UNIVERSIDADE FEDERAL DE UBERLÂNDIA
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Poliploidia e variações reprodutivas em Bombacoideae (Malvaceae): distribuição geográfica, filogeografia e tamanho do genoma

Aluna: Rafaela Cabral Marinho

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_____
(Profª Drª Ana Maria Bonetti)

Dedico esta tese a minha mãe
A minha família e a amigos que foram o meu alicerce durante essa jornada.
Ao meu amor, por sua determinação em me apoiar e me fazer feliz.

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Sumário

RESUMO

O surgimento da apomixia está relacionado a mudanças climáticas que influenciariam a rápida expansão deste modo de reprodução assexual, atualmente encontrada no Cerrado, com significativa frequência. Em sua maioria, as espécies apomíticas são poliplóides e são comumente associadas com a presença de sementes poliembriônicas. Por isso, neste estudo a presença de poliembrionia implica no desenvolvimento de embriões extranumerários formados a partir de processos apomíticos. Estes fenômenos podem influenciar a estrutura genética levando a consequências ecológicas e evolutivas e devem ser melhor compreendidos para as plantas tropicais. A espécie *Eriotheca pubescens* (subfamília Bombacoideae, Malvaceae) possui ampla distribuição nesse bioma e apresenta um mosaico reprodutivo e citológico. A distribuição geográfica destes padrões não é claramente delimitada, por isso, o primeiro capítulo da tese visou ampliar o conhecimento dos mosaicos reprodutivos e citológicos num contexto geográfico no Complexo Tricoma Estrelado (*E. pubescens* + *E. estevesiae*). Para isso, foi feita a avaliação da presença de poliembrionia associada ao modo de reprodução assexual e usamos medidas morfométricas do estômato para estimar a ploidia. Neste estudo, nós verificamos que as medidas de estômatos funcionaram apenas em parte como estimador de ploidia numa escala regional. A poliembrionia foi encontrada na metade das populações sendo que todas são hexaplóides e apresentaram estômatos relativamente grandes. As populações monoembriônicas de *E. estevesiae*, consideradas diplóides, apresentaram os menores estômatos, enquanto estômatos de tamanho intermediário foram observados em uma população tetraplóide (CRI), já descrita em um estudo anterior. No entanto, uma variação não esperada no tamanho de estômato não permitiu uma relação clara com a ploidia em cinco populações monoembriônicas. No segundo capítulo nós utilizamos sequências de regiões do DNA plastidial para entender a distribuição dos sistemas reprodutivos e citológicos do Complexo Tricoma Estrelado num contexto filogeográfico, comparando os padrões de distribuição com outras espécies de Cerrado e ainda verficando a similaridade genética entre as espécies que formam o complexo. Foi possível verificar que existe uma estruturação maior nas populações poliembriônicas/hexaplóides e que os padrões filogeográficos são semelhantes a alguns já descritos para outras

espécies e associados às mudanças paleoclimáticas e distribuição da vegetação de Cerrado. O estudo mostrou que as duas espécies estudadas compartilham um haplótipo predominante e uma população de *E. estevesiae* apresenta um haplótipo exclusivo. No terceiro capítulo foi apresentada uma compilação de dados do tamanho do genoma e número cromossômico em espécies do clado Malvatheca, juntamente com a apresentação de novos dados de tamanho de genoma para espécies de Bombacoideae e determinação do número de cromossomos de uma espécie recém-descrita do gênero *Eriotheca*. Neste estudo nós encontramos dados mais robustos para subfamília Malvoideae e o tamanho médio do genoma é significativamente maior em Bombacoideae. Uma forte correlação entre o tamanho do genoma e o número cromossômico foi encontrada apenas em espécies de *Eriotheca* que apresentam séries poliplóides incompletas. Comparando o tamanho do genoma (1Cx) dos principais gêneros que formam o clado Pachira, foi possível verificar que o gênero *Eriotheca* possui menor tamanho de genoma médio do que *Pachira* indicando uma origem genômica diferente entre os gêneros que têm sido incluídos em um clado. O número cromossômico obtido para *E. estevesiae* foi 2n=2x=92 cromossomos, sendo que esse resultado foi importante porque corroborou os dados de medidas morfométricas de estômatos descritos no primeiro capítulo e também completa a série poliplóide proposta para o complexo formado por *E. pubescens* e *E.estevesiae.*

Palavras-chave: Malvaceae, duplicação do genoma, filogeografia, apomixia, *Eriotheca*.

Abstract

The emergence of apomixis is related to climatic changes that would influence the rapid expansion of this mode of asexual reproduction, currently found in the Cerrado, with significant frequency. Most apomictic species are polyploid and are commonly associated with the presence of polyembryonic seeds. Therefore, in this study the presence of polyembryony implies the development of extra embryos formed from apomitic processes. These phenomena can influence the genetic structure leading to ecological and evolutionary consequences and should be better understood for tropical plants. The species *Eriotheca pubescens* (subfamily Bombacoideae, Malvaceae) has a wide distribution in this biome and presents a reproductive and cytological mosaic. The geographical distribution of these patterns is not clearly defined, so the first chapter of this study was aimed increasing the knowledge of the reproductive and cytological mosaics in a geographic context in the Stellate Trichoma Complex (*E. pubescens* + *E. estevesiae*). For this, we evaluated the presence of polyembryony associated with the asexual reproduction mode and we used morphometric measures of the stomata to estimate ploidy. In this study, we found that stomata measurements worked only partly as a ploidy estimator on a regional scale. Polyembryony was found in half of the populations, all of which are hexaploid and have relatively large stomata. The monoembryonic populations of *Eriotheca estevesiae*, considered diploid, presented the smallest stomata, while stomata of intermediate size were observed in a tetraploid population (CRI), already described in an earlier study. However, an unexpected variation in stomata size did not allow a clear relationship with ploidy in five monoembryonic populations. In the second chapter, we used sequences from regions of plastid DNA to understand the distribution of the reproductive and cytological systems of the Stellate Trichome Complex in a phylogeographic context, comparing distribution patterns with other Cerrado species and still verifying the genetic similarity between the species that form the complex. It was possible to verify that there is a bigger structuring in the polyembryonic/hexaploid populations and that the phylogeographic patterns are similar to some already described for other species and associated to the paleoclimatic changes in distribution of the Cerrado vegetation. The study showed that the two species studied share a predominant

haplotype and a population of *E. estevesiae* presents a unique haplotype. In the third chapter a compilation of genome size and chromosome number data was presented in species of the Malvatheca clade, together with the presentation of new genome size data for Bombacoideae species and determination of the number of chromosomes of a newly described species of genus *Eriotheca*. In this study we found more robust data for the subfamily Malvoideae than in Bombacoideae and the average size of the genome is significantly larger in Bombacoideae. A strong correlation between genome size and chromosome number was found only in *Eriotheca* species with incomplete polyploid series. Comparing the genome size (1Cx) of the main genera that form the clade Pachira, it was possible to verify that the *Eriotheca* genus has a smaller size of average genome than *Pachira* indicating a different genomic origin between the genera that have been included in a clade. The chromosome number obtained for *E. estevesiae* was 2n=2x=92 chromosomes, and this result was important because it corroborated the data of morphometric measures of stomata described in the first chapter and also completes the polyploid series proposed for the complex formed by *E. pubescens* and *E. estevesiae*.

Key words: Malvaceae, genome duplication, Phylogeography, apomixis, *Eriotheca*.

1. Introdução

A evolução e diversificação das Angiospermas parecem ter envolvido mudanças drásticas no tamanho do genoma associadas ou não a processos de hibridação. A poliploidia ou duplicação completa do genoma (Whole genome duplication - WGD) parece ter marcado etapas cruciais do desenvolvimento das plantas com flores e definiram novas linhagens e possibilidades evolutivas (Jiao *et al.* 2011; Otto e Whitton 2000; Ramsey e Schemske 1998; Soltis *et al.* 2014).

Após uma extensiva análise genômica em diversas plantas importantes para compreensão do processo de diversificação das Gimnospermas e das Angiospermas, dois eventos de WGD parecem marcar estes grupos. Um evento apareceu no ancestral comum de todas as plantas com semente e outro no ancestral comum a todas as Angiospermas. A presença de um subconjunto de genes duplicados pode ter contribuído para o surgimento de inovações nestes ancestrais, alguns deles com um papel importante na reprodução e no desenvolvimento das flores (Jiao *et al.* 2011).

Nas Angiospermas, cerca de 70% das espécies são poliplóides ou se originaram desse tipo de evento (Masterson 1994), apesar de variações desta estimativa terem sido propostas nos últimos anos (Soltis *et al.* 2014). Sabe-se que este processo geralmente resulta em especiação, e a duplicação completa do genoma é reconhecida como a maior força evolutiva nas plantas e tem sido discutida e estudada á mais de um século (Soltis *et al.* 2014). Estes eventos de duplicação podem causar mudanças fenotípicas, fisiológicas, genômicas e ecológicas nos poliplóides quando são comparados com seus progenitores diplóides (Balao *et al.* 2016; Husband *et al.* 2016; Soltis *et al.* 2014, 2016; Vallejo-Marín *et al.* 2016). Por isso, autores ressaltam a necessidade de estudos relacionando essas variações em clados poliplóides (Husband *et al.* 2016; Soltis *et al.* 2016). Os melhores modelos poliplóides por vezes não foram estudados em campo e não possuem o progenitor diplóide, por isso, a difícil compreensão dos fenômenos relacionados a eventos de poliploidia se deve, dentre outros fatores, a ausência de conjuntos de dados completos para os sistemas poliplóides (Soltis *et al.* 2016).

Processos de poliploidização não somente levam a especiação, mas também estão associados a mudanças nos sistemas reprodutivos. Neste contexto, um amplo espectro de sistemas de reprodução pode ser encontrado nas Angiospermas, desde sistemas estritamente sexuados que requerem polinização cruzada e fertilização, até sistemas autógamos, que dependem apenas de fluxo de pólen dentro da mesma flor ou entre ramos. Ainda são encontrados os modo de reprodução assexual, podendo ser por propagação vegetativa ou por apomixia, que é a formação de sementes por vias assexuais (Richards 2003; Koltunow 2011; Hörandl 2010; Vallejo-Marín *et al.* 2016). Estudos recentes têm mostrado que estes processos assexuais podem ser comuns em alguns grupos de Angiospermas e associados à poliploidia (Hojsgaard *et al.* 2014; Mendes-Rodrigues *et al.* 2012; Vallejo-Marín *et al.* 2016). Portanto, há evidências de que a estrutura e o potencial evolutivo das populações de plantas com flores são diretamente influenciados pela presença destes diferentes sistemas de reprodução (Richards 2003).

1.1 A influência da poliploidia (Whole genome duplication – WGD) na diversificação das Angiospermas

A poliploidia é a presença de mais de dois conjuntos cromossômicos em uma célula (Soltis *et al.* 2009, 2014, 2016). Apesar do importante papel na evolução das Angiospermas já citado anteriormente, ela pode ser limitada devido ao difícil estabelecimento de neopoliplóides. Por isso, são necessárias barreiras reprodutivas capazes de gerar um isolamento entre diplóides e poliplóides ao longo do tempo (Husband *et al.* 2016).

Os poliplóides estão relacionados com os principais clados de Angiospermas e parecem ter surgido várias vezes na evolução deste grupo de plantas (Soltis *et al.* 2014). Estes mesmos autores questionam: Populações com origens genômicas diferentes podem intercruzar, ou elas são linhagens reprodutivamente isoladas? Estudos recentes descobriram que eventos de duplicação total do genoma geram significantes e imediatas divergências fenotípicas e isolamento reprodutivo (Husband *et al.* 2016), que podem ser suficientes para levar essas populações isoladas à especiação.

No entanto, outros estudos têm mostrado que existe fluxo gênico entre indivíduos de ploidias diferentes (ex. Cushman *et al.* 2017). Desta forma, a evolução dos poliplóides deve ser estudada caso a caso, ampliando os conhecimentos gerais sobre estas alterações no modo e história de vida destes organismos.

É notório que alguns citótipos com estrutura genética e ploidia diferentes podem coexistir em uma população, não sendo distintos taxonomicamente, apesar de em muitos casos estarem isolados reprodutivamente (Soltis *et al.* 2016). Com isso, o conhecimento desses citótipos, sua distribuição geográfica e sistemas de reprodução são importantes para deduções a respeito do processo evolutivo das plantas (Balao *et al.* 2009, 2016).

Neopoliplóides formados artificialmente comumente apresentam uma similaridade morfológica com seus congeneres naturais, mas diferem em características associadas ao tamanho celular. O aumento no tamanho celular é considerado uma consequência imediata logo após um processo de duplicação genômica (Husband *et al.* 2016; Soltis *et al.* 2016), sendo que este fenômeno é também observado em citótipos poliplóides naturais (ex. Marinho *et al.* 2014a). No entanto, outras características como tamanho da folha e altura da planta, putativamente associadas ao tamanho celular, não monstraram diferenças claras entre diplóides e poliplóides em alguns organismos, demonstrando que estas divergências morfológicas não devem ser adquiridas apenas pela duplicação completa do genoma (Husband *et al.* 2016).

O estabelecimento de um novo poliplóide entre congêneres diplóides é crucial para a poliploidização e as múltiplas origens dessas linhagens auxiliam devido esse processo devido à amplitude genética e ecológica dos novos citótipos formados (Balao *et al.* 2016; Husband *et al.* 2016). Assim sendo, algumas características reprodutivas podem contribuir para o estabelecimento e persistência destes organismos, como a presença de um ciclo de vida longo, propensão à apomixia, e a capacidade de formar populações uniparentais (Otto e Whitton 2000; Soltis *et al.* 2014).

1.2 Alternativas reprodutivas: apomixia associada a poliembrionia

1.2.1 Apomixia

Apomixia é a formação assexual de sementes provenientes dos tecidos do óvulo, sem fecundação para a formação de um embrião (Bicknell e Koltunow 2004; Carman 1997). Este processo combina os benefícios de dispersão por meio de semente com a reprodução assexual (Mogie 1992). Deste modo, desde as últimas décadas do século passado, há um profundo interesse nos aspectos ecológicos das plantas apomíticas (Hojsgaard *et al.* 2014).

Hojsgaard *et al.* (2014) fizeram uma revisão recente que compilou dados filogenéticos de mais de 70 famílias de Angiospermas com a presença de apomixia. Foi possível afirmar que este fenômeno parece ter surgido várias vezes nos grandes clados, apesar da sexualidade ainda ser considerada uma característica ancestral nas Angiospermas. A presença da apomixia em 19% das famílias estudadas demonstrou um papel evolutivo positivo e importante na evolução deste grupo de plantas, e a presença desta forma assexual de reprodução em famílias com altos níveis de diversidade pode sugerir que este fenômeno também possa frequentemente contribuir para o processo de diversificação (Hojsgaard *et al.* 2014).

A apomixia pode ser de modo geral, subdividida em dois tipos, que se distinguem pela origem dos embriões: a apomixia gametofítica quando o embrião é originado de um gametófito e a esporofítica quando o embrião é originado do tecido do esporófito (Koltunow 1993; Hand e Koltunow 2014; Mendes-Rodrigues 2010). Na apomixia esporofítica, que também é chamada de embrionia adventícia, os embriões são formados do tecido nucelar ou tegumentar e sua formação pode ocorrer simultaneamente com embriões sexuais. Portanto, as espécies exibindo esse modo de reprodução normalmente produzem múltiplos embriões por semente (Hojsgaard *et al.* 2014; Koltunow 1993; Koltunow e Grossniklaus 2003; Mendes-Rodrigues 2010, 2012). Esse parece ser o tipo de apomixia mais comum entre as Angiospermas, especialmente em ambientes tropicais (Carman 1997; Hojsgaard *et al.* 2014; Mendes-Rodrigues 2010; Mendes-Rodrigues *et al.* 2012).

Este modo de reprodução assexual poderia levar a um declínio da variabilidade genética intrapopulacional, pois a planta-mãe pode produzir uma

progênie geneticamente idêntica a ela, formando assim uma população clonal (Richards 2003; Hörandl 2010). Apesar da formação de embriões clonais, eventos de mutação, sexualidade facultativa, colonização por diferentes clones e retrocruzamentos com indivíduos diplóides sexuados podem gerar variação genética nas espécies apomíticas (Paun *et al.* 2006).

A apomixia pode ser considerada obrigatória, excluindo a possibilidade de eventos de sexualidade, ou facultativa alternando estes momentos com a reprodução sexual, sendo que a primeira é considerada um evento raro (Hojsgaard *et al.* 2014; Koltunow 1993; Koltunow e Grossniklaus 2003; Richards 2003). Estudos nas últimas décadas têm mostrado que sistemas mistos de reprodução podem explicar a variabilidade genética encontrada em populações apomíticas, podendo gerar uma progênie com embriões zigóticos e apomíticos (Hand e Koltunow 2014; Koltunow 1993).

A apomixia pode surgir a partir de uma desregulação do processo sexual, causado por eventos de hibridação entre espécies que diferem no tempo de desenvolvimento dos estágios iniciais do saco embrionário ou da formação do endosperma (Carman 1997; Koltunow e Grossniklaus 2003). Sendo assim, estima-se que possa ocorrer uma desestabilização de um genoma híbrido impossibilitando o pareamento durante a meiose. Por isso, a maioria dos apomíticos são poliplóides (Carman 1997), sendo que as múltiplas cópias do genoma possibilitam o pareamento dos cromossomos e a manutenção desta desregulação do sistema sexual, favorecendo a apomixia por várias gerações (Carman 1997, 2007; Koltunow 1993). Sendo assim, a poliploidia é considerada a responsável pelo grande sucesso da distribuição de alguns grupos apomíticos (Richards 2003). Portanto, este fenômeno pode funcionar como um trampolim reprodutivo para novas espécies e gêneros com ampla hibridação, poliploidia, rearranjos genéticos e genômicos (Hojsgaard *et al.* 2014).

Os apomíticos são considerados exploradores pioneiros de nichos ecológicos e geográficos, com uma tendência a ocupar uma distribuição geográfica maior do que seus progenitores diplóides e a colonizar áreas que passaram por glaciações, possibilitando novas hibridações e poliploidizações, num processo denominado partenogênese geográfica (Bierzychidek 1985; Horandl 2006; Horandl e Hojsgaard 2012). Este fenômeno ocorre devido a vários

fatores como vantagens vindas através da poliploidia, melhor habilidade de colonização devido à reprodução uniparental, dentre outros. Portanto, é possível supor que o aumento de indivíduos apomíticos em áreas recém-colonizadas impedia por competição, o estabelecimento de indivíduos sexuais (Horandl 2006). Com isso, a apomixia pode promover especiação por replicação clonal de genótipos ecologicamente bem sucedidos (Cushman *et al.* 2017).

1.2.2 Poliembrionia em apomíticos

Além da poliploidia, a apomixia está comumente associada a eventos de poliembrionia. A poliembrionia é definida como ocorrência de mais de um embrião por semente ou por óvulo (Mendes-Rodrigues 2010). Estudos realizados comprovaram a relação entre apomixia, poliploidia e a poliembrionia (Carman 1997; Mendes-Rodrigues 2010, 2012; Sampaio 2010). A formação de embriões adventícios requer um endosperma funcional que raramente ocorre sem fertilização (Koltunow e Grossniklaus, 2003). Por isso, embriões zigóticos podem ser formados por meio sexual e embriões adventícios por meio assexual, produzindo uma semente poliembriônica com embriões originados de formas distintas (Carman 1997; Mendes-Rodrigues *et al.* 2005, 2010).

A desvantagem da poliembrionia é a competição por nutrição entre os embriões, levando à diminuição no peso e consequentemente, no tamanho das plântulas, podendo dificultar o estabelecimento (Mendes-Rodrigues *et al.* 2010). E a principal vantagem é o aumento das chances de sucesso de pelo menos um embrião por semente se estabelecer. Ainda, a presença de muitos embriões compensa uma eventual produção de poucos frutos e há um aumento no sucesso reprodutivo quando plântulas estão se desenvolvendo em grupo ao invés de isoladas (Mendes-Rodrigues *et al.* 2010).

Estudos desenvolvidos em ambientes tropicais têm indicado a ocorrência de apomixia com alta frequência de poliembrionia em espécies amplamente distribuídas no Cerrado brasileiro, onde mosaicos de sistemas de reprodução indicam a co-ocorrência de indivíduos/populações diplóides, monoembriônicos e sexuados com outros indivíduos/populações poliplóides, poliembriônicos e predominantemente apomíticos (Mendes-Rodrigues *et al.* 2005, 2010, 2012; Salomão e Allem 2001; Sampaio 2010).

1.3 Bioma Cerrado

Esse bioma é definido com base na variação climática, fatores edáficos e regimes de fogo, variando de áreas abertas com arbustos dispersos e pequenas árvores a áreas muito fechadas com árvores de floresta (Simon e Pennington 2012). O regime de fogo traz grandes desafios para as plantas que devem tolerar ainda um ambiente com grandes variações climáticas e solos pobres e ácidos (Simon e Pennington 2012).

O Cerrado é o segundo maior bioma da América do Sul, classificado como um *hot spot* mundial devido à sua grande biodiversidade com mais de quatro mil espécies de plantas que não são encontradas em nenhum outro lugar do planeta (Myers *et al.* 2000; Strassburg *et al.* 2017). Desde a década de 70, está sendo rapidamente modificado e fragmentado devido às grandes áreas de plantações e pastoreio (Ratter *et al.* 1997). Estima-se que apenas 19,8% da sua cobertura vegetal esteja inalterada com uma taxa de desmatamento de 1% ao ano, sendo que esta taxa é muito maior do que a encontrada em outros biomas brasileiros (Strassburg *et al.* 2017).

Projeções recentes mostram que um grave evento de extinção pode ocorrer nestas áreas caso políticas públicas de conservação não sejam adotadas. Com expectativas de intenso crescimento agropecuário, estima-se que cerca de 30% das áreas de Cerrado restantes sejam destruídas até 2050. Este mesmo trabalho sugere um plano de ação que envolve, dentre outras questões a identificação de áreas críticas para conservação e restauração do bioma (Strassburg *et al.* 2017).

O Cerrado, bioma onde é encontrada a maioria das espécies do presente estudo, é dominado por savanas tropicais que ocupam o Brasil Central, apresentando a maior área e mais rica flora entre as formações deste tipo em todo o mundo. Estudos filogenéticos consideram que os clados de vários grupos bem representados no Cerrado são de origem recente, com filogenias datadas de menos de 10 milhões de anos (Simon e Pennington 2012). Com isso, a flora lenhosa encontrada neste bioma, parece ter sido influenciada por mudanças paleoclimáticas e glaciações, durante o período do Pleistoceno (Ramos *et al.* 2007, 2009). Estas mudanças climáticas recentes parecem também ter

influenciado o surgimento de alternativas reprodutivas que foram documentadas em espécies endêmicas deste bioma (Marinho *et al.* 2014b) e em outros biomas do planeta (Carman 1997; Hörandl 2006).

As previsões futuras da sobrevivência do Cerrado nos levam a acreditar que nossas ações e as políticas públicas de conservação precisam mudar, ampliando a proteção a este bioma. A expansão do agronegócio, o desenvolvimento de infraestrutura, a baixa proteção legal e o pequeno incentivo para a conservação podem levar a um evento de extinção sem precedentes (Strassburg *et al.* 2017).

1.3.1 Estudos filogeográficos no Cerrado

Em concordância, a maioria dos estudos que realizaram análises filogeográficas em plantas ocorrentes no Cerrado aponta a relevância dessa discussão para a conservação deste bioma fortemente ameaçado (Collevatti *et al.* 2003, 2009; Ramos *et al.* 2007, 2009; Resende-Moreira *et al.* 2017; Ribeiro *et al.* 2016). A conservação dos biomas brasileiros é sempre um desafio, e os dados de filogeografia podem auxiliar no conhecimento de linhagens desconhecidas e na definição de um padrão geral de distribuição das linhagens, para enfim traçar melhores planos de conservação (Miyaki 2009).

Os estudos filogeográficos passaram a ser realizadas com dados multiloci e as previsões para os próximos anos é que os estudos passem a ser genômicos (MiyakI 2009). Ensaios multidisciplinares unindo dados geológicos, ecológicos, climáticos e genéticos podem auxiliar na construção de padrões históricos. Assim, a filogeografia busca avaliar a congruência entre as linhagens genéticas e a distribuição espacial (Avise 2000; Miyaki 2009).

Essa discussão pode ser ampliada quando se conhece os padrões filogeográficos de organismos aparentados que possuem uma distribuição geográfica semelhante (Miyaki 2009), como o realizado com duas espécies arbóreas de *Hymeneae* e outro com duas espécies de *Anonna*, encontradas no Cerrado brasileiro (Ramos *et al.* 2009; Ribeiro *et al.* 2016).

No entanto, alguns cenários filogegráficos descritos para plantas arbóreas nem sempre são concordantes. Estas variações demonstram a necessidade de mais evidências e ressaltam a necessidade de unir informações de mais de uma

espécie para auxiliar na definição dos padrões filogeográficos para o Cerrado (Ribeiro *et al.* 2016). Assim, a história evolutiva será melhor compreendida se espécies com a mesma distribuição geográfica e com diferentes dispersores de pólen e sementes forem investigadas (Resende-Moreira *et al.* 2017).

Estudos recentes estão mostrando que as mudanças climáticas que ocorreram no Quaternário afetaram a diversidade genética das plantas de várias formas (Collevatti *et al.* 2012; Lima *et al.* 2017; Novaes *et al.* 2013; Ribeiro *et al.* 2016). Depois do último máximo glacial, com a melhora das condições climáticas a pradaria remanescente foi substituída por diferentes formas de vegetação de Cerrado (Ramos *et al.* 2009). Em *Eugenia dysenterica* as linhagens encontradas se dispersaram do Brasil Central em direção ao oeste e do norte em direção ao sudeste do Brasil. Com a sobreposição deste padrão em outras plantas, as populações do centro geográfico, foram consideradas as mais antigas (Lima *et al.* 2017). Do mesmo modo, os resultados de *H. courbaril* sugerem que, durante o último máximo glacial, as populações se refugiaram ao norte e em locais de baixa elevação e a expansão das populações para o sul do Cerrado aconteceu depois do restabelecimento das condições climáticas favoráveis (Ramos *et al.* 2009).

A história biogeográfica do Cerrado brasileiro ainda está sendo construída, mas algumas conclusões já foram propostas. Dentre elas, a possibilidade de uma concordância da estrutura filogeográfica com os padrões fitogeográficos (Ratter *et al.* 2003) em populações de *Dalbegia miscolobium e* parcialmente em *Annona coriacea* (Novaes *et al.* 2013; Ribeiro *et al.* 2016). Nessa proposta o Cerrado brasileiro foi dividido em 4 regiões fitogeográficas e 2 disjunções: Sul, Central e Sudeste, Norte e Nordeste e Centro-Oeste, sítios mesotróficos do Extremo leste e Disjunção Amazônica (Ratter *et al.* 2003).

O padrão de diversidade de *D. miscolobium* no sul do Cerrado é muito semelhante ao relatado para outras arbóreas (Novaes *et al.* 2013). Nesta região, populações de *D. miscolobium* apresentam baixa diversidade genética e provavelmente são descendentes de populações do norte. Esse padrão também aponta para uma recente expansão das plantas de Cerrado ao sul (Novaes *et al.* 2013; Ramos *et al*. 2009; Ribeiro *et al.* 2016).

Em espécies que compartilham a mesma distribuição geográfica é possível que desenvolvam diferentes tolerâncias para estresse devido às variadas

pressões seletivas. Em *Annona coriacea* uma forte separação leste e oeste parece ter ocorrido entre 2.06 (populações do leste) e 3.19 milhões de anos (populações do oeste), período que corresponde ao início do pleistoceno marcado por fortes oscilações climáticas (Ribeiro *et al.* 2016), um evento importante na similar separação das linhagens de várias espécies de Cerrado (Collevatti *et al.* 2009; Novaes *et al.* 2013; Ramos *et al.* 2007; Ribeiro *et al.* 2016). Já em *Annona crassiflora* não foram econtradas variações genéticas em nenhuma das populações amostradas. Essa diferença observada mostra que essas espécies desenvolveram alternativas adaptativas e evolutivas distintas (Ribeiro *et al.* 2016).

Situação semelhante ao relatado em *Annona coriaceae* foi relatada também para *Lychnophora ericoides,* um arbusto endêmico do Cerrado com distribuição disjunta das populações devido a glaciações do Quaternário. Estas populações mostraram alta diferenciação genética entre as populações do leste e oeste, sendo que as do leste parecem ser mais antigas (Collevatti *et al.* 2009).

Um cenário distinto foi proposto para *Caryocar brasiliense,* em que múltiplas linhagens atuaram para a origem da espécie. As populações ficaram refugiadas durante o último máximo glacial em locais mais úmidos e logo após a estabilização climática, migraram para áreas mais favoráveis e nesse período pode ter ocorrido contato entre diferentes linhagens (Collevatti *et al.* 2003).

Os estudos que visam à compreensão da origem e história biogeográfica do Cerrado envolvem espécies de plantas com características ecológicas diferentes, principalmente relacionadas ao modo de dispersão. No entanto, o nível de diversidade e a separação espacial encontrada é similar, mostrando que provavelmente, a intensa fragmentação do Cerrado está interferindo nestes fatores relacionados principalmente à dispersão (Resende-Moreira *et al.* 2017).

Marcadores genéticos plastidiais têm sido amplamente utilizados em estudos filogeográficos. Essas regiões possuem uma taxa de mutação lenta (Ramos *et al.* 2009) e têm se mostrado eficientes para propor teorias a respeito da origem e diversificação dos biomas brasileiros (Collevatti *et al.* 2009; Novaes *et al.* 2013; Ramos *et al.* 2007, 2009; Ribeiro *et al.* 2016).

Estes marcadores plastidiais são ainda interessantes por serem menos influenciados por mudanças de ploidia e formas de reprodução. O genoma plastidial é basicamente matrilinear (Bock 2007) e a distribuição de citótipos deve

ser influenciada principalmente pela dispersão das sementes. Desta maneira, os estudos filogeográficos das plantas de Cerrado descritos anteriormente raramente levararam em conta os mecanismos reprodutivos envolvidos e os possíveis mosaicos de ploidia descritos para muitos grupos de plantas de Cerrado. Muitas espécies de Cerrado apresentam números cromossômicos relativamente altos (Forni-Martins 2000; Morawetz 1986) e, como mencionado, estudos recentes têm mostrado uma associação entre ploidia e apomixia em vários grupos de plantas do Cerrado (Mendes-Rodrigues 2017; Sampaio 2010). Apesar de não afetar a herança plastidial via sementes, é possível que as mudanças reprodutivas afetem a velocidade relativa de distribuição dos citótipos e a filogeografia destas plantas.

1.4 Objeto de estudo

Eventos de WGD associados à irradiação das Angiospermas no final do Cretáceo parecem ter dado origem a grupos importantes dentro da família Malvaceae (Marinho *et al.* 2014b). Esta família possui 9 subfamílias: Malvoideae, Bombacoideae, Tilioideae, Dombeyoideae, Sterculioideae, Brownlowideae, Helicteroideae, Grewioideae e Byttnerioideae (Alverson *et al.* 1999; Bayer *et al.* 1999). Malvatheca é um clado fortemente sustentado por dados moleculares, formado pelas antigas famílias Malvaceae e Bombacaceae (Baum *et al.* 2004; Duarte *et al.* 2010).

O número cromossômico foi considerado uma característica com forte sinal filogenético para a filogenia de Malvaceae s.l. mostrando distinções nos números cromossômicos entre estas subfamílias. A subfamília Bombacoideae apresenta os maiores números cromossômicos e parece ter surgido há aproximadamente 60 milhões de anos, mas os gêneros atualmente conhecidos divergiram a menos de 35 milhões de anos (Marinho *et al.* 2014b).

Recentemente, um estudo com análises filogenéticas verificou que a evolução de caracteres morfológicos relacionados com as sementes e frutos também confirmam o monofiletismo do core Bombacoideae (Carvalho-Sobrinho *et al.* 2016). Bombacoideae se distingue das demais subfamílias por possuir cromossomos pequenos e numerosos (Baum e Oginuma 1994; Morawetz 1986). Apresenta cerca de 17 gêneros e 160 espécies e é representada principalmente por espécies arbóreas neotropicais (Carvalho-Sobrinho *et al.* 2016).

Entretanto, os estudos filogenéticos recentes não levaram em conta a variação cromossômica marcante e com forte sinal filogenético que parece caracterizar a família Malvaceae *sensu lato* (Baum e Oginuma 1994, Marinho *et al.* 2014b). Um exemplo deste aparente desinteresse pode ser observado quando foram analisadas as relações filogenéticas dos genêros que compoem Bombacoideae (Carvalho-Sobrinho *et al.* 2016; Duarte *et al.* 2011). O clado *Pachira* foi proposto utilizando dados moleculares pelos autores acima citados e é formado principalmente por espécies integrantes de dois gêneros: *Pachira* e *Eriotheca.* Os mesmos discordam quanto ao monofiletismo do gênero *Eriotheca* e não consideram diferenças de número cromossômico e do tamanho do genoma descritos para estes grupos. Além disso, há diferenças morfológicas claras entre os gêneros do Clado Pachira, principalmente, com relação às características florais (Duarte 2010), que indicam a separação entre estes grupos.

Eriotheca é um pequeno gênero Neotropical que possui cerca de 24 espécies distribuídas na América do Sul e 16 espécies que ocorrem em biomas brasileiros como a Floresta Atlântica, Amazônia e no Cerrado (Carvalho-Sobrinho *et al.* 2016; Duarte *et al.* 2010; Simon e Pennington 2012). Alguns estudos recentes têm mostrado grande variação cromossômica e genômica, associada às alternativas de sistemas de reprodução e ocorrência de apomixia (Marinho *et al.* 2014b; Mendes-Rodrigues *et al.* 2005; Oliveira *et al.* 1992). Além de espécies amplamente distribuídas em áreas de Cerrado, algumas novidades taxonômicas têm sido descritas (Carvalho-Sobrinho *et al.* 2015; Duarte e Esteves 2011) que sugerem uma diversidade maior do que anteriormente descrito.

A variação morfológica floral presente entre as espécies de *Eriotheca* é uma característica determinante nas relações filogenéticas, apesar de que o androceu e a estrutura floral variam menos que nos outros grupos da família (Duarte *et al.* 2010). A morfologia floral de *Eriotheca pubescens* (Mart. & Zucc.) Schott & Endl e *Eriotheca gracilipes* (K. Schum.) A. Robyns, espécies filogeneticamente próximas, é semelhante, diferindo basicamente no tamanho. *E. gracilipes* apresenta flores menores e nectários extra-florais na base do receptáculo, que é glabro e avermelhado e em *E. pubescens* o cálice é cinza piloso e sem nectários (Oliveira *et al.* 1992).

Para *E. pubescens* foram descritas populações autocompatíveis e poliembriônicas (Oliveira *et al.* 1992), caracterizadas como apomíticas e pseudogâmicas a partir de estudos histológicos (Mendes-Rodrigues *et al.* 2005). Para essa espécie, até o momento apenas uma população autoincompatível e monoembriônica foi documentada (Mendes-Rodrigues 2010). Em contraste, apesar da autoincompatibilidade e monoembrionia serem predominantes nas populações de *E. gracilipes*, a poliembrionia e apomixia foram documentados para alguns indivíduos da espécie (Mendes-Rodrigues 2010). A ocorrência de apomixia nestes grupos pode indicar uma associação com a poliploidia, fenômeno comum entre as espécies do Cerrado (Mendes-Rodrigues *et al.* 2005; 2010; Morawetz 1986; Oliveira *et al.* 1992; Sampaio 2010).

As espécies *E. pubescens* e *E. gracilipes* quando foram estudadas quanto ao número cromossômico por Marinho *et al.* (2014b), apresentaram, não só os maiores números cromossômicos documentados em todos os gêneros que pertencem a subfamília Bombacoideae, como também variações neste número (2n = 92,184 e 276) mostrando que nestas espécies são encontrados citótipos neopoliplóides. Estes eventos em *Eriotheca* parecem ser recentes, com menos de 10 milhões de anos como sugerido por análises filogenéticas (Marinho *et al.* 2014b).

1.5 Objetivos gerais

Este estudo teve como objetivo geral relacionar os mosaicos reprodutivos e citológicos associados à presença de fenômenos de duplicação total do genoma (*whole genome duplication* - WGD), com a distribuição geográfica, os padrões filogeográficos encontrados em plantas que pertencem à subfamília Bombacoideae, aqui tratadas como Complexo Tricoma Estrelado. Além disso, avaliamos as mudanças no tamanho do genoma com as relações filogenéticas no clado Malvatheca. O trabalho de tese está então subdividido em três objetivos específicos que constituem capítulos até certo ponto independentes.

1 - Verificar a distribuição geográfica dos padrões de embrionia e citológico em populações do Complexo Tricoma Estrelado – Capítulo 1.

2 - Conhecer os padrões filogeográficos deste complexo e compará-los com os padrões reprodutivos e citológicos e com as teorias filogeográficas propostas para outras espécies de Cerrado – Capítulo 2.

3 - Analisar a evolução do número cromossômico e do tamanho de genoma no clado Malvatheca (Malvoideae + Bombacoideae, Malvaceae) e mais especificamente no clado *Pachira* (*Eriotheca*+ *Pachira*, Bombacoideae) – Capítulo 3.

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CAPÍTULO 1

Stomata measurements for different cytotypes in species of *Eriotheca* that formed the Stellate Trichomes Complex (Bombacoideae – Malvaceae)

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Abstract

The emergence of apomixis seems to be influenced by climatic changes that allowed the rapid geographic expansion, which has being increasingly found in Cerrado. Most apomictic species are polyploid and commonly associated with polyembryonic seeds, with multiples embryos formed from apomictic and/or sexual processes. The species *Eriotheca pubescens* has a wide distribution in this biome and presents reproductive and cytological mosaic, and recent studies have shown that part of their populations form a new species called *Eriotheca estevesiae*. We aim here to demonstrate the range-wide and diverse distribution of the reproductive and cytological mosaics based on stomata measurements and polyembryonic estimates of populations of *E. pubescens* and *E. estevesiae,* called here the Stellate Trichome Complex, in the Cerrado of Central Brazil. Leaf and seed material from individuals of 19 populations were collected as a function of wide distribution that comprises a large part of the Brazilian Cerrado. The seeds were evaluated for the presence of polyembryony and ploidy was estimated from stomatal measurements. Slides from abaxial epidermis of leaflets were made for 3 to 5 individuals from each population and the height and width of 20 to 50 stomata were measured. Monoembryonic seeds were found in 8 populations, polyembryonic in 8 and three populations could not be verified. It was possible to find a complexity that was not observed in previous studies. Therefore, five different groups were found based on geographical, embriony, and ploidy pattern. Four groups included monoembryonic populations, while all the polyembryonic populations form a homogeneous group**.** In group 1 (formed by *E. estevesiae* populations*)* all the populations were considered diploid. Group 2 was formed by a monoembryonic and tetraploid population (CRI), and groups 3 and 4 included populations that did not have the level of ploidy determined only by stomata morphometric measurements. There was a geographical separation in the embryony patterns. No variation in cytological or reproductive features was observed among *E. estevesiae* populations. In *E. pubescens*, the polyembryonic pattern seems to be predominant in the southern populations in agreement with theories of geographic parthenogenesis and apomixis, which indicate a tendency to be

pioneer explorers of new habitats, made available after the last glacial maximum period.

Key-words: *Eriotheca*, apomixis, ploidy, geographical distribution, Cerrado.

Introduction

Sporophytic apomixis is the asexual formation of seeds from maternal tissues, without fertilization and meiosis (Bicknell and Koltunow 2004, Carman 1997). This process combines the benefits of seed dispersal with asexual reproduction (Mogie 1992). The apomictic species are commonly polyploid (Carman 1997; Horandl and Hojsgaard 2012) and events of whole genome duplication (WGD) are considered to be responsible for the great success of distribution of some apomictic groups when compared to sexual counterparts (Bierzhchudek 1985; Richards 2003). The association of polyploidy with the presence of apomixis can promote the development of new functions and gene combinations, giving more ecological and physiological flexibility to the polyploid (Richards 2003; Horandl and Hojsgaard 2012). Studies have shown that polyploidy is a frequent and important phenomenon in the evolution of plants because it can be a rapid form of adaptation and speciation (Otto and Whitton 2000; Ramsey and Schemske 1998; Soltis *et al.* 2009, 2014).

Although the sexual reproduction is more frequent and can be considered an ancestral feature in Angiosperms, apomixis is common in several families and has been associated with different life forms and habitats (Carman 1997; Hojsgaard *et al.* 2014; Horandl and Hojsgaard 2012). This asexual mode of reproduction has been described for some groups of Cerrado plants, such as Malvaceae (Oliveira *et al.* 1992; Mendes-Rodrigues *et al.* 2005), Bignoniaceae (Sampaio 2010) and Melastomataceae (Goldenberg and Shepherd 1998; Mendes-Rodrigues *et al.* 2012). The high frequency of polyembryony is often associated with apomixis and polyploidy in this biome (Mendes-Rodrigues *et al.* 2005, 2012; Salomão and Allem 2001; Sampaio 2010), a phenomenon that can generate significant ecological, genetic, and evolutionary consequences (Hojsgaard *et al.* 2014; Hörandl 2010; Paun *et al.* 2006).

Eriotheca (Schott &Endl) is a small Neotropical genus within the subfamily Bombacoideae (Malvaceae). It has 24 species distributed in South America and with about 18 species in Brazil, some of them, as *Eriotheca pubescens* (Mart. & Zucc.) Schott & Endl, restricted to Cerrado areas (Duarte 2010; Simon and Pennington 2012; Flora do Brasil 2020 in construction). Previous studies have

shown the occurrence of reproductive mosaics in *E. pubescens*, with apomitic and sexual populations (Mendes-Rodrigues *et al.* 2005, 2010), apparently with different geographical distribution.

Most populations of *E. pubescens* have been reported as self-compatible, apomictic, polyembryonic, and hexaploid (Marinho *et al.* 2014b; Mendes-Rodrigues *et al.* 2005; Oliveira *et al.* 1992). Only one population has been described as self-incompatible, monoembryonic and tetraploid (Marinho *et al.* 2014b; Mendes-Rodrigues 2010). But only a few populations have been studied so far, encompassing a small part of the species distribution.

The geographical distribution of *E. pubescens* comprises Goiás, Minas Gerais, São Paulo, Mato Grosso and Mato Grosso do Sul states, a large part of Cerrado of the Brazil Central (Duarte 2010, Flora do Brasil 2020 in construction). The Cerrado is the second largest biome in South America, classified as a hot spot with more than four thousand species of plants not found anywhere else (Myers *et al.* 2000; Strassburg *et al.* 2017). Recent projections show that a serious extinction event may occur in this biome if public conservation actions are not adopted, and one of the suggested strategies is the identification of critical areas for conservation and restoration of the biome, that has been rapidly changing and fragmented due to large areas of plantation and pastureland (Ratter *et al.* 1997; Strassburg *et al.* 2017).

E. pubescens can be differentiated from other *Eriotheca* by the occurrence of distinctive stellate trichomes on the abaxial leaf surface. Another recent study described plants from Tocantins state, sharing this same morphological characteristic*,* as comprising a new species, called *E. estevesiae* (Carvalho-Sobrinho *et al.* 2015). In recent phylogenetic analysis, this new species was considered sister of *E. pubescens* and these species were separated based on molecular and morphological characteristics such as some variations in leaf morphology, absence of stellate trichomes in pedicels and calyces, smaller flowers and capsules, variations in the color of the ferruginous trichomes, presence of irregularly peltate scales, and variations in density and size of the stomata (Carvalho-Sobrinho *et al.* 2015). However, although some of these characteristics may be influenced by modifications in genome size (Husband *et*

al. 2016; Marinho *et al.* 2014a; Soltis *et al.* 2016), these recent phylogenetic analysis have not discussed polyploidy effects on the phylogeny and diversification of these *Eriotheca* species.

Actually, genome size and chromosome number seem to be important for the evolution of the Bombacoideae as a whole and for the *Eriotheca* in particular (Marinho *et al.* 2014b; chapter 3). The distribution of reproductive and genome size mosaics may help to understand the evolution of the group and to foresee species distribution under the disturbance pressure now in action. But chromosome number, genome size and anatomical studies are complex in large scale, relatively expensive, and require extensive collection of seeds and leaves. On the other hand, other vegetative or reproductive characters, such as stomata and pollen size as well as polyembryony occurrence, may be used as proxies of ploidy or breeding system in the species (Marinho *et al.* 2014a; Mendes-Rodrigues *et al.* 2005), and readily used to map reproductive and cytotype/genome size variation in the species.

We aim here to evaluate the range-wide and diverse distribution of the cytological and reproductive mosaics based on stomata measurements and the presence of polyembryony in populations of *E. pubescens* and *E. estevesiae*, treated here as the Stellate Trichome Complex, in the Cerrado of Brazil Central.

Methods

We collected leaves and seeds of 92 individuals in 19 populations. Four populations were collected in Tocantins (*E. estevesiae*) and the others in Goiás, Minas Gerais and São Paulo states (*E. pubescens*) covering the most wide distribution of the species, which comprises a large part of the Brazilian Cerrado (Tab. 1 and Fig. 1). The elevation ranged from 330 to 1178m and representative vouchers for some of the sampled populations were deposited in the Herbarium Uberlandense (HUFU - Universidade Federal de Uberlândia - HUFU 00015010; HUFU 00015011; HUFU 00015012; HUFU 00015070; HUFU 00015073; HUFU00015078; HUFU15079). The seeds were dissected to evaluate the presence of polyembryony or monoembryony, and stomatal measurements were taken from the stored dried leaves.

For the analysis of the number of embryos, the collected seeds (at least ten seeds per population; number of individuals ranged from 1 to 5 – Tab.1) were placed inside transparent plastic germination boxes (Gerbox), on filter paper moistened with distilled water. Imbibed seeds were dissected and the embryos were counted to determine the percentage of seeds with extranumerary embryos. The appearance of seeds with more than one embryo was considered an evidence of polyembryonic population, and indicated the presence of apomixis (Mendes-Rodrigues *et al.* 2011).

The protocol used for the preparation of the foliar printing for visualization and measurement the stomata were described in Marinho *et al.* (2014a). Stomata slides were made for 3 to 5 individuals from each population and the height and width of 20 to 50 stomata were measured. A nested ANOVA comparison of measurements averages with GLM and confidence intervals of 95% were carried out using the SPSS 20.0 (IBM Corp. Released 2011) in order to know if the groups clearly differed from each other.

For this study, we included the morphometric data of the two populations (CAT and CRI) previously described for the number of chromosomes with ploidy level and stomata width and height in Marinho *et al.* (2014a). These populations were used as reference to estimate the level of ploidy of the populations analyzed in this study. In this previous work, all height and width values have

been reduced by 26% due to a scale error used to perform all measurements. This error does not imply any change in the discussion of the previous work, because all the measurements were submitted to the same scale error, but we had to adjust those measurements in order to compare to the new data obtained here.

Results

In the 19 populations studied, monoembryonic seeds were found in eight, polyembryonic seeds in another eight, and for three populations embryony pattern could not be directly verified due to the absence of fruits at the time of collection (NAT, GUR and SER) (Fig. 1 and Table 1). The geographical distribution of the monoembryonic populations comprised a large part of Minas Gerais state, more specifically the populations of the Central and Northern regions (5 populations). One monoembryonic population was located in the extreme east of Goiás state (CRI), the other two monoembryonic populations were located in the Tocantins state and belonged to *E. estevesiae* (other two populations at this area were not observed for polyembryony but also belonged to *E. estevesiae*).

Most of the polyembryonic populations were located in the Goiás state (6 populations), and only two were located in the southern of Minas Gerais state (Fig 1). Altogether, the percentage of polyembryony was high, ranging from 55.17%, in the population located near from Formiga (FOG) city in the South of Minas Gerais, to 99.58% in the population near from Luziânia (LUZ) city in Goiás (Table 1).

The morphometric data of stomata size showed a higher diversity and complexity than we previously predicted. We observed at first, an overlap between averages in width and height of the stomata in polyembryonic and some monoembryonic populations (Fig 2 A and B).

We then refined the analysis by grouping populations based on their location, embryony pattern, and stomatal measurements. Therefore, five different groups could be distinguished, most of them comprised monoembryonic populations (1-4 groups), while all the polyembryonic formed a homogeneous group (group 5). These groups were clearly and significantly

different considering both stomatal measurements (Fig. 2 C and D). The GLM test showed that all groups were different from each other for both measures, height (X^2 = 89368,075; P<0.001) and width (X^2 = 14345,050; P<0,001). The nested ANOVA for the measures of stomata height showed that most of the variation (76.36%) was explained by differences among groups, a very small part was due to differences among individuals (6,78%), and the remaining was due to other factors (16.85%). When we analyzed the width data, most of the variation was still explained by the differences among the five groups (51.17%), a small part was due to the individuals (12.88%), and in this case a larger part was due to other factors (35.95%).

The group 1 was composed by the four *E. estevesiae* populations located in Tocantins (Fig 1), which had the lowest stomata heights and widths (NAT, SAN, GUR and ARE) (Tab. 1). Based on the stomata size, when compared to the reference monoembryonic population (CRI), these populations presented stomata with 22.32% reduction in height and 7.45% of width.

The groups 2 and 3 included five monoembryonic populations that were located in the central and northern regions of the Minas Gerais state (Group 2 VMI, CAN and PIR; Group 3 VAL, FRS). They were not very distant but these groups were separated geographically by the São Francisco river. They presented stomata sizes bigger than those found in the also monoembryonic reference population of Cristalina-GO (CRI – Group 4). For these groups it was not possible to estimate the ploidy based only in stomata measures (Fig 2 C and D).

Group 5 included all polyembryonic and putatively hexaploid populations, and one population was not verified for the presence of polyembryony (Tab.1). When we compared the polyembryonic populations with the CAT population (reference for polyembryonic populations), there was no significant difference in stomatal size (mean height of the polyembryonic populations = 31.90 and mean height of the reference population 32.35) (Fig.2).

A scatter diagram of the measures of stomata width and height showed that individuals of group 2 and 3 were the largest among all populations (Highest monoembryonic – PIR = 35.01 ± 4.46), including polyembryonic ones (Highest polyembryonic – FOS = 33.87 ± 3.27). On the other extreme, the individuals of

E. estevesiae were the smallest and clearly separated group (Group 1) (Smallest population - NAT = 19.59 ± 2.50) (Fig.3).

Discussion

There was no predominance of polyembryonic or monoembryonic populations in the Stellate Trichome Complex. Nevertheless, it was possible to observe a geographical separation in the embryony patterns (Fig.1). Apparently the polyembryonic populations occurred mostly in southern and western areas while monoembryonic individuals occurred mostly in the Northern portion of the sampled region. The exception of this affirmation was the monoembryonic population of Cristalina (CRI), which despite presenting sexuality as a form of reproduction (Mendes-Rodrigues *et al.* 2017), was located near to the polyembryonic/apomitic populations. This pattern, to a certain extent, concurs with phytogeographic theories that Southmost areas of Cerrado are relatively recent, probably colonized after the last glacial maximum (LGM) (Lima *et al*. 2017; Novaes *et al*. 2013; Ramos *et al.* 2009; Ribeiro *et al.* 2016). This recent colonization may be analogous to the geographic parthenogenesis described to apomictic species and population in Europe, there apomictic individuals/populations colonized Northern areas of receding glaciers taking advantage of their uniparental reproduction ability (Horandl 2006).

In polyembryonic populations analyzed here, the percentage of polyembryonic seeds was as high as observed by Mendes-Rodrigues *et al.* (2005). This characteristic increases the chances of survival of progenies and is clearly linked to apomixis in *Eriotheca* (Mendes-Rodrigues 2010). Moreover, cytological studies, although limited to some of the populations so far, consistently showed that polyembryony and apomixis are associated with hexaploid cytotypes (Marinho *et al.* 2014b; Mendes-Rodrigues *et al.* 2017).

However, the morphometric studied carried out here failed to show a direct relationship between stomata size, cytology, and breeding features of *E. pubescens* due to the high variation found in height and width within the monoembryonic populations. Marinho *et al.* (2014a) observed a clear association between the stomata measures and the level of ploidy in populations with distint pattern of embryony, with the presence of a sexual system (monoembryonic) with tetraploid (2n=4x=184) individuals, and the

presence of apomixis (polyembryonic) with hexaploid (2n=6x=276) individuals in *E. pubescens*. But analyzing only stomata as an estimate of ploidy on a larger scale, we found more complex variations of stomata size in some monoembryonic populations.

Therefore, the five distinct groups we proposed in this study resulted in relatively homogeneous groups. Most variance was due to differences between groups (76.26% and 51.17%, respectively), and variance within groups was much lower (16.85% and 12.88%, respectively).

However, data was more consistent for the stomata height than for stomata width. When we evaluated the width data, we observed that there was a greater influence of other factors on the morphometric data (35.95%). Probably, one of these factors may be related to the rehydration process prior to the preparation of the foliar printing slides. The opening of the stomata could be influenced by factors related to absorption and loss of water (Kerbauy 2004), although rehydration has been controlled, may have affected the increase or reduction of guard cells causing greater interference in width than height measurements (Fig. 2)

Using all measures of stomata (height and width), spatial and reproductive data, the five groups can be clearly observed and distinct (Fig 1 and 2). Nevertheless, individuals from different groups overlapped (Fig. 3), and the increased in stomatal size previously predicted, was not always observed. Actually, the measurements of groups 1, 4 and 5 appear to be congruent with the initial idea, but the groups 2 and 3 presented stomata way out of the predicted sizes.

The group 1 appears more geographically distant (state of Tocantins) and also presented clearly smaller stomata. This group included plants of *E. estevesiae*, species phylogenetically close to *E. pubescens* (Carvalho-Sobrinho *et al.* 2015). One of the characteristics used in the taxonomic separation of these species were the stomata size (Carvalho-Sobrinho *et al.* 2015), but the reduction observed could be related to the lower level of ploidy. The stomatal size of these populations indicated that this population was diploid with a reduction in height size by more than 20% and 7% in width. This estimate was confirmed from counts of the chromosome number in some individuals of *E.*

estevesiae, which showed diploid species (2n = 2x = 92) (Chapter 3). Although the embryonic pattern has not been evaluated for two populations of *E. estevesiae* (NAT e GUR), the stomata measurements of all populations were close, suggesting the same level of ploidy and also the absence of a polyembryonic pattern that would be associated with larger sizes of stomata and consequently in the increase of ploidy level.

The group 4 was formed by only one monoembryonic and tetraploid population (CRI) (data obtained in Marinho *et al.* 2014a) that was used to estimate the ploidy of the monoembryonic groups. This was the only monoembryonic population present in the Goiás state and was the closest to the polyembryonic ones. One phylogeographic study evaluated these same populations and the propositions from geographic barrier analysis showed that this population was geographically and genetically close to a polyembryonic population (CAT) (Chapter 2). This demonstrates that these phylogeographic data are more related to geographic patterns and that these reproductive differences do not reflect the phylogeographic ancient history of these plants.

The polyembryonic populations form a homogeneous group, without wide variations in the measures of stomata height and width, composed of 8 populations (Group 5). High polyembryony rates observed for these *E. pubescens* populations have been related to the apomixis (Mendes-Rodrigues *et al.* 2005). Based on the comparison with the reference population (CAT), previously described for chromosome number and cytometry (2n = 6x = 276), we propose that populations in Group 5 are also hexaploid (Table 1, Fig 1and 2).

In a polyembryonic population (SER), the embryony pattern was not verified, but an absence of variation in the size of the stomata in comparisons with the other polyembryonic populations suggests that this population is also hexaploid and possibly polyembryonic and correctly included in Group 5. This group had populations that were distributed in an ample territory that comprises great part of the state of Goiás and South of the state of Minas Gerais.

Groups 2 and 3, located in the north and northeast of Minas Gerais, presented unexpectedly high stomata height and width. As they were formed by monoembryonic and sexual populations and this pattern tends to be associated

with lower levels of ploidy (Marinho *et al.* 2014b) we expected that the size of stomata would be closer to monoembryonic and tetraploid population (CRI - the reference population), or close to that of diploid populations within group 1. Instead, group 2 formed by three populations located between the city of Varjão de Minas and Pirapora, and group 3 formed by two populations located in the northeast of the same state, presented stomatal sizes close or even higher than the polyembryonic populations (Fig. 3). Between the two groups, there was the São Francisco River that could be considered as a possible geographical barrier. In spite of this, a phylogeographic study using cpDNA data, carried out with the same populations, showed no geographical barriers or haplotypes unique to these groups or between them (Chapter 2). Phylogeographic studies using cpDNA show an ancient phylogeographic history because they have a slower mutation rate than other markers (Bock 2007; Collevatti *et al.* 2003; Ramos *et al.* 2009), so probably the selective pressures that have acted in these groups causing these variations in stomatal size may be more recent and unrelated to changes in genome size (Husband *et al.* 2016). For these reasons it is not possible to estimate the ploidy only using the stomatal measurements in these groups, therefore, other methodologies such as flow cytometry and chromosome number counting will be necessary.

Apomixis is generally associated with polyploidy in Angiosperms (Hojsgaard *et al.* 2014; Mendes-Rodrigues *et al.* 2012; Vallejo-Marín *et al.* 2016). The combination of these two characteristics may have provided for these plants greater genetic and ecological flexibility, such as independence of pollinators and genetic diversity associated with polyploidy. These advantages made these plants good invaders of new habitats. Therefore, these individuals were expected to have a greater geographical distribution, when compared to diploids individuals which require sexual reproduction. This process was described as geographic parthenogenesis and seems to have been important for the nowadays distribution of many European apomictic plant complexes (Bierzychidek 1985; Horandl 2006; Horandl and Hojsgaard 2012).

Although breeding system is yet to be documented for *E. estevesia*, it is monoembryonic and has a geographic distribution restricted to the Tocantins state. The distribution of the remaining *E. pubescens* populations (15

populations) corroborates with the geographic parthenogenesis theory, with southern most populations being polyembryonic, apomitic and polyploid.

The apomitic are considered pioneer explorers of ecological and geographic niches and colonize areas that have gone through glaciations, allowing new hybridizations and polyploidizations (Bierzychidek 1985; Horandl 2006; Horandl and Hojsgaard 2012), and providing greater geographical distribution to apomictic species complexes. This phenomenon occurs due to several factors such as advantages coming through the polyploidy, better colonization skills due to uniparental reproduction, introgression of apomixis in sexual individuals, among others (Horandl 2006).

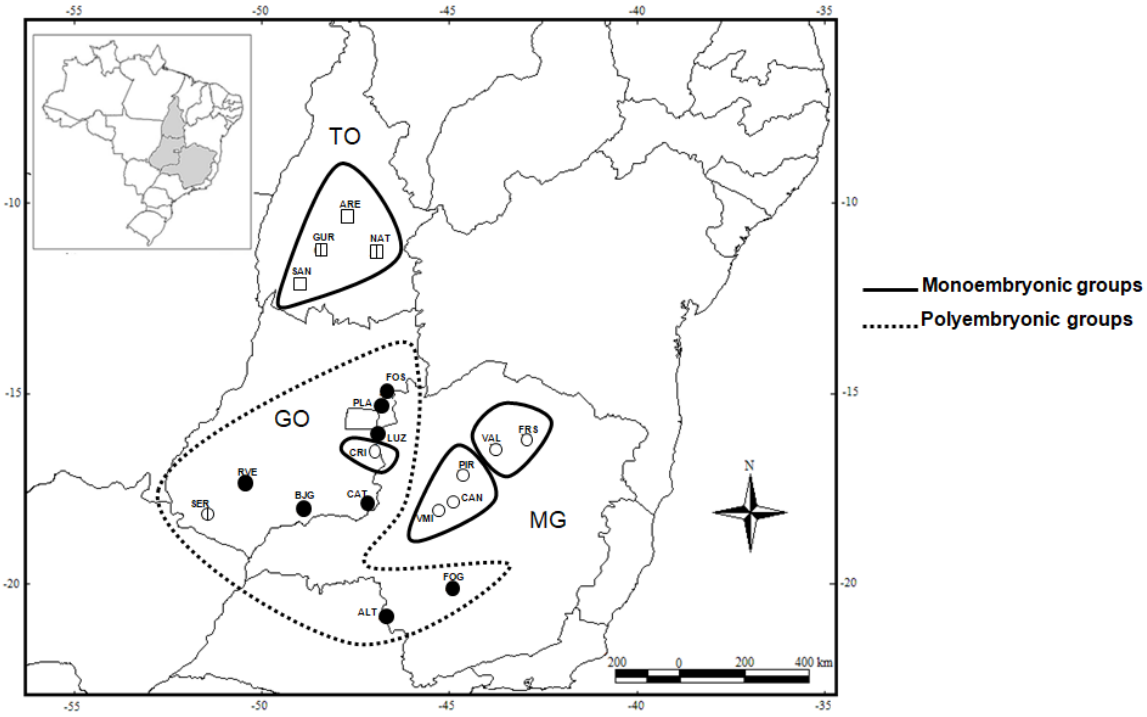

Figure 1 Distribution map of the 19 populations of Stellate Trichomes Complex. The black circles represent polyembryonic populations and white circles represent monoembryonic populations of *Eriotheca pubescens*. The white squares represent populations of *Eriotheca estevesiae*. The populations marked with white square or circle filled with a dash were not evaluated for the presence of polyembryonic seeds.

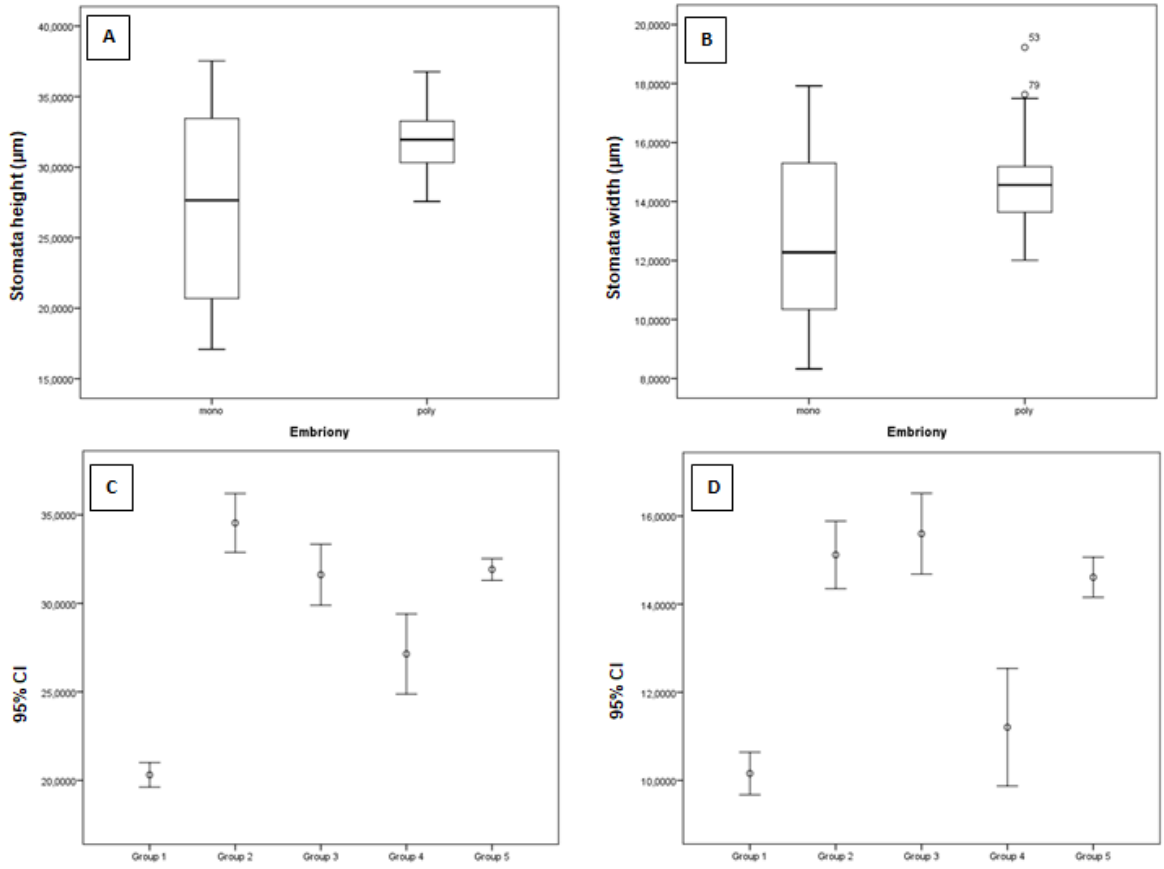

Figure 2 Stomata measurements for 19 populations of Stellate Trichomes Complex. Figure A and B showed the stomata height and width for Monoembryonic and Polyembrionic populations. C: Mean stomata height for groups 1-5 (76.36% of the variance was due groups and only 6.78% of the variance was due to individuals). D: Mean stomata width for groups 1-5 (51.17% of the variance was due groups and only 12.88% of the variance was due to individuals).

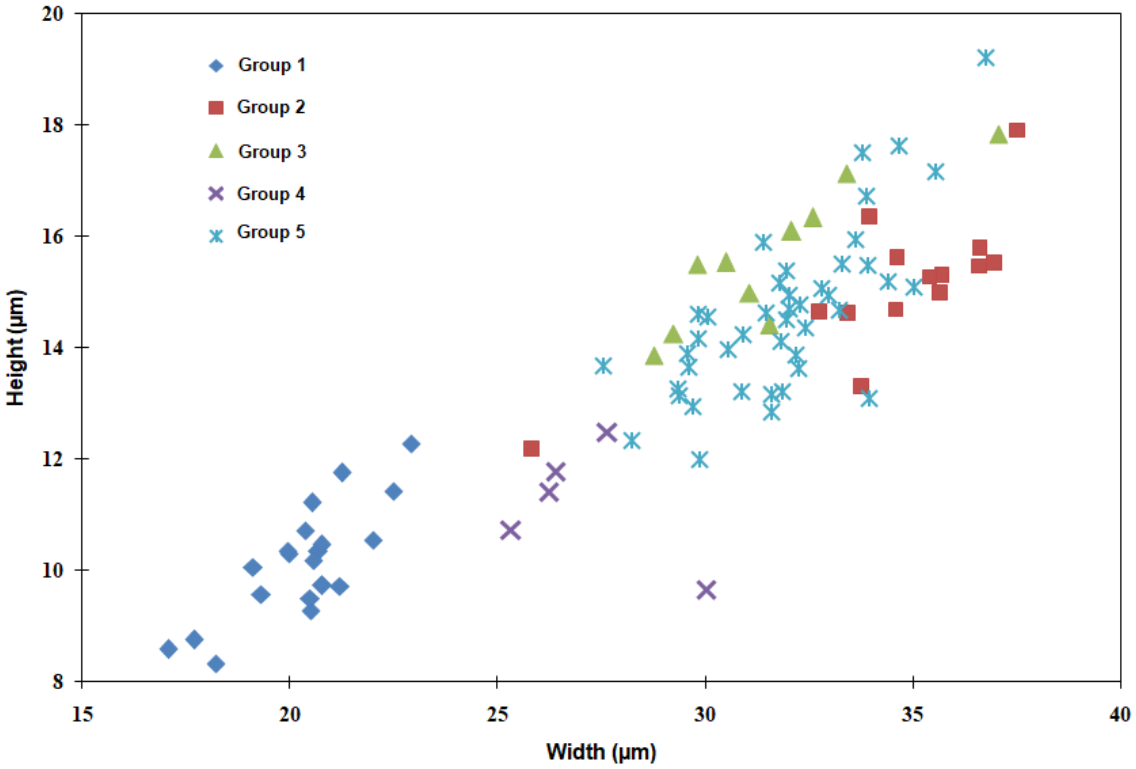

Figure 3 Scatter diagram of 92 individuals of 19 populations of Stellate Trichomes Complex. Five groups were used for separated the populations, the groups 1- 4 were formed by monoembryonic and group 5 by polyembryonic populations.

Table 1 Localities, states of population that belonging **stellate trichomes complex**, with the code of each population, geographical coordinates, embryonic pattern, percentage of polyembryony, number of seeds evaluated, averages and standard deviations of the values of the height and width of the stomata and estimated ploidy.

Species	Group	Locality/State	Population code	Latitude (S)	Longitude (W)	Pattern	Percentage of polyembryony	Number of seeds evaluated (number of individuals)	Mean values and standard deviation of stomatal height	Mean values and standard deviation of stomatal width	Estimated Ploidy
E.estecesiae	**1**	Natividade/TO	NAT	11°38'28.1"	47°40'31.8"	NE*	NE*	NE*	19,59 ± 2,50	9,09 ± 1,41	Diploid
		Sandolândia/TO	SAN	12°25'55.8"	49°47'47.8"	Mono	0	72 (2)	20,81 ± 2,08	10,34 ± 1,43	Diploid
		Gurupi/TO	GUR	11°32'07.4"	49°01'09.2"	NE*	NE*	NE*	20,57 ± 2,41	10,66 ± 1,61	Diploid
		Areias/TO	ARE	10°48'16.2"	48°21'87.9"	Mono	0	66 (2)	20,50 ± 2,83	10,63 ± 2,02	Diploid
E.pubescens	**2**	Pirapora/MG	PIR	17°24'33.7"	45°01'47.6"	Mono	0	418(4)	35,01 ± 4,46	15,72 ± 2,63	ND**
		Vista Alegre/MG	VAL	16°53'16.8"	44°10'51.5"	Mono	0	78(4)	34,86 ± 3,72	14,66 ± 2,33	ND**
	3	Francisco Sá/MG	FRS	16°27'57.0"	43°26'12.2"	Mono	0	10(4)	34,38 ± 3,88	14,95 ± 2,34	ND**
		Varjão de Minas/MG	VMI	18°18'45.2"	45°58'06.0"	Mono	0	38 (1)	32,10 ± 4,36	15,82 ± 2,80	ND**
		Canoeiro/MG	CAN	18°04'33.1"	45°29'56.5"	Mono	0	22 (1)	31,10 ± 3,72	15,33 ± 2,04	ND**
	4	Cristalina/GO	CRI	16°52'35.1"	47°40'42.8"	Mono	0	3833 (5)	26,43 ± 3,07	11,55 ± 3,39	Tetraploid
		Serranópolis/GO	SER	18°28'06.0"	52°05'18.8"	NE*	NE*	NE*	33,04 ± 4,69	16,38 ± 3,41	Hexploid
		Bom Jesus de Goiás/GO	BJG	18°14'41.3"	49°41'55.3"	Poli	93,55	31 (1)	32,32 ± 3,69	14,07 ± 2,73	Hexploid

E.pubescens	**5**	Rio verde/GO	RVE	17°48'50.7"	51°04'44.3"	Poli	100	167 (5)	31,87 ± 2,69	14,42 ± 1,95	Hexploid
		Altinópolis/SP	ALT	21°01'30.7"	47°13'26.1"	Poli	94,74	57(1)	32,64 ± 3,63	15,16 ± 2,73	Hexploid
		Formiga/MG	FOG	20°25'47.6"	45°24'49.2"	Poli	55,17	29(1)	30,63 ± 3,10	13,96 ± 1,99	Hexploid
		Formosa/GO	FOS	15°28'71.7"	47°06'07.6"	Poli	93,24	148 (3)	33,87 ± 3,27	15,72 ± 2,54	Hexploid
		Planaltina/GO	PLA	15°40'96.1"	47°30'33.8"	Poli	64,06	128 (2)	31,30 ± 3,02	13,64 ± 2,29	Hexploid
		Luziânia/GO	LUZ	16°16'29.6"	47°37'50.6"	Poli	99,58	240 (2)	29,75 ± 2,53	13,48 ± 2,16	Hexploid
		Catalão/GO	CAT	18°08'42.0"	47°54'23.5"	Poli	78,17	252(5)	32,39 ± 4,38	14,46 ± 2,73	Hexploid

***** NE - Presence and percentage of polyembryonic were not evaluated. ** ND - Ploidy was not defined considering the stomata size

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CAPÍTULO 2

Phylogeography of the Stellate Trichomes Complex (Bombacoideae – Malvaceae)

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Abstract

The whole genome duplication (WGD) is recognized as a major evolutionary force in plants and these events can cause phenotypic, physiological, reproductive, genomic, and ecological changes in polyploids. Apomixis is defined as the asexual formation of seeds and recent studies have shown that an association between polyploidy and apomixis is common in some groups of plants. Glaciations and climatic changes during the Pleistocene period seem to have influenced the emergence of this reproductive alternatives in Cerrado biome. In the present study, we aimed to investigate the genetic structure among populations of *Eriotheca pubescens* and *E. estevesiae* that formed the stellate trichome complex, relating with variations in reproductive system and cytology. Our study addresses the following specific questions: (1) Are the structure and possible migration routes of these populations similar to those already described for other Cerrado species? (2) Are the distinct patterns of embryony and ploidy already described for the stellate complex genetically different? (3) Are there evidences of genetic similarity among the *E. pubescens* and *E. estevesiae*? We used three regions of cpDNA in 14 populations collected in TO, GO and MG states. We found eight haplotypes, two were widely distributed in most populations (Hap 1 and Hap 2). Other unique haplotypes were found and were located on the periphery of the geographic distribution of the complex. The PLA and FOG populations had the presence of unique haplotypes and did not share any of the predominant haplotypes indicating a possible isolation of the populations for a long time. The spatial analyzes showed that some differences found here were similar with others studies in Cerrado plants, with geographic and genetic separation north-south and east-west. We also showed a geographic separation between the monoembryonic and polyembryonic pattern and a predominance of exclusive haplotypes in polyembryonic populations (SER, FOG and PLA). This scenario suggests that these reproductive variations associated with polyploidy may had arisen several times in this group of plants and helped the expansion to new areas after the last glacial period. We also found a genetic similarity between species which corroborates the initial idea that the populations of *E. pubescens*, the most widely distributed, may have originated from the restricted populations of *E. estevesiae* located in the north.

Key-words: *Eriotheca*, cpDNA, reproductive mosaics, polyploidy, Cerrado.

Introduction

The whole genome duplication (WGD) is recognized as a major evolutionary force in plants and has been discussed and studied for more than a century (Soltis *et al.* 2014). These events can cause phenotypic, physiological, reproductive, genomic, and ecological changes in polyploids when compared to their diploid ancestors (Balao *et al.* 2016; Husband *et al.* 2016; Soltis *et al.* 2014, 2016). For the establishment and evolution of newly formed polyploid, reproductive barriers are necessary to generate isolation between diploids and polyploids (Husband *et al.* 2016). In this context, a broad spectrum of breeding systems can be found in polyploid Angiosperms, including both sexual reproduction and apomixis. Apomixis is defined as the asexual formation of seeds (Richards 2003; Koltunow 2011; Hörandl 2010; Vallejo-Marín *et al.* 2016), and recent studies have shown that an association between polyploidy and apomixis is common in some groups of plants (Hojsgaard *et al.* 2014; Mendes-Rodrigues *et al.* 2012; Vallejo-Marín *et al.* 2016). The apomictic species are commonly polyploid (Carman 1997) and this phenomenon is considered to be responsible for the great success of the distribution of them (Horandl 2006; Horandl and Hojsgaard 2012). Therefore, it is evident that the structure and evolutionary potential of flowering plants populations are directly influenced by the presence of both polyploidy and apomixis (Richards 2003).

Glaciations and climatic changes during the Pleistocene period seem to have influenced the emergence of reproductive alternatives, especially in temperate environments (Carman 1997; Hörandl 2006). Apomixis is frequent in these environments where climatic fluctuations have enabled events of polyploidization and hybridization generating a rapid expansion of apomictic populations (Carman 1997; Hörandl 2006). The woody flora found in Cerrado Biome seems also to be influenced by these paleoclimatic changes, which may explain the distribution of genotypes, probably favoring the differential distribution of haplotypes as a function of their adaptation to the environment (Ramos *et al.* 2009; Ribeiro *et al.* 2016).

The Cerrado is the second largest biome in South America, classified as a worldwide hot spot due to its great biodiversity with more than four thousand species of plants not found anywhere else (Myers *et al.* 2000; Strassburg *et al.*

2017). Since the 70's it has been rapidly changing and fragmented due to large areas of plantation and pastureland, with a deforestation rate of 1% per year, much higher than that found in other Brazilian biomes (Ratter *et al.* 1997; Strassburg *et al.* 2017). Recent projections show that an unprecedent extinction event may occur in this biome if public conservation strategies are not adopted. With expectations of intense agricultural growth, it is estimated that around 30% of the remaining Cerrado areas will be destroyed by 2050 (Strassburg *et al.* 2017).

The savanna areas that occupy the central of Brazil have the highest and richest species area in the tropics. Phylogenetic studies consider the Cerrado plants clades relatively recent, mostly derived less than 10 million years ago (Simon and Pennington 2012). Some species of the genus *Eriotheca* Schott & Endl. (subfamily Bombacoideae - Malvaceae) are found in this bioma and are good objects of study because they accumulate variations in the reproductive and cytological systems. These events in *Eriotheca* appear to be also recent, less than 10 million years, as suggested by phylogenetic analyzes (Marinho *et al.* 2014; Simon and Pennington 2012).

In *E. pubescens* (Mart. & Zucc.) Schott & Endl, most populations are self-compatible, apomictic and hexaploid (Oliveira *et al.* 1992; Mendes-Rodrigues *et al.* 2005, 2010). Until now, few populations have been described as self-incompatible, monoembryonic and tetraploid (Marinho *et al.* 2014; Mendes-Rodrigues 2010). *E. pubescens* flowered from August to September and seeds are wind-dispersed at the end of the dry season (Oliveira *et al.* 1992). Another recently described species, *E. estevesiae*, previously recognized as *E. pubescens,* has a restricted distribution in Tocantins state, also in Cerrado areas (Carvalho-Sobrinho *et al.* 2015).

A phylogenetic analysis for this genus included a key of identification and described in detail the geographical distribution of several species, including *E. pubescens* (Duarte 2010). The morphological characteristic that separates *E. pubescens* from the other species studied was the presence of stellate trichomes, structure also found in *E. estevesiae*. In new phylogenies, these species appears as sisters (Carvalho-Sobrinho *et al.* 2015), therefore we will use the nomenclature Stellate Trichomes Complex to indicate the presence of these two species in the analyzes.

In the present study, we aimed to investigate the genetic structure patterns among populations of species forming the Stellate Trichome Complex, taking into account variations in reproductive system and cytology. Our study addresses the following specific questions: (1) Are the structure and possible migration routes of these populations similar to those already described for other Cerrado species? (2) Are the distinct patterns of embryony, breeding system, and ploidy already described for the stellate complex genetically different? (3) Are there evidences of genetic similarity among the *E. pubescens* and *E. estevesiae*?

Methods

Population sampling and evaluation of the reproductive system of Stellate Trichomes Complex

We collected leaves of *E. pubescens* and *E. estevesiae* in fourteen different locations covering most of their entire geographic distribution. We used the data from the embryonic pattern and ploidy level described previously (Chapter 1) for the populations used here.

For phylogeographic analyzes, we collected leaves from four to 9 individuals per population, totalling 97 individuals (Fig. 1; Table 1). Populations NAT and SAN occurred in Tocantins state and belonged to *E. estevesiae.* Populations PIR, VAL, FRS, VMI and CAN were collected along the central and northeast region of Minas Gerais state. CRI and PLA populations were collected at the east, and SER, BJG, RVE and CAT populations in the south of Goiás state. Finally, FOG population was collected in the southern region of Minas Gerais state (Fig. 1; Table 1). The elevation ranged from 330 to 1178m and some vouchers for all sampled populations were deposited in the Herbarium Uberlandense (HUFU - Universidade Federal de Uberlândia, HUFU 00015010; HUFU 00015011; HUFU 00015012; HUFU 00015070; HUFU 00015073; HUFU00015078; HUFU15079).

DNA extraction, amplification and sequencing

DNA was extracted from leaves stored in silica gel, using the DNAeasy Plant Mini Kit following the manufacturer's recommendations. Three intergenic cpDNA regions which presented polymorphisms and presented good amplifications in previous tests (*trn*S-*trn*G*, trn*L-E-*trn*L-F and *rpl32-trn*L) were used for amplifications. The polymerase chain reactions (PCR) were performed in a volume of 25 µl containing 20-25ng template DNA, 0.7 unit Taq polymerase (Sigma), 1x buffer with 1.5 mM MgCl2, 0.2 mM dNTP, 0.10 µM of each primer. The PCR products were verified by 1% agarose gel electrophoresis with 0.5x TBE buffer, stained in ethidium bromide solution and photodocumented for quantification. The PCR's were performed with a 1 min incubation at 96 °C, followed by 35 cycles of 45 seconds at 94 °C for denaturing, 1 min at 52°C (*rpl*32-*trn*L; Shaw *et al.* 2007) or 58°C (*trn*S-*trn*G - Hamilton 1999*, trn*L-E-*trn*L-F; Taberlet *et al.* 1991) for annealing and 1 min at 72 ° C for extension, and a final extension

at 72 °C for 7 min. PCR products were visualized on 1% agarose gels and purified with polyethylene glycol protocol (PEG 20%). The sequencing reactions were made by Macrogen Inc. (Seoul, South Korea - http://dna.macrogen.com) using the same primers used for the PCR.

Diversity, haplotype network and population structure

The consensus sequences were obtained using the package PHRED/PHRAP/CONSED (Ewing and Green 1998; Ewing *et al.* 1998; Gordon *et al.* 1998). An alignment matrix was constructed from consensus sequences using Clustal W implemented in MEGA 6 software (Tamura *et al.* 2013) and manually edited to minimize errors. Events with more than one base pair of insertion or deletion were treated as single mutation events. Variations in microsatellite sequences were removed from analyze keeping only bases with high quality.

The cpDNA haplotypes were established using sequences aligned with the software DNAsp 5.10 (Librado and Rozas 2009). The Nucleotide (μ) and haplotype (h) diversity indexes were calculated using Arlequin software (Excoffier *et al.* 2005). Phylogenetic relationships among haplotypes were inferred by the method Median joining (Bandelt *et al.* 1999) implemented in the Network 4.6 software (Forster *et al.* 2004).

The Spatial Analysis of Molecular Variance was performed by software SAMOVA (Dupanloup *et al.* 2002) to relate geographic and genetic distance. This method was used for estimate the number of distinct genetic groups (K) ranging from 1 to 6. These different groups were tested and the highest value of F_{ct} (differentiation among groups) was used to choose the best K. The geographic barriers that may had separated the Samova groups were estimated using Monmonier Maximum Difference Algorithm (Monmomier 1973), implemented in the BARRIER 2.2 program (Manni and Guérard 2004).

The genetic differentiation among the populations in different groups was assessed by the molecular variance analysis (AMOVA) using the Arlequin software (Excoffier *et al.* 2005). The groups considered in AMOVA were chosen according to the result in SAMOVA and also according to the presence and absence of polyembryony in the populations.

Results

Genetic diversity and phylogeographical structure

The alignment of plastid DNA regions *trn*S-*trn*G*, trn*L-E-*trn*L-F and *rpl32-trn*L produced a concatenated sequence of 1957 bp for Stellate Tricomes Complex. A total of 8 haplotypes were obtained, only three substitution and three indels were found when insertions and deletions were removed in microsatellite regions (Table S1).

The location, population code, geographic coordinates, pattern of embryony, sample size, haplotype diversity (*h*), nucleotide diversity (*μ*) and haplotype in each population are presented in Table 1. In general, low intrapopulation diversity was found, since only one population had three different haplotypes (PLA) and the other populations had either one or two haplotypes (Table 1 and Fig 1A). The haplotype and nucleotide diversity ranged from 0.0000 to 0.5714 and 0.0000 to 0.0004, respectively (Table 1).

In phylogenetic relationships only one mutational step separated all haplotypes based in Median-Joining method (Fig. 1B). In Stellate Tricome Complex two haplotypes were more frequent, the predominant Hap 1, and Hap 2 presented in almost all populations, except in FOG and PLA (Fig. 1B). Some haplotypes were unique in populations such as Hap 3 (SER), Hap 4, 5 and 6 (PLA), Hap 7 (FOG) and Hap 8 (NAT) (Table 1 and Fig.1B). One of these haplotypes (hap 8) was exclusive to *E. estevesia* (only in NAT) individuals, although the two populations (NAT and SAN) presented other haplotypes common to the *E. pubescens* populations.

In SAMOVA results, k=2 (F_{ct} = 0.70838) and k=5 (F_{ct} = 0.81825) presented the highest values of F_{ct} (Table S2). In k=2 the population FOG, located in the south of Minas Gerais state, was separated into one group alone and the others into another group. In k=5 the Group 1 had two populations CRI and CAT, group 2 only PLA, group 3 BJG, VMI, CAN, RVE, SER, PIR, FRS, VAL and SAN, group 4 only FOG and group 5 only NAT. The results obtained through Monmonier's algorithm showed that possible barriers can separate the previously predicted groups through SAMOVA. When 5 barriers were considered this analysis showed

three coincidence (1- CRI,CAT; 2 -PLA; 3 -FOG), while the others present some differences.

These two groupings (k=2 and k=5) were also used for the AMOVA and the results showed a high percentage of variation when considering the first grouping (70.84%) and even higher when second grouping (81.82%), thus, these results support the geographical groups proposed in SAMOVA (Table 2).

Associations of phylogeographical structure, pattern of embryony and ploidy

In the analysis of the embryonic pattern and level of ploidy, polyembryony was predominant in populations of southern Minas Gerais state and Goiás state. The population located in the northeast region of Goiás state (PLA) was an exception. All polyembryonic populations were considered hexaploid (Chaper 1) and contained most of the exclusive haplotypes (H3, H4, H5, H6 and H7). The only exclusive haplotype (H8) in monoembryonic was found in one population of Tocantins state (NAT), part of *E. estevesiae* species and considered diploid (2n=2x=92) (Chapter 1 and 3).

As for the other monoembryonic population, Cristalina (CRI) is tetraploid (2n=2x=184 Chapter. 1, Marinho *et al.* 2014), while for the populations located at central and northeast of Minas Gerais state it was not possible to obtain clear ploidy level. These monoembryonic populations did not have exclusive haplotypes, shared only the predominant haplotypes (H1 and H2), and showed low rates of genetic diversity (Tab.1).

This association data showed that no exclusive haplotype separates polyembryonic and monoembryonic populations (Fig. 1A). For *E. pubescens* populations, the AMOVA considering one group formed by polyembryonic populations and the second by monoembryonic, the highest percentage of variation occurred among populations within groups (81.71%) and there was no variation among groups (Tab.2).

Discussion

We analyzed the phylogeographic, reproductive and cytological patterns of two endemic species of Brazilian Cerrado. We found some congruence between the phylogeographic pattern here and some propositions in other Cerrado plants. Some distinct genetic structure feature the reproductive and cytological variations already described for Stellate Tricome Complex.

Eight haplotypes were found in all three plastid regions analyzed. Two haplotypes were widely distributed in most populations (Hap 1 and Hap 2) and one of them was also present in populations that correspond to the *E. estevesiae* species. This indicates that these species shared a phylogeographic history which corroborates recent phylogenetic analyzes (Carvalho-Sobrinho *et al.* 2015). However, they did not share the same geographical distribution and one of this population presented one exclusive haplotype (Hap 8). The *E. estevesiae* are located only in the northern region of Brazil (Tocantins state) and these populations were previously identified as *E. pubescens* due to morphological similarities (Duarte 2010).

Other unique haplotypes were found and were located on the periphery of the geographic distribution of the complex, and at the center of the distribution only the predominant haplotypes were found (Hap 1 e 2) (Fig.1A). The PLA population had the highest genetic diversity with the presence of three unique haplotypes. This and the FOG population were the only that did not share any of the predominant haplotypes. Probably these populations went through a long period of isolation from the others analyzed, similar to the proposal by Ribeiro *et al.* (2016) in another Cerrado species.

The SER population shared one predominant haplotype (Hap1) and one exclusive haplotype. In this same region another phylogeographic study carried out with a *Qualea* species, showed a population that presented high diversity and exclusive haplotypes. This same study showed that this region had suitable areas for refuge in Last Glacial Maximum which may explain this variation (Buzatti 2016).

The spatial analyzes showed two propositions of geographic barriers: a grouping formed by two geographic barriers, separating the FOG population from all the others and a second grouping that proposes five geographic barriers (1-CRI and CAT; 2-PLA; 3- BJG, VMI, CAN, RVE, SER, PIR, FRS, VAL and SAN; 4-

FOG; 5-NAT). The AMOVA analyzes showed the highest variation were 81.82% among the groups proposed by 5 barriers.

Although temporal analyzes were not shown in this paper, the spatial analysis mentioned above showed pattern found were common to some Cerrado plants that were also analyzed using plastid markers. In this study, we found a spatial separation between north and south populations proposed by Samova's analyzes (k = 2 and k = 5) and by the barriers. Several studies have shown that there was a recent colonization of the southern region in Cerrado from the northern populations (Collevatti *et al.* 2003; Ramos *et al.* 2007 and 2009; Novaes *et al.* 2010 and 2013). This suggests that northern populations representing here specimens belonging to *E. estevesiae* may have originated the more widely distributed populations of *E. pubescens* in the Southern Cerrado. This proposal can be sustained by evidences of polyploidy events that allowed changes in the reproductive system of these plants that may have favored the greater dispersion of the apomitic populations. These whole genome duplication events were less than 10 million years old in *E. pubescens* (Marinho *et al.* 2014), which corresponds to the Pleistocene period and the intense diversification that occurred in Cerrado Biome (Simon and Pennington 2012). These events of genome duplication and the emergence of reproductive alternatives that have been proposed here are often associated with speciation in flowering plants (Husband *et al.* 2016; Soltis *et al.* 2014).

An east-west spatial separation was also identified by the same analyzes described above, and also confirmed by the result of amova which showed a strong separation of groups proposed by samova in these species as well as in other Cerrado phylogeographic studies (Collevatti *et al.* 2003; Ramos *et al.* 2007, 2009; Resende *et al.* 2017). The eastern populations have lower diversity indexes, did not have exclusive haplotypes and shared only the predominant haplotypes previously described. In the western populations these predominant haplotypes were also shared and a exclusive haplotype was present in the SER population. In this same region another population (RVE) did not share this exclusive haplotype, probably this happened due to the lower number of individuals sampled.

A population that is located to the east of Goiás state (PLA) presented high genetic diversity in comparison to the others. In this population three exclusive

haplotypes and none of the two predominant haplotypes were found. This shows that this population can be isolated from the others for a long period (Buzatti 2016) and more attention should be given in future studies for a better understanding.

Reproductive and cytological pattern in phylogeographic structure

This is the only phylogeographic study for Cerrado plants which also evaluated ploidy and reproductive systems mosaics in populations, phenomena that appear to be common in plants of this biome (Allem 2004; Mendes-Rodrigues *et al.* 2005, 2010; Sampaio 2010).

The barrier analyzes showed a geographic separation between the monoembryonic and polyembryonic populations, with the exception of CAT (poly) and CRI (mono) populations. These populations are geographically close and shared the same haplotype (H2). Despite this clear separation, the AMOVA analysis showed that most of the genetic variation found was among the populations within the groups in *E. pubescens*. Probably this result reflects the exclusive haplotypes found in polyembryonic populations.

The polyembryonic populations SER, FOG and PLA presented these exclusive haplotypes. This scenario suggests that these reproductive variations represented by the presence of polyembryony (apomixis) associated with polyploidy may had arisen several times in this group of plants.

In spite of the smallest genetic diversity of monoembryonic populations in *E. pubescens*, with the absence of exclusive haplotypes, they present a considerable geographic extension, covering great part of the center and northeast of the state of Minas Gerais and one population in Goiás. There is evidence of polyploidy in at least one of them (CRI - tetraploid), so this genomic duplication event may have influenced the expansion of these populations. The opposite was found in the monoembryonic populations of *E. estevesiae*, the presence of a diploid genome and the absence of reproductive alternatives may have limited the expansion of these populations into new areas.

The life form, reproductive system, seed dispersal mode, and geographic distribution are also factors that directly influence genetic diversity (Hamrick and Godt 1989, 1996; Nybom and Bartish 2000). Contrary to what we found, polyembryony and apomixis could lead to a decline in variability due to uniparental reproduction and clone formation (Hörandl 2010; Richards 2003). Despite this,

mutation events, facultative sexuality, colonization by different clones and backcrosses with sexual diploid individuals can generate genetic variation in the apomictic polyploid species (Paun *et al.* 2006). This association may also promote the development of new functions and gene combinations, giving more ecological and physiological flexibility for these species. Thus, polyploidy is considered to be responsible for the great success of the distribution of some apomictic groups (Richards 2003). This phenomenon can act as a reproductive trampoline in new species and genera with broad hybridization, polyploidy, genetic and genomic rearrangements (Hojsgaard *et al.* 2014).

Therefore, the apomictic are considered explorers pioneers of ecological and geographic niches, with a tendency to occupy a greater geographical distribution than their diploid progenitors and to colonize areas that have passed through glaciations, allowing new hybridizations and polyploidizations, process denominated geographic parthenogenesis (Bierzychidek 1985; Horandl 2006; Horandl and Hojsgaard 2012). This phenomenon occurs due to several factors such as advantages coming through the polyploidy, better colonization skills due to uniparental reproduction, introgression of apomixis in sexual individuals among others. Therefore, it is possible to suppose that the increase of apomictic individuals in newly colonized areas prevented by competition the establishment of sexual individuals (Horandl 2006). Thus, apomixis can promote speciation by clonal replication of ecologically successful genotypes (Cushman *et al.* 2017).

In view of all these data, we believe that phylogeographic studies that address important ecological characteristics for plant evolution should be carried out in Cerrado species. This intensely threatened biome needs efficient conservation policies and this work demonstrates that the integration of different characteristics is complex but necessary to draw up new conservation strategies.

Table 1 Location, population code, altitude, pattern of embryony and ploidy level, number of individuals per population and genetic diversity indices of 14 populations of Stellate Trichomes Complex.

Locality/State	Population code	Latitude (S)	Longitude (W)	Altitude (m)	Pattern of embryony and ploidy level	Size sample	*h*	*π*	Haplotypes
1. Natividade/TO	NAT	11°38'28.1"	47°40'31.8"	330	NV*/diploid	8	0.5714	0.0002	Hap1; Hap8
2. Sandolândia/TO	SAN	12°25'55.8"	49°47'47.8"	296	Mono/diploid	8	0.000	0.0000	Hap1
3. Pirapora/MG	PIR	17°24'33.7"	45°01'47.6"	554	Mono/ND**	7	0.000	0.0000	Hap1
4. Vista Alegre/MG	VAL	16°53'16.8"	44°10'51.5"	956	Mono/ND**	8	0.000	0.0000	Hap1
5. Francisco Sá/MG	FRS	16°27'57.0"	43°26'12.2"	800	Mono/ND**	8	0.000	0.0000	Hap1
6. Varjão de Minas/MG	VMI	18°18'45.2"	45°58'06.0"	949	Mono/ND**	7	0.4762	0.0002	Hap1; Hap2
7. Canoeiro/MG	CAN	18°04'33.1"	45°29'56.5"	802	Mono/ND**	4	0.000	0.0000	Hap1
8. Cristalina/GO	CRI	16°52'35.1"	47°40'42.8"	1167	Mono/tetraploid	7	0.000	0.0000	Hap2
9. Serranópolis/GO	SER	18°28'06.0"	52°05'18.8"	742	Poli/hexaploid	9	0.3889	0.0001	Hap1; Hap3
10. Bom Jesus de Goiás/GO	BJG	18°14'41.3"	49°41'55.3"	686	Poli/hexaploid	7	0.2857	0.0001	Hap1; Hap2
11. Rio verde/GO	RVE	17°48'50.7"	51°04'44.3"	786	Poli/hexaploid	6	0.000	0.0000	Hap1
12. Formiga/MG	FOG	20°25'47.6"	45°24'49.2"	919	Poli/hexaploid	6	0.000	0.0000	Hap7
13. Planaltina/GO	PLA	15°40'96.1"	47°30'33.8"	1178	Poli/hexaploid	8	0.4643	0.0004	Hap4; Hap5; Hap6
14. Catalão/GO	CAT	18°08'42.0"	47°54'23.5"	-	Poli/hexaploid	4	0.0000	0.000	Hap2

* Not verified, ** Not defined by previous study

Table 2. Analysis of molecular variance (AMOVA) of cpDNA data in populatios of Stellate Tricomes Complex

Source of variation	**d.f.**	**Sum of squares**	**Variance components**	**Percentage of variation**
Among groups: **(SAMOVA k=2)**	1	9.500	0.73079 Va	70.84
Among populations within groups	12	17.326	0.19132 Vb	18.55
Within populations	83	9.091	0.10953 Vc	10.62
Among groups: **(SAMOVA k=5)**	4	25.871	0.49091 Va	81.82
Among populations within groups	9	0.956	-0.00049 Vb	-0.08
Within populations	83	9.091	0.10953 Vc	18.26
Among groups: **Embriony (Mono; Poli - for *E. pubescens*)**	1	1.157	-0.03033 Va	-7.66
Among populations within groups	10	22.665	0.32355 Vb	81.71
Within Populations	69	7.091	0.10277 Vc	25.95

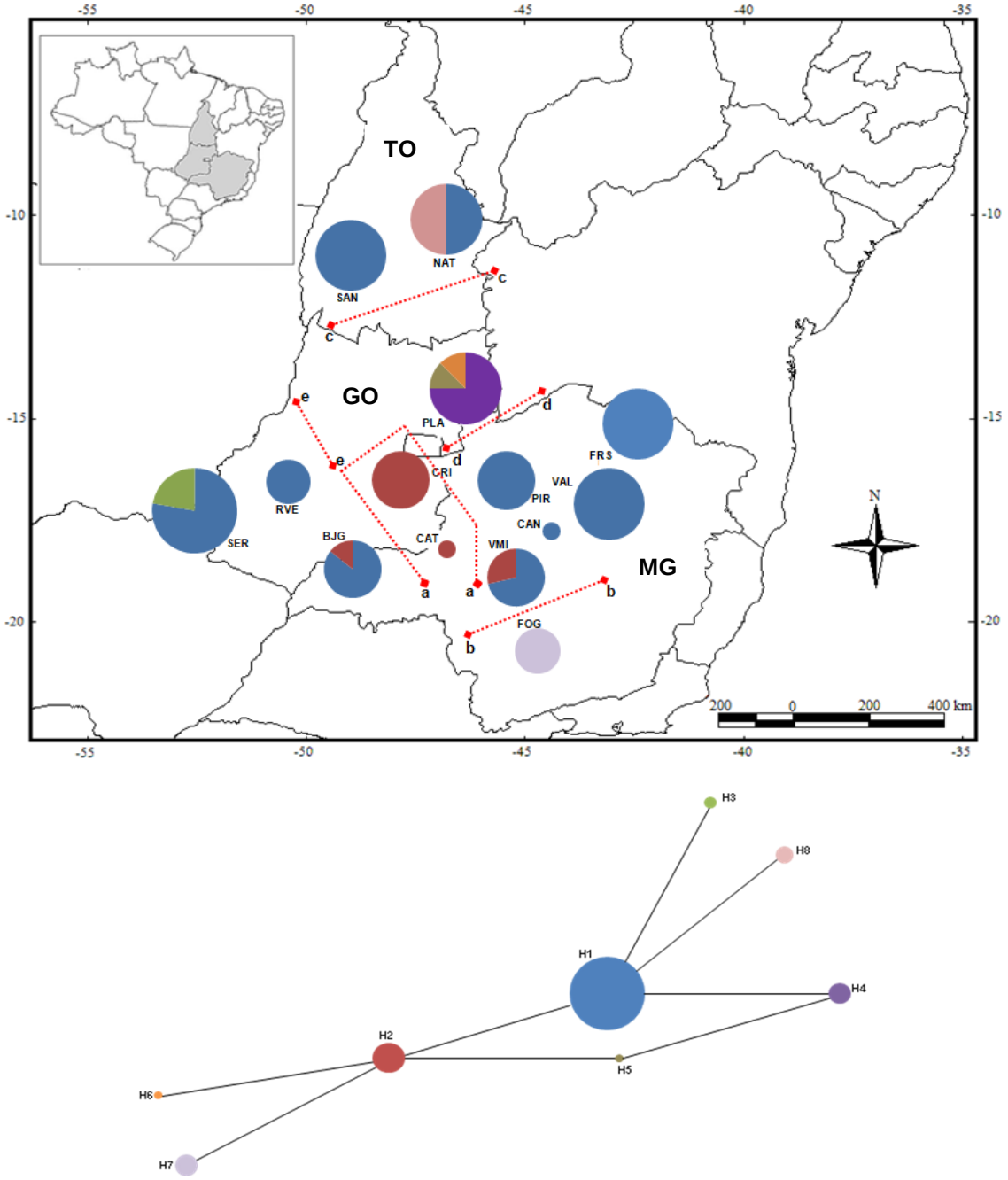

Figure 1 Geographical ditribution map of plastid DNA haplotypes for 14 populations of Stellate Trichomes Complex. A: The size of each circle is proportional to the number of individuals sampled, and the red lines show 5 barriers proposed by the Barrier program. B: The median-joining haplotype network, the length of links between the haplotypes is proportional to the number of mutational steps (Table S1).

Table S1. Mutations observed in the cpDNA regions trnS-trnG and rpl32-trnL for Stellate Trichomes Complex and distribution and frequency of the resulting haplotypes. In the trnl-E trnl-F region only SSR sites were polymorphic and therefore removed from the analysis.

	Polymorphic sites							Populations sampled											
	rpl32-trnL (767bp)		*trnS-trnG* (774bp)																
	1 1 8 6 7	2	1 7 1	1 9 1	6 8 6	7 2 9													
Haplotype	S	Id	S	Id	S	Id		BJG	VMI	CAN	RVE	SER	PIR	FRS	VAL	PLA	FOG	SAN	NAT	CRI	CAT	Total
H1	A	—	A	—	C	—		6	5	4	6	7	7	8	8			8	4			63
H2	.	—	.	—	A	—		1	2											7	4	14
H3	.	T	.	—	C	—						2										2
H4	.	T	.	A	.	—										6						6
H5	.	—	C	—	A	—										1						1
H6	.	—	A	—	.	—										1						1
H7	T	—	.	—	.	—											6					6
H8	.	—	.	—	C	C													4			4
							TOTAL	7	7	4	6	9	7	8	8	8	6	8	8	7	4	97

Id, indel; S, substitution. Length of indels are as follows, according to order of mutations: 6bp, 9bp.

Table S2 Statistic Fs generated by Spatial Molecular Variance Analysis (Samova) from chloroplast DNA data for k=2 until k=6 in Stellate Trichomes Complex.

k	F_{sc}	F_{st}	F_{ct}	**Grupos**
2	0.63592	0.89383	0.70838	1. FOG 2. BJG, VMI, CAN, RVE, SER, PIR, FRS, VAL, PLA, SAN, NAT, CRI, CAT
3	0.62734	0.83113	0.54686	1. BJG, VMI, CAN, RVE, SER, PIR, FRS, VAL, PLA, SAN, CRI, CAT 2. FOG 3.NAT
4	0.42835	0.82572	0.69514	1.BJG,VMI,CAN,RVE,SER,PIR,FRS,VAL,PLA,SAN 2. FOG 3. CRI,CAT 4. NAT
5	-0.00449	0.81743	0.81825	1. CRI,CAT 2.PLA 3.BJG, VMI,CAN,RVE,SER,PIR,FRS,VAL,SAN 4.FOG 5.NAT
6	-0.05944	0.79005	0.80183	1. BJG,VMI,CAN,RVE,PIR,FRS,VAL,SAN 2.CRI,CAT 3.FOG 4.PLA 5.SER 6.NAT

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CAPÍTULO 3

Evolution of genome size, reproductive system of the Malvatheca clade (Malvaceae)

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Abstract

The evolution and diversification of Angiosperms seem to have involved drastic changes in genome size. The complete genome duplication (WGD) seems to have marked crucial stages in the development of flowering plants and defined new lineages and evolutionary possibilities. WGD events associated with irradiation of the angiosperms seem to have originated important groups within the Malvaceae family, such as Bombacoideae and Malvoideae that form Malvatheca clade, strongly supported by molecular data.The study aims to evaluate the influence of genome size variation on evolution and phylogenetic relationships of the taxa belonging to the Malvatheca clade, with emphasis in species of the Cerrados of Central Brazil. We used published data in the Plant DNA C-values Database and other more recent articles for compilate cytometry data for broaden the knowledge and discussion about the evolution of this characteristic in this group. We estimated the genome size of some species belonging to the Bombacoideae subfamily using standard protocol previously described with some modifications to increase the purity of the solution, and counted the number of chromosomes of *Eriotheca estevesiae* in some metaphases. We found a higher chromosome number and genome size in Bombacoideae species and the correlation of this data was strong only in *Eriotheca* species. When we compared the genome size of the subfamilies the higher mean was in Bombacoideae and the higher variation was in Malvoideae. In the two subfamilies the genome size increased and decreased and were more conserved in smaller taxa like in *Eriotheca*. When the haploid genome of this genus was compared with *Pachira* (Pachira clade), we observed significant differences between the size of the genome showing a possible different origin between them. Furthermore, the chromosome number counts performed on *E. estevesiae* (2n = 92) helped us to propose a complete polyploid series for *E. pubescens* considering that both form a Stellate Trichome Complex. We conclude that the genome size and chromosome number data, although presenting a great diversity, appear to be important in defining and understanding the evolution of subfamilies, genera and the emergence of neopolyploid *Eriotheca* discussed in this paper.

Key words: Polyploidy, Malvoideae, Bombacoideae, *Pachira* clade, *E. estevesiae*.

Introduction

The evolution and diversification of Angiosperms seem to have involved drastic changes in genome size (Bennett and Leitch 2005) associated or not with hybridization processes. The complete genome duplication (WGD) seems to have marked crucial stages in the development of flowering plants and defined new lineages and evolutionary possibilities (Otto and Whitton 2000; Ramsey and Schemske 1998; Soltis and Soltis 2000, 2014).

In Angiosperms, about 70% of the species are polyploid or originated from polyploidy (Masterson 1994). For the establishment and evolution of newly formed polyploids, reproductive barriers are required that cause isolation between diploids and polyploids over time (Husband *et al.* 2016), usually resulting in speciation and increasing biodiversity (Soltis *et al.* 2014). The establishment of a new polyploid is crucial to its success, and some reproductive traits may contribute to the persistence of these organisms, such as propensity for apomixis or self-compatibility (Otto and Whitton 2000; Soltis *et al.* 2014).

WGD events associated with irradiation of the Angiosperms at the end of the Cretaceous seem to have originated important groups within the Malvaceae family (Marinho *et al.* 2014). This family has 9 subfamilies including Bombacoideae and Malvoideae that form the Malvatheca clade (Alverson *et al.* 1999; Baum *et al.* 1998, 2004; Bayer *et al.* 1999). This clade is strongly supported by molecular data and is formed by the old families Malvaceae and Bombacaceae, currently considered subfamilies (Baum *et al.* 1998, 2004; Duarte 2010). Recently, a study with phylogenetic analyzes verified that the evolution of morphological characters related to seeds and fruits also confirm the monophyly of the core Bombacoideae (Carvalho-Sobrinho *et al.* 2016).

The subfamily Bombacoideae presents about 17 genera and 160 species and is represented mainly by Neotropical, polyploid tree species with small and numerous chromosomes (Baum and Oginuma 1994; Carvalho-Sobrinho *et al.* 2016; Gibbs *et al.* 1988; Marinho *et al.* 2014; Morawetz 1986). However, phylogenetic studies did not take into account the marked chromosome variation, with a strong phylogenetic signal, probably due to duplication events of the genome, and which seems to characterize the Malvaceae family sensu lato (Baum and Oginuma 1994; Marinho *et al.* 2014).

An example of this apparent disinterest with cytogenetic and genomic changes is the discussion about the phylogenetic relationships of the genera that make up Bombacoideae (Carvalho-Sobrinho *et al.* 2016; Duarte *et al.* 2011). The monophyletic clade *Pachira* Aubl. was proposed using molecular data by the authors mentioned above and it is formed mainly by species belonging to the genus *Pachira* and *Eriotheca* Schott & Endl.. The studies disagree on the monophyly of the last genus but never discussed the diferences in chromosome number which have already been reported.

Eriotheca is a small neotropical genus that has about 24 species distributed in South America and about 18 species in Brazil (Flora do Brasil 2020 em construção), and studies have shown great chromosome variation and genomics, associated to alternatives of reproductive systems and occurrence of apomixis (Marinho *et al.* 2014; Mendes-Rodrigues 2010; Oliveira *et al.* 1992). Adventitious embryony, the apomixis type present in *Eriotheca* species, is the most common form of apomixis in Angiosperms (Hojsgaard *et al.* 2014), and it is frequent in environments where climatic fluctuations have enabled events of polyploidization and hybridization, generating a rapid expansion of populations (Carman 1997; Hörandl 2006). However, the influence of these processes on the diversity of the plants of the region and the possible impacts of the environmental degradation on the ecology, distribution, and conservation of this diversity is still under discussion.

Most of *Eriotheca* species occur in tropical forests, but some are found in Cerrado areas (Duarte *et al.* 2011; Simon and Pennington 2012). In addition to species widely distributed in the region, some taxonomic novelties have been described (Carvalho-Sobrinho *et al.* 2015; Duarte and Esteves 2011), suggesting a greater diversity than previously considered.

The Cerrado is the second largest biome in South America, classified as a worldwide conservation hot spot due to its great biodiversity (Myers *et al.* 2000; Simon and Pennington 2012). The woody flora, found in the Cerrado, seems to be influenced by paleoclimatic changes (Ramos *et al.* 2007, 2009) and probably triggered the emergence of apomixis as a reproductive alternative associates with polyploidy (Mendes-Rodrigues *et al.* 2017). Glaciations during the Pleistocene

period also influenced the emergence of these reproductive alternatives elsewhere, especially in temperate environments (Carman 1997; Hörandl 2006).

The study aims to evaluate the influence of genome size variation on evolution and phylogenetic relationships of the taxa belonging to the Malvatheca clade, with emphasis in species found in Cerrado of Brazil Central. In specific, the objectives is to compile genome size data (2C values) for Malvatheca clade and contribute with new genome size data for some species of Bombacoideae for help with the elucidation of phylogenetic conflits still presented. We also sought to determine the number of chromosomes of *Eriotheca estevesiae*, a recently described species, and compare the level of ploidy with the basic number proposed for the Bombacoideae subfamily.

Methods

We used both chromosome number and flow cytrometry data to evaluate changes in genome size for some species belonging to the Malvatheca clade (Malvaceae). A compilation of cytometry data was made to broaden the knowledge and discussion about the evolution of this characteristic in the Malvatheca clade and we used published data in the Plant DNA C-values Database and other more recent articles. We estimated the genome size of some species belonging to the subfamily Bombacoideae and further checked the level of ploidy of selected species considering the basic number of chromosomes n=44, suggested by Costa *et al.* (2017). We also checked the ploidy level of some critical populations that belong to the Stellate Trichomes Complex, and defined the number of chromosomes of *E. estevesiae*, following protocols described in Marinho *et al.* (2014) and detailed below whenever necessary.

Cytometry analyzes

Cytometric analyzes may be used as an indirect estimate of ploidy or for determining genome size in species yet unknown to *Eriotheca* and other genera of Bombacoideae. The flow cytometry study involved the species *Eriotheca candolleana*, *Eriotheca estevesiae*, *Eriotheca parvifolia*, *Eriotheca* sp., *Pachira aquatica*, *Pachira glabra*, *Pachira* sp., *Pseudobombax longiflorum*, *Pseudobombax minimum* and *Pseudobombax tomentosum*. Specimens of *Eriotheca* sp. and *Pachira* sp., were sent for identification with a specialist and it was suggested that they may be species not yet described.

Fresh young leaves of each species were collected most of them in the Cerrado areas near the city of Uberlândia-MG. Seeds of some species such as *Eriotheca estevesiae*, *Eriotheca parvifolia, Eriotheca* sp. *Pseudobombax minimum*, had already been collected in expeditions to Tocantins, Bahia and northern Minas Gerais, respectively. The collection sites had the coordinates established using GPS and voucher materials were collected, herborized with usual techniques, and deposited in the herbarium of the Federal University of Uberlândia (HUFU).

Seeds were placed in germination boxes (Gerbox®) with filter paper moistened with water. Previous studies have shown that it is difficult to use expanded leaves for flow cytometry analysis of DNA content, so soon after the

germination of the seeds, they were transplanted to greenhouse until seedling stage and young foliar tissue were readily used for the flow cytometry analyses.

Freshly collected leaves, both from field adults and seedlings, were used for the analyses following usual methodology (Dolezel *et al.* 1989; Dolezel and Bartos 2005). Estimates of genome size were performed with at least three replicates for each species, on the same equipment (Table 1). The fresh leaf tissue were prepared following a protocol adapted from Dolezel *et al.* (1989), aiming at obtaining a cleaner nuclear suspension (Marinho *et al.* 2014). About 25-50 mg of leaf tissue were fragmented using a razor blade on a petri dish containing 1 ml of LB01 buffer, on ice. When required, about 0.5 to 1 ml of buffer was added to improve the flowability of the suspension, then, it was filtered on CellTric filters (Partec GMbH, Munster, Germany) with 30mm membranes. The product should be extremely clean and was filtered as many times as necessary. After filtering, 50 µL of propidium iodide (50 µg / mL) and 50 µL RNase (50 µg / mL) was added. Until the moment of the analysis in the equipment, the samples were kept on ice and protected from light and as soon as possible the analyzes were performed on a BD FACS Canto II flow cytometer. The species *Pisum sativum* (2C = 9.09 pg), *Zea mays* (2C = 5.43 pg) or *Vicia faba* (2C = 26.90pg) (Dolezel *et al.* 1998) were used as the standards for estimating the amount of DNA. The absolute DNA amount of each species was calculated through the various measurements that were made. The 2C and 1Cx size of DNA (*sensu* Greilhuber *et al.* 2005) and the coefficient of variation were compared. The 2C values were the total genome size measured and 1Cx was considered haploid genome size (2C value divided by the ploidy level).

Results

In the compilation of genome size and chromosome number for species of the Malvatheca clade, we found 88 species with proper estimates and chromosome numbers. Only 28 species were included in the Bombacoideae subfamily and most of the species were part of the Malvoideae subfamily. The majority of Malvoideae data was for the genus *Gossipium* (41 species), and in Bombacoideae was for the genus *Eriotheca*, including the cytotypes found originally here (8 species) (Tab S1). The genome size and chromosome number data plotted showed a moderate correlation for the Malvatheca clade (R^2 = 0.3897). For the Malvoideae, the correlation was very weak (R^2 = 0.0551) and the opposite was found in Bombacoideae, where this correlation was strong (R^2 = 0,8191). When these data were plotted only for *Eriotheca* species the correlation was even stronger (R^2 = 0,9912) (Fig.1). The mean genome sizes of Bombacoideae (mean 2C value = 3.58pg) and Malvoideae (mean 2C value = 3.33pg) were significantly different and we noted that the 2C value is higher in Bombacoideae, and we found a greater amplitude in Malvoideae data (Fig 2).

Most of the species that belong to the Malvoideae subfamily have already been documented to the level of ploidy. In some species of the Bombacoideae this characteristic had not been described, so we use the basic number for determine and majority was diploid with some exceptions (*Adansonia digitata* 2n=4x*, Eriotheca gracilipes* 2n=6x*, Eriotheca macrophylla* 2n=6x, *Eriotheca obcordata* 2n=4x*, Eriotheca pubescens* 2n=6x) (Tab S.1).

In the new genome size data obtained in this study, the lowest genome size found was in *Pseudobombax longiflorum* 2C = 2.70pg (3.21%) and the highest in an *Eriotheca* sp. 2C = 10.75pg (3.62%) (Tab.1). This high value of genome size found in *Eriotheca* sp. was close to that found in hexaploid specimens of *Eriotheca pubescens* (Tab. S1).

For Bombacoideae studied here, in *Eriotheca* species the basic genome size varied from 1Cx = 1.66pg in *E. estevesia* to 1Cx = 1.83pg in *E. candolleana*. For the genus *Pachira* the genome size ranged from 1Cx = 1.81pg in *P. glabra* to 1 Cx = 2.64pg in *Pachira sp.,* and in *Pseudobombax* the smallest genome size was found in *P. longiflorum* 1Cx = 1.35pg and the highest in *P. tomentosum* 1Cx = 1.59pg (Tab.1). Using all compiled data, the genera *Eriotheca* and *Pachira*, which

form a putatively clade, presented a significantly different haploid genome size (1Cx) (Fig. 3). The 1Cx values for *Pachira* were much larger than those for *Eriotheca,* without overlap between the average values of each genus.

Few cells in the metaphase stage were found in the slides of radicles of *E. estevesia*. Only five metaphases contained condensed and scattered chromosomes good enough for counting. The number of chromosomes varied in some metaphases, but the most common was 2n = 92, and the level of ploidy was estimated at 2n = 2x according to the basic number of chromosomes proposed for the subfamily (Tab. S1). For this species, the genome size estimated was 2C=3.33pg (4.64%), close to that found for the other diploid species of *Eriotheca*.

Discussion

The compilation of genome size and chromosome number data showed that the Malvoideae subfamily has a larger number of species with descriptions of these informations than the Bombacoideae subfamily. This greater interest is due to the high number of species with economic interest linked to the cultivation of cotton (*Gossypium*). The knowledge of the genome size of these species is important for sequencing of the complete genome now facilitated with the use of next-generation sequencing (Collevatti and Dornellas 2016). This fact emphasizes the need of more studies that describe this information in more species that belong to the subfamily Bombacoideae, since they are also important data to study the evolution of these species (Costa *et al.* 2017; Marinho *et al.* 2014).

In general, it was possible to observe a weak correlation between the chromosome number and genome size (2C) as found in other studies (Collevatti and Dornellas 2016; Costa *et al.* 2017) and in agreement with Soltis *et al.* (2003) which emphasizes that the genome size may vary regardless of chromosome number. In Malvatheca subfamilies polyploidy events marked the development of the taxa studied here as well as in most angiosperms. Polyploidy and the presence of transposable elements are some of the phenomena responsible for increasing the diversity of genome size in plants (Bennet and Leitch 2005; Soltis *et al*. 2003). Some studies have shown that events of increase and decrease of genome size occurred several times in plants (Bennet and Leitch 2005; Collevatti and Dornellas 2016; Silva *et al*. 2017;Soltis *et al.* 2003), as can also be visualized inside of each subfamily here.

The ancestor of angiosperms had a small genome size and over time with the events cited previously and DNA self-replication, there was a increased in a particular way in each group of plants (Bennet and Leitch 2005; Soltis *et al*. 2003). In *Theobroma* ssp. for example, the small genome (1C = 0.46pg) found is similar to that of other species of the Byttnerioideae, showing that this basal subfamily present in Malvaceae s.l. has a small genome and the increase of 2C-value occurred in more specialized subfamilies such as Malvoideae and Bombacoideae (Silva *et al.* 2017).

Events of whole genome duplication are probably involved in the evolution of these groups, a first one can be proposed in the separation between this

subfamilies that form the clade Malvatheca and events may be linked more specifically and recently to the species of *Eriotheca* which present more than level of ploidy (Marinho *et al.* 2014). The correlation between genome size and chromosome number was extremely strong when we observed *Eriotheca* data due to the presence of two incomplete polyploid series, one in *E. gracilipes* (2x and 6x) and another in *E. pubescens* (4x and 6x). These data also explain the strong correlation found when we analyzed Bombacoideae, since when we remove the data for *Eriotheca*, the correlation becomes also weak for the Bombacoideae (data not shown).

In Bombacoideae subfamily, the monophyletic *Pachira* clade s.l., is frequently discussed in studies of phylogenetic and chromosomal evolution (Carvalho-Sobrinho *et al.* 2016; Costa *et al.* 2017; Marinho *et al.* 2014). Our results showed that the genome size of these two genera was significantly different. Therefore, we believe that a greater effort to sample the genome size and chromosome number may help us to better establish the evolutionary relationships and supporting the hypothesis of distinct origins of these two genera.

The species *E. estevesia* was recently described by Carvalho-Sobrinho *et al.* (2015) and up to now observed only in the Tocantins state. These specimens, previously treated as *E. pubescens* were studied here for the first time with chromosomes number and genome size. Although few metaphases were found so far, it was possible to suggest that it is a diploid species with 2n = 2x = 92 chromosomes. Marinho *et al.* (2014) proposed an incomplete polyploid series for *E. pubescens* with tetraploid specimens (1Cx = 1.73pg and 2C = 6.91pg) and hexaploids (1Cx = 1.70pg and 2C = 10.23pg). The data of chromosome number and genome size of *E. estevesiae* (1Cx = 1.66pg and 2C = 3.33pg) complement this series, so it is possible to suggest that *E. estevesiae* diploid populations may have originated populations of *E. pubescens*.

The phylogeographic study carried out with populations of both species, included in the *Eriotheca* stellate trichome complex (Chapter 2), indicates some similarities in cpDNA haplotypes. The polyploidy, and possibly apomixis (in hexaploid), may have favored the expansion of tetraploid and hexaploid *E. pubescens* population to other regions of the country and populations of

E. estevesiae (sexual and diploids) have so far been restricted in the north of the country probably due to the absence of these phenomena.

The new WGD event (or events) which resulted in the neopolyploid populations of *Eriotheca* may be characteristic of these neotropical species and associated to recent paleoclimatic changes and expansion of the Cerrado Biome (see also Chapter 2). Changes in chromosome number and breeding systems are also characteristic of some european groups and may have triggered the geographic partenogenesis events which helped to colonize Northern areas after glacial cycles during the Pleistocene (Horandl 2006). Neither polyploidy nor apomixis alone seem to be able to trigger extended range and geographic parthenogenesis, but paleoclimatic changes may have created new opportunities for the establishment of neopolyploid and apomictic *Eriotheca* in Central Brazil, possibly from diploid and sexual populations of the Stellate Trichome Complex.

Similar expansion associated with polyploidy was proposed to *Adansonia* trees in the African continent (Pettigrew *et al.* 2012) although more recent studies failed to find genome size differences between the putative diploid *A. kilima* and the widespread tetraploid *A. digitata* (Crom *et al.* 2016). Two genome size results were described for *A. digitata*: 2C = 3.34pg (160 chromosomes) and 7.70pg (Costa *et al.* 2017 and Ohri 1996 in Plant DNA C-values Database, respectively). In our data we did not find any species with high chromosome number (2n=160) and small genome size (2C=3.34pg), close to that of diploid plants of other genera in Bombacoideae. Therefore, we suggest greater attention and caution in the treatment and discussion of these data.

We conclude that the genome size and chromosome number data, although presenting a great diversity, appear to be important in defining and understanding the evolution of subfamilies, genera and the emergence of neopolyploid *Eriotheca* discussed in this paper. Marinho *et al.* (2014) showed that the chromosome number has a strong phylogenetic signal for the family Malvaceae s.l. and Costa *et al.* (2017) showed a weaker phylogenetic signal for genome size and chromosome number in Bombacoideae. This last result probably is due to variations of increase and decrease of the genome previously discussed and that emphasize the necessity of more studies with new data for a better understanding of these phenomena in the evolution of this groups. The higher

chromosome counts and genome size estimated by flow cytometry suggests that the ancient WGD that possibly originated the Bombacoideae (Marinho *et al.* 2014) was somewhat conserved in most trees of this group.

Table 1. Genome size 1Cx, 2C, coefficient of variation, ploidy level and number of replicates for some Bombacoideae species.

Species	Voucher	1Cx (pg)	2C (pg)	CV (%)	Ploidy	N replicate
Eriotheca candolleana	HUFU00015091	1.83	3.66	2.60	2	5
Eriotheca sp.	HUFU00015077	1.79	10.75	3.62	6	5
Eriotheca estevesiae	HUFU00015079	1.66	3.33	4.64	2	8
Eriotheca parvifolia	HUFU00015028	1.80	3.60	4.43	2	7
Pachira glabra	HUFU00015396	1.81	3.63	3.77	2	6
Pachira aquática	HUFU00015391	2.39	4.79	3.70	2	7
Pachira sp.	HUFU00015399	2.64	5.29	3.18	2	5
Pseudobombax longiflorum	HUFU00015385	1.35	2.70	3.21	2	4
Pseudobombax minimum	HUFU00015387	1.47	2.95	4.59	2	7
Pseudobombax tomentosum	-	1.59	3.18	3.38	2	5

1Cx = 2C pg/ ploidy. The 2C and CV % was obtained by means of replicates

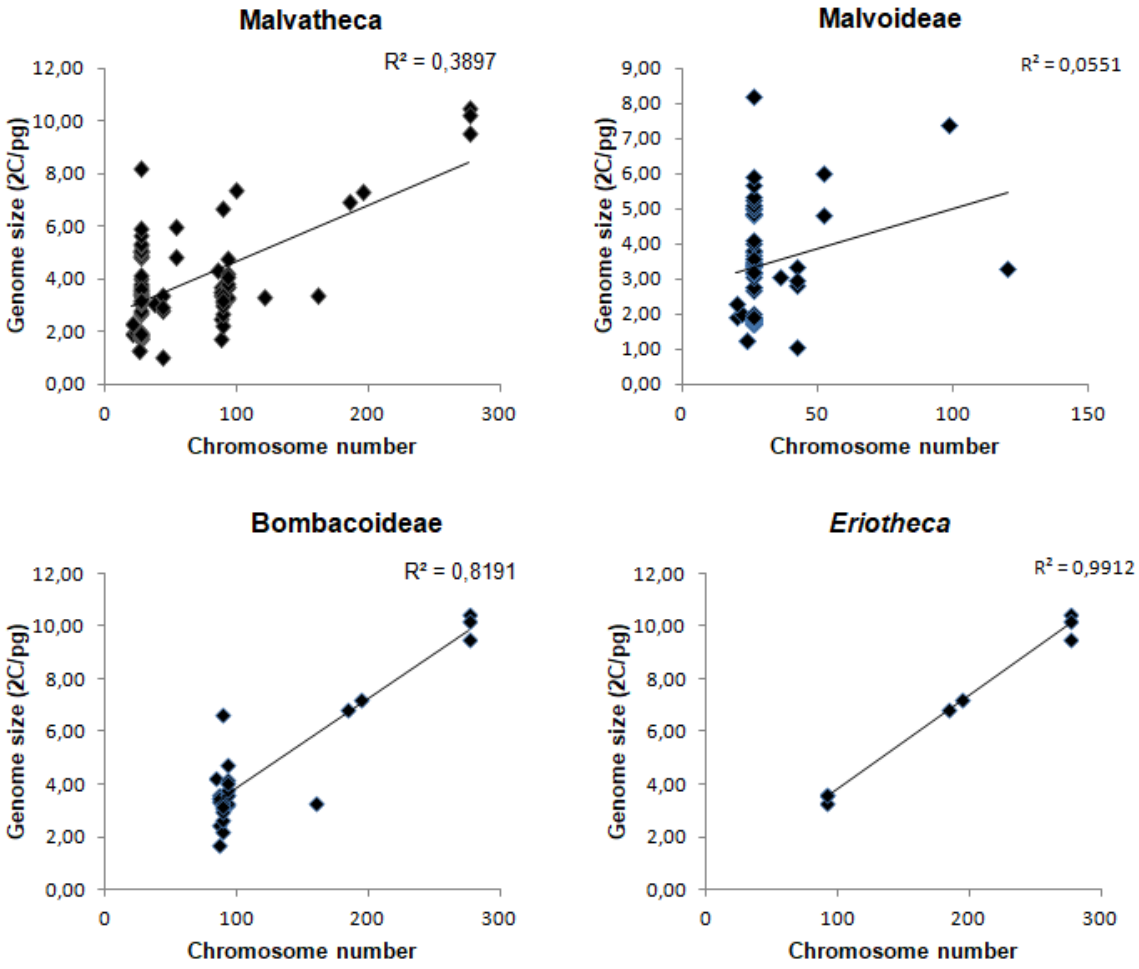

Figure 1 Data compiled from the literature and obtained in this study of genome size and chromosome number were plotted for the Malvatheca clade, Malvoideae subfamily, Bombacoideae subfamily and for the *Eriotheca*.

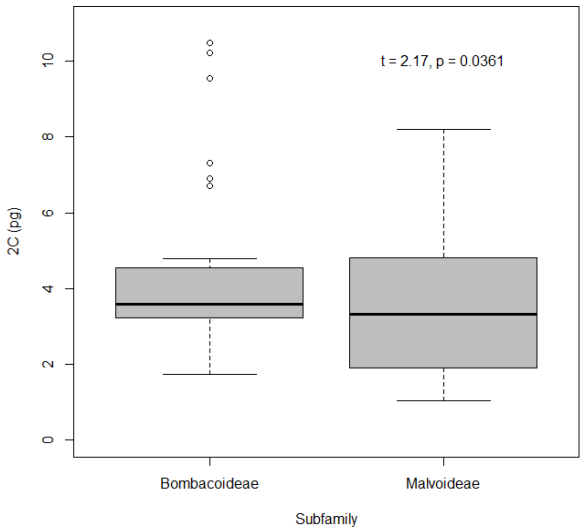

Figure 2 Genome sizes (2C value) for 88 species of Bombacoideae (28 species) and Malvoideae (60 species). The mean genome sizes of Bombacoideae was 2C = 3.58pg and Malvoideae was 2C = 3.33pg.

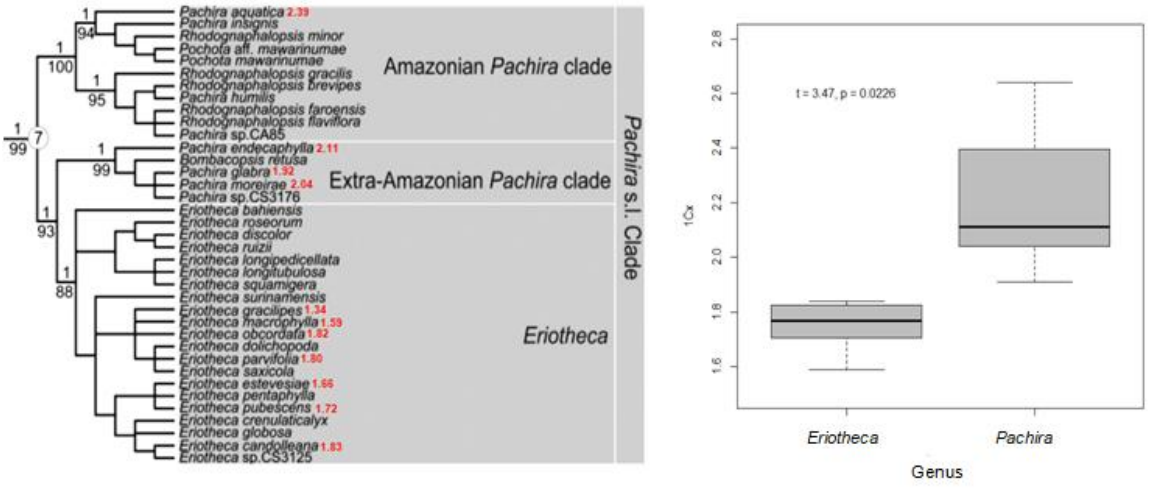

Figure 3 A: Phylogenetic relationships of Bombacoideae lineages modified of Carvalho-Sobrinho *et al.* (2016). The red numbers represent the 1Cx value for *Pachira* and *Eriotheca*. B: Basic genome size of *Eriotheca* and *Pachira* genus (Pachira clade) with mean 1Cx= 1.77pg and 2.11pg respectively.

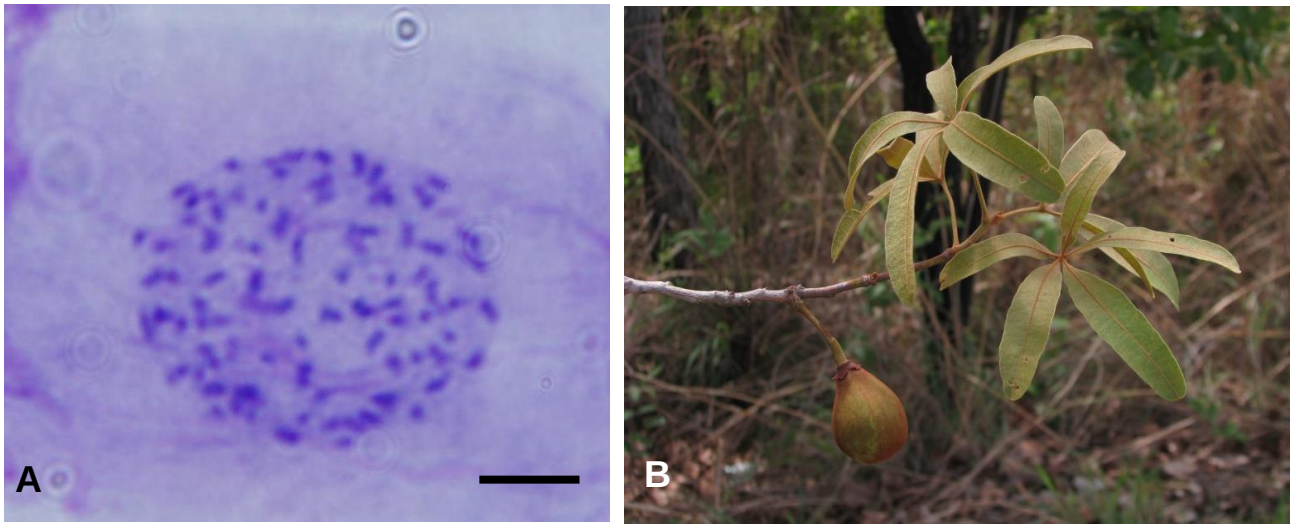

Figure 4 New count of chromosome number for *Eriotheca estevesiae*. A: Mitotic metaphase showed 2n = 92 chromosomes. B: Photograph showed a branch with ferrugineous fruit.

Table S1. Compilation of genome size and chromosome number with ploidy level and original reference for species belonging to the subfamily Malvoideae and Bombacoideae (Malvatheca clade).

Subfamily	Species	Chromosome number	Ploidy level	2C (pg)	Original Reference
Malvoideae	*Abelmoschus esculentus*	120	-	3,30	*Bennett et al. 1999*
Malvoideae	*Abutilon theophrasti*	42	6	2,80	Bennett *et al.* 1998
Malvoideae	*Cienfuegosia tripartita*	20	2	1,90	Wendel *et al.* 2002
Malvoideae	*Cienfuegosia yucatanensis*	22	2	2,00	Wendel *et al.* 2002
Malvoideae	*Cienfuegosia hitchcockii*	20	2	2,30	Wendel *et al.* 1999
Malvoideae	*Gossypioides kirkii*	24	2	1,25	Wendel *et al.* 1999
Malvoideae	*Gossypioides raimondii*	26	2	2,00	Wendel *et al.* 2002
Malvoideae	*Gossypioides herbaceum*	26	2	3,70	Wendel *et al.* 2002
Malvoideae	*Gossypium thurberi*	26	2	1,72	Hendrix & Stewart, 2005
Malvoideae	*Gossypium gossypioides*	26	2	1,72	Hendrix & Stewart, 2005
Malvoideae	*Gossypium trilobum*	26	2	1,74	Hendrix & Stewart, 2005
Malvoideae	*Gossypium armourianum*	26	2	1,75	Hendrix & Stewart, 2005
Malvoideae	*Gossypium klotzschianum*	26	2	1,80	Hendrix & Stewart, 2005
Malvoideae	*Gossypium raimondii*	26	2	1,80	Hendrix & Stewart, 2005
Malvoideae	*Gossypium turneri*	26	2	1,86	Hendrix & Stewart, 2005
Malvoideae	*Gossypium davidsonii*	26	2	1,86	Hendrix & Stewart, 2005
Malvoideae	*Gossypium harknessii*	26	2	1,86	Hendrix & Stewart, 2005
Malvoideae	*Gossypium aridum*	26	2	1,88	Hendrix & Stewart, 2005
Malvoideae	*Gossypium laxum*	26	2	1,91	Hendrix & Stewart, 2005
Malvoideae	*Gossypium lobatum*	26	2	1,91	Hendrix & Stewart, 2005
Malvoideae	*Gossypium longicalyx*	26	2	2,68	Hendrix & Stewart, 2005
Malvoideae	*Gossypium capits-virdis*	26	2	2,75	Hendrix & Stewart, 2005
Malvoideae	*Gossypium anomalum*	26	2	2,78	Hendrix & Stewart, 2005
Malvoideae	*Gossypium somalense*	26	2	3,06	Hendrix & Stewart, 2005
Malvoideae	*Gossypium stocksii*	26	2	3,13	Hendrix & Stewart, 2005
Malvoideae	*Gossypium triphyllum*	26	2	3,35	Kadir, 1976
Malvoideae	*Gossypium areysianum*	26	2	3,40	Hendrix & Stewart, 2005
Malvoideae	*Gossypium herbaceum*	26	2	3,41	Hendrix & Stewart, 2005
Malvoideae	*Gossypium arboreum*	26	2	3,43	Hendrix & Stewart, 2005
Malvoideae	*Gossypium barbosanum*	26	2	3,50	Kadir, 1976
Malvoideae	*Gossypium bickii*	26	2	3,59	Hendrix & Stewart, 2005
Malvoideae	*Gossypium nelsonii*	26	2	3,59	Hendrix & Stewart, 2005
Malvoideae	*Gossypium australe*	26	2	3,75	Hendrix & Stewart, 2005
Malvoideae	*Gossypium incanum*	26	2	3,80	Kadir, 1976
Malvoideae	*Gossypium robinsonii*	26	2	3,99	Hendrix & Stewart, 2005
Malvoideae	*Gossypium sturtianum*	26	2	4,12	Hendrix & Stewart, 2005
Malvoideae	*Gossypium hirsutum*	52	4	4,80	Hendrix & Stewart, 2005
Malvoideae	*Gossypium darwinii*	26	2	4,83	Hendrix & Stewart, 2005
Malvoideae	*Gossypium mustelinum*	26	2	4,85	Hendrix & Stewart, 2005
Malvoideae	*Gossypium tomentosum*	26	2	4,87	Hendrix & Stewart, 2005
Malvoideae	*Gossypium barbadense*	26	2	5,01	Hendrix & Stewart, 2005

Malvoideae	*Gossypium rotundifolium*	26	2	5,01	Hendrix & Stewart, 2005
Malvoideae	*Gossypium exiguum*	26	2	5,03	Hendrix & Stewart, 2005
Malvoideae	*Gossypium pilosum*	26	2	5,10	Hendrix & Stewart, 2005
Malvoideae	*Gossypium cunninghamii*	26	2	5,11	Hendrix & Stewart, 2005
Malvoideae	*Gossypium enthyle*	26	2	5,26	Hendrix & Stewart, 2005
Malvoideae	*Gossypium marchantii*	26	2	5,35	Hendrix & Stewart, 2005
Malvoideae	*Gossypium nobile*	26	2	5,68	Hendrix & Stewart, 2005
Malvoideae	*Gossypium barbadense*	52	4	6,00	Bennett *et al.* 1982
Malvoideae	*Hampea appendiculata*	26	2	5,90	Wendel *et al.* 2002
Malvoideae	*Hibiscus cannabinus*	36	-	3,05	Bennett *et al.* 1999
Malvoideae	*Kokia drynarioides*	24	2	1,25	Wendel *et al.* 1999
Malvoideae	*Kydia calycina*	98	-	7,40	Ohri and Kumar, 1986
Malvoideae	*Lavatera arbórea*	42	6	3,35	Ceccarelli *et al.* 1998
Malvoideae	*Lebronnecia kokioides*	26	2	3,60	Wendel *et al.* 2002
Malvoideae	*Malva parviflora*	42	2	1,05	Bidak and Brandham, 1995
Malvoideae	*Malva sylvestris*	42	6	2,95	Ceccarelli *et al.* 1998
Malvoideae	*Thespesia lampas*	26	2	3,20	Wendel *et al.* 2002
Malvoideae	*Thespesia thespesioides*	26	2	3,20	Wendel *et al.* 2002
Malvoideae	*Thespesia populnea*	26	2	8,20	Wendel *et al.* 2002
Bombacoideae	*Adansonia digitata*	160	4	3,34	Costa *et al.* 2017
Bombacoideae	*Bombax ceiba*	92	2	3,25	Ohri, 2002
Bombacoideae	*Cavanillesia umbellata*	88	2	6,70	Costa *et al.* 2017
Bombacoideae	*Ceiba erienthos*	86	2	3,40	Costa *et al.* 2017; Figueredo *et al.*2016
Bombacoideae	*Ceiba glaziovii*	86	2	3,66	Costa *et al.* 2017; Figueredo *et al.*2016
Bombacoideae	*Ceiba pentandra*	86	2	3,50	Costa *et al.* 2017; Figueredo *et al.*2016
Bombacoideae	*Ceiba speciosa*	86	2	2,50	Costa *et al.* 2017; Figueredo *et al.*2016
Bombacoideae	*Ceiba speciosa*	86	2	1,73	Ohri, 2002
Bombacoideae	*Ceiba pentandra*	86	2	3,50	Ohri *et al.* 2004
Bombacoideae	*Eriotheca candolleana*	92	2	3,66	Marinho *et al.* 2014;This study
Bombacoideae	*Eriotheca estevesiae*	92	2	3,33	This study
Bombacoideae	*Eriotheca gracilipes*	92	2	3,68	Marinho *et al.* 2014
Bombacoideae	*Eriotheca gracilipes*	276	6	10,48	Marinho *et al.* 2014
Bombacoideae	*Eriotheca macrophylla*	276	6	9,54	Costa *et al.* 2017
Bombacoideae	*Eriotheca obcordata*	194	4	7,30	Costa *et al.* 2017
Bombacoideae	*Eriotheca pubescens*	184	4	6,91	Marinho *et al.* 2014
Bombacoideae	*Eriotheca pubescens*	276	6	10,23	Marinho *et al.* 2014
Bombacoideae	*Ochroma pyramidale*	84	2	4,30	Costa *et al.* 2017
Bombacoideae	*Pachira aquática*	92	2	4,79	This study; Costa *et al.* 2017
Bombacoideae	*Pachira endecaphylla*	92	2	4,22	Costa *et al.* 2017
Bombacoideae	*Pachira glabra*	92	2	3,82	Costa *et al.* 2017
Bombacoideae	*Pachira moreirae*	92	2	4,08	Costa *et al.* 2017
Bombacoideae	*Pochota fendleri*	88	2	2,26	Costa *et al.* 2017
Bombacoideae	*Pseudobombax longiflorum*	88	2	2,70	This study; Costa *et al.* 2017; Marinho *et al.* 2014
Bombacoideae	*Pseudobombax marginatum*	88	2	3,14	Costa *et al.* 2017
Bombacoideae	*Pseudobombax parvifolium*	88	2	3,36	Costa *et al.* 2017

Bombacoideae	*Pseudobombax simplicifolium*	88	2	3,02	Costa *et al.* 2017
Bombacoideae	*Pseudobombax tomentosum*	88	2	3,18	This study; Costa *et al.* 2017

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Considerações Finais

Este estudo teve como objetivo geral relacionar os masaicos reprodutivos e citológicos associados à presença de fenômenos de duplicação total do genoma (WGD), com a distribuição geográfica, os padrões filogeográficos encontrados em plantas que pertencem à subfamília Bombacoideae, aqui tratadas como Complexo Tricoma Estrelado. Além disso, relacionamos mudanças no tamanho de genoma com as relações filogenéticas no clado Malvatheca, formado pela subfamília Bombacoideae e Malvoideae.

Para isso, nós coletamos exemplares de *Eriotheca pubescens* e de *Eriotheca estevesiae* e as utilizamos como fonte de estudo porque são espécies endêmicas do Cerrado Brasileiro e com mosaicos reprodutivos e citológicos bem descritos. *E. pubescens* é uma espécie caracterizada por apresentar cromossomos pequenos e numerosos o que dificultou um conhecimento mais amplo da distribuição geográfica dessas variações citológicos em trabalhos anteriores. Por isso, utilizamos a estimativa de ploidia através da medida morfométrica do estômato por ser um método que apresentou bons resultados em testes anteriores, em menor escala, para populações tetraplóides e hexaplóides desta espécie.

Neste primeiro estudo, conseguimos avaliar os padrões de embrionia (neste caso refletindo a presença ou não de reprodução assexual) e a ploidia em 19 populações amplamente distribuídas nos estados de Tocantins, Goiás e Minas Gerais. Resultados interessantes mostraram que as populações poliembriônicas apresentaram sempre uma alta porcentagem de sementes com embriões extranumerários e ainda formaram um grupo homogêneo hexaplóide baseado nas medidas morfométricas dos estômatos. Já nas populações monoembriônicas, nós não conseguimos inferir a ploidia de 5 populações por apresentarem um padrão anormal do tamanho dos estômatos provavelmente relacionado a variações ambientais. Estudos futuros, com ferramentas mais precisas de definição de ploidia como citometria de fluxo e/ou contagem do número cromossômico deverão ser realizados para preencher essa lacuna.

Apesar disso, estes dados nos auxiliaram na descoberta de um padrão diplóide, até agora desconhecido no complexo de espécies estudadas. As quatro

populações de *E. estevesiae* são restritas a região localizada no estado do Tocantins e foram avaliadas com metodologia usual para contagem do número cromossômico no terceiro capítulo desta tese (2n=92), que confirmou a ploidia inferida a partir dos dados de morfometria do estômatos.

Apesar da estimativa de ploidia através do tamanho dos estômatos não ter funcionado para todas as populações estudadas, esta ferramenta nos auxiliou a conhecer a distribuição geográfica dos diferentes níveis de ploidia da maior parte das populações. Este estudo confirmou ainda a existência de uma associação entre a poliploidia e poliembrionia, ressaltando a presença da reprodução sexual em indivíduos com ploidias menores.

A partir destes resultados, nós relacionamos estas descobertas com padrões filogeográficos obtidos para a maioria destas populações descritas anteriomente e ainda comparamos com as teorias filogeográficas propostas para outras espécies de Cerrado. No segundo capítulo, nós encontramos que a maior parte da variação genética, estimada por regiões de cpDNA, estava presente nas populações poliembriônicas. Foram encontrados dois haplótipos predominantes entre as populações que formam o Complexo Tricoma Estrelado sedo que um deles é compartilhado entre *E. pubescens* e *E. estevesiae* o que nos sugere um possível origem comum e fortelece a teoria, proposta no primeiro capítulo, que as populações poliplóides e amplamente distribuídas de *E. pubescens* provavelmente surgiram das populações diplóides e sexuadas de *E. estevesiae*.

Esta teoria também pode ser sustentada quando nós relacionamos os nossos resultados com os padrões filogeográficos encontrados em outras espécies de Cerrado, que sugerem que durante o último máximo glacial, as plantas deste Bioma parecem ter se refugiado em regiões ao Norte e depois da melhora climática houve uma recolonização das regiões mais ao sul. Nós ainda encontramos uma diferenciação genética das populações do leste e oeste corroborando também outros padrões filogeográficos. Não há uma separação filogeográfica marcada entre os padrões reprodutivos e citológicos, mas sugirimos que a maior variação genética marcada pela presença de diferentes haplótipos em populações poliembriônicas/hexaplóides pode ter sido favorecida pela presença de apomixia, que dá a esses organismos melhores condições de explorar áreas após perturbações climáticas.

Estes dois estudos (Capítulos 1 e 2) mostraram que variações cromossômicas e alterações no genoma plastidial foram influenciadas em um nível intraespecífico pela biologia reprodutiva destas plantas. Sendo assim, o terceiro estudo (Capítulo 3) traz num contexto filogenético mais amplo, a influência da evolução do número cromossômico e do tamanho de genoma no clado Malvatheca (Malvoideae + Bombacoideae, Malvaceae) e mais especificamente no clado Pachira (*Eriotheca*+ *Pachira*, Bombacoideae).

A compilação de dados de tamanho de genoma e número cromossômico realizada neste estudo mostrou que não há, de forma geral, uma correlação forte entre estes fenômenos, sendo que o contrário só aconteceu quando nós estudamos separadamente o gênero *Eriotheca*. Essa forte correlação entre estes fenômenos neste gênero é devida, dentre outros fatores, as séries poliplóides observadas em *E. pubescens* e *E. gracilipes*.

Foi possível propor que apesar do clado Malvatheca ter sido considerado monofilético, estas subfamílias apresentam tamanhos de genoma (2C) distintos, sendo que o maior é encontrado em Bombacoideae e o menor em Malvoideae. Malvoideae pode ser ainda caracterizada por apresentar uma grande amplitude entre os dados encontrados sugerindo que, assim como em Bombacoideae, eventos de duplicação, autoreplicação do DNA, elementos de transposição e outros fenômenos podem influenciar no aumento e diminuição do genoma de forma específica em cada subgrupo destas plantas.

Uma menor variação entre os dados de tamanho de genoma haplóide (1Cx) foi encontrada quando avaliamos os gêneros que compõem o possivelmente monofilético clado Pachira. *Eriotheca* apresentou um valor médio do tamanho do genoma menor do que em *Pachira* sugerindo que estes gêneros estão evoluindo de formas distintas e separadamente, o que não sustenta o monofiletismo proposto.

Todas estas discussões ressaltam a necessidade de estudos multidisciplinares, como o aqui apresentado, para auxiliar na compreensão de eventos ecológicos, genéticos e reprodutivos das plantas. Ainda com estes dados é possível auxiliar a construção de estratégias de conservação importantes e necessárias para biomas ameçados, como o Cerrado.