspecific variation in genome size in angiosperms: identifying its existence. **Annals of Botany,** v.95, n.1 p.91-98.

Grossniklaus U. *et al.* (2003) Engineering of apomixis in crop plants: what can we learn from sexual model systems? In Plant Biotechnology 2002 and Beyond. proceeding so the 10th IAPTC&B Congress (ed.Vasil, I.K.), pp. 309-314. Kluver Academic Publishers, Boston, U

Hammatt N., Blackhall N.W., Davey M.R. (1991). Variation in the DNA content of *Glycine* species. **Journal of Experimental Botany,** n.42, p.659–665.

Hanna W.W., Bashaw E.C. (1987). Apomixis: its identification and use in plant breeding. **Crop Science**, Madison, v. 27, n.6, p. 1136-1139.

Hardie D.C., Gregory T.R., Hebert P.D.N. (2002). From pixels to pictograms a beginers' guide to genome quantification by Feulgen image analysis densitometry. J Histochem **Cytochem** , v. 6, n. 50, p.735–749.

Heslop-Harrison J.S. (2000). Comparative Genome Organization in Plants: From Sequence and Markers to Chromatin and Chromosomes. **The Plant Cell**, v.12, n.5, p. 617–635.

Heslop-Harrison J.S. (2012). Genome evolution: extinction, continuation or explosion **Current Opinion in Plant Biology,** v.15, n.2, p.115–121.

Hirsch A.M. *et al.* (2001). Embryo rescue from interspecific crosses in the genus Actinidia (kiwifruit). **Plant Cell Reports,** v.6, n.20, p.508-516.

IBGE. (2007). Instituto Brasileiro de Geografia e Estatística. Censo Agropecuário 2006: resultados preliminares. Rio de Janeiro: IBGE, 141p.

INTERNATIONAL BRACHYPODIUM INITIATIVE (2010). Genome sequencing and analysis of the model grass ***Brachypodium distachyon***. **Nature**, v.463, n7282, p.763–768

Ishigaki G. *et al.* (2009). Induction of tetraploid ruzigrass (*Brachiaria ruziziensis*) plants by colchicine treatment of *in vitro* multiple-shoot clumps and seedlings. **Grassland Science,** v.55, n.3, p.164-170, 2009.

Ishigaki G. *et al.* (2010). Estimation of genome size in *Brachiaria* species, **Grassland Science** v. 56, n.4, p. 240-242.

Karia C.T., Duarte J.B., Araújo A.C.G. Desenvolvimento de cultivares do gênero Brachiaria (trin.) Griseb no Brasil. Planaltina, DF: Embrapa Cerrados, 2006. 57 p. (Embrapa Cerrados. Documentos, 163).

Kazazian H.H. (2004). Mobile elements: drivers of genome evolution. **Science,** v.13, n.6, p. 1626-1632.

Keller-Grein G., Mass B.L., Hanson, J. (1996). Natural variation in Brachiaria and existing germoplasma collections. In Brachiaria: biology, agronomy and improvement (J.W. Miles, B.L. Maass & C.B. Valle, eds.). Centro Internacional de Agricultura Tropical – CIAT; Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA, Cali; Brasília, p. 17-42.

Kelley A.M. *et al.* (2009). A survey of apomixis and ploidy levels among *Poa* L. (Poaceae) using flow cytometry. **Crop Sci**. n.49, p.1395-1402.

Kolano B. *et al*. (2013). Isolation and characterization of reverse transcriptase fragments of LTR retrotransposons from the genome of *Chenopodium quinoa* (Amaranthaceae). **Plant Cell Reports**, v.32, n.10, p1575-1588.

Kubis S., Schmidt T., Heslop-Harrison J.S. (1998). Repetitive DNA elements as a major component of plant genomes. **Annals of Botany,** v.82, p.45–55.

Latado R.R *et al.* (2007). Plantas autotetraploides de citros sob tratamento in vitro com colchicina. Pesquisa Agropecuária Brasileira, v.42, p.1429- 1435.

Langdon T *et al.* (2000). Retrotransposon evolution in diverse plant genomes. **Genetics**, v.156, p.313–325.

Letterielo G. *et al.* (1999) Citologia e modo de reprodução de acessos pentaplóides de *Brachiaria brizantha*. In: Anais da 36a Reunião Anual da Sociedade Brasileira de Zootecnia. Porto Alegre CD- Rom

LI B. et al. (2013). Wheat centromeric retrotransposons: the new ones take a major role in centromeric structure. **The Plant Journal: for cell and molecular biology,** v.73, n.6, p.952–965.

Lysák M. e Doležel, J. (1998). Estimation of nuclear DNA content in Sesleria (Poaceae). **Caryologia** v. 2, n. 52, p. 123-132.

Maass B.L. **Identifying and naming Brachiaria species**. In: MILES, J.W. et al. (Ed.). Brachiaria: biology, agronomy, and improvement. Cali: CIAT, 1996. p. 9-13.

Macas J. *et al.* (2011). Next Generation Sequencing-Based Analysis of Repetitive DNA in the Model Dioceous Plant **Silene latifolia**. **PLoS ONE.** v.6, n.12, e27335.

Macedo M.C.M. Pastagens no ecossistema Cerrado: evolução das pesquisas para o desenvolvimento sustentável. In: SIMPÓSIOS - A PRODUÇAO ANIMAL E O FOCO NO AGRONEGÓCIO, 2005, Goiânia. Anais da 42 Reunião da Sociedade Brasileira de Zootecnia, 2005. p. 56-84.

Martel E. *et al.* (1997). Genome size variation and basic chromosome number in pearl millet and fourteen McLEISH, related Pennisetum species. **Journal of Heredity**, v.2, n.88, p.139-143.

Matzk F., Meister A., Shubert., I. (2000). An efficient screen for reproductive pathways using mature seeds of monocots and dicots**. Plant Journal**, v.21, p. 97-108.

Mendes-Bonato A.B *et al*. (2002). Chromosome number and microsporogenesis in *Brachiaria brizantha* (Gramineae). **Euphytica**, v.125, n.3, p. 419-425.

Mendes-Bonato A.B *et al.* (2004). Abnormal pollen mitoses (PM I and PM II) in an interspecific hybrid of *Brachiaria ruziziensis* and *Brachiaria decumbens* (Gramineae). **Journal Genetic,** v.83, n.3, p.279-283.

Mendes-Bonato A.B *et al.* (2006a). Chromosome number and meiotic behavior in *Brachiaria jubata* (Gramineae). **Journal Genetic,** n.85, n.1, p.83-87.

Mendes-Bonato A.B *et al.* (2006b). Cytogenetic evidence for genome elimination during microsporogenesis in interspecific hybrid between *Brachiaria ruziziensis* and *Brachiaria brizantha* (Poaceae). **Genetetics and Molecular Biology**, v.29, n.4, p. 711-714.

Mendes-Bonato A.B., Pagliarini M.S, Valle C.B. (2007). Meiotic arrest compromises pollen fertility in an interspecific hybrid between *Brachiaria ruziziensis* x *Brachiaria decumbens* (Poaceae:Paniceae). **Brazilian Archives of Biology and Technology,** v.50, n.5, p. 831-837.

Misset M.T. e Gourret, J.P. (1996). Flow cytometric analysis of the different ploidy levels observed in the genus Ulex L Faboideae-Genisteae in Brittany (France). **Botanica Acta,** v.1, n.109, p. 72-79.

Morgan E.R *et al.* (1995). Interspecific hybridization between *Limonium pergrinum* Bergius and *Limonium purpuratum* L. **Euphytica,** v.83, p.215–244.

Morgan-Richards M., Trewick S.A., Chapman H., Krahulcov A. (2004). Interspecific hybridization among Hieracium species in New Zealand: evidence from flow cytometry. **Heredity,** v.93, p.34−42.

Morrone O., Zuloaga F.O. (1992). Revisión de las especies sudamericanas nativas e introducidas de los géneros Brachiaria y Urochloa (Poaceae: Panicoideae: Paniceae). **Darwiniana,** v.31, n.1/4, p.43–109.

Moscone E. *et al.* (2003). Analysis of nuclear DNA content in *Capsicum* (Solanaceae) by flow cytometry and feulgen densitometry. **Annals of Botany**, v.92, n.1, p. 21–29.

Murray B.G. (2005). When does intraspecific C-value variation become taxonomically significant? **Annals of Botany**, n.95, n.1, p.119-125.

Nielen S. *et al.* (2010). Physical mapping of rDNA genes corroborates allopolyploid origin in apomictic *Brachiaria brizantha*. **Sex Plant Reprod** n.23, n.1, p.45-51.

Noirot M. *et al.* (2003). Effects of caffeine and chlorogenic acid on propidium iodide accessibility to DNA: consequences on genome size evaluation in coffee trees. **Annals of Botany** n.92, n.2, p. 259–264, 2003.

Ozaki Y.K. Narikiyo C., Okubo H. (2004). Ploidy variation of progenies from intra–and inter–ploidy crosses with regard to trisomic production in asparagus (Asparagus officinalis L.). **Sexual Plant Reproduction,** v.17, n.4, p.157–164

Ozkan H. *et al.* (2010). Genome size variation in diploid and tetraploid wild wheats. **AoB Plants**, v.2010.

Penteado M.I.O. *et al*. (2000) Determinação de poliploidia e avaliação da quantidade de DNA total em diferentes espécies de gênero *Brachiaria*. Boletim de Pesquisa 11, Embrapa Gado de Corte, Campo Grande, MS, Brazil.

Peterson-Burch B.D., Nettleton D., Voytas D.F. (2004). Genomic neighborhoods for Arabidopsis retrotransposons: a role for targeted integration in the distribution of the Metaviridae. **Genome Biololgy,** v.5, n.10, p.R78.

Pillay M. *et al.* (2006). Ploidy and genome composition of *Musa* germplasm at the International Institute of Tropical Agriculture (IITA). **African Journal of Biotechnology**, v. 5, n. 13, p. 1224-1232.

Pinheiro A.A. *et al.* (2000). Duplication of the chromosome number of diploid Brachiaria brizantha plants using colchicine. **Plant Cell Reports** v.19, n.3, p.274-278.

Pozarowski P., Darzynkiewicz Z. (2004). Analysis of cell cycle by flow cytometry. **Methods in Molecular Biology**, v.281, p.301-312

Renvoize S.A., Clayton W.D., Kabuye C.H.S. (1996) Morphology, taxonomy, and natural distribution of Brachiaria (Trinius) Griseberg. In: MILES, J. W. et al. (Ed.). *Brachiaria*: biology, agronomy, and improvement. Cali: CIAT. pp 1-17.

Risso-Pascotto C. *et al.* (2003). Chromosome number and microsporogenesis in a pentaploid accession of *Brachiaria brizantha* (Graminae). **Plant Breeding**, v. 122, n. 1, p.136-140.

Risso-Pascotto C., Pagliarini M.S., Valle C.B, Jank L. (2004). Asynchronous meiosis in an interspecific hybrid of *Brachiaria ruziziensis* and *B. brizantha*. **Plant Cell Reports,** v.23, n.5, p.304-310.

Risso-Pascotto C., Pagliarini M.S, Valle C.B. (2006). A new basic chomossome number for the genus *Brachiaria* (Trin.) Griseb. (Poaceae:Panicoideae:Paniceae). **Genetic Resources and Crop Evolution**, v.53, n.1, p 7-10.

Risso-Pascotto C., Pagliarini M.S, Valle C.B. (2009). Microsporogenesis in *Brachiaria bovonei* (Chiov.) Robyns and *B. subulifolia* (Mez.) Clayton (Poaceae). **Scientia Agricola** v.66, n.5, p.691-696.

Roux N., Toloza A., Radecki Z., Zapata-Aria F., Doležel, J. (2003). Rapid detection of aneuploidy in *Musa* using flow cytometry. **Plant Cell Reports**, v.21, n.5, p. 483-490.

Sabharwal P.S. e Doležel J. (1993). Interspecific hybridization in *Brassica*: Application of flow cytometry for analysis of ploidy and genome composition in hybrid plants. **Biologia Plantarum,** v.35, n.3, p. 169–177.

Sano E.E., Barcellos A.O., Bezerra H.S. (1999). Área e distribuição espacial de pastagens cultivadas no Cerrado. Planaltina: Embrapa Cerrados, 21p. (Embrapa Cerrados. Boletim de Pesquisa, 3).

Sharma A.K. e Sem S. (2002). Chromosome botany, Sience Publishers, Enfield, NH, USA, 155p.

Shelton M. *Brachiaria decumbens.* 2007. Disponível em:<http://www.fao.org/ag/AGP/AGPC/doc/Gbase/data/Pf00 0188.HTM >. Acesso em: 30 jan. 2007.

Shirasuna R.T. Lista de espécies da flora do Brasil:*Urochloa humidicola*(Rendle) Morrone e Zuloaga. Rio de Janeiro: Jardim Botânico, 2010 Disponível em <>/. Acesso em: 20 out. 2013

Schifino-Wittmann M.T. (2001). Determinação da quantidade de DNA nuclear em plantas. **Ciência Rural**, v.31. n. 5, p. 897-902.

Schimidt T., Heslop-Harrison J.S. (1998). Genomes, genes and junk: the large-scale organization of plant chromosomes. **Trends in Plant Science,** v.3, n.5, p.195-199.

Šmarda P e Bureš P. (2006). Intraspecific DNA content variability in *Festuca pallens* on different geographical scales and ploidy levels. **Annals of Botany**, v.98, n.3, p.665–678.

Smarda P. e P. Bures. *et al.* (2008). "Intrapopulation genome size dynamics in *Festuca pallens*." **Annals of Botany**, v.4, n.102, p.599-607. http://www.ncbi.nlm.nih.gov/pubmed/18684733.

Suda J., Kyncl T., Freiová R**.** (2003). Nuclear DNA amounts in Macaronesian Angiosperms. **Annals of Botany, v.**92, n.1, p. 153-164.

Suzuki G. *et al.* (2012). Random BAC FISH of monocot plants reveals differential distribution of repetitive DNA elements in small and large chromosome species[J]. **Plant Cell Reports**, v.31, n.4, p.621-628.

Spillane C., Curtis M.D., Grossniklaus U. (2004). Apomixis Technology Development – Virgin Births in Farmers Fields. **Nature Biotechnology,** v.22, n.6, p. 687-691.

Stebbins G.L. (1985). Polyploidy, hybridization, and the invasion of new habitats. **Annals of the Missouri Botanical Garden**. v.72, n.4, p. 824–832.

Swenne A. Louant B., Durjardin M. (1981). Induction par la colchicine de formes autotetraploïdes chez *Brachiaria ruziziensis* Germain et Evrard (Graminée). **Agronomie Tropicale,** v.36, n.2, p.134-114.

Thibault J. (1998). Nuclear DNA amount in pure species and hybrid willows (*Salix*): a flow cytometric investigation. **Canadian Journal of Botany,** v.76, n.1, p.57–165, 1998.

Timbó A.L.O., Pereira R.C., Santos V.B. *et al.* (2012). Histogram score contributes for reliability of DNA content estimatives in *Brachiaria spp*. **Ciência e Agrotecnologia,** v.36, n.6, p. 599-607.

Timbó A.L.O., Pereira R.C., Souza Sobrinho F., Davide L.C. (2014). Nuclear DNA content and chromosome number in *Brachiaria* spp. genotypes. **Ciência Agronomica,** v.45, n.1, p. 62-67.

Tohme J., Palcios N., Lenis S. Applications of biotechnology to Brachiaria. In: MILLES, J. Maas, B. L.; VALLE, C. B. do (Eds.). *Brachiaria*: biology, agronomy and improvement. Cali: CIAT; Brasília: EMBRAPACNPGC, 1996. p. 147-163. (CIAT Publication, 256).

Thibault J. (1998). Nuclear DNA amount in pure species and hybrid willows (Salix): a flow cytometric investigation. **Canadian Journal of Botany,** v.76, n.1, p.157–165.

Tsukahara S. *et al.* (2012). Centromere-targeted *de novo* integrations of an LTR retrotransposon of *Arabidopsis lyrata*. **Genes & Development,** v. 26, n.7, p.705-713.

Utsunomiya K.S., Pagliarini M.S., Valle C.B. (2005). Microsporogenesis in tetraploid accessions of *Brachiaria nigropedata* (Ficalho & Hiern) Stapf (Gramineae), **Biocell**

v.29, n.3, p. 295–301.

Valle C.B., Singh R.J., Miller, D.A. (1987). Pachytene chromosomes of *Brachiaria ruziziensis* Germain et Evrard. **Plant Breeding**, v. 78, n.1, p. 75-78.

Valle C.B., Coleção de germoplasma de espécies de Brachiaria no CIAT. Estudos básicos visando ao melhoramento genético. Campo Grande: Embrapa Gado de Corte, 1990. 33 p. (Embrapa Gado de Corte. Documentos, 46).

Valle C.B., Savidan Y.H (1996) Genetics, cytogenetic and reproductive biology of *Brachiaria.* In: MILES, J. W.; MASS, B. L.; VALLE, C. B. do (Ed.). ***Brachiaria:*** biology, agronomy and improvement. Cali: CIAT; Brasília, pp 147-163.

Valle C.B., Jank L., Resende R.M.S. (2009). O melhoramento de forrageiras tropicais no Brasil, **Ceres.** v.56, n.4, p. 460-472.

Valle C.B., Pagliarini M.S. Biology, cytogenetics, and breeding of *Brachiaria*. (2009) In: Singh RJ (ed) Genetics resources, chromosome engineering, and crop improvement. CRC Press, Boca Raton, pp 103-151.

Voglmayr, H., Greilhuber, J. (1998). Genome size determination in Peronosporales (Oomycota) by Feulgen image analysis. **Fungal Genetics and Biology,** v.25, n.3, p. 181–195.

Yang G.L., Koo D.H., Li Y., Zhang X., Luan F., Havey M.J., Jiming Jang., Weng Y. Chromosome rearrangements during domestication of cucumber as revealed by high-density genetic mapping and draft genome assembly. **The plant Journal**, v.71, n.6, p.895–906.

Walker D.J., Monino I., Correal E. (2006). Genome size in (L.) C.H. Stirton (Fabaceae) populations: separation of "true" differences from environmental effects on DNA determination. **Environmental and Experimental Botany ,** v.55, n.3, p. 104-116.

Wang K., Zhang W., Jiang Y., Zhang T. ((2013). Systematic Application of DNA Fiber-FISH Technique in Cotton. **PLoS ONE**, v.8, n. 9, 2013.

Willard H.F., e Waye, J.S. (1987). Hierachical order in chromosome-specific human alpha satellite DNA. **Trends in Genetics**. v.3, p. 192–198.

Manuscrito 1 - Euphytica

DNA C-values and karyotype features highlight the genomic relationships among species of *Bracharia* (Poaceae)

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Abstract

The plant breeding programs have frequently used information about genomes and chromosomes, especially those coming from DNA C-values and karyotypes, which provide data on ploidy level in species with agronomic importance. *Brachiaria* (Poaceae) comprises a group of forage species, marked by facultative apomixis and polyploidy, that have direct interest to the Brazilian economy. Aiming to differentiate the species and polyploid accesions of *Brachiaria*, this study determined the DNA C-values and the karyotype features of 10 accesions, belonging to five species from the Germplasm Bank of Embrapa Beef Cattle ando one *Uroclhoa mosambiencis*. Chromosome numbers of accesions ranged from $2n = 18$ to $2n = 54$, including all the ploidy levels $2x$, $4x$, $5x$, $6x$ and $9x$. The chromosomes presented higher condensation in proximal regions in prometaphase, with terminal regions of the long and short arms always more decondensed. Additionally, karyotypes presented a set of chromosomes that condensed earlier than others at prometaphase. Estimates on karyotype asymmetry

showed that there are differences between classifications obtained according to indexes proposed by Zarco and Stebbins. The asymmetry indexes A1 and A2 provides a more accurate results when species of *Brachiaria* with different ploidy levels were tested. The results showed some intra and interspecific variations in DNA C-values in samples with same and different chromosome numbers, that are associated with polyploidy and/or drive of repetitive DNA elements in *Brachiaria*. This study provide information that can be used to optimize the selection/elimination of materials that are used in the *Brachiaria* breeding projects, and thereby save time quality, when compared with just phenotypic evaluation.

Keywords: chromosomes • flow cytometry • forage plants • intra- and interspecific variation • karyotypes • ploidy levels

Introduction

The genus *Brachiaria*, belongs to the Poaceae family, and comprises about 100 species (Renvoize et al. 1996). Species of *Brachiaria* are the most cultivated forage crop in Brazil, where the production of comercial seeds is about 100 tons/year (Embrapa 2011). According to Valle et al. (2009), the the Brazilian Company "Embrapa Beef Cattle" has a germplasm bank with about 450 accesions of *Brachiaria* available for the breeding programs.

About 40% of *Brachiaria* species were studied using cytogenetic methods and, for most of them, the information is limited to records of chromosome numbers and meiotic behavior (Bernini and Marin-Morales 2001; Utsunomiya et al. 2005; Mendes-Bonato et al. 2006; Risso-Pascotto et al. 2009; Boldrini et al. 2011). Studies of physical mapping of chromosomes using repetitive DNA probes (Nielen et al. 2010), as well as those related to the DNA C-values (Ishigaki et al. 2010; Timbó et al. 2012; 2014) has been recently published, but they are still insufficient for a broader understanding of genomes and karyotypes of *Brachiaria*.

The basic chromosome number of the genus has been subject of much speculation. There are reports of x = 7 and 9, with a predominance of x = 9 (Dujardin 1979; Basappa et al. 1987; Valle and Savidan 1996). Risso-Pascotto et al. (2006) reported x = 6 in *B. dictyoneura* with $2n$ = 24, and the occurrence of quadrivalent associations. Species of *Brachiaria* are predominantly polyploids with $2n = 4x = 36$, with facultative apomixis, such as *B. decumbens* and *B. brizantha* (Basappa et al. 1987; Utsunomiya et al. 2005; Pagliarini et al. 2008). Of all species used in the *Brachiaria* breeding, *B. ruziziensis* is the most important because it is predominantly sexual and diploid, with $2n = 2x = 18$, and it is used as female parent in the selection of new cultivars (Valle and Savidan 1996; Valle and Miles 2001). However, the crossing between accesions of *B. ruziziensis* with tetraploids of *B. decumbens* and/or *B. brizantha* has produced interspecific hybrids with meiotic irregularities, that are responsible for different degrees of infertility (Valle and Pagliarini 2009).

The comparative analyses using cytogenetic information and flow cytometry data seems to be a good strategy for germplasms characterization aiming at plant breeding programs. Previous studies showed that species of *Brachiaria* exhibited intra- and interspecific variations in the DNA C-values, but without a perfect relation between these variations and chromosome numbers (Penteado et al. 2000). We believe that comparative analysis using differents ploidy levels and DNA C-values, is necessary to improve the genotypes evaluation criteria. Comparisons analysis using accesions of *Brachiaria* from the Brazilian germoplasm collections can be useful to guide *Brachiaria* breeders in the characterizing of materials, in order to eliminate contaminates, optimizing time and costs of crossings since it requires large number of samples in field experiments.

Materials and Methods

Plant material

Ten accesions of *Brachiaria* and one of *Urochloa mosambicensis* from the germplasm banks at Embrapa Beef Cattle, Campo Grande, MS, Brazil were used in this study. The accesions belong to five different species: *Brachiaria brizantha* (B178), *B. ruziziensis* (R134 and R46), *B. humidicola* (cultivar Llanero, H13, H16 and H112), *Brachiaria decumbens* (D4, D5 and D62), *Urochloa mosambicensis* (U) (2n = 28) (Table 1).

Karyotype analysis

Root tips were pretreated with a solution containing 2 mM 8-hydroxyquinolin, 1% colchicine and 5% dimethylsulfoxide for 24 h at 9 °C. Samples were fixed in ethanol:acetic acid (3:1, v:v) for 12 h, and stored at - 20 °C until use. Roots were digested using a solution containing 4% cellulase, 40% pectinase and 4% hemicellulase at 37 °C, hydrolyzed in 1 M HCl for 10 min at 60 °C and then exposed to Feulgen methodology. Meristems were isolated and squashed in a drop of 45% acetic acid. Coverslips were removed after freezing in liquid nitrogen, and slides mounted permanently with Entellan (Merck). Light microscopy images were acquired in grayscale using a Leica DM 4500 B microscope, equipped with a Leica DFC 300FX camera.

Idiograms (supplementary figures) were organized comparing the chromosome measurements of, at least, five metaphases. Measurements were used to obtain: i) size of largest arms (SLA) and size of smallest arms (SSA), ii) chromosome lengths (CL = SLA + SSA), iii) relative lengths of chromosomes (RL = [CL / ΣCL]×100) and iv) centromeric indexes (CI = [BC / (BC + BL)] x100). All these data were used to

calculate the haploid set sizes (HSS = ΣCL / 2), asymmetry indexes (Zarco 1986; Stebbins 1971) and to determinate the chromosome types. These last ones were organized according to arms ratios (AR), which was calculated by long arm length/short arm length (Levan et al. 1964), with modifications. Based on the RA, chromosome were classificated into: i) M or metacentrics (1 < RA < 1,5), ii) M-SM or metacentrics to submetacentrics (1,5 < RA < 3,5), iii) SM or submetacentrics (3,5 < RA < 7) and iv) SM-A or submetacentrics to acrocentrics (RA > 7).

DNA C-value estimation

Young leaves (30 mg) of three samples from each accessions/species(*B. brizanta*, *B. ruziziensis*, *B. humidicola*, *B. decumbens*, and *Urochloa mosambicensis*) were macerated in a 1 mL of a buffer containing $MgSO_4$ (9.53 mM $MgSO_4.7H_2O$, 47.67 mM KCl, 4.77 mM HEPES, 6.48 mM DTT and 0.1% Triton X-100, pH 8.0, according to (Arumuganathan and Earle 1991), or LB01 (15 mM TRIS, 2 mM Na_2EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 15 mM β-mercaptoethanol and 0.1% Triton X-100, pH 7.5), according to Doležel et al. (1989). Nuclear suspensions were filtered through a 50 µm nylon mesh into a microtubes, and after treated with 25 µL of 1 $mg.mL^{-1}$ propidium iodide and 2.5 µL and l U/ml of RNase.

Estimates of DNA content were made in picograms (pg) in, at least, ten thousand nuclei for each sample, using a FACSCalibur flow cytometer (Becton Dickinson). Comparisons were evaluated using the external *Solanum lycopersicum* with 2C = 1.96 DNA C-values relative to the amount in haploid genome or *n* (C) and relative to the amount in diploid genome or 2*n* (Cx) were converted to mega base pairs (Mbp), such as proposed by (Doležel et al. 2007). Results were obtained from completely randomized experiments, with three replications using data with coefficients of variation ≤ 0.05, based in SISVAR software, Tuckey test ($p < 0.05$) and Pearson correlation with dates of

karyotipe asymmetry and haploid set size.

Results and Discussion

Different accesions of *B. ruziziensis*, *B. brizantha*, *B. humidicola*, *B. decumbens* and *Urochloa mosambicensis* were used for obtaining chromosome numbers and measurements, karyotypes determination and asymmetry indexes, and DNA C-values. Chromosome numbers ranged from $2n = 18$ to $2n = 54$, including all the ploidy levels $2x$, $4x$, $5x$, $6x$ and $9x$ (Table 1 and Figs. 1, 2 and 3), corroborating the occurrence of different chromosome basic numbers in *Brachiaria,* such as $x = 7$ and 9, with a predominance of $x = 9$, such as proposed by Valle and Savidan (1996). The basic number $x = 6$, as seen in *B. humidicola* H16 e H13, was recently described for the accesions of *B. dictyoneura* with $2n = 24$ and *B. humidicola* with $2n = 42$ (Risso-Pascotto et al. 2006; Boldrini et al. 2009, respectively).

All the chromosome counting are in agreement with the literature (Basappa 1987; Penteado et al. 2000; Bernini and Marin Morales 2001; Timbó et al. 2012), however, some karyotype features of *Brachiaria* could be better clarified and discussed after our results. According to Gaut (2002), different x basic chromosome numbers found in grasses can be associated with events of polyploidy followed by aneuploidy or hybridizations.

The chromosome condensation in prometaphase-metaphase was different within and between karyotypes. In general, chromosomes present higher condensation in the proximal regions in prometaphase, with the end regions of the long and short arms always more descondensed (Figs. 1-3). We observed that the karyotypes presented a set of chromosomes wich condensed earlier than others at prometaphase. In this context, the accession D4 of *B. decumbens* with $2n = 18$ (Fig. 3A) showed five prematurely

condensed chromosomes (PCCs), while *B. ruziziensis* (accession R46) with 2*n* = 36 showed eighteen PCC (Fig 1C). The Table 1 and Figs. 1-3 show a comparative profile the chromosome condensations from prometaphase to metaphase, including all the samples tested by cytogenetic analysis.

Prematurely condensed chromosomes can be associated with cell signaling for chromatin condensation or other epigenetic modifications. High fidelity of chromosome condensation is essential for accurate transmission and differentiation of the genome into daughter cells, however this process tolerates some degree of structural heterogeneity between homologous chromosome (Khan et al, 2014; Londoño-Vallejo et al, 2001). The occurrence of PCCs in *Brachiaria*, suggesting existence of differences in the structure or behavior of chromosomes/genomes, could reinforce the existence of hybrids in the evolutionary history in this genus. In fact, some allopolyploids were proposed for the genus *Brachiaria* (Mendes-Bonato et al. 2002, 2006; Utsunomiya et al. 2005; Risso-Pascotto et al. 2006; Boldrini et al. 2009; Nielen et al. 2010; Akiyama et al. 2010), but a good illustrative case was reported by Mendes et al. (2006), which studied the meiotic behavior of two pentaploid accesions with (2*n* = 5*x* = 45) based in *x*= 9. These authors proposed these accesions are all allo-autopentaploids, with a tetraploid portion arising from an unreduced sperm cell (2*n* = 4*x* = 36, BBBB genome) from a male apomitic donor crossed with a female sexual diploid species, providing a reduced egg cell (*n* = 9, X genome).

In general, the metaphasic chromosomes were identified as submetacentric and a few of them as submetacentric to acrocentric (Figs. 5-7). Regarding the chromosome measurements, our results showed a range in the sizes from 0.47 µm in the accession B178 of *Brachiaria brizantha* (Fig 7A) to 4.52µm in the access R46 of *B. ruziziensis* (Fig 6B), representing a variation of about 9.6 times. The greatest intraspecific variation

(about 8.10 times) was observed in *Brachiaria brizantha* (accession B178), and the lowest (2.87 times) in the access R46 of *B. ruziziensis* (Figs 5-7). When chromosome measurements were used to estimate karyotype asymmetry, the accesions D4 of *B. decumbens* and R134 of *B. ruziziensis* with 2*n* = 18 were included in the category 1A, presenting more symmetrical and primitive karyotypes (Figs 5A and C, respectively), considering the criteria proposed by Stebbins (1971). Of all accesions analyzed, B178 of *B. brizantha* was the only one a classification 1C (Fig 7A). The other accesions presented karyotypes of type 1B (Table 1).

The first group includes the accesions D5 (*B. decumbens*) and R134 (*B. ruziziensis*) with symmetrical karyotypes. In the second, composed by the accesions D4 of *B. decumbens* and R46 of *B. ruziziensis;* D62 of *B. decumbens*; H112, H16 and cv. lallnero of *B. humidicola,* samples showed with intermediate levels of asymmetry. The third group was composed by the accesions B178 of *B. brizantha*, *Urochloa mosambicensis* and H13 of *B. humidicola* (Table 1 and Fig 7). When results of karyotype asymmetry based on Zarco (1986) and Stebbins (1971) were compared, it was possible to note some differences in the karyotype classifications. The accesions D4 and D5 of *B. decumbens*, H13 of *B. humidicola* and *U. mosambicensis* were not coincident (Table 1). Apparently, Zarco's indexes were more useful in the comparasion of species with different ploidy levels. In Stebbin´s classification, the symmetry is characterized by the predominance of chromosomes similar in length and with centromere in metacentric and submetacentric positions and, therefore, can not accurately categorize when species have very similar karyotypes. Probably for this reason, it didn't identify the karyotype variations in *Urochloa mosambicensis* and *B. humidicola,* H13 (Table 1). Bernini and Marin Morales (2001) also evaluated the asymmetry indexes A1 and A2 for access R134, D5 and H13, and they proposed a

tendency for asymmetry in direction of polyploidy in *Brachiaria.* Although these authors have used more condensed chromosomes in relation to chromosomes analyzed by us, our results have shown symmetrical karyotypes in diploid accesions, except for D4 of *B. decumbens,* and asymmetry in polyploids (Fig. 8).

DNA values obtained for the 11 accesions were within the quality criteria adopted by Timbó et al. (2012), with a coefficient of variation lower than 5% (Dolezel and Bartos 2005). These data can be seen in the Table 1 and Figure 4 (supplementary). Seemingly, the haploid set sizes (HSSs) and C-values (Table 1) were increased according to the chromosome number, and the asymmetry index was greater when DNA C-value was also higher, such as observed in the correlations between C*x* and A2 (inter-chromosomal asymmetry) (Table 2). Variations of 1.23×, 1.12× and 1.4× in HSS for the accesions D5 of *B. decumbens*, R134 of *B. ruziziensis* and H13 of *B. humidicola* are similar to those found by Bernini and Morales-Marin (2001) for the same accesions. These variations could be explained by several factors such the differences in the condensation of chromosomes in metaphases evaluated and change in the total content of chromatin (Guerra 2000). According Speranza et al. (2003), the condensation pattern can also interfere with our perception about the correct positioning of centromeres, since the longest less condensed arms keep shortening as condensation proceeds.

The DNA C-values and HSSs of the tetraploids R46 (*B. ruziziensis*) and D62 (*B. decumbens*) were almost double those for accesions D4, D5 and R134, all diploids. However, this trend was not detected for the remainder of the polyploids (Table 1). Similar results were reported by Ishigaki et al. (2009), also with species of *Brachiaria*. Significant variations in the DNA amount were noted when higher chromosome numbers were compared, except for the accession H16 of *B. humidicola* with $2n = 6x = 36$, for wich DNA content was 3.65 pg and HSS was 41.35 µm (Table 1). Comparated

to the hexaploid H13 access of *B. humidicola*, wich exhibited 4.39 pg of DNA content ($p < 0.05$) and HSS with 36.03 μm, lower than accession H16, being the only accession that showed intraspecific differences in the same ploidy level and chromosome basic number. These results seem to be contrary of the expected in relation to positive Pearson correlations ($p<0.05$) between C*x* with HSS (Table 1). The positive correlation between the DNA content with HSS normally indicates an increase of both HSS and DNA content. Especially for the hexaploid H13 access, we have doubts if there was a misidentification of this accession (this can happen often in genus *Brachiaria* because of the seeds dispersal by wind and similar phenotype) or if this is an exceptional case of large variation in DNA content.

The accesions B178 of *B. brizantha* with $2n = 5x = 45$, H112 ($2n = 9x = 54$) and cultivar Llanero ($2n = 9x = 54$) of *B. humidicola* showed the highest DNA C-values (4.73, 4.81 and 5.81 pg, respectively), when they were compared with the others accesions with different chromosome numbers. Interspecific variations in the DNA C-value was observed also between samples with the same chromosome number. This difference reached about 1.83 times between the accesions H16 and H13 ($2n = 6x = 36$) and D62 and R46 with $2n = 4x = 36$ (Table 1). Our results are in agreement with Penteado et al. (2000), Ishigaki et al. (2010) and Timbó et al. (2012), who found intraspecific and interspecific variations 1.05× 1.4× in *Brachiaria* samples with 2*n*, 4*n*, 5*n*, 6*n* and 7*n*. These interspecific variations (for instance, from 118.5 Mpb to 4088,02 Mpb between *B. ruziziensis* and *B. decumbens*) could be due to the action of allo and autopolyploidy, but multiple events can also act in the expansion or contraction of the DNA C-value (Bennett and Leitch 2005). Some studies have reported the presence of different amounts of transposable elements between individuals of the same species (Baucom et al. 2009), which consequently generate intraspecific variation.

Hybridization and allopolyploidization, for example, have been considered as potential genomic shocks involved in the accumulation and reorganization of transposable elements in plants (Parisod et al. 2010).

A better understanding of variations on the karyotype features and DNA amount, is important for the interpretation of the genomic specificities in *Brachiaria*, whose species and accesions are target of evaluation before large field experiments. In this case, both cytogenetic and flow cytometry tools allow for the detection of interspecific hybrids, and other genome variations. These procedures have been used successfully in several hybridization studies, such as in *Elytrigia repens* × *E. intermedia* (Mahelka et al. 2005) and *Hienacium* (Suda et al. 2007). The joint use of these two tools showed that species and accesions of *Brachiaria* exhibit marked differences in: i) chromosome numbers, ii) chromosome condensation patterns (intra-karyotypes), probably associated to epigenetic modifications, and iii) karyotype asymmetry and DNA C-values, associated to neopoliploydy and/or drive of repetitive elements. This study sought to reevaluate cytometry, cytogenetic aspects and DNA content in order to improve the understanding of genome and karyotype organization in *Brachiaria*, given the limitations resulting from small chromosomes and difficultying to detecting centromeres. Results this work contributed to improve knowledge on the organization of karyotypes for future studies in order to anchor tags generated by molecular cytogenetics, likes FISH and GISH.

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References

Arumuganathan K, Earle ED (1991) Estimation of nuclear DNA content of plants by flow cytometry. Plant. Mol. Biol. 9:229–241.

Akiyama Y, Yamada-Akiyama H, Ebina M (2010) Morphological diversity of chromosomes bearing ribosomal DNA loci in *Brachiaria* species. J. Japan. Soc. Grassl. Sci. 56:217–223.

Baucom RS, Estill JC, Leebens-Mack J, Bennetzen JL (2009) Natural selection on gene function drives the evolution of LTR retrotransposon families in the rice genome. Genome Res. 19: 243–254.

Basappa GP, Muniyamma M, Chinnappa CC (1987) An investigation of chromosome numbers in the genus *Brachiaria* (Poaceae:Paniceae) in relation to morphology and taxonomy. Can J Bot 65:2297–2309.

Bennet MD, Leitch IJ (2005) Nuclear DNA amounts in angiosperms - progress, problems and prospects. Ann Bot 95:45–90.

Bernini C, Marin-Morales MA (2001) Karyotype analysis in *Brachiaria* (Poaceae) species. Cytobios 407:157–171.

Boldrini KR, Pagliarini MS, Valle CB (2009) Meiotic behavior of a nonaploid accession endorses x = 6 for *Brachiaria humidicola* (Poaceae). Genet Mol Res 8:1444–1450.

Boldrini KR, Adamowski EV, Silva N, Pagliarini MS, Valle CB (2011) Meiotic behavior in nonaploid accessions of *Brachiaria humidicola* (Poaceae) and implications for breeding. Genet Mol Res 10:169–176.

Doležel J, Binarová P, Lucretti S (1989) Analysis of nuclear DNA content in plant cells by flow cytometry. Biol Plant 31:113–120.

Doležel J, Bartoš J (2005) Plant DNA flow cytometry and estimation of nuclear genome size. Ann Bot 95:99–110.

Doležel J, Greilhuber J, Suda J (2007) Estimation of nuclear DNA content in plants using flow cytometry. Nat Protoc 2:2233–2244.

Dujardin M (1979) Additional chromosome numbers and meiotic behavior in tropical African grasses from western Zaire. Can J Bot 57:864–876.

EMBRAPA (2011) Banco de notícias Embrapa Gado de Corte, 18 de julho de 2011. Available at: http://www.cnpgc.embrapa.br/index.php?pagina=bancodenoticias/2011/18072011_curso.html. Acessed on 08 September in 2011.

Gaut BS (2002) Evolutionary dynamics of grass genomes. New Phytol. 154:15–28.

Guerra, Marcelo (2000) Patterns of heterochromatin distribution in plant c hromosomes. Genet Mol Biol 23:1029–1041.

Huziwara Y (1962) Karyotype analysis in some genera of Compositae. VIII.Further studies on the chromosomes of Aster. Am J Bot 49:116–119.

Ishigaki G, GondoT, Ebina M, Suenaga K, Akashi R (2010) Estimation of genome size in Brachiaria species. J Jpn Grassi Sci 56:240–242.

Khan WA, Rogan PK, Knoll HM (2014) Localized, non-random differences in chromatin accessibility between homologous metaphase chromosomes. Mol Cytogenetic 7:1-70.

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromossomes. Hereditas 52: 201–220.

Londoño-Vallejo JA, DerSarkissian H, Cazes L, Thomas G (2001) Differences in telomere length between homologous chromosomes in humans. Nucleic Acids Res 29:3164–3171.

Mahelka V, Suda Jarolímová JV, Trávníček P, Krahulec F (2005) Genome size discriminates between closely related taxa *Elytrigia repens* and *E. intermedia*

(Poaceae: Triticeae) and their hybrid. Folia Geobot 40:367–384.

Mendes DV, Boldrini KR, Mendes-Bonato AB, Pagliarini MS, Valle C. B. (2006) Cytological evidence of natural hybridization in *Brachiaria brizantha* Stapf (Gramineae). Bot J Linn Soc 150:441–446.

Mendes-Bonato AB, Pagliarini MS, Forli F, Valle CB, Penteado MIO (2002) Chromosome number and microsporogenesis in *Brachiaria brizantha* (Gramineae). Euphytica 125:419–425.

Mendes-Bonato AB, Risso-Pascotto C, Pagliarini MS, Valle CB (2006) Chromosome number and meiotic behavior in *Brachiaria jubata* (Gramineae). J Genet 85:83–87.

Nielen S, Almeida LM, Carneiro VTC, Araujo ACG (2010) Physical mapping of rDNA genes corroborates allopolyploid origin in apomictic *Brachiaria brizantha*. Sex Plant Reprod 23:45–51.

Pagliarini MS, Pascotto C, Souza-Kaneshim AM, Valle CB (2008) Analysis of meiotic behavior in selecting potential genitors among diploid and artificially induced tetraploid accessions of *Brachiaria ruziziensis* (Poaceae). Euphytica 164:181–187.

Parisod C, Alix K, Just J, Petit M, Sarilar V, Mhiri C, Ainouche M, Chalhoub B, Grandbastien MA (2010) Impact of transposable elements on the organization and function of allopolyploid genomes. New Phytol 186: 37–45.

Paszko B (2006) A critical review and a new proposal of karyotype asymmetry indices. Plant Syst Evol 258:39–48.

Penteado MIO, Santos ACM, Rodrigues IF (2000) Determinação de poliploidia e avaliação da quantidade de DNA total em diferentes espécies de gênero *Brachiaria*. Boletim de Pesquisa 11, Embrapa Gado de Corte, Campo Grande, MS, Brazil.

Renvoize SA, Clayton WD, Kabuye CHS (1996) Morphology, taxonomy, and natural distribution of *Brachiaria* (Trinius) Griseberg. In: MILES, J. W. et al. (Ed.).

Brachiaria: biology, agronomy, and improvement. Cali: CIAT. pp 1-17.

Risso-Pascotto C, Pagliarini MS, Valle CB (2006) A new basic chromosome number for the genus *Brachiaria* (Trin.) Griseb. (Poaceae:Panicoideae:Paniceae). Genetic Resour Crop Ev 53:7–10.

Risso-Pascotto C, Pagliarini MS, Valle CB (2009) Chromosome number and microsporogenesis of two accessions of *Brachiaria* dura Stapf (Poaceae). Biota Neotrop 9:257–261.

Shirasuna RT (2010) Lista de espécies da flora do Brasil: *Urochloa humidicola* (Rendle) Morrone e Zuloaga. Rio de Janeiro: Jardim Botânico. Available at http: //floradobrasil.jbrj.gov.br/2010/FB087091. Accessed on 21 June in 2013

Stebbins GL (1971) Chromosomal evolution in higher plants. Addison Wesley, New York.

Suda J, Krahulcová A, Trávníček P, Rosenbaumová R, Peckert T, Krahulec F. (2007) Genome size variation and species relationships in *Hieracium* sub-genus *Pilosella* (Asteraceae) as inferred by flow cytometry. Ann Bot 100:1323–1335.

Speranza P, Vaio M, Mazzella C (2003) Karyotypes of two cytotypes of *Paspalum quadrifarium* Lam. (Poaceae): an alternative technique for small chromosomes in plants. Genet Mol Biol 26:449–503.

Timbó ALO, Pereira RC, Santos VB, Sobrinho FS, Davide LC (2012) Histogram score contributes for reliability of DNA content estimatives in *Brachiaria* spp. Ciência e Agrotecnologia. 36:599–607.

Timbó ALO, Pereira RC, Sobrinho FS, Davide LC (2014) Nuclear DNA content and chromosome number in *Brachiaria* spp. genotypes. Rev Ciênc Agron 45:62–67.

Utsunomiya KS, Pagliarini MS, Valle CB (2005) Microsporogenesis in tetraploid accessions of *Brachiaria nigropedata* (Ficalho & Hiern) Stapf (Gramineae). Biocell

29:295–301.

Valle CB, Savidan YH (1996) Genetics, cytogenetic and reproductive biology of Brachiaria. In: MILES, J. W.; MASS, B. L.; VALLE, C. B. do (Ed.). *Brachiaria*: biology, agronomy and improvement. Cali: CIAT; Brasília, pp 147-163.

Valle CB, Miles JW (2001) Breeding of apomictic species. In: CYMMYT. The flowering of apomixis: from mechanisms to genetic engineering. CYMMYT/IRD, Mexico, DF, Mexico, pp 137–152.

Valle CB, Pagliarini MS (2009) Biology, cytogenetics, and breeding of *Brachiaria*. In: Singh RJ (ed). Genetics resources, chromosome engineering, and crop improvement. CRC Press, Boca Raton, pp 103-151.

Zarco R (1986) A new method for estimating karyotype asymmetry. Taxon 35:526–530.

Table 1 - DNA C-values and karyotype features of species and acessions of *Brachiaria* and *Uroclhoa*.

Species/Accesions	C-value (pg)	Mbp C-^{1}	Mbp Cx-^{1}	2*n*	PL	PCC	HSS	A1	A2	A
B. ruziziensis R134	1.54 ± 0.04(a)	753.06	753.06	18	2x	4	20,57	0.20	0.17	1A
B. decumbens D4	1.59 ± 0.10(a)	777.51	792.18	18	2x	5	20,54	0.24	0.20	1A
B. decumbens D5	1.63 ± 0.11(a)	797.07	797.07	18	2x	2	22,12	0.20	0.27	1B
B. ruziziensis R46	2.99 ± 0.19(b)	1462.11	731.06	36	4x	18	43,91	0.24	0.28	1B
B. decumbens D62	2.99 ± 0.10(b)	1462.11	731.06	36	4x	5	34,13	0.25	0.24	1B
Uroclhoa mosambicensis	3.27 ± 0.10(bc)	1599.03	799.51	28	4x	4	22,68	0.32	0.29	1B
B. humidicola H16	3.65 ± 0.06(c)	1784.85	594.95	36	6x	10	41,35	0.24	0.29	1B
B. humidicola H13	4.39 ± 0.13(d)	2146.71	715.57	36	6x	10	36,03	0.30	0.22	1B
B. humidicola H112	4.81 ± 0.01(e)	2352.09	522.69	54	9x	10	48,42	0.24	0.26	1B
B. brizantha B178	4.73 ± 0.04(e)	2312.97	925.19	45	5x	8	45,92	0.31	0.35	1C
B. humidicola cv. Llanero	5.81 ± 0.02(f)	2841.09	937.35	54	9x	10	49,72	0.24	0.32	1B

C-value = DNA amount estimated in picograms and standard deviation (SD). Letters after SD indicate similarities after Tuckey test at 5%; Mbp C^{-1} = gametic DNA content with a chromosome number *n*; Mbp Cx^{-1} = DNA content in each genome; 2*n* = diploid number; PL = ploidy level; PCC = prematurely condensed chromosomes; HSS = haploid set size; A1 and A2 correspond to asymmetry indexes estimated according to Zarco (1986), and A, correspond to asymmetry indexes estimated according to Stebbins (1971).

Table 2 Pearson' s correlation coefficient evaluated with karyotype asymmetry indexes A1 and A2 (Zarco 1986), with the DNA content (Mbp C^{-1}) and haploid set size (HSS).

	A1	**A2**	**HSS**	**Mbp Cx^{-1}**
A1	1			
A2	0.41	1		
HSS	0.23	0.63	1	
Mbp Cx^{-1}	0.36	0.73*	0.95**	1

A1 = chromosomal asymmetry index, A2 = inter- chromosomal asymmetry index, HSS = haploid set sizes, Mbp Cx^{-1} = DNA content in each genome, *p<0,05 ; **p<0,0001

Figure 1 – Prometaphases (A, C, E, G) and metaphases (B, D, F, H) of four access of *Brachiaria*: **A and B)** access R134 of *B. ruziziensis* with 2*n* = 2*x* = 18, **C and D)** access R46 of *B. ruziziensis* with 2*n* = 4*x* = 36, **E and F)** access B178 of *B. brizantha* with 2*n* = 5*x* = 45, and **G and H)** *Urochloa mosambicensis* with 2*n* = 28. Observe that prometaphase chromosomes exhibit a proximal condensation with terminal regions more descondensed (arrowhead). Arrows indicate prematurely condensed chromosomes (PCCs).

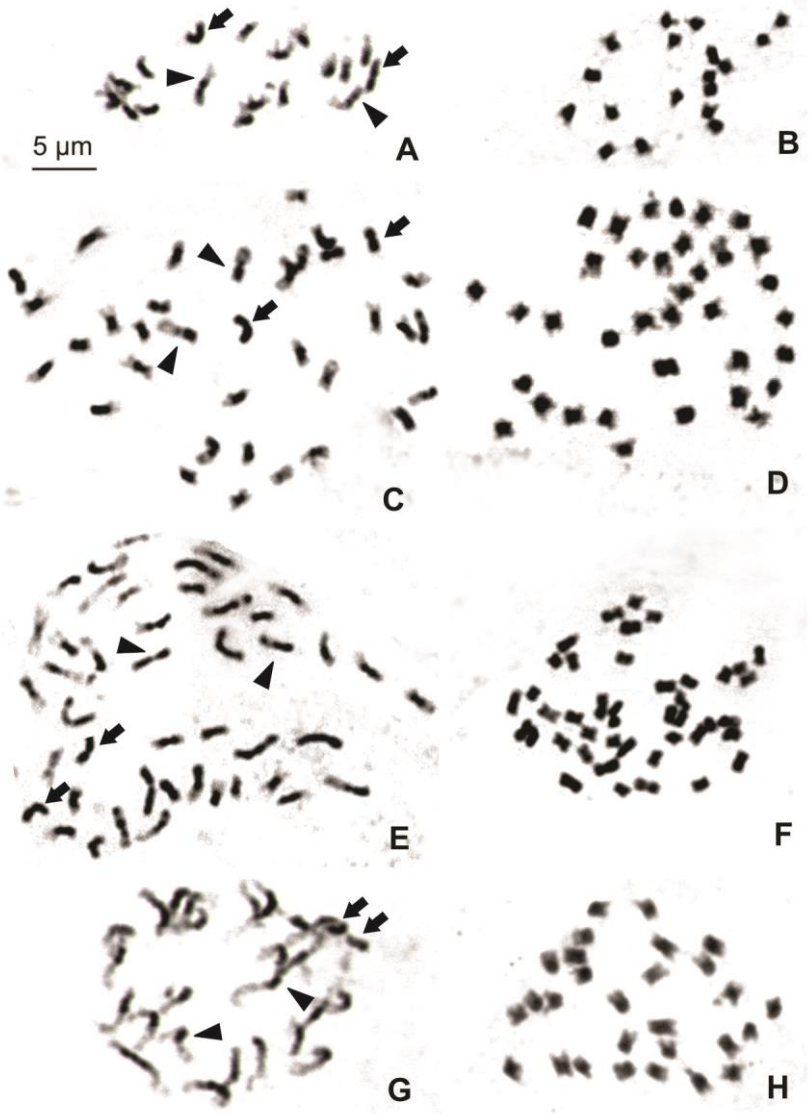


Figure 2 – Prometaphases (A, C, E, G) and metaphases (B, D, F, H) of four access of *Brachiaria*: **A and B)** access H16 of *B. humidicola* with 2*n* = 4*x* = 36, **C and D)** access H13 of *B. humidicola* with 2*n* = 4*x* = 36, **E and F)** access H112 of *B. humidicola* with 2*n* = 9*x* = 54, and **G and H)** *cv.* Llanero with 2*n* = 9*x* = 54. Observe that prometaphase chromosomes exhibit a proximal condensation with terminal regions more descondensed (arrowhead). Arrows indicate prematurely condensed chromosomes (PCCs).

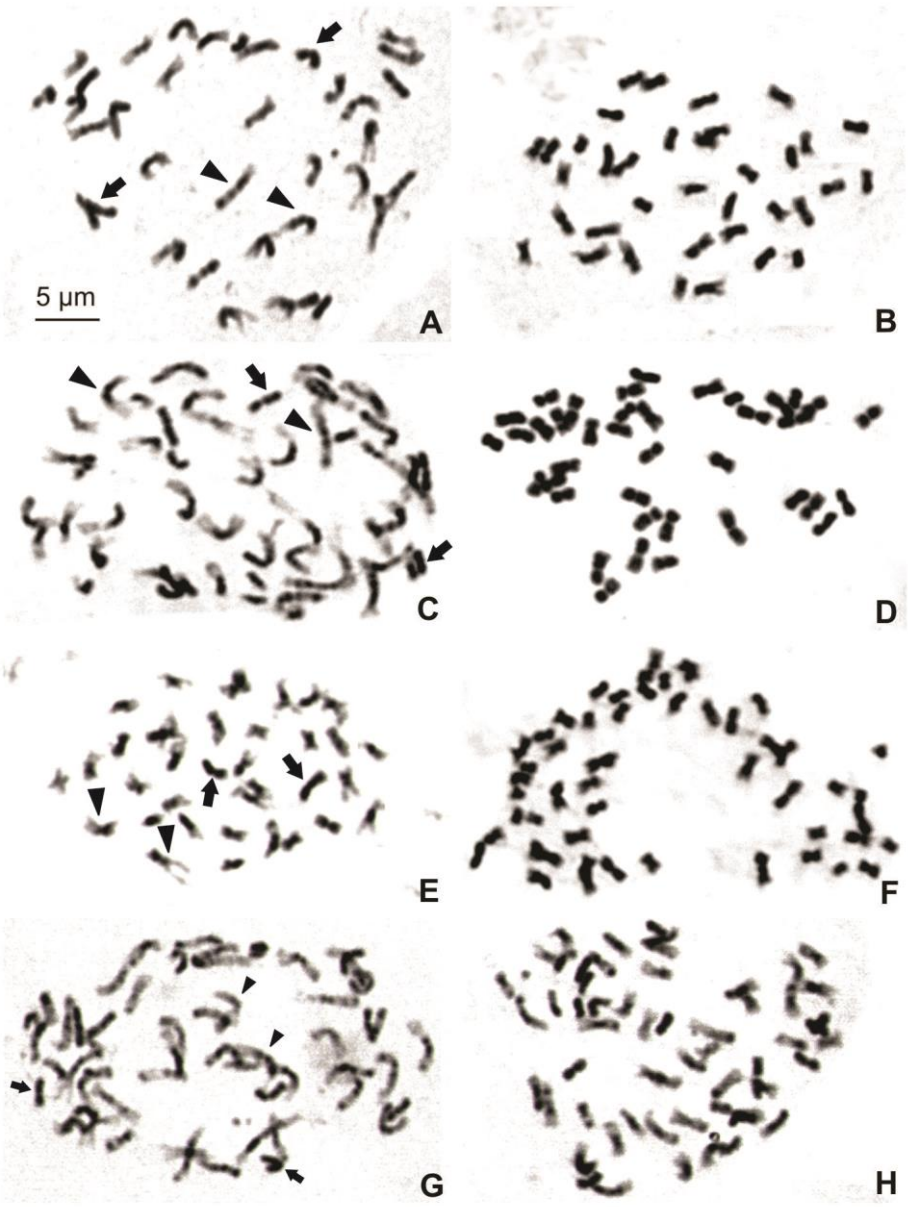


Figure 3 – Prometaphases (A, C, E) and metaphases (B, D, F) of three access of *Brachiaria*: Accesions D4 **(A and B)** and D5 **(C and D)** of *B. decumbens* with $2n = 2x = 18$, and **E and F)** access D62 of *B. decumbens* with $2n = 4x = 36$. Arrows represent part of the chromosomes with condensed early, condensation that are possible formed by the nucleolar organizer regions. Note in promethafases the terminal chromosome regions more descondensed. In some cases, there are also the prematurely condensed chromosomes (PCCs) in all the samples (arrowheads).

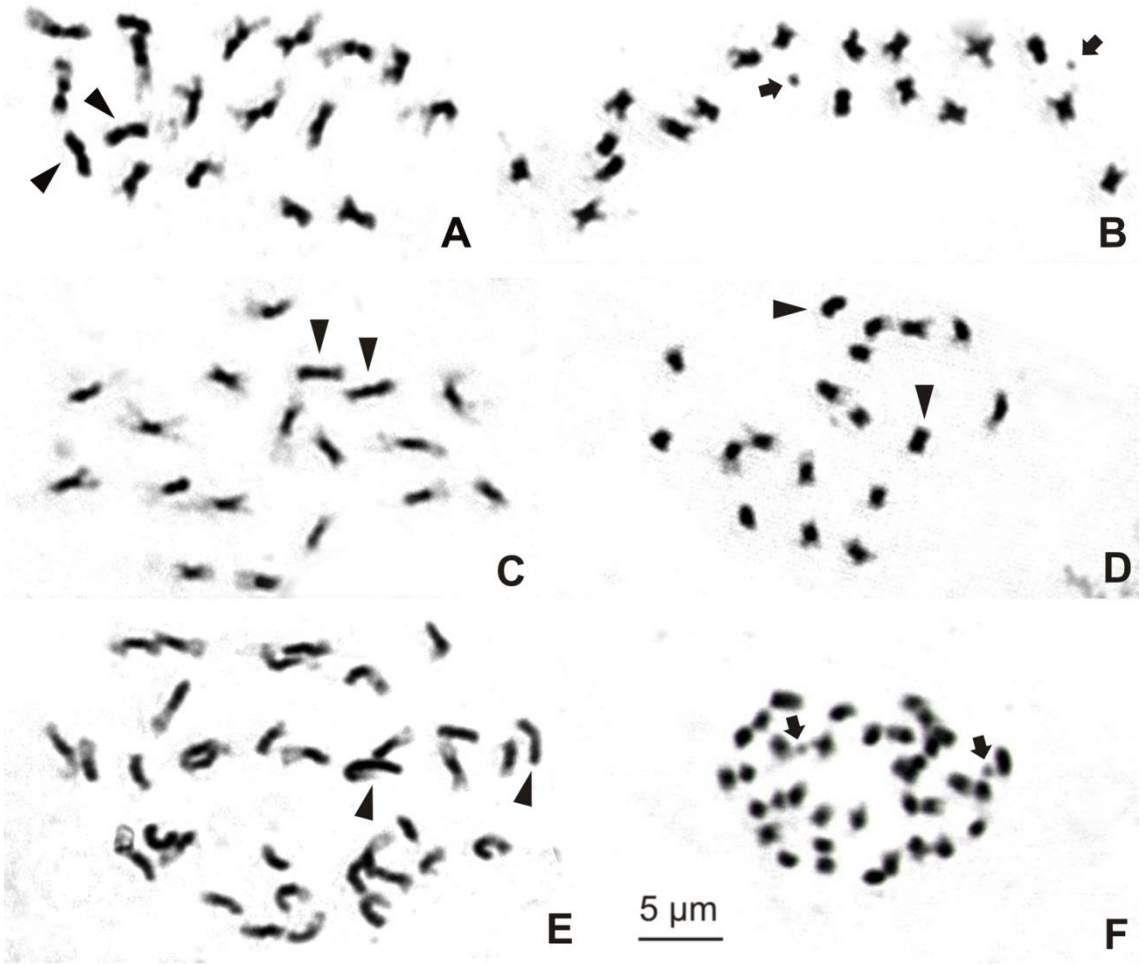


Figure 4 supplementary – Histograms obtained from flow cytometry, which represent variations on the DNA content in *Brachiaria*. From **A** to **F**, the first peak in each histogram correspond to G1 peak of the external pattern (*Solanum lycopersum*) and the second peak is the peak G1 sample for the *Brachiaria*. In **G** and **H,** the first peak in each histogram refers to the G1 peak of *Brachiaria* species and the second corresponds to G1 peaks of internal pattern *Raphanus sativus*. **A)** *B. ruziziensis* - R134, **B)** *B. ruziziensis* - R46, **C)** *B. humidicola* - H16, **D)** *B. humidicola*- H112, **E)** *cv.* Llanero, **F)** *B. brizantha*- B178, **G)** *B. ruziziensis* – R46, and **H)** *B. decumbens* with overlapping peaks in the histograms of the analysis of DNA content.

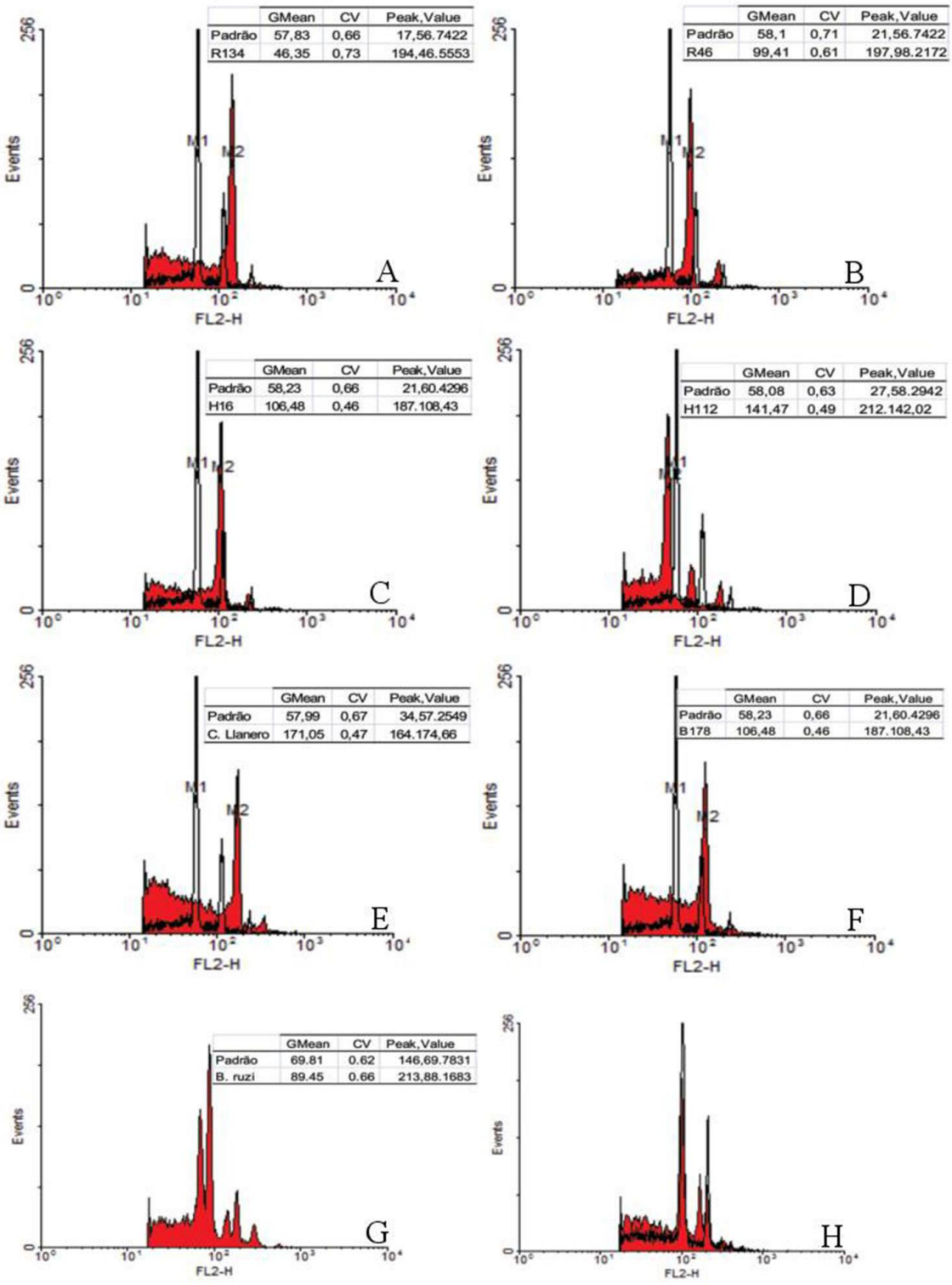
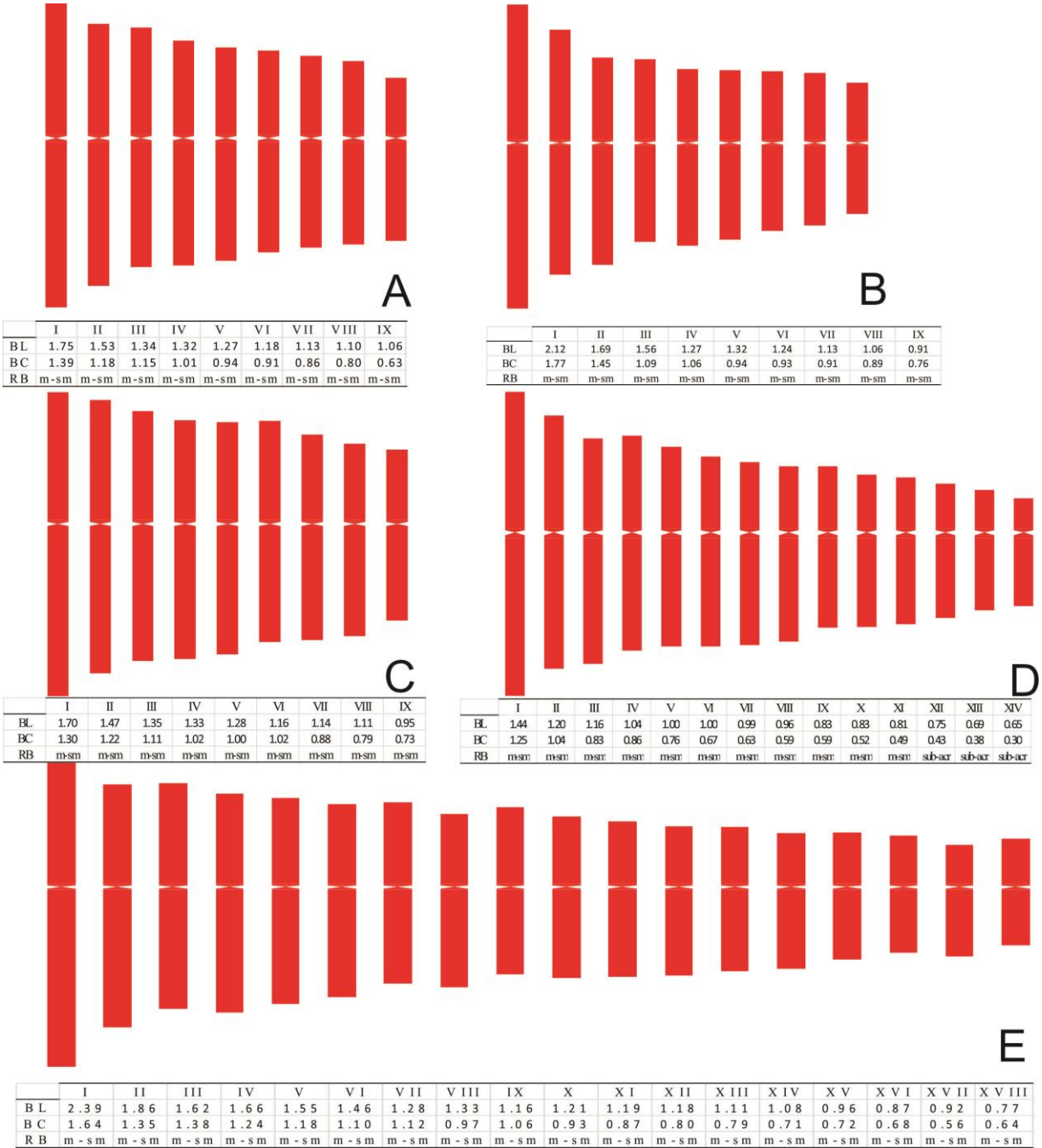

GMean CV Peak,Value
Padrão 57,83 0,66 17,56.7422
R134 46,35 0,73 194,46.5553
A
GMean CV Peak,Value
Padrão 58,1 0,71 21,56.7422
R46 99,41 0,61 197,98.2172
B
GMean CV Peak,Value
Padrão 58,23 0,66 21,60.4296
H16 106,48 0,46 187.108,43
C
GMean CV Peak,Value
Padrão 58,08 0,63 27,58.2942
H112 141,47 0,49 212.142,02
D
GMean CV Peak,Value
Padrão 57,99 0,67 34,57.2549
C. Llanero 171,05 0,47 164.174,66
E
GMean CV Peak,Value
Padrão 58,23 0,66 21,60.4296
B178 106,48 0,46 187.108,43
F
GMean CV Peak,Value
Padrão 69.81 0.62 146,69.7831
B. ruzi 89.45 0.66 213,88.1683
G
H
Events
FL2-H
M1
M2

Figure 5 supplementary – Idiograms representing karyotypes of *Brachiaria* accesions, including measurements of long arm (BL), short arm (BC) and chromosome types according to arm ratio (AR), i.e. m-sm (metacentric to submetacentric) and sm-a (submetacentric to acrocentric). Accesions D4 with 2*n* = 18 **(A)**, D5 with 2*n* = 18 **(B)**, R134 with 2*n* = 18 **(C),** *Urochloa mosambicensis* with 2*n* = 28 **(D)** and H16 with 2*n* = 36 **(E)**.



	I	II	III	IV	V	VI	VII	VIII	IX
BL	1.75	1.53	1.34	1.32	1.27	1.18	1.13	1.10	1.06
BC	1.39	1.18	1.15	1.01	0.94	0.91	0.86	0.80	0.63
RB	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm

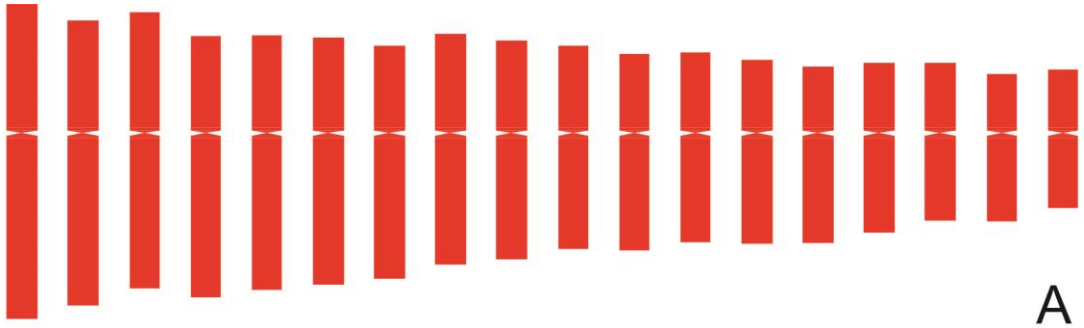
	I	II	III	IV	V	VI	VII	VIII	IX
BL	2.12	1.69	1.56	1.27	1.32	1.24	1.13	1.06	0.91
BC	1.77	1.45	1.09	1.06	0.94	0.93	0.91	0.89	0.76
RB	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm

	I	II	III	IV	V	VI	VII	VIII	IX
BL	1.70	1.47	1.35	1.33	1.28	1.16	1.14	1.11	0.95
BC	1.30	1.22	1.11	1.02	1.00	1.02	0.88	0.79	0.73
RB	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm

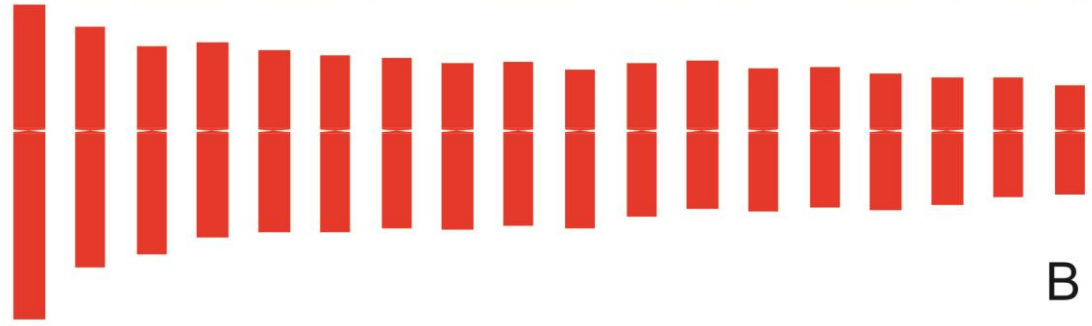
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV
BL	1.44	1.20	1.16	1.04	1.00	1.00	0.99	0.96	0.83	0.83	0.81	0.75	0.69	0.65
BC	1.25	1.04	0.83	0.86	0.76	0.67	0.63	0.59	0.59	0.52	0.49	0.43	0.38	0.30
RB	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	sub-acr	sub-acr	sub-acr

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
BL	2.39	1.86	1.62	1.66	1.55	1.46	1.28	1.33	1.16	1.21	1.19	1.18	1.11	1.08	0.96	0.87	0.92	0.77
BC	1.64	1.35	1.38	1.24	1.18	1.10	1.12	0.97	1.06	0.93	0.87	0.80	0.79	0.71	0.72	0.68	0.56	0.64
RB	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm

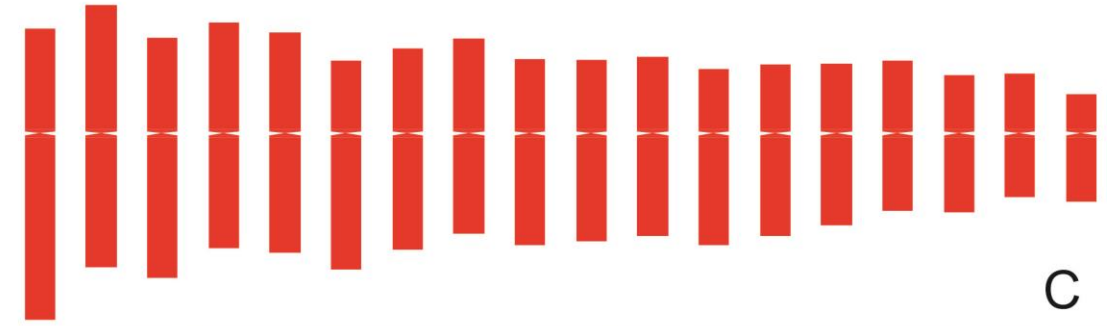
Figure 6 supplementary – Idiograms representing karyotypes of *Brachiaria* accesions, including measurements of long arm (BL), short arm (BC) and chromosome types according to arm ratio (AR), i.e. m-sm (metacentric to submetacentric) and sm-a (submetacentric to acrocentric). Accesions H13 **(A)**, R46 **(B)** and D62 **(C)**, all with 2*n* = 36.



	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
BL	1.49	1.39	1.25	1.32	1.26	1.22	1.17	1.06	1.02	0.93	0.94	0.88	0.89	0.88	0.80	0.71	0.71	0.60
BC	1.04	0.91	0.97	0.78	0.79	0.77	0.70	0.80	0.75	0.70	0.64	0.65	0.59	0.54	0.56	0.57	0.48	0.52
RB	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm

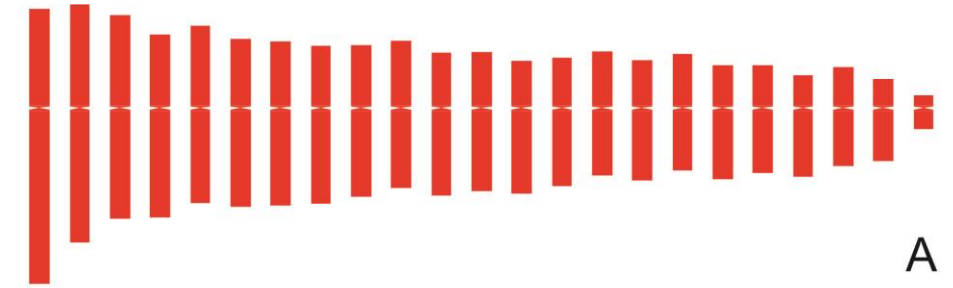


	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
BL	2.703	1.966	1.768	1.525	1.466	1.451	1.409	1.415	1.363	1.403	1.24	1.124	1.16	1.098	1.148	1.066	0.945	0.913
BC	1.818	1.49	1.217	1.263	1.152	1.084	1.041	0.981	0.992	0.878	0.983	1.012	0.893	0.922	0.821	0.772	0.766	0.662
RB	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm

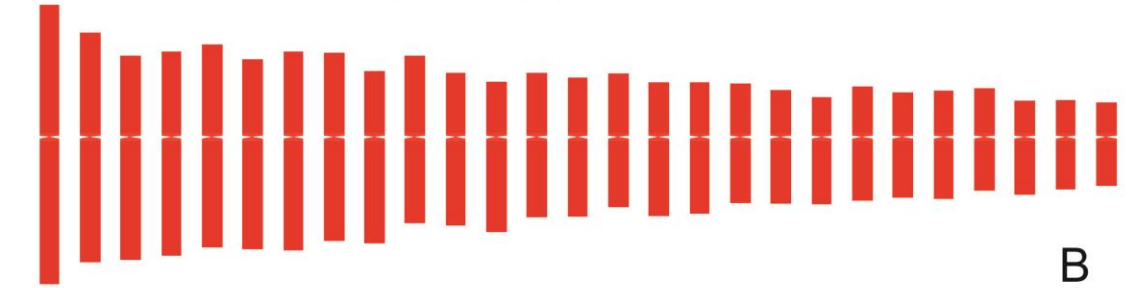


	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
BL	1.86	1.34	1.44	1.15	1.19	1.36	1.16	1.00	1.12	1.08	1.03	1.11	1.03	0.91	0.77	0.79	0.64	0.69
BC	1.05	1.28	0.95	1.10	1.01	0.72	0.85	0.95	0.74	0.74	0.77	0.64	0.69	0.70	0.72	0.58	0.60	0.39
RB	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	sm-a

Figure 7 supplementary – Idiograms representing karyotypes of *Brachiaria* accesions, including measurements of long arm (BL), short arm (BC) and chromosome types according to arm ratio (AR), i.e. m-sm (metacentric to submetacentric) and sm-a (submetacentric to acrocentric). Accesions B178 with 2*n* = 45 **(A)**, *cv.* Llanero with 2*n* = 54 **(B)** and H112 with 2*n* = 54 **(C)**.



	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI	XXII	XXIII
BL	2.44	1.86	1.53	1.52	1.32	1.37	1.35	1.32	1.23	1.11	1.21	1.15	1.19	1.09	0.94	1.01	0.87	0.99	0.90	0.96	0.81	0.74	0.29
BC	1.37	1.44	1.29	1.02	1.14	0.96	0.93	0.86	0.88	0.93	0.77	0.78	0.66	0.70	0.78	0.67	0.75	0.59	0.60	0.46	0.57	0.40	0.18
RB	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	sm-a	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	sm-a	m-sm	sm-a	m-sm



	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI	XXII	XXIII	XXIV	XXV	XXVI	XXVII
BL	1.80	1.53	1.51	1.45	1.35	1.37	1.38	1.27	1.30	1.06	1.09	1.16	0.98	0.98	0.86	0.97	0.93	0.81	0.82	0.82	0.78	0.74	0.76	0.66	0.71	0.64	0.60
BC	1.62	1.28	0.99	1.05	1.14	0.96	1.05	1.03	0.81	0.99	0.79	0.68	0.79	0.73	0.78	0.67	0.67	0.66	0.58	0.49	0.62	0.55	0.57	0.60	0.45	0.46	0.43
RB	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	sm-a	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	sm-a	m-sm	sm-a	m-sm	m-sm	m-sm	m-sm	m-sm

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII	XIX	XX	XXI	XXII	XXIII	XXIV	XXV	XXVI	XXVII
BL	1.71	1.40	1.31	1.38	1.39	1.25	1.17	1.19	1.07	1.05	1.08	1.10	1.08	1.00	1.12	1.02	0.95	0.94	0.85	0.79	0.74	0.71	0.71	0.73	0.66	0.72	0.56
BC	1.33	1.16	1.10	0.90	0.86	0.95	1.02	0.95	0.96	0.86	0.80	0.72	0.71	0.76	0.59	0.63	0.67	0.63	0.62	0.63	0.63	0.63	0.61	0.58	0.56	0.44	0.46
RB	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	sm-a	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm	m-sm

Manuscrito 2

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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)
IRAP (Inter-Retroelement Amplified Polymorphism)
PPT (Polypurine Tract)
LTRs (Long Terminal Repeats)
LTR-RTs (Retrotransposons with LTR)
TEs (Transposable Elements)
POL (Polygenic String)
FISH (Fluorescent *In Situ* Hybridization)
CRM (Centromere-specific retrotransposons of Maize)

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 and removal events in distinct chromosome regions (Bennetzen and Wang 2014).

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: *copia* (Pseudovirideae) and *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). *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 *Alstroemeria* (Kuipers et al. 1998). In *Arabidopsis*, *copia* elements are clustered at centromeres (Heslop-Harrison et al. 2003) and also occur along the chromosomes, while *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 *gypsy* LTR-RTs called Centromeric Retrotransposons (CRs, called CRM in Maize), suggesting that 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 ploidies to 2n=10x=90. Four of the species, *B. 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 (BLAST*x*, *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 available in the Table 1. Inserts of the p*Ta*794 clone containing the 5S rDNA sequence of *T. aestivum* (Gerlach and Dyer 1980) and the pLT11 clone containing the telomeric insert $(TTTAGGG)_n$ of p*At*T4 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 equally.

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 (p*Ta*794) of *T. aestivum* and $TTTAGGG_{(n)}$ (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 transciptase 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; 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* super-family 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 exemplifed 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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References

Adams KL, Wendel JF. 2005. Polyploidy and genome evolution in plants. Current Opinion in Plant Biology 8: 135-141. DOI: 10.1016/j.pbi.2005.01.001

Akiyama Y, Yamada-Akiyama H, Ebina M. 2010. Morphological diversity of chromosomes bearing ribosomal DNA loci in Brachiaria species. Grassland Sci 56(4): 217-223.

Alsayied NF, Fernández JA, Schwarzacher T, Heslop-Harrison JS. 2015. Diversity and relationships of *Crocus sativus* and its relatives analysed by inter-retroelement amplified polymorphism (IRAP). Annals of Botany 116: 359-368.

Bennetzen JL, Wang H. 2014. The contributions of transposable elements to the structure, function, and evolution of plant genomes. Annual Review of Plant Biology 65: 19.1-19.26. DOI: 10.1146/annurev-arplant-050213-035811.

Bertioli DJ, Vidigal B, Nielen S, Ratnaparkhe MB, Lee T-JH, Leal-Bertioli SCM, Kim C, Guimaraes PM, Seijo G, Schwarzacher T, Paterson AH, Heslop-Harrison P, Araujo ACG. 2013. The repetitive component of the A genome of peanut (*Arachis hypogaea*) and its role in remodelling intergenic sequence space since its evolutionary divergence from the B genome. Ann Bot 112 (3): 545-559. doi: dx.doi.org/10.1093/aob/mct128

Boldrini KR, Micheletti PL, Gallo PH, Mendes-Bonato AB, Pagliarini MS, Valle CB. 2009. Origin of a polyploid accession of Brachiaria humidicola (Poaceae: Panicoideae: Paniceae). Genet Mol Res 8: 888-895

Casacuberta E, González J. 2013. The impact of transposable elements in environmental adaptation. Mol Ecol 22: 1503-1517.

Chavanne F, Zhang D, Liaud M, Cerff R (1998) Structure and evolution of Cyclops: a novel giant retrotransposon of the *Ty*3/*Gypsy* family highly amplified in pea and other legume species. Plant Molecular Biology 37: 363-375. DOI: 10.1023/A:1005969626142

Cheng Z, Murata M. 2003. A centromeric tandem repeat family originating from a part of *Ty*3/*gypsy*-Retroelement in Wheat and its relatives. Genetics 164: 665-672.

D'Hont A, Denoeud F, Aury J-M, Baurens, FC, Carreel F, Garsmeur O, ... & Jaillon O. 2012. The banana (Musa acuminata) genome and the evolution of monocotyledonous plants. Nature, 488(7410), 213-217.

Daron J, Glover N, Pingault L, Theil S, Jamilloux V, Paux E, Barbe V, Mangenot S, Alberti A, Wincker P, Quesneville H, Feuillet C, Choulet F. 2014. Organization and

evolution of transposable elements along the bread wheat chromosome 3B. Genome Biol. 15(12):546. doi:10.1186/s13059-014-0546-4

Edgar. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32(5): 1792-1797.

Estep MC, DeBarry JD, Bennetzen JL. 2013. The dynamics of LTR retrotransposon accumulation across 25 million years of panicoid grass evolution. Heredity 110: 194-204. DOI: 10.1038/hdy.2012.99.

Gaeta RT, Pires JC, Iniguez-Luy F, Leon E, Osborn TC. 2007. Genomic changes in resynthesized *Brassica napus* and their effect on gene expression and phenotype. Plant Cell 19; 3403-3417.

Gao D, Jiang N, Wing RA, Jiang J, Jackson SA. 2015. Transposons play an important role in the evolution and diversification of centromeres among closely related species. Frontiers in plant science 6 in press.

Gao X, Hou Y, Ebina H, Levin HL, Voytas DF. Chromodomains direct integration of retrotransposons to heterochromatin. Genome Res. 2008;18:359–369

Gerlach WL, Dyer TA. 1980. Sequence organization of the repeating units in the nucleus of wheat which contain 5S rRNA genes. Nucleic Acids Research 8(21): 4851-4865

Gorinšek B, Gubenšek F, Kordiš D. 2004. Evolutionary genomics of chromoviruses in eukaryotes. Mol Biol Evol 21(5): 781-798.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Regev A. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29(7): 644-652

Gregory RT (2005) The C-value enigma in plants and animals: A review of parallels and an appeal for partnership. Annals of Botany 95: 133-146. DOI:10.1093/aob/mci009

Greilhuber J, Borsch T, Müller K, Worberg A, Porembski S, Barthlott W. 2006. Smallest angiosperm genomes found in Lentibulariaceae with chromosomes of bacterial size. Plant Biology 8: 770-777. DOI: 10.1055/s-2006-924101

Hansen CN, Heslop-Harrison JS. 2004. Sequences and phylogenies of plant pararetroviruses, viruses and transposable elements. Advances in Botanical Research 41: 165-193

Heitkam T, Holtgräwe D, Dohm JC, Minoche AE, Himmelbauer H, Weisshaar B, Schmidt T. 2014. Profiling of extensively diversified plant LINEs reveals distinct plant-specific subclades. Plant J 79: 385-397

Heslop-Harrison JS, Brandes A, Schwarzacher T. 2003. Tandemly repeated DNA sequences and centromeric chromosomal regions of Arabidopsis species. Chromosome Research 11: 241-253.

Heslop-Harrison JS, Schmidt T. 2012. Plant Nuclear Genome Composition. In: Encyclopedia of Life Sciences. eLS 2012, John Wiley & Sons Ltd: Chichester http://www.els.net/ http://dx.doi.org/10.1002/9780470015902.a0002014.pub2

Heslop-Harrison JS. 2012. Genome evolution: extinction, continuation or explosion? Current Opinion in Plant Biology 15: 115-121.

Houben A, Schroeder-Reiter E, Nagaki K, Nasuda S, Wanner G, Murata M, Endo TR. 2007. CENH3 interacts with the centromeric retrotransposon *cereba* and GC-rich satellites and locates to centromeric substructures in barley. Chromosoma, 116(3), 275-283.

Janicki M, Rooke R, Yang G. 2011. Bioinformatics and genomic analysis of transposable elements in eukaryotic genomes. Chromosome Research 19: 787-808. DOI: 10.1007/s10577-011-9230-7

Jurka J, Kapitonov VV, Pavlicek A, Klonowski P, Kohany O, Walichiewicz J. 2005. Repbase Update, a database of eukaryotic repetitive elements. Cytogenet Genome Res. 110(1-4):462-7

Kamm A, Doudrick RL, Heslop-Harrison JS and Schmidt T. 1996. The genomic and physical organization of Ty1-copia-like sequences as a component of large genomes in *Pinus elliottii* var. *elliottii* and other gymnosperms. Proceedings of the National Academy of Sciences 93: 2708-2713

Kejnovsky E, Hawkins JS, Feschotte C. 2012. Plant Transposable Elements: Biology and Evolution, Wendel JF et al. (eds.), Plant Genome Diversity Volume 1

Kuipers AGJ, Heslop-Harrison JS, Jacobsen E. 1998. Characterisation and physical localisation of Ty1-copia-like retrotransposons in four *Alstroemeria* species. Genome 41: 357-367

Lerat E. 2010. Identifying repeats and transposable elements in sequenced genomes: how to find your way through the dense forest of programs. Heredity 104: 520-533. doi: 10.1038/hdy.2009.165

Lisch D, Bennetzen JL. 2011. Transposable element origins of epigenetic gene regulation. Curr. Opin. Plant Biol. 14: 156-161. DOI:10.1016/j.pbi.2011.01.003.

Llorens C, Futami R, Covelli L, Dominguez-Escriba L, Viu JM, Tamarit D, Aguilar-Rodriguez J, Vicente-Ripolles M, Fuster G, Bernet GP, Maumus F, Munoz-Pomer A, Sempere JM, LaTorre A, Moya A. 2011. The Gypsy database (GyDB) of mobile genetic elements: Release 2.0. Nucleic Acids Research (NARESE) 39 (suppl 1): D70-D74. DOI: 10.1093/nar/gkq1061.

Llorens C, Muñoz-Pomer A, Bernad L, Botella H, Moya A. 2009. Network dynamics of eukaryotic LTR retroelements beyond phylogenetic trees. Biology Direct 4:41. doi:10.1186/1745-6150-4-41

Ma X-F, Gustafson JP. 2008. Allopolyploidization-accommodated genomic sequence changes in triticale. Ann. Bot. 101: 825-832.

Marín I, Lloréns C. 2000. Ty3/Gypsy Retrotransposons: description of new *Arabidopsis thaliana* elements and evolutionary perspectives derived from comparative genomic data. Molecular Biology and Evolution 17: 1040-1049.

Mendes-Bonato AB, Pagliarini MS, Forli F, Valle CB, de Oliveira Penteado MI. 2002. Chromosome numbers and microsporogenesis in Brachiaria brizantha (Gramineae). Euphytica 125(3): 419-425

Menzel G, Heitkam T, Seibt KM, Nouroz F, Müller-Stoerme M, Heslop-Harrison JS, Schmidt T. 2015. The diversification and activity of hAT transposons in Musa genomes. Chromosome Research 22: 559–571. doi: 10.1007/s10577-014-9445-5

Nagaki K, Neumann P, Zhang D, Ouyang S, Buell CR, Cheng Z, Jiang J. 2005. Structure, divergence, and distribution of the CRR centromeric retrotransposon family in rice. Mololecular Biology and Evolution 22: 845-855. DOI: 10.1093/molbev/msi069

Nakamura T, Miranda CH, Ohwaki Y, Valéio JR, Kim Y, Macedo M. 2005. Characterization of nitrogen utilization by Brachiaria grasses in Brazilian Savannas (Cerrados). Soil Science & Plant Nutrition 51(7): 973-979

Neale DB, Wegrzyn JL, Stevens KA, Zimin AV, Puiu D, Crepeau MW, Cardeno C, Koriabine M, Holtz-Morris AE, Liechty JD, Martínez-García PJ, Vasquez-Gross HA, Lin BY, Zieve JJ, Dougherty WM, Fuentes-Soriano S, Wu LS, Gilbert D, Marçais G, Roberts M, Holt C, Yandell M, Davis JM, Smith KE, Dean JF, Lorenz

WW, Whetten W, Sederoff R, Wheeler N, McGuire PE, Main D, Loopstra CA, Mockaitis K, deJong PJ, Yorke JA, Salzberg SL, Langley CH. 2014. Decoding the massive genome of loblolly pine using haploid DNA and novel assembly strategies. Genome Biol. 4;15(3):R59.

Nielen S, Almeida LM, Carneiro VT, Araujo ACG. 2010. Physical mapping of rDNA genes corroborates allopolyploid origin in apomictic *Brachiaria brizantha*. Sexual Plant Reproduction 23(1): 45-51

Nouroz F, Shumaila N, Heslop-Harrison JS. 2015. Evolutionary genomics of miniature inverted-repeat transposable elements (MITEs) in *Brassica*. Mol Gen Genomics in press. Doi: /10.1007/s00438-015-1076-9.

Parisod C, Alix K, Just J, Petit M, Sarilar V, Mhiri C, Ainouche M, Chalhoub B, Grandbastien MA. 2010. Impact of transposable elements on the organization and function of allopolyploid genomes. New Phytol. 186: 37-45.

Pearce SR, Li D, Flavell AJ, Harrison G, Heslop-Harrison JS, Kumar A. 1996. The Ty1-copia group retrotransposons in *Vicia* species: copy number, sequence heterogeneity and chromosomal localisation. Molecular and General Genetics MGG 250: 305-315.

Pellicer J, Fay MF, Leitch IJ (2010) The largest eukaryotic genome of them all? Botanical Journal of the Linnaean Society 164: 10-15. DOI: 10.1111/j.1095-8339.2010.01072.x

Pereira V. 2004. Insertion bias and purifying selection of retrotransposons in the *Arabidopsis thaliana* genome. Genome biology: 5(10), R79

Piednoe M, Carrete-Vega G, Renner SS. 2013. Characterization of the LTR retrotransposon repertoire of a plant clade of six diploid and one tetraploid species. The Plant Journal 75: 699-709. DOI: 10.1111/tpj.12233

Renny-Byfield S, Wendel JF 2014. Doubling down on genomes: polyploidy and crop plants. American J Bot 101(10): 1711-1725

Renvoize SA, Clayton WD, Kabuye CH. 1996. Morphology, taxonomy, and natural distribution of Brachiaria (Trin.) Griseb. In: Brachiaria: biology, agronomy, and improvement CIAT publication no. 259. Miles JW, Maass BL, Valle CB, Kumble V (eds.) CIAT, Campo Grande, Brazil

Richards EJ, Ausubel FM. 1988. Isolation of a higher eukaryotic telomere from Arabidopsis thaliana. Cell 53(1): 127-136

Schwarzacher T, Heslop-Harrison JS. 2000. Practical in situ hybridization. Oxford: Bios. 203+xii pp

Takeda S, Sugimoto K, Otsuki H, Hirochika H. 1998. Transcriptional activation of the tobacco retrotransposon Tto1 by wounding and methyl jasmonate. Plant Mol Biol 36: 365-376

VanBuren R, Zeng F, Chen C, Zhang J, Wai CM, Han J, Aryal R, Gschwend AR, Wang J, Na JK, Huang L, Zhang L, Miao W, Gou J, Arro J, Guyot R, Moore RC, Wang ML, Zee F, Charlesworth D, Moore PH, Yu Q, Ming R. 2015. Origin and domestication of papaya Yh chromosome. Genome Res. 25:524-533

Verde I, et al. 2013. The high-quality draft genome of peach (*Prunus persica*) identifies unique patterns of genetic diversity, domestication and genome evolution. Nature Genetics 45: 487-494. DOI: 10.1038/ng.2586

Vicient CM, Jääskeläinen MJ, Kalendar R, Schulman AH. 2001. Active retrotransposons are a common feature of grass genomes. Plant Physiol. 125: 1283-1292

Vogel JP, Garvin DF, Mockler TC, Schmutz J, Rokhsar D, Bevan MW, Barry K et al. 2010. Genome sequencing and analysis of the model grass *Brachypodium distachyon*. Nature 463: 763-768. DOI: 10.1038/nature08747

Weber B, Schmidt T. 2009. Nested Ty3-gypsy retrotransposons of a single *Beta procumbens* centromere contain a putative chromodomain. Chromosome Res 17(3), 379-396.

Wicker T, Sabot F, Hua-Van A, Bennetzen J, Capy P, Chalhoub B, Flavell A, Leroy P, Morgante M, Panaud O, Paux E, San-Miguel P, Schulman AH. 2007. A unified classification system for eukaryotic transposable elements. Nature Reviews Genetics 8: 973-982. DOI:10.1038/nrg2165

Willing EM, Rawat V, Mandáková T, Maumus F, James GV, Nordström KJ, ... & Schneeberger K. 2015. Genome expansion of Arabis alpina linked with retrotransposition and reduced symmetric DNA methylation. Nature Plants, 1(2) Article number: 14023

Wright DA, Voytas DF. 1998. Potential Retroviruses in Plants: Tat1 Is Related to a Group of Arabidopsis thaliana Ty3/gypsy Retrotransposons That Encode Envelope-Like Proteins. Genetics 149: 703–715.

Zhong CX, Marshalla JB, Topp C, Mroczek R, Kato A, Nagaki K, Birchler JA, Jiang J, Dawe RK. 2002. Centromeric retroelements and satellites interact with maize kinetochore protein CENH3. Plant Cell 14: 2825-2836. DOI:/10.1105/tpc.006106

Zonneveld BJM. 2010. New record holders for maximum genome size in eudicots and monocots. Journal of Botany 2010: Article ID 527357 (4 pages). DOI:10.1155/2010/527357

Zou J, Fu D, Gong H, Qian W, Xia W, Pires JC, Li RY, Long Y, Mason AS, Yang T, Lim YP, Park BS and Meng J. 2011. De novo genetic variation associated with

retrotransposon activation, genomic rearrangements and trait variation in a recombinant inbred line population of *Brassica napus* derived from interspecific hybridization with *Brassica rapa*. Plant Journal 68: 212-224.

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.

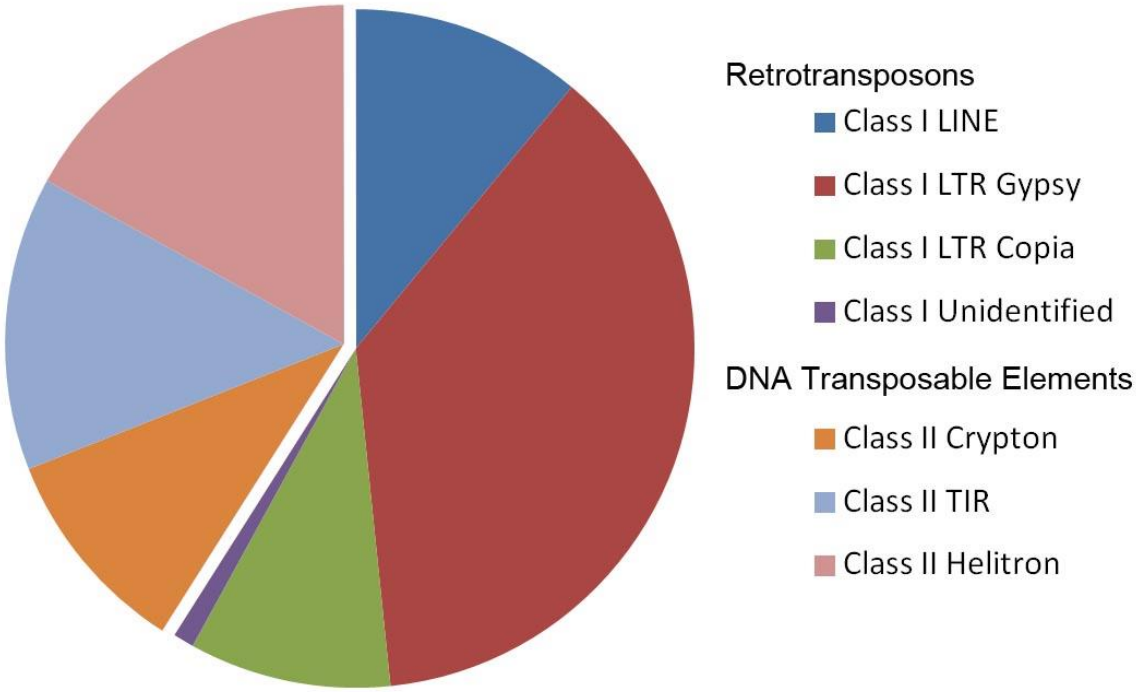


Figure 2. Cladogram showing the relationships between *Ty*1-*copia* and *Ty*3-*gypsy* retrotransposable element superfamilies identified in the transcriptome of *B. decumbens* within contigs/unigenes (black names) or as characteristic domains (red). Arrows indicate the *gypsy* LTR-RTs sequences used to design primers for *in situ* hybridization (Table 1).

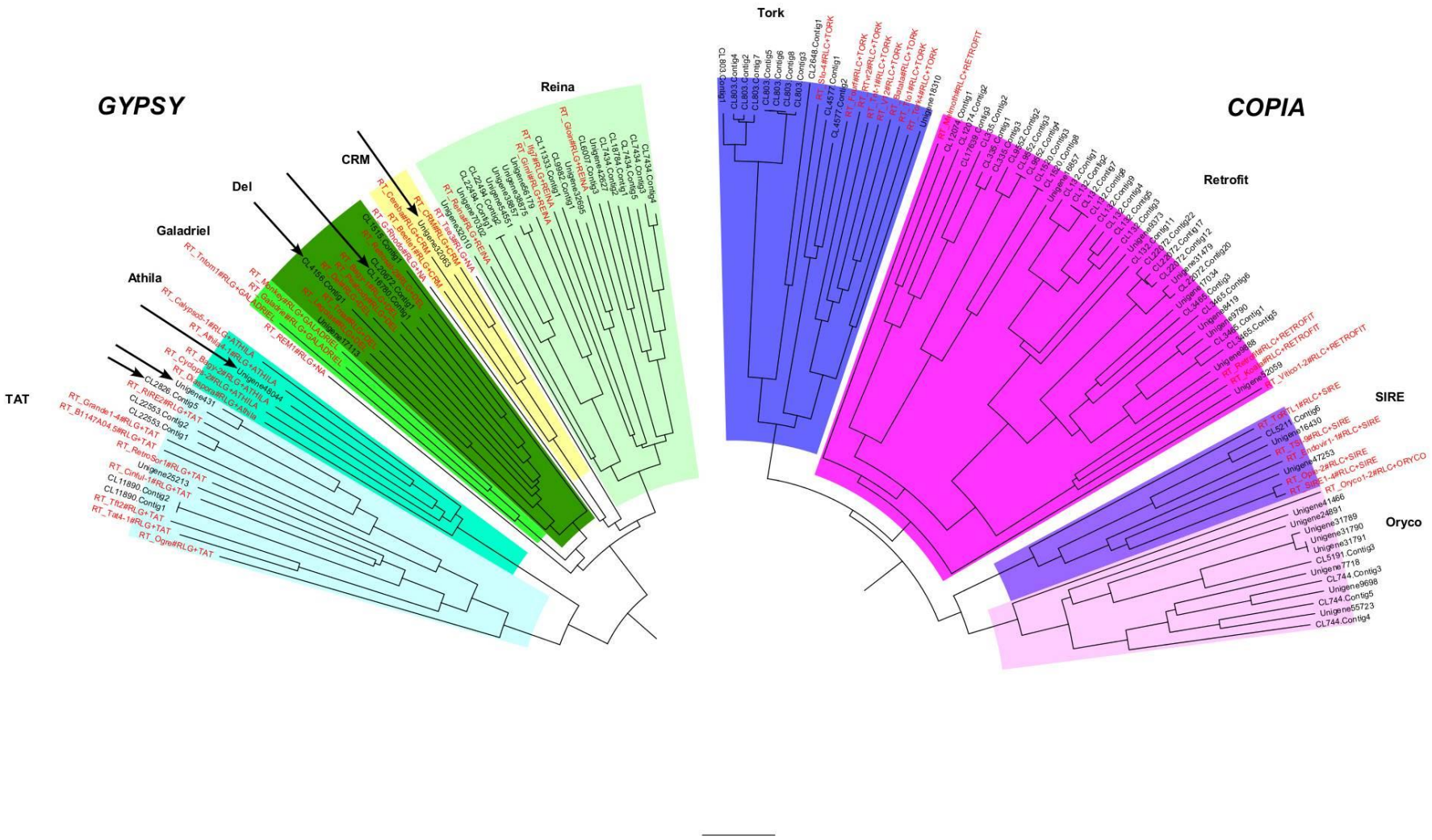

GYPSY
COPIA
Tork
Reina
CRM
Del
Galadriel
Athila
TAT
Retrofit
SIRE
Oryco
0.06

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.

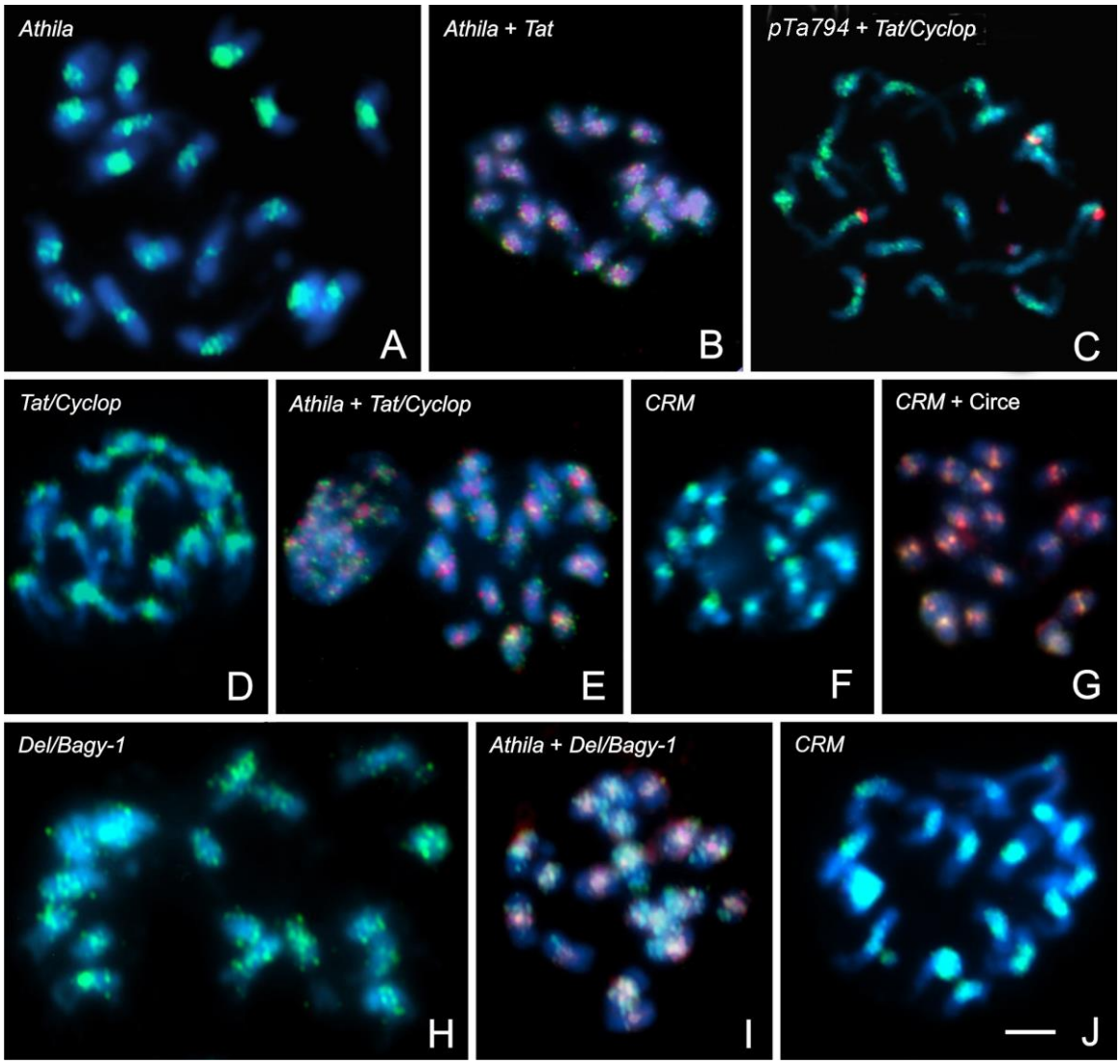


Figure 4. *In situ* hybridization of *gypsy* retrotransposon probes to chromosomes in polyploids of *B. humidicola* (A, C) and *B. brizantha* (B, D): **A)** partial metaphase hybridized with telomeric probe (red), which shows dot-like signals distally positioned, and INT-*Tat/Cyclops* probe (green) along 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 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 p*Ta*794 probe (green), showing six interstitial-proximal signals, and INT-*Tat-cyclop* probe (red), with signals predominantly centromeric-pericentromeric and few interstitial. Bar = 5 μm.

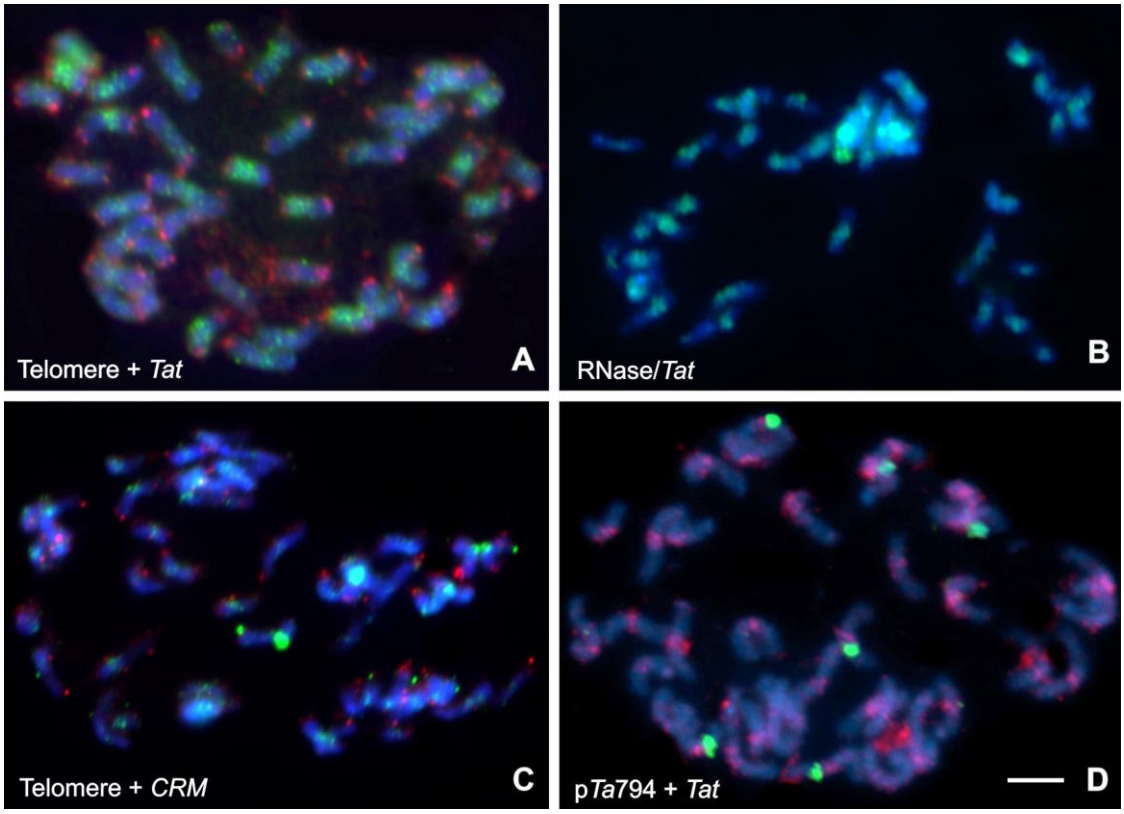
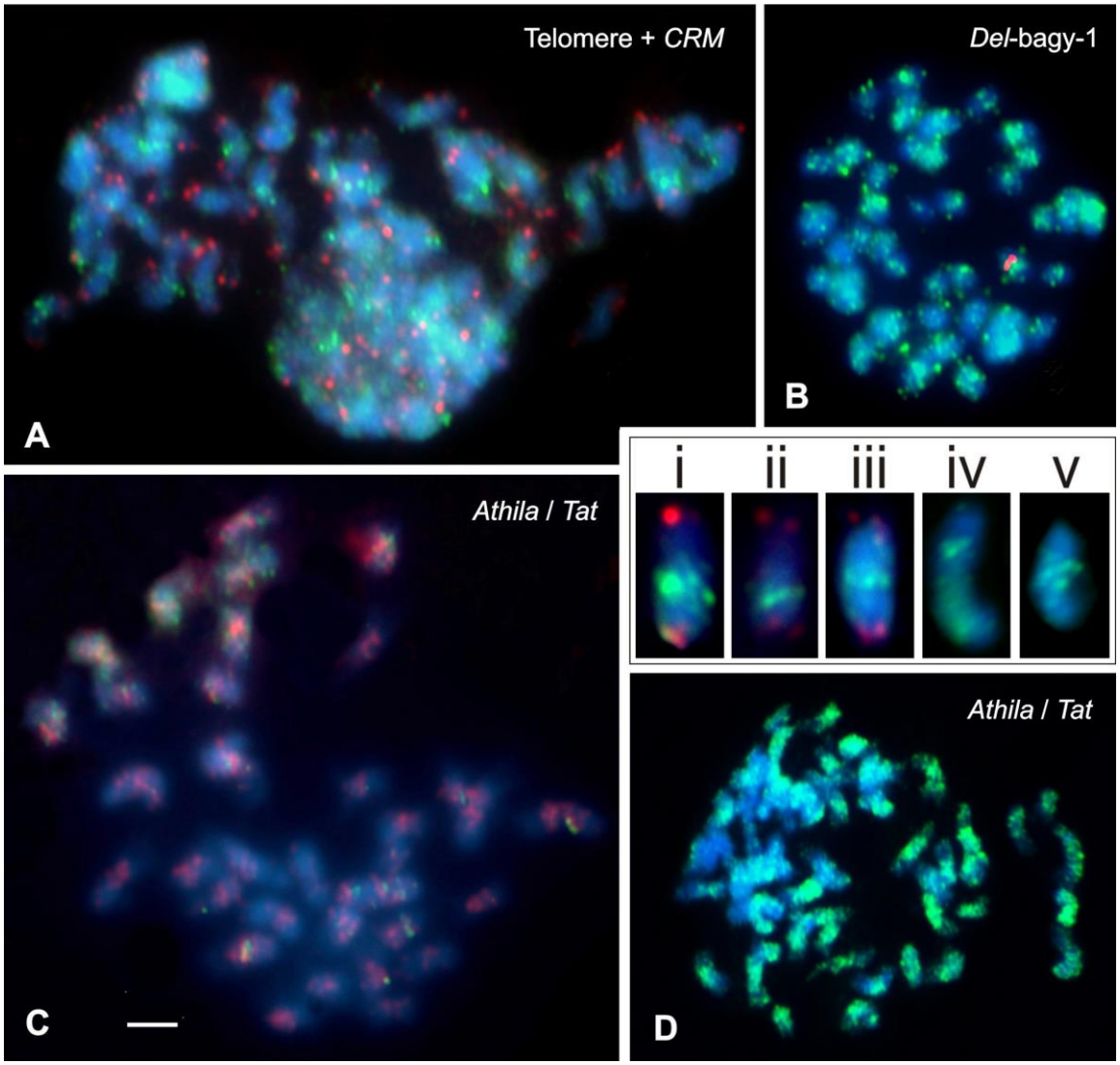


Figure 5. *In situ* hybridization of *gypsy* retrotransposon probes to chromosomes in polyploids of *B. brizantha*: **A)** metaphase and nucleus hybridized with telomeric probe (red), which shows dot-like signals terminally positioned, and RT-*CRM* probe (green) with signals concentrated in centromeric-pericentromeric regions. In the box inset below (i, ii and iii) telomeric dots and proximal *CRM* signals are presented. **B)** metaphase hybridized with RT-*Del/baggy*-1 probe (green), showing dispersed signals along chromosomes. Box below shows two chromosomes with more interstitial signals

(iv) and more interstitial to pericentromeric ones (v). **C)** metaphase hybridized with RT-*Athila* (red) and RT-*Tat* (green) probes, showing RT-*Athila* accumulated in centromeric-pericentromeric regions and RT-*Tat* as dot-like in interstitial positions. **D)** metaphase hybridized with INT-*Tat/Cyclop* probe (green), showing signals predominantly scattered along chromosomes, with some of them exhibiting accumulation of signals in proximal regions. Bar = 5 μm.



Conclusão Geral

O uso da ferramenta de citometria de fluxo foi eficaz na discriminação de indivíduos, facilitando a detecção de materiais contaminados em programas de melhoramento de *Bracharia* que utlizam diferentes espécies diploides e poliploides. As sondas hibridizadas de retrotransposons, INT-Tat-cyclop, RT-Athila e RT-CRM mostraram sinais preferencialmente nas regiões centroméricas-pericentroméricas e Del/bagy-1 em regiões interticiais e espalhados nos cromossomos. Esses elementos devem ser avaliados com mais critério, sobretudo Athila e CRM, já que foram de distribuição proximal. Há na literatura inúmeras evidências de perda de cromossomos na meiose decorrente de problemas na segregação, ou seja, problemas envolvendo a região centromérica. Asim sendo, nosso estudo vem contribuir para direcionar estudos futuros sobre a relação desses LTR-RTs na fertilidade dos híbridos itnerespecíficos e funcionamento centromérico. Além disso os elementos tranponíveis podem fornecer marcas para definir relações genômicas e fornecer infomações para compreensão do pareamento e recombinações na meiose.

Materiais suplementares

Table 1 - Estimate the amount of DNA, C Value, Value C_x, chromosome number (2n) and ploidy level (x) in *Brachiaria sp.* same letter in the column do not differ statistically at 5% probability by Skkot Knot.

Acesso	DNA (pg)*	**Mbp C-^{1}	Mbp Cx-^{1}	(2n)	x
R134(a)	1.54 ± 0.04	753.06	753.06	18	2x
D4 (a)	1.59 ± 0.10	777.51	792.18	18	2x
D5 (a)	1.63 ± 0.11	797.07	797.07	18	2x
B105(b)	2,12 ±0.01	1036.68	1036.68	18	2x
N(c)	2,81±0,01	1374.09	687.05	36	4x
J11(c)	3,02±0.09	1476.78	738.39	36	4x
R46(d)	2.99 ± 0.19	1462.11	731.06	36	4x
D62(d)	2.99 ± 0.10	1462.11	731.06	36	4x
U(d)	3.27 ± 0.10	1599.03	799.51	28	4x
Piata(e)	3,5±0.12	1711.50	855.75	36	4x
B193(e)	3,61±0.2	1545.24	706.12	45	5x
Basilisk(e)	3,67±0.13	1794.63	897.32	36	4x
B72(e)	3,71±0.05	1814.19	907.10	36	4x
ESP103(e)	3,77±0,18	1843.53	921.77	36	4x
H16(e)	3.65 ± 0.06	1784.85	594.95	36	6x
DT158(f)	4±0.05	1956.00	978.00	36	4x
Tupi(f)	4,18±0,02	2044.02	1022.01	36	4x
H13(f)	4.39 ± 0.13	2146.71	715.57	36	6x
B140(g)	4,43±0.11	2166.27	1073.36	36	4x
B183(g)	4,59±0.33	2244.51	897.80	45	5x
H112(g)	4.81 ± 0.01	2352.09	522.69	54	9x
B178(g)	4.73 ± 0.04	2312.97	925.19	45	5x
H30(h)	5,29±0,02	2586.81	739.09	42	7x
LL(i)	5.81 ± 0.02	2841.09	937.35	54	9x
H6(i)	6,02±0.16	2943.78	654.17	54	9x
H38(j)	6,27±0.03	3066.03	681.34	54	9x
B ruziziensis. (a)	1,48±0.03	724.35	724.35	18	2x
B.decumbens. (b)	2,9±0.02	1418.1	354.52	36	4x
B. brizantha. (c)	3,47±0.00	1696.01	424	36	4x
Timbó et al. 2012 (Tampão-MGSO4, Padrão- Raphanus Sativus cv. Saxa)					
B. ruziziensis (a)	1.51±0.02	738.39	738.39	18	2x
Híbrido natural (b)	2.58±0.03	1261.62	841.08	24	3x
B. brizantha (Marandu) (c)	3.48±0.07	1701.72	850.86	36	4x
B. ruzizizensis "Iracema" (b)	2.89±0.06	1413.21	706.65	36	4x
Ishigaki et al. (2009; 2010) (Tampão - Tris-HCl e Padrão-Oriza sativus)					
B ruziziensis	1.26	615± 13	615± 13	18	2x
B.deumbens	3.34	1633± 17	817± 6	18	4x
B. brizantha	2.87	1404± 13	702± 7	36	4x
B. brizantha.	3.56	1743± 15	697± 6	45	5x
B. humidicola.	3.99	1953± 16	651± 5	36	6x

**Médias seguidas de ** Mbp= Mega pares de bases.

Table 2 - Asymmetry indexs calculated A1 and A2 (Zarco, 1986), CV_{CI} and CV_{CL} (Paszko, 2006), TF% (Huziwara, 1962) for the accessions of *Brachiaria brizantha* (B178), *B. ruziziensis* (R134 and R46), *B. humidicola* (H13, H16, H112), *B. decumbens* (D4 and D5), *Urochloa mosambicensis* and *cv*. Llanero (*B. humidicola*).

Access	**A1**	**A2**	**CTLH**	**TF%**	**CV_{cl}**	**Cv_{CI}**	**AI**	**A**
D4	0.24	0.2	20.54	42.8	20	5.78	1.156	1A
Genótipo 1	0.23	0.09	22.69	43.41	9	7	0.63	1A
R134	0.20	0.17	20.57	44.1	17	3.35	0.5695	1A
D5	0.20	0.27	22.12	44.25	27	4.38	1.1826	1B
U	0.32	0.29	22.68	40.12	29	10.49	3.0421	1B
D62	0.25	0.24	34.13	42.43	24	11.45	2.748	1B
H13	0.3	0.22	36.03	40.93	22	6.18	1.3596	1B
H16	0.24	0.29	41.35	42.87	29	5.78	1.6762	1B
R46	0.24	0.28	43.91	42.85	28	5.01	1.4028	1B
B178	0.31	0.35	45.92	40.47	35	9.41	3.2935	1C
H112	0.24	0.26	48.42	42.85	26	7.79	2.0254	1B
cv Llanero	0.24	0.32	49.72	42.76	32	7.29	2.3328	1B

Tabela 3 - Correlações lineares de Pearson realizadas entres os índices de assimetria dos cariótipos A1 e A2 (Zarco 1986), CV_{cL} e CV_{cI} (Paszko 2006) e TF% (Huziwara, 1962), com o conteúdo de DNA C*x* (pg) e comprimento total do lote haploide (CTLH).

	A1	**A2**	**CTLH**	**TF%**	**CV_{CL}**	**Cv_{CI}**	**AI**	**C_x**
A1	1							
A2	0.41	1						
CTLH	0.23	0.63	1					
TF%	-0.99**	-0.44	-0.24	1				
CV_{CL}	0.41	1**	0.63	-0.44	1			
Cv_{CI}	0.66	0.3	0.23	-0.68*	0.3	1		
AI	0.7*	0.75*	0.47	-0.71*	0.72*	0.84**	1	
C_x	0.36	0.73*	0.95**	-0.38	0.73*	0.32	0.67**	1

*p<0,05 ; **p<0,0001