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Citogenômica de *Pterodon pubescens* e citogenética comparativa com *P. emarginatus* (Leguminosae)

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Nível: Mestrado

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Citogenômica de *Pterodon pubescens* e citogenégica comparativa com *P. emarginatus* (Leguminosae)

Dissertação apresentada ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal de Goiás, como requisito parcial para a obtenção do título de Mestre em Genética e Biologia Molecular.

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ATA DE DEFESA DE DISSERTAÇÃO

Ata nº **082** da sessão de Defesa de Dissertação de **VICTÓRIA BORGES ALBERNAZ**, que confere o título de Mestre(a) em **GENÉTICA E BIOLOGIA MOLECULAR**, na área de concentração em **Genética de Populações e Evolução Molecular**.

Ao/s **cinco dias do mês de março de dois mil e vinte**, a partir das **8:30 horas**, no **miniauditório** do **CERCOMP**, realizou-se a sessão pública de Defesa de Dissertação intitulada **"Citogenômica comparativa de *Pterodon pubescens* e *P. emarginatus* (Leguminosae)"**. Os trabalhos foram instalados pela Orientadora, Professora Doutora **Thannya Nascimento Soares (ICB/UFG)** com a participação dos demais membros da Banca Examinadora: Professora Doutora **Andrea Pedrosa-Harand (CB/UFPE)**, membro titular externo, cuja participação ocorreu através de videoconferência e a Doutora **Rosana Pereira Vianello (EMBRAPA)**, membro titular externo. Durante a arguição os membros da banca **fizeram** sugestão de alteração do título do trabalho para **Citogenômica de *Pterodon pubescens* e citogenética comparativa com *P. emarginatus* (Leguminosae)**. A Banca Examinadora reuniu-se em sessão secreta a fim de concluir o julgamento da Dissertação, tendo sido a candidata **aprovada** pelos seus membros. Proclamados os resultados pela Professora Doutora **Thannya Nascimento Soares**, Presidente da Banca Examinadora, foram encerrados os trabalhos e, para constar, lavrou-se a presente ata que é assinada pelos Membros da Banca Examinadora, ao(s) **cinco dias do mês de março de dois mil e vinte**.

TÍTULO SUGERIDO PELA BANCA

Citogenômica de *Pterodon pubescens* e citogenética comparativa com *P. emarginatus* (Leguminosae)

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Sumário

Resumo

O gênero *Pterodon* Vogel (Leguminosae) possui apenas quatro espécies, das quais *P. pubescens* (Benth.) Benth. e *P. emarginatus* Vogel (ambas conhecidas como "sucupira branca") são as mais próximas filogeneticamente. Estas espécies apresentam ampla distribuição geográfica no Brasil e possuem o número cromossômico 2*n* = 16 com cromossomos pequenos e morfologicamente similares. O objetivo do presente trabalho foi realizar uma análise comparativa do tamanho do genoma, o padrão de bandeamento e a composição de elementos repetitivos nos cromossomos de *P. pubescens* e *P. emarginatus*, visando aprofundar os conhecimentos citogenômico e evolutivo destas espécies. Para isso, foi realizada a caracterização citogenética pela análise do número e morfologia cromossômica, bandeamento CMA e DAPI, FISH com DNAr 5S e 35S e determinação do tamanho do genoma por citometria de fluxo. Além disso, utilizamos o sequenciamento do genoma de *P. pubescens* por NGS (*Next Generation Sequencing*) usando a plataforma Illumina para caracterizar as frações repetitivas genômicas, utilizando uma plataforma Galaxy/RepeatExplorer-Elixir. Os elementos mais abundantes do genoma de *P. pubescens* foram localizados nos cromossomos por hibridização fluorescente *in situ* (FISH) e transferidos para *P. emarginatus*. As espécies apresentaram cariótipos muito semelhantes com: (i) bandas $CMA^+/DAPI^-$ na região terminal de dois pares de cromossomos e na região pericentromérica de todos os cromossomos; (ii) dois pares de sítios de DNAr 35S co-localizados com as bandas CMA^+ terminais; (iii) um par de sítios de DNAr 5S localizados na região proximal de um par cromossômico. O tamanho do genoma de *P. pubescens* e *P. emarginatus* também foi semelhante, 1C = 0,665 pg e 1C = 0,620 pg, respectivamente. A fração repetitiva representou 26,4% do genoma obtido de *P. pubescens*, sendo que os retrotransposons Ty3-Athila (24,24%), Ty3-Tekay (21,93%) e Ty1-Ale (3,37%) são os elementos mais abundantes. DNAs satélites de baixa abundância foram identificados: PubSat1-254 (2,09%), PubSat2-76 (2,06%), PubSat3-216 (0,58%), PubSat4-138 (0,23%). A hibridização *in situ* revelou que todas as sequências repetitivas analisadas estão colocalizadas com as bandas CMA^+ proximais em ambas espécies, exceto o retroelemento Ty1-Ale, que também está disperso na eucromatina dos cromossomos de *P. pubescens*. A similaridade citomolecular observada aqui sugere que os genomas de *P. pubescens* e *P. emarginatus* apresentam frações repetitivas altamente semelhantes, o que corrobora sua proximidade filogenética. No entanto, a recente expansão do

elemento Ty1-Ale no genoma de *P. pubescens* sugere algum grau de diferenciação nas frações repetitivas desses genomas.

Palavras-chave: DNAsat, FISH, NGS, sequências repetitivas, sucupira-branca, retroelementos

Abstract

The genus *Pterodon* Vogel (Leguminosae) has only four species, of which *P. pubescens* (Benth.) Benth. and *P. emarginatus* Vogel (both known as "white sucupira") are the closest phylogenetically. These species have a wide geographical distribution in Brazil and have chromosome number 2*n* = 16 with small and morphologically similar chromosomes. The objective of the present work was to carry out a comparative analysis of the genome size, the banding pattern and the composition of repetitive elements in the chromosomes of *P. pubescens* and *P. emarginatus*, aiming to enhance the cytogenomic and evolutionary knowledge of these species. For this, cytogenetic characterization was performed by analyzing the number and chromosomal morphology, CMA and DAPI banding, hybridization with DNAr 5S and 35S and determining the genome size by flow cytometry. In addition, we used *P. pubescens* genome sequencing by NGS (Next Generation Sequencing) using the Illumina platform to characterize repetitive genomic fractions, using a Galaxy/RepeatExplorer-Elixir platform. The most abundant elements of the *P. pubescens* genome were located on the chromosomes by fluorescent in situ hybridization (FISH) and transferred to *P. emarginatus*. The species showed very similar karyotypes with: (i) CMA^{+}/$DAPI^{-}$ bands in the terminal region of two chromosome pairs and pericentromeric region of all chromosomes; (ii) two pairs of 35S rDNA sites co-located with the terminal CMA^{+} bands; (iii) a pair of 5S rDNA sites located in the proximal region of a chromosomal pair. The genome size of *P. pubescens* and *P. emarginatus* was also similar, 1C = 0.665 pg and 1C = 0.620 pg, respectively. The repetitive fraction represented 26,4% of the *P. pubescens* sequenced genome, with Ty3-Athila Ty3-Athila (24,24%), Ty3-Tekay (21,93%) and Ty1-Ale (3,37%) retrotransposons being the most abundant elements. Low abundance satellite DNAs were identified: PubSat1-254 (2,09%), PubSat2-76 (2,06%), PubSat3-216 (0,58%), PubSat4-138 (0,23%). *In situ* hybridization reveal that all analyzed repeats were enriched in proximal CMA^{+} heterochromatin in both species, except for the Ty1-Ale retroelement, which was also dispersed also in the euchromatin of *P. pubescens* chromosomes. The cytomolecular similarity observed here suggests that the genomes of *P. pubescens* and *P. emarginatus* have highly similar repetitive fractions, which corroborates their phylogenetic proximity. However, the recent expansion of the Ty1-

Ale element in the *P. pubescens* genome suggests some degree of differentiation in the repetitive fractions of these genomes.

Keywords: FISH, NGS, satDNA, repetitive sequences, "white sucupira**”**

Introdução Geral

Família Leguminosae

Leguminosae é a terceira maior família de plantas, com aproximadamente 770 gêneros e mais de 19.500 espécies, sendo as famílias Orchidaceae e Asteraceae as únicas que a superam em número de espécies (Cardoso et al. 2012; LPWG 2017). A família Leguminosae apresenta uma importância econômica e ecológica (Lewis et al. 2005; Yahara et al. 2013), perdendo apenas para a família das gramíneas em importância econômica (Lewis et al. 2013). Economicamente, essa família é valorizada pela extensa produção de diferentes tipos de alimentos, madeiras, combustíveis, medicamentos e produtos químicos (Lewis et al. 2005; Cardoso et al. 2012; Yahara et al. 2013; LPWG 2017). As espécies que compõem a família Leguminosae estão presentes em quase todos os biomas da Terra, desde florestas tropicais até desertos, e ecologicamente, a maioria das leguminosas se destacam por serem responsáveis pela fixação de nitrogênio atmosférico através de bactérias simbióticas presentes nas raízes (McKey 1994; Yahara et al. 2013; DRYFLOR 2016; LPWG 2017).

A família Leguminosae foi recentemente dividida em seis subfamílias (Cercidoideae, Detarioideae, Duparquitioideae, Dialioideae, Caesalpinioideae e Papilionoideae) (LPWG 2017). As leguminosas pertencentes à subfamília Papilionoideae formam um grupo diverso, contendo aproximadamente 14.000 espécies e 484 gêneros (Lewis et al. 2005; Lewis et al. 2013; Cardoso et al. 2015). Essa diversificação dentro desse grupo pode ser evidenciada pela morfologia floral, que inclui não apenas as flores papilionadas, mas também exemplos de flores não-papilionadas, de perda de pétalas, de pétalas indiferenciadas, numerosos estames livres, cálice fechado e simetria radial (Leite et al. 2014b). A subfamília Papilionoideae (Leguminosae) inclui três tribos distintas, Angylocalyx, Dipterygeae e Amburana, que compõem o clado ADA (Cardoso et al. 2012; Cardoso et al. 2013; Cardoso et al. 2015). Dipterygeae é um grupo monofilético e compreende quatro gêneros: *Dipteryx* Schreb., *Monopteryx* Spruce ex Benth, *Pterodon* Vogel e *Taralea* Aubl. (Cardoso et al. 2012; Cardoso et al. 2015).

Pterodon emarginatus e *P. pubescens*

O gênero *Pteroron* pode ser distinguido por apresentar características marcantes, tais como fruto em formato de criptosâmara com glândulas de óleo no epicarpo, folhas com raque marginada, semente lisa com rafe aparente, hilo na posição lateral coberto

por um arilo e um embrião liso (Leite et al. 2014a). Ao longo dos anos esse gênero apresentou diferentes números de espécies variando entre seis, três e duas espécies dependendo da base de dados que fosse consultada (Rocha 2006). Essa discrepância em relação ao número de espécies do gênero é causada principalmente pela delimitação das espécies *Pterodon pubescens* (Benth.) Benth. e *Pterodon emarginatus* Vogel, por já terem sido consideradas como uma espécie apenas, possuindo duas morfos (Rocha 2006). Segundo o REFLORA (Carvalho, em construção), o gênero Pterodon possui quatro espécies *Pterodon. abruptus* (Moric.) Benth, *Pterodon. apparicioi* Pedersoli, *P. pubescens* e *P. emarginatus*.

As espécies *P. pubescens* e *P. emarginatus* são as únicas do gênero que não são endêmicas do Brasil, mas são amplamente distribuídas pelo Brasil, ocorrendo nos estados de Rondônia, Tocantins, Bahia, Maranhão, Piauí, Goiás, Distrito Federal, Mato Grosso, Mato Grosso do Sul, Minas Gerais e São Paulo (Carvalho, em construção). Ambas as espécies ocorrem no Cerrado (*lato sensu*), Floresta Ciliar ou Galeria, exceto Floresta Estacional Semidecidual onde somente *P. emarginatus* é encontrada (Carvalho, em construção).

Em Afonso 1997, foi relatado períodos distintos de floração (*P. emarginatus* com pico em agosto enquanto *P. pubescens* em setembro), mas em campo é observada a floração simultânea, com capacidade de formar híbridos naturais (Rocha 2006). No estudo de Rocha 2006, foram encontrados três indivíduos com características morfológicas intermediárias entre as duas espécies. Nesse estudo foram utilizados marcadores RAPD e caracteres morfológicos para verificar se a variação encotrada dentro da espécie *P. emarginatus* (uma morfo com flores rosas e a outra com flores roxas) poderia ser um indicativo de que *P. emarginatus* engloba mais de uma entidade taxonômica. Os resultados moleculares e morfológicos suportam a premissa de que são na verdade duas espécies distintas. As diferenças morfológicas entre *P. pubescens* e *P. emarginatus* são discretas, de folhas pusbescentes com 6 a 19 folíolos ou folhas glabras com 4 a 10 folíolos, respectivamente. Os indivíduos de *P. emarginatus* apresentam, em geral, folíolos grandes e mais largos, botões florais com base mais estreita do que o ápice, que é arredondado (Rocha 2006). Em contrapartida, os botões florais em *P. pubescens* são alongados, com a base da mesma largura que o ápice (Rocha 2006). A diferença morfológica de maior destaque são as flores de coloração do róseo claro, quase branco, a róseo escuro em *P. pubescens* e roxo, em *P. emarginatus* (Rocha 2006).

A polinização é realizada por abelhas e as sementes são dispersas pelo vento (Almeida et al. 2003). Em *P. emarginatus*, as flores são polinizadas por *Bombus morio*, enquanto as flores de *P. pubescens* são polinizadas por *Bombus attratus* (Afonso 1997). As diferenças de polinizadores e picos florais, corroboram a delimitação como espécies distintas, mas é provável a hibridização entre *P. pubescens* e *P. emarginatus*, principalmente em regiões que ocorrem as duas espécies. No estudo de Lima 2019, foi evidenciado uma região com essas espécies em condições de provável simpatia, no município de Itacaiú-GO e na região da bacia do Araguaia (entre latitudes -14,70 e -15,80).

Estudos com o gênero *Pterodon* têm sido relacionados principalmente ao potencial medicinal do óleo extraído de sementes. Em *P. pubescens*, o óleo apresentou atividade anti-*Trypanosoma cruzi* (Menna-Barreto et al. 2008), bem como anti-inflamatória (Hoscheid et al. 2013) e antinociceptiva (Coelho et al. 2005). Atividade anti-inflamatória e antinociceptiva também foram confirmadas para *P. emarginatus* (de Moraes et al. 2009). Trabalhos que abrangem outras áreas da ciência têm sido realizados, como genética de populações para acessar à diversidade genética e estrutura em populações naturais de *P. emarginatus* (Pinto 2017). Nesse estudo de Pinto 2017 foram testados 25 iniciadores transferidos de *Dipteryx alata* Vogel em 12 populações naturais foram genotipadas para 6 locos microssatélites. Os resultados desse estudo permitem concluir que as populações apresentaram nível intermediário de diversidade genética, maior variabilidade dentro de populações do que entre populações e a espécie apresentam um sistema misto de acasalamento. No estudo de Melo (2018), 27 marcadores microssatélites foram desenvolvidos para *P. pubescens* e 26 marcadores polimórficos foram transferidos para espécies de *P. emarginatus*, considerando que serão úteis para estudos de genética de populações devido ao alto potencial de discriminação individual encontrado. Um estudo filogeográfico comparado entre *P. pubescens* e *P. emarginatus* concluiu que as espécies estão em processo de separação recente, por isso foi encontrado um alto compartilhamento de haplótipos (Lima 2019). Esse resultado segue o padrão de recente diversificação evidenciado para outras leguminosas: *Astragalus* (Scherson et al. 2008), *Abarema* (Iganci et al. 2016), *Canavalia* (Snak et al. 2016), *Chamaecrista* ser. Coriaceae (Rando et al. 2016), *Lupinus* (Drummond 2008) e *Phaseolus* (Delgado-Salinas et al. 2007).

O gênero *Pterodon*, assim como o clado ADA, é pouco estudado do ponto de vista citogenético, sendo conhecido apenas o número cromossômico de duas espécies, *Pterodon pubescens* (Benth.) Benth. e *Pterodon polygalaeflorus* (Benth.) Benth. (= *Pterodon emarginatus* Vogel) ambos com 2*n* = 16 e cromossomos pequenos (Bandel 1974; Coleman e DeMenezes 1980). Para algumas espécies do clado ADA também são conhecidos os números cromossômicos, como *Angylocalyx oligophyllus* (Baker) Baker (2*n* = 26), Dipteryx odorata (Aubl.) Willd. (2*n* = 32), Dipteryx alata (2*n* = 16, Taquary 2017), Amburana cearensis (Allemao) A. C. Sm. (2*n* = 22), Myrospermum frutescens Jacq. (2*n* = 26), Myrospermum sousanum A. Delgado & M. C. Johnst. (2*n* =26) Myroxylon balsamiferum Harms (2*n* = 26), Myroxylon pereirae Klotzsch (2*n* = 28), Myroxylon pereirae Royle (2*n* = 28) (Rice et al. 2015).

Citogenética clássica, molecular e Citogenômica

A Citogenética consiste no estudo de cromossomos isolados ou em conjunto, condensado ou distendido, tanto no que se refere a morfologia, organização, função, estrutura e replicação (Guerra 2004). Estes cromossomos, na maioria das vezes, são cromossomos mitóticos de eucariotos. A caracterização do cariótipo diz respeito a análise de características cromossômicas como: número cromossômico, tamanho dos cromossomos, posição do centrômero e presença de constrições secundárias (Guerra 2004). Essa caracterização pode ser feita utilizando técnicas convencionais, gerando fórmulas cariotípicas para as espécies (Malik and Thomas 1966; Weiss et al. 2003).

A fórmula cariotípica para alguns gêneros de plantas que possuem número cromossômico variável, permite a diferenciação entre espécies (Stace 1978; Martel et al. 1997). No entanto, existem gêneros que possuem espécies com número cromossômico e morfologia similares (Siljak-Yakovlev et al. 2003; de Souza Almeida et al. 2007; Viana and Souza 2012). Esses casos necessitam de técnicas de coloração diferencial, sendo o bandeamento com fluorocromos CMA (cromomicina A3) e DAPI (4', 6-diamidino- 2-fenilindol), os mais utilizados para o bandeamento cromossômico em plantas. Os fluorocromos CMA e DAPI marcam as regiões ricas em GC e AT, respectivamente, e são empregados para análise da heterocromatina (Guerra 2000). Esses fluorocromos são úteis para diferenciar espécies com número cromossômico estável (da Costa Silva et al. 2015; Mendonça de Almeida et al. 2016; Van-Lume et al. 2017; Ortiz et al. 2017).

As técnicas convencionais, como CMA/DAPI, permitem a verificação da localização da heterocromatina, mas não se pode definir quais os tipos de elementos repetitivos estão presentes. No entanto, com a integração de dados genômicos e citogenéticos foi originada a citogenômica (Talukdar and Sinjushin 2015), que possibilitou a caracterização dos elementos repetitivos presentes. A identificação da composição e proporção de elementos da fração repetitiva de genomas eucarióticos que estão geralmente associados à heterocromatina, como elementos transponíveis (TEs), DNA satélite (DNAsat) e DNA ribossomal (DNAr) (Belyayev et al. 2018, Pamponét et al. 2019; Bolsheva et al. 2019; Samoluk et al. 2019; Van-Lume et al. 2019), foi facilitada pelo sequenciamento de nova geração (NGS) (Ansorge 2009) juntamente com análises bioinformáticas apropriadas (*RepeatExplorer*, Novák et al. 2013). Após a identificação dos elementos presentes no genoma, a verificação da distribuição desses elementos nos cromossomos é feita pela técnica de hibridização *in situ* fluorescente (FISH), técnica convencional da Citogenética molecular. A Citogenética molecular e Citogenômica forneceram novas possibilidades de estudo na evolução cromossômica e organização do genoma, o que também contribui para uma melhor caracterização do cariótipo (Siljak-Yakovlev et al. 2014).

A técnica de FISH consiste na hibridização de uma sequência repetitiva ou não repetitiva de DNA (sonda) à uma região específica do cromossomo (Guerra 2004). As sequências repetitivas de DNA representam uma grande parte do genoma de plantas, podendo representar mais que 80% do genoma total [*Triticum aestivum* L. (Li et al. 2004); *Zea mays* L. (Mehrotra and Goyal 2014)]. O DNA repetitivo pode ser classificado geralmente pelos seus padrões de sequência e localização. Os DNA satélites, por exemplo, são formados por sequências repetitivas (monômeros) em tandem, geralmente gênero-específicas, e são um dos principais componentes das regiões heterocromáticas, podendo estar localizados nas regiões centroméricas e subteloméricas, mas também em posições intercalares (Mehrotra e Goyal 2014). Como as regiões centroméricas são os locais onde se encontram mais agrupamentos de sequências repetitivas em tandem, tanto para plantas quanto para animais (Melters et al. 2013), a função mais aceita do DNAsat está relacionada ao centrômero, que possui um papel essencial na segregação correta dos cromossomos na mitose e meiose (Plohl et al. 2014).

O DNAsat é um dos principais componentes da heterocromatina, e diferenças na composição dessa região podem estar relacionadas com eventos de especiação e isolamento reprodutivo (Bachmann et al. 1989; Plohl et al. 2014). A região do

centrômero acumula diferenças, pois, apesar da sua função ser crítica e conservada, o DNA centromérico subjacente (e.g DNA satélite e LTR-retrotransposons) evoluem rapidamente (Plohl et al. 2014; Rosin and Mellone 2017), levando à uma compatibilidade reduzida em cromossomos homólogos de híbridos (Henikoff 2001). Esse evento de redução de compatibilidade pode gerar um isolamento pós-zigótico, desencadeando a especiação (Henikoff 2001; Fishman and Saunders 2008).

Os DNA ribossomais (DNAr) também fazem parte da fração repetitiva do genoma e são formados por sequências repetitivas em tandem altamente conservadas que codificam RNA ribossomais (RNAr) e por regiões espaçadoras intergênicas (Volkov et al. 2017). Existem dois tipos de DNAr: 35S e 5S, porém existem quatro tipos regiões codificadoras de RNAr (18S, 5.8S, 26S e 5S) que são os constituintes essenciais do ribossomo de todos eucariotos (Hemleben V, Volkov RA, Zentgraf U 2004). Os locos do DNAr 45S consistem em unidades repetidas em tandem das sequências 18S, 5.8S e 26S, sequências transcritas internas (ITS1 e ITS2) e espaçadores gênicos (IGS) compõem um loco gênico (Siljak-Yakovlev et al. 2014). Os genes transcritos por essas regiões formam a região organizadora no nucléolo (NOR), e podem ser reconhecidas por análises cromossômicas citológicas como constrições secundárias na meiose/mitose (Hemleben V, Volkov RA, Zentgraf U 2004). Ao contrário do conhecimento limitado que temos sobre a maioria das sequências repetitivas, cujas funções ainda não estão bem definidas, os DNArs 5S e 35S são vitais para os organismos por fornecerem os rRNAs necessários para a montagem de ribossomos funcionais, que representam mais de 90% de todo o RNA transcrito de uma célula (Volkov et al. 2017). Nos eucariotos, a quantidade de cópias de DNAr excede o número necessário de RNA transcritos, por isso grande parte das cópias é silenciada transcricionalmente (Volkov et al. 2017).

Os elementos transponíveis, por sua vez, estão geralmente dispersos por todo o genoma e são classificados com base nos domínios enzimáticos conservados e estrutura (Pamponét et al. 2019). Retrotransposons (classe 1) e transposons de DNA (classe 2) são os dois tipos presentes nos genomas eucarióticos. Os retrotransposons são caracterizados por apresentarem RNA intermediário transcrito reversamente e um mecanismo de "*copy and paste*", enquanto os transposons possuem DNA intermediário e frequentemente um mecanismo de transposição "*cut and paste*", porém existem outros mecanismos a depender da família do retrotransposon (Finnegan 1989; Feschotte e Pritham 2007). Dentre os retrotransposons existem duas subclasses, aquelas flanqueadas por repetições terminais longas (RT-LTR) e aquelas que podem ou não ter repetições

terminais curtas (não RT-LTR) (Finnegan 1989; Gaiero et al. 2019). Os retrotransposons LTR-RT quando autônomos possuem pelo menos cinco domínios de proteína: GAG, protease (PROT), transcriptase reversa (RT), ribonuclease H (RH) e integrasse (INT). Esses retrotransposons estão agrupados em superfamílias e vários estudos mostram que as superfamílias Ty3/*gypsy* e Ty1/*copia* são as mais abundantes em plantas (Won et al. 2018; Said et al. 2018; González et al. 2018; Liu et al. 2019; Hloušková et al. 2019). Além dos RT-LTRT representarem tipicamente a maior fração da maioria dos genomas vegetais, há indícios de que seja o principal determinante da variação no tamanho dos genomas (Wessler 2006; Zuccolo et al. 2007; Macas et al. 2015), devido às novas cópias de elementos geradas que, após a integração, aumentam o tamanho do genoma (Galindo-González et al. 2017; Neumann et al. 2019). O tamanho genômico de espécies de plantas pode variar de 63 a 149.000 Mb (Heslop-Harrison and Schwarzacher 2011; Bennett and Leitch 2011).

Os retrotransposons comumente são encontrados dispersos pelo genoma, mas também podem estar concentrados nas regiões centroméricas e pericentroméricas de cromossomos de plantas. O grupo denominado de cromovírus, por exemplo, podem ser encontrados nos centrômeros. Esse grupo é formado pelos elementos Chlamyvir, Tcn1, Tekay, Reina, CRM e Galadriel pertencentes à superfamília Ty3/*gypsy* e possuem cromodomínios (CHD e CHDCR) (Hřibová et al. 2010; Neumann et al. 2019). A presença de cromodomínio nesse grupo é o que os diferencia do restante dos elementos denominados como não-cromovírus (Phygy, Selgy, Athila, Tat, Ogre, Retand)(Hřibová et al. 2010; Neumann et al. 2019).

Em estudos citogenéticos comparativos, a caracterização da distribuição física da heterocromatina em genomas de plantas utilizando técnicas de bandeamento cromossômico tem contribuído para diferenciação de espécies, especialmente em grupos com números cromossômicos estáveis (de Souza Almeida et al. 2007; da Costa Silva et al. 2015; Van-Lume et al. 2017). A caracterização da composição da heterocromatina pode ajudar a aprofundar as análises comparativas, identificando algumas classes de repetições que enriquecem as bandas heterocromáticas (Belyayev et al. 2018; González et al. 2018; Van-Lume et al. 2019; Peška et al. 2019). O conhecimento sobre a distribuição e abundância dessas sequências repetitivas (DNAsat, DNAr e TEs) pode contribuir para a compreensão sobre a evolução de genomas, como processos expansão e retração dos genomas, reestruturação de cromossomos (fusão, fissão, translocação e inversão), assim como processos de especiação e hibridização (Mehrotra e Goyal 2014;

Garrido-Ramos 2015). No entanto, observa-se que os tipos de sequências de DNA que compreendem bandas heterocromáticas ainda são pouco conhecidos na maioria dos estudos que exploraram a distribuição física da heterocromatina (Bolsheva et al., 2019; Gaiero et al. 2019). Portanto, não é claro se essas bandas localizadas em posições cromossômicas semelhantes dentro e entre as espécies são compostas de repetições semelhantes (Liu et al. 2019).

Considerando a proximidade filogenética entre *P. pubescens* e *P. emarginatus*, a análise citogenômica comparativa pode contribuir para o entendimento do relacionamento evolutivo destas espécies.

Objetivos

Objetivo geral

Realizar uma análise comparativa do tamanho do genoma, o padrão de bandeamento e a composição de elementos repetitivos nos cromossomos de *P. pubescens* e *P. emarginatus*, visando aprofundar os conhecimentos citogenômico e evolutivo destas espécies.

Objetivos específicos:

- Conhecer o tamanho genômico de *P. pubescens* e *P. emarginatus*;
- Selecionar as sequências repetitivas mais abundantes do genoma de *P. pubescens* para desenvolver sondas a hibridação *in situ* por fluorescência (FISH) e transferi-las para *P. emarginatus*;
- Determinar a distribuição física e composição da heterocromatina por meio da coração CMA/DAPI e pelo uso de sondas de regiões repetitivas para *P. pubescens* e *P. emarginatus*;
- Comparar os padrões de distribuição dos elementos repetitivos nos cariótipos de *P. pubescens* e *P. emarginatus*.

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Cytogenomic analysis of *Pterodon pubescens* (Benth.) Benth. and comparative cytogenetics with *P. emarginatus* Vogel (Leguminosae, Papilionoideae) reveals similarity in their genomes repetitive fraction

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Main Conclusion: Comparative analysis of *P. pubescens* and *P. emarginatus* reveals highly similar chromosomal repeats distribution. However, expansion of Ty1/*copia*-Ale in *P. pubescens* euchromatin represents a genomic mark that differentiates their karyotypes.

The genus *Pterodon* presents currently four species, of which *P. pubescens* and *P. emarginatus* (both known as “sucupira branca") are more morphologically and phylogenetically related. These species have a wide geographical distribution in Brazil and both present the karyotype 2*n* = 16 with small and morphologically similar metacentric/ submetacentric chromosomes. The objective of the present work was to characterize the repetitive fraction of *P. pubescens* by genome skimming, aiming to perform a comparative cytogenomic analysis between this species and *P. emarginatus*. The species presented similar karyotypes with: (i) CMA^{+}/$DAPI^{-}$ bands in the terminal region of two chromosome pairs and proximal region of all chromosomes; (ii) two pairs of 35S rDNA sites co-localized with the terminal CMA^{+} bands; and (iii) one pair of 5S rDNA sites located in the proximal region of one chromosomal pair. The genome size of *P. pubescens* was similar to *P. emarginatus* - 1C = 0.665 pg and 1C = 0.620 pg, respectively. The repetitive fraction represented 26.4% of the *P. pubescens* sequenced genome, with Ty3/*gypsy*-Athila (24.24%), Ty3/*gypsy*-Tekay (21.93%), Ty3/*gypsy*-Ogre (6.72%) and Ty1/*copia*-Ale (3.37%) retrotransposons being the most abundant elements. Additionally, low abundance of satellite DNAs were identified: PubSat1-254 (2.09%), PubSat2-76 (2.06%), PubSat3-216 (0.58%), and PubSat4-138 (0.23%). *In situ*

hybridization revealed that all analyzed repeats were enriched in proximal CMA^+ heterochromatin in both species, except the Ale retroelement, which was dispersed also in the euchromatin of *P. pubescens* chromosomes. The cytomolecular similarity observed here shows that the genomes of *Pterodon* species share highly similar repetitive fractions, which corroborates their phylogenetic proximity. However, the Ty1/*copia*-Ale dynamic reinforce the idea that different families of LTR-RTs have independent activity on genomes and chromosomes being a genomic mark that differentiates karyotypes from *P. pubescens* and *P. emarginatus* and corroborate the delimitation of both species.

Keywords: FISH, NGS, satDNA, repetitive sequences, "sucupira branca"

Abbreviations

CMA: Chromomicin A3

Cy3-dUTP: 5-amino-propargyl-2′-deoxyuridine 5′- triphosphate coupled to red cyanine fluorescent dye

DAPI: 4',6-diamidino-2-phenylindole

FISH: Fluorescent *in situ* hybridization

NGS: Next-generation sequencing

rDNA: Ribosomal DNA

TEs: Transposable elements

RT: Retrotransposons

LTR: Long terminal repeat

Introduction

Repetitive DNA sequences represent large fractions of plant nuclear genomes, which can be divided into two large groups (e.g. de Souza et al. 2018; Bolsheva et al. 2019). The first group consists of elements dispersed throughout the genome, called transposable elements (TEs). There are two classes of TEs: retrotransposons (class I) and transposons (class II) (Finnegan 1989; Bourque et al. 2018; Won et al. 2018). Retrotransposons (RTs) are divided into two subclasses; those flanked by long terminal repeats (LTR-RT) and those that may or may not have short terminal repeats (non-LTR-RT). LTR retrotransposons Ty3/*gypsy* and Ty1/*copia* are the most abundant superfamilies in plants nuclear genome (Won et al. 2018; Said et al. 2018; González et al. 2018; Liu et al. 2019; Hloušková et al. 2019). The second repetitive group consists of tandem-organized sequences (as satellite DNA), with copies located adjacent to each other, and predominantly concentrated in functionally important regions in the chromosomes: centromeres, telomeres, and sometimes chromosomal interstitial regions (e.g. Garrido-Ramos 2015; Belyayev et al. 2018; Bolsheva et al. 2019).

Repetitive elements of the genome can modify the karyotype patterns and plant genome through genetic changes generated mainly by the activity of TEs (Oliver et al. 2013; Horváth et al. 2017). In general, the repetitive elements are enriched in heterochromatic regions (González et al. 2018; Li et al. 2019; Samoluk et al. 2019) and it has been observed that TEs expansion have a high impact on genome size and that it can cover half, or even more, of the total amount of nuclear DNA of many plant (Fu et al. 2019), (Schnable et al. 2009), (Oliver et al. 2013) and (Mehra et al. 2015).

In addition to classical cytogenetic parameters (Zoldos et al. 1999; Carvalho and Guerra 2002; Pinto et al. 2016), TEs have been used in comparative cytogenomic studies to understand inter and intraspecific variability in plants (Deng et al. 2019; Tomás et al. 2016; Samoluk et al. 2019 ; Bolsheva et al. 2019; Van-Lume et al. 2019;

Siljak-Yakovlev et al. 2003; Almeida et al. 2007; Viana and Souza 2012; da Costa Silva et al. 2015; Van-Lume et al. 2017; Ortiz et al. 2017; Mata-Sucre et al. 2020).)

Comparative cytogenetic studies have usually been performed using chromosome banding (e.g. C-banding and double staining with the fluorochromes chromomycin A3 [CMA] and 4', 6-diamidino-2-phenylindole [DAPI]) and/or fluorescent *in situ* hybridization (FISH) with ribosomal DNA (rDNA) probes, respectively, evaluating the distribution of heterochromatin and the number and position of ribosomal loci along the chromosomes in plant karyotypes. These techniques are powerful for comparative cytogenetic studies, especially for plant groups with slight differentiation in chromosome morphology and stable chromosome numbers (Siljak-Yakovlev et al. 2003; De Souza Almeida et al. 2007; Viana and Souza 2012; da Costa Silva et al. 2015; Van-Lume et al. 2017; Ortiz et al. 2017; Mata-Sucre et al. 2020). However, many studies involving close related species were not able to distinguish karyotypes by these conventional banding techniques (Zoldos et al. 1999; De Carvalho and Guerra 2002). NGS sequencing technologies has recently emerged as a versatile source of sequencing genomic data, specially enabling the genome skimming approach (Dodsworth et al. 2019). Hence, the advent of genome skimming allowed coupled with the development of bioinformatic analysis (e.g. RepeatExplorer, Novák et al. 2013) has integrated genomics and cytogenetics (cytogenomics) allowing to determine the composition of the repetitive fraction of the genome and the association of repetitive elements with heterochromatin

Pterodon Vogel is a legume genus of the subfamily Papilionoideae which can be distinguished from other members of the tribe Dipterygeae by conspicuous morphological features. Among these, we can highlight the cryptosamara-shaped fruit with oil glands on the epicarp, the vented rachis leaf, the seed testa smooth, the apparent

raphe with the hilum in the lateral position covered by an aryl, and a smooth embryo (Leite et al. 2014; Pinto et al. 2014). The species of the genus presents medicinal potential through the oil extracted from its seeds (Oliveira et al. 2016; Faria et al. 2017; Outuki et al. 2018). The genus is currently composed by only four species: *P. abruptus* (Moric.) Benth, *P. apparicioi* Pedersoli, *P. pubescens* (Benth.) Benth. and *P. emarginatus* Vogel. The last two are not endemic to Brazil, nor Brazilian savannah, but occur in Bolivia and are widely distributed throughout Brazil, occurring in the states of Rondônia, Tocantins, Bahia, Maranhão, Piauí, Goiás, Distrito Federal, Mato Grosso, Mato Grosso do Sul, Minas Gerais and São Paulo (Flora do Brasil 2020, constantly updated).

Despite the easy differentiation when species are blooming, for many years it was difficult to delimit the four species of the genus. Over the years, this genus has taxonomic inconstancies, showing different species numbers varying from six, three and two species depending on the database (Rocha 2006). This taxonomic discrepancy is mostly due to the delimitation of *P. pubescens* and *P. emarginatus*, as they have been considered as one species, with two morphotypes (Rocha 2006). Apparently, there is no hybridization between natural populations of *P. pubescens* (with pink flowers and hairy leaves) and *P. emarginatus* (with purple flowers and glabrous leaves), although three individuals carrying intermediate morphological characteristics were observed (Rocha 2006). The possibility of natural hybridization may be linked to the recent divergence of the species (Lima 2019), and can be better studied with additional tools to classical taxonomy, such as cytogenetics and genomics approaches (see Chase et al. 2003; Chester et al. 2010; Da Silva et al. 2017; Marques et al. 2018; Silva et al. 2018) .

Pterodon has been poorly studied from a cytogenetic point of view. The chromosome number is the only cytogenetic feature described for the species *P.*

pubescens and *Pterodon polygalaeflorus* (Benth.) Benth. (= *P. emarginatus*), both having 2*n* = 16 and presenting small chromosomes (Bandel 1974; Coleman and DeMenezes 1980). Therefore, the two *Pterodon* species, *P. pubescens* and *P. emarginatus*, represent an interesting case to test comparative cytogenomic to evaluate further genomic characteristics that can support the circumscription of both species.

Thus, we aim to analyze the abundance and composition of repetitive elements in *P. pubescens* and perform a comparative cytogenetic analysis between this species and *P. emarginatus*. Here we address three questions: (1) Do the usual cytomolecular techniques (CMA/DAPI banding, 5S and 35S rDNA and genome size) allow distinguishing the two species karyotypes? (2) What kind of repetitive elements are located along the chromosomes of these species? (3) How similar is the heterochromatic composition of these genomes?

Material and Methods

Genome size estimative

The nuclear DNA content of *P. pubescens* e *P. emarginatus* was estimated by flow cytometry. Seeds of *P. pubescens* were collected in Goiânia (LAT -16.577/ LON -49.273, the plant material collected was deposited at the Herbarium of the Federal University of Goiás - Voucher: 61013) and *P. emarginatus* of Alvorada do Norte (LAT -14.586/ LON -46.609), Goiás, Brazil. All seeds were germinated, grown, and maintained in the experimental garden of the Laboratory of Plant Cytogenetics and Evolution, Recife, Pernambuco, Brazil. Young leaves were collected and nuclei suspensions were prepared using the one-step protocol adapted from Doležel et al. (2007). Approximately 25–50 mg of leaf tissue was chopped with a razor blade on a

Petri dish (kept on ice) containing 1 mL of isolation buffer Marie (Marie and Brown 1993). The solution was filtered through a 30 mm mesh filter and mixed with 50 μL propidium iodide (1 mg / mL) for nuclei staining. The absolute DNA amount of a sample was calculated based on the values of the G1 peak means: "[2C sample DNA content (pg)] = [(sample G1 /standard G1)] x [2C standard DNA content (pg)]", where "G1" refers to the mean fluorescence value emitted by nuclei in the G1 stage of interphase and "2C standard" refers to the absolute DNA content of the internal standard used in the measurement (Doležel and Bartoš 2005). Leaves of *Solanum lycopersicum* L. was used as internal standard (Doležel et al. 1998).

Flow cytometry measurements were performed using a Cyflow Space flow cytometer (SYSMEX, Norderstedt, Germany) equipped with a green laser (532 nm). Three independent estimates of DNA using three individuals were performed on different days. Relative fluorescence histograms were analyzed using FloMax 2.3 software (PARTEC, Muster-Germany), and measurements that generated peaks with a coefficient of variation (CV) greater than 5% were discarded.

Repeat Identification and Annotation

The DNA used for Next-Generation Sequencing (NGS) was extracted from leaves of *P. pubescens* collected in Goiânia (LAT -16.577/ LON -49.273, the plant material collected was deposited at the Herbarium of the Federal University of Goiás - Voucher: 61013), using the cetyltrimethylammonium bromide (CTAB) method, described by Doyle and Doyle (1987). NGS of the *P. pubescens* genome was done using Illumina platform. DNA libraries were constructed using Nextera DNA Library prep kit and sequencing was performed on Illumina MiSeq using 600-cycle Miseq Reagent v3 kit (2× 250bp, paired reads). A fasta file containing 3,337,638 reads was used as input in

cluster analysis with the RepeatExplorer pipeline (Novak et al. 2013, http://repeatexplorer.umbr.cas.cz/). In order to identify and classify repetitive sequences in the *P. pubescens* genome, paired reads were analyzed using graph-based comparative clustering. Reads were assigned to the same clusters if they shared 90% similarity in a 55% minimum sequence overlap. The clusters were classified based on similarity search in Viridiplantae version 3.0 database implemented in RepeatExplorer. The TAREAN tool was used to identify and characterize satellite DNA sequences (Novak et al. 2017). Clusters that potentially had satellite DNA sequences were examined manually using the Dot-plot genomic similarity tool in Geneious Prime 2019.2.1 (https://www.geneious.com) (Kearse et al. 2012). Protein domains were identified using NCBI's conserved domain database (Derbyshire et al. 2015), to identify putative TE domains elements.

Primers design, PCR-amplification of satellites DNAs and retrotransposons families

Primers were designed using the Primer 3 tool (Untergasser et al. 2012) included in Geneious version 7.2.1. (Kearse et al. 2012). For satellite DNA, primers were designed based on K-mer information from most conserved regions and in facing towards leaving a maximum of 4-5 bp distance between forward and reverse (Table 1). In addition, three LTR-RT (Ty3/*gypsy*-Tekay, Ty3/*gypsy*-Athila and Ty1/*copia*-Ale) specific primers were designed for the integrase domain. All primers had produced successfully amplified products in PCR reaction (Online Resource 1), and a DNA fragment of expected size from each region were used for sequence confirmation by Sanger sequencing on the ABI-3500 platform (Applied Biosystems).

For the PCR-amplification, the DNA used was extracted from leaves of *P. pubescens* collected in Pontalina (LAT -17.520 / LON -49.444), Goiás, Brazil. The

samples were stored in freezer at -80 °C and subsequently the DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1987). PCR reactions were carried out with final concentration of 20 ng DNA, 0.4 μM forward and reverse primers, 0.1 μM from each dNTPs, 1x PCR buffer, 2 μM $MgCl_2$, 0.025 U/μL Taq polymerase and ultrapure water to complete the final volume of 25 μL. DNA amplification was performed on the Applied Biosystems® Veriti® thermal cycler, which was programmed for PCR reactions of: 94°C for 3 min, 30 × (94°C 1 min, 55°C 1 min, 72°C 1 min) and 72°C for 10 min. For probe synthesis, amplification tests were used to determine the best annealing temperature for *P. pubescens.* The probes were produced and transferred for *P. emarginatus*.

Slide preparation and chromosome banding

Seeds of *P. pubescens* were collected in Goiânia (LAT -16.577/ LON -49.273) and seeds of *P. emarginatus* were collected in Alvorada do Norte (LAT -14.586/ LON -46.609) and Planaltina (LAT -15.600/ LON -47.658), Brasília-DF, Brazil. The material collected in Goiânia and Planaltina were deposited at the Herbarium of the Federal University of Goiás (Voucher: 61013 and 68411, respectively). Seeds of these two species are enclosed by the woody wrap of the fruit and a layer of oily glands. Sowing is most effective when fruits are manually opened, but the germination rate is still low. After removal of this woody wrap, seeds were washed once with a 4% hypochlorite solution for 10 min, and twice with distilled water for 5 min. Root tips obtained from germinated seeds were pretreated with 2 mM 8-hydroxyquinoline for 24 h at 10 °C, fixed in ethanol-acetic acid (3:1 v/v) and stored at -20 °C.

Root tips were digested using a solution containing 2% cellulase and 20% pectinase (w/v) for 120 minutes at 37 °C and mitotic preparations were performed

according to Carvalho and Saraiva (1993). The CMA/DAPI double staining technique was used for fluorochrome banding, following Souza et al. (2015). The slides were stained with CMA (0.1 mg/mL) for 30 min and mounted in DAPI (2 µg/mL): McIlvaine pH 7.0 buffer with glycerol (1:1) and aged for 3 days before analysis in a Leica DMLB epifluorescence microscope. Metaphases cells images were captured with a Cohu CCD video camera using Leica QFISH software and were optimized for better contrast and brightness using Adobe Photoshop CS6.

In situ *hybridization*

PCR products were labeled by *Nick translation* (Invitrogen or Roche Diagnostics) with Cy3-dUTP (GE). Labeled probes were used to locate repetitive elements by FISH technique following the protocol described by Ribeiro et al. (2017). The hybridization mixture consisted of 50% (v/v) formamide, 10% (v/v) dextran sulfate, 2× SSC and 5 ng/mL of each probe. The slides were denatured at 75 °C for 5 min. The final stringency was of approx. 76% and some hybridizations were made with low stringency. Slides were counterstained in 2 µg/mL DAPI in Vectashield (Vector). Images of the best cells were captured as described above. The *Lotus japonicas* (Regel) K. Larsen D2 (Pedrosa et al. 2002) and *Triticum aestivum* pTa71 (Gerlach and Bedbrook 1979) probes, labeled with Cy3-dUTP and digoxigenin-11-dUTP (Roche), were used to locate 5S and 35S rDNA sites, respectively.

Ty1/copia-Ale Sequencing

Since repeat Ty1/*copia*-Ale is the most divergent in the cytogenomic comparison (see Results), a sequencing approach was used to estimate the integrase sequence variability of the Ty1/*copia*-Ale elements in natural populations of *P. emarginatus* and *P.*

pubescens. Sanger sequencing was performed on the ABI-3500 platform (Applied Biosystems) including 19 populations for *P. pubescens* and 11 for *P. emarginatus* [one individual per population was used] encompassing a wide distribution of species (Online Resource 2). Each individual sequence (forward and reverse) was analyzed in software BioEdit version 7.0.5.3 (Hall 1999), then a consensus sequence was assembled for each individual and all sequences were aligned using ClustalW.

Estimating the age of **Pterodon**

To estimate the age of *P. emarginatus* and *P. pubescens* divergence, we performed a molecular phylogeny of the clade Dipterygeae based on the *mat*K gene, the plastid spacer *trn*L–*trn*F, and the ITS (ITS1– 5.8S–ITS2) of nuclear rDNA. We sampled three of the genus *Pterodon*, eight species of the genus *Dipteryx* and as outgroup *Taralea rigida*. All sequences were obtained from GenBank. All the sequences were aligned using MUSCLE (Edgar 2004) as a plugin in Geneious v.9.1.8 (Kearse et al. 2012) with subsequent manual adjustments. We used jModelTest v.2.1.6 to assess the best model of DNA substitution for each individual locus (Darriba et al. 2012) through the Akaike information criterion (Akaike 1974). The best fitting model was GTR for all loci. Phylogenetic relationships were inferred using the BI approach implemented in MrBayes v.3.2.6. (Ronquist et al. 2012). All analyses were performed for each region separately. Four independent runs with four Markov Chain Monte Carlo (MCMC) runs were conducted, sampling every 1000 generations for 3 000 000 generations. Each run was evaluated in Tracer v.1.6 (Rambaut et al. 2014) to determine that the estimated sample size (ESS) for each relevant parameter was >200, and a burn-in of 25 % was applied. The majority rule consensus tree and posterior probability (PP) were visualized and edited in FigTree v.1.4.2 (Rambaut 2014).

Divergence time estimates were performed in BEAST v.1.8.3 (Drummond and Rambaut 2007; Drummond et al. 2012) fixing the tree topology of the Bayesian analyses. An uncorrelated relaxed lognormal clock (Drummond et al. 2006) and a Yule Process speciation model (Gernhard 2008) were applied. Two independent runs of 10,000,000 generations each were performed, sampling every 10,000 generations for consensus *mat*K+ *trn*L–*trn*F+ ITS loci. In order to verify the effective sampling of all parameters and assess the convergence of independent chains, we examined their posterior distributions in Tracer v.1.6, and the MCMC sampling was considered sufficient at an ESS >200. After removing 25% of samples as burn-in, the independent runs were combined and a maximum clade credibility (MCC) tree was constructed using TreeAnnotator v.1.8.2. (Drummond et al. 2012). Calibrations were performed using the secondary calibrations of Kumar et al. (2017) for the *Pterodon*/*Dipteryx* divergence approx. 4.21 million years ago (Mya).

Results

Genome size and characterization of the repetitive genomic fraction of P. pubescens

Considering the genome size of *P. pubescens* as 1C = 650.37 Mbp (0.665 pg) (Online Resource 3) we obtained a 0.2× coverage. A total of 266 clusters, containing 135 to 9,669 reads, and corresponding to at least 0.01% of the genome were used for annotation of moderately to highly abundant repetitive elements. The repetitive fraction represented 26.4% of the total genome sequenced. The analysis revealed that the retrotransposons lineages Ty3/*gypsy*-Athila (24.24%), Ty3/*gypsy*-Tekay (21.93%) and Ty1/*copia*-Ale (3.37%) were the most abundant of Ty3/*gypsy* and Ty1/*copia* superfamilies, respectively (Fig. 1, Table 1). A total of 2.55% of the repetitive fraction was represented by 5S and 35S rDNA. Four clusters showed a characteristic ring graph

pattern (Online Resource 4), which is used to classify the repeat families as DNA satellites. These satellite DNA showed an abundance of 4.96% in the genome and were named PubSat1-254, PubSat2-76, PubSat3-216 and PubSat4-138 (Fig. 1, Table 1).

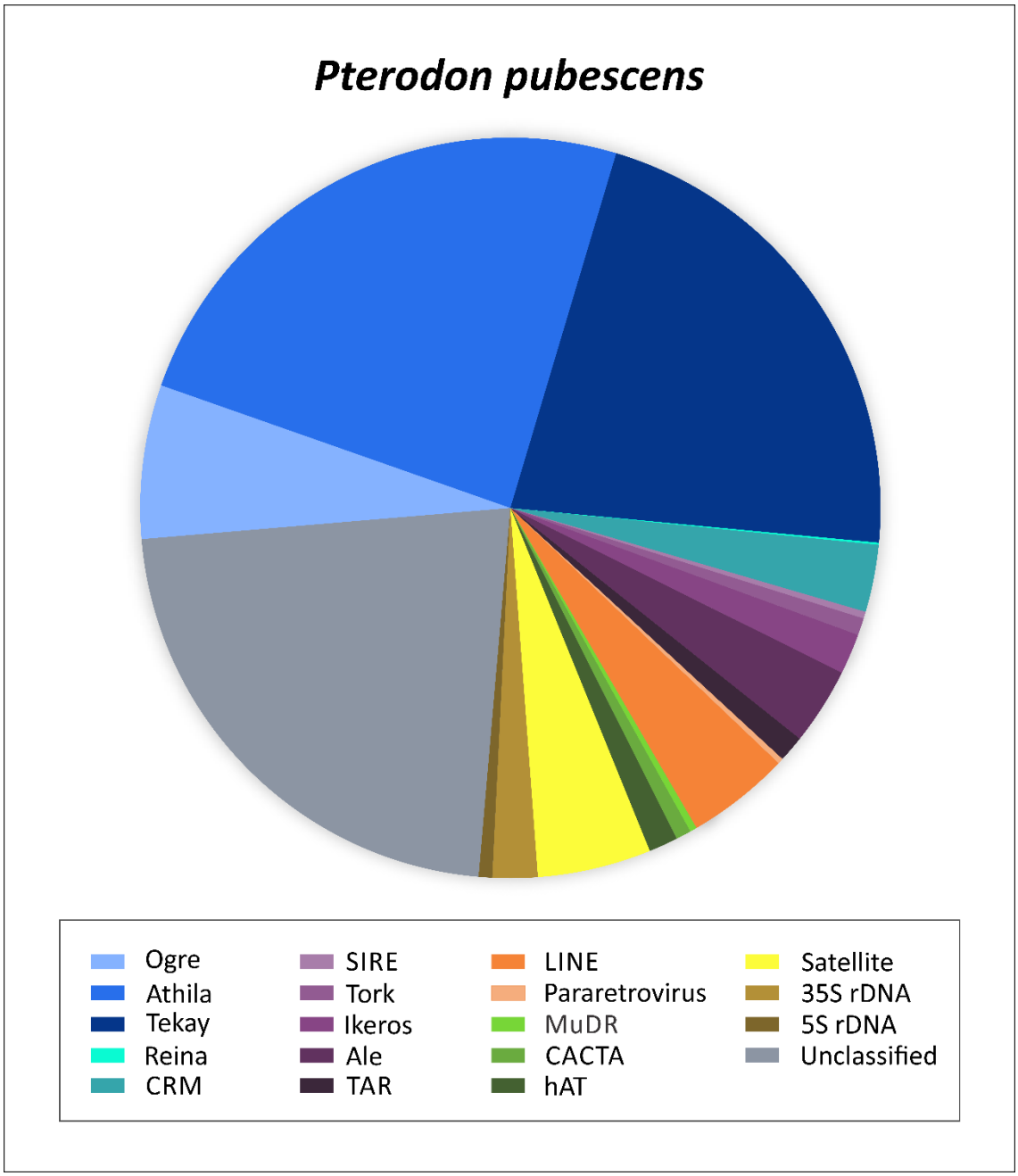

Figure 1: Relative abundances of different kinds of repetitive DNA sequences in the genome of *P. pubescens*, based on the graph-based clustering analysis

Table 1. Information of primers sequences designed for FISH and PCR amplification of Satellite DNAs and TE fragments identified in the genome of *P.pubescens*

Analysis type	Name	Monomer/ FS (pb)	Genome proportion	Classification	Primers Forward/Reverse
TAREAN	PubSat1-254	254	2.09%	Satellite	GGGCGAAAGTTATCAACCTA TGGTTGATAACTGCATGTGA
TAREAN	PubSat2-76	76	2.06%	Satellite	GTTGGGGCTTGAAAAGAAAA CCCCCATTTTCATTGTTCTG
TAREAN	PubSat3-216	216	0.58%	Satellite	ACTTGTGTCTTCTGGTTGAT ACACACCAGTAGCAGAAAAT
TAREAN	PubSat4-138	138	0.23%	Satellite	CCAACCTTCCAAGTAAGCAA TTGCACACACTACAACTTGT
Protein domains tools	Ty3/*gypsy*-Athila	329	24.24%	Ty3/*gypsy*-Athila	CATTTGTCTGAGGGTGGTAA GGGGTATTGATTTCATGGGT
Protein domains tools	Ty3/*gypsy*-Tekay	215	21.93%	Ty3/*gypsy*-Tekay	GTGAAGGCTGAACATCAAAG TGGGCATACTGAGATACACT
Protein domains tools	Ty1/*copia*-Ale	242	3.37%	Ty1/*copia*-Ale	AGCAGAGTCTCCTTTTTCAA CGCAAAATACGAATGGTCTT

FS = fragment size or monomer obtained after PCR for probe used in FISH experiments

Comparative genome size, CMA/DAPI and rDNA mapping

The genome sizes of *P. pubescens* and *P. emarginatus* were similar, with 1C = 0.665 pg and 1C = 0.620 pg, respectively (Online Resource 3). *P. pubescens* and *P. emarginatus* showed 2*n* = 16 with chromosome morphology very similar, predominantly meta/submetacentric. Only one acrocentric pair (VIII) was observed in *P. pubescens* and two (VII and VIII) in *P. emarginatus* (Fig. 2a-b). The $CMA^+/DAPI^-$ bands were observed in the terminal region of two chromosome pairs (I and VIII) and in the pericentromeric regions of all chromosomes (Fig. 2). In *P. pubescens*, the chromosome pairs III and V showed CMA^+ band extending along most of the long and short arms, respectively (Fig. 2a). One pair of 5S rDNA site, located in the proximal region of pair III, and two pairs of 35S rDNA, located in the terminal region of pairs I and VIII, were observed in both species (Fig. 2). This site on pairs I and VIII were colocalized with terminal $CMA^+/DAPI^-$ bands (Fig. 2a-b).

Chromosomal distribution of repeats

The three RT probes (Ty3/*gypsy*-Tekay, Ty3/*gypsy*-Athila and Ty1/*copia*-Ale) were *in situ* hybridized and showed labels in the chromosomes of both species (Fig. 2). In general, the retrotransposons were enriched in centromeric and pericentromeric CMA^+ heterochromatin on most chromosomes (Fig. 2). For instance, in *P. pubescens* and *P. emarginatus*, Ty3/*gypsy*-Tekay signals were concentrated in the proximal region of all chromosomes, with weak labelling on chromosome pair VI, which also showed a weaker CMA^+ band (Fig. 2).

In situ hybridization of Ty3/*gypsy*-Athila revealed small dot-like signals in the proximal CMA^+ heterochromatin (Fig. 2). The short arm of the acrocentric pair VIII

showed a stronger Ty3/*gypsy*-Athila label, revealing a higher richness of this element in this chromosomal pair on both species and colocalized with 35S rDNA signal. In *P. emarginatus*, absence of Ty3/*gypsy*-Athila signals was observed in chromosomal pair VI (Fig. 2b). The chromosomal distribution of Ty1/*copia*-Ale was the most dissimilar between the karyotypes of *P. pubescens* and *P. emarginatus*. As expected based in the chromosomal location of other repeats, *P. emarginatus* showed a Ty1/*copia*-Ale distribution restricted to the centromeric and pericentromeric region of all chromosomes (Fig. 2b). On the other hand, in *P. pubescens* chromosome pairs I, II and VIII were completely labeled, revealing a expansion of the labeling for the euchromatin (Fig. 2a). Chromosomal pairs VI, characterized by low amount of proximal CMA^+ heterochromatin, also showed weak or absent Ty1/*copia*-Ale signals (Fig. 2a). We investigated whether this expansion of Ty1/*copia*-Ale in *P. pubescens* was accompanied sequence diversification. Despite the difference in the distribution of Ty1/*copia*-Ale, the sequencing of the Ty1/*copia*-Ale element in a wide populational sample including individuals of both species did not reveal any polymorphisms, indicating that the expansion of these elements in the *P. pubescens* genomes was not accompanied by incremental sequence-level variability, at least, in the integrase domain. Comparative *in situ* hybridizations have been performed using satellite probes too, and the only two showed signals: DNA PubSat2-76 and PubSat4-138 (Fig. 3). Like RTs, satellite DNA were enriched in CMA^+ pericentromeric heterochromatin in both species (Fig. 3).

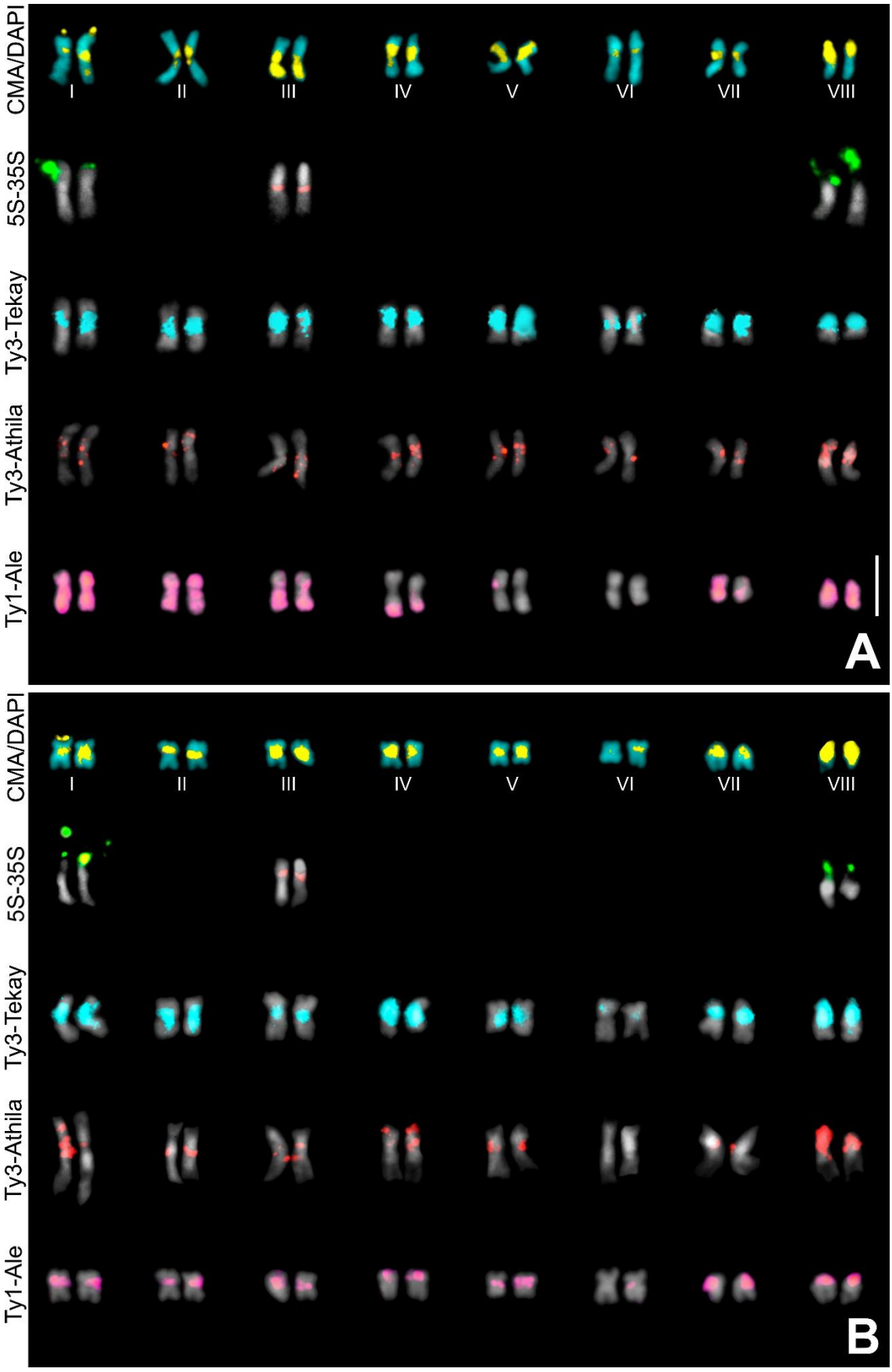
CMA/DAPI
5S-35S
Ty3-Tekay
Ty3-Athila
Ty1-Ale
I
II
III
IV
V
VI
VII
VIII
A
B

Figure 2: Karyograms showing the relative chromosome length, position of centromeres, CMA/DAPI banding, *in situ* hybridization with 5S (red) and 35S (green) rDNA and three retrotransposons (Ty3/*gypsy*-Athila, Ty3/*gypsy*-Tekay, Ty1/*copia*-Ale) probes in *P. pubescens* (A) and *P. emarginatus* (B). Chromosome labelled with different probes are from different metaphase cells. Bar in A represents 5 μm

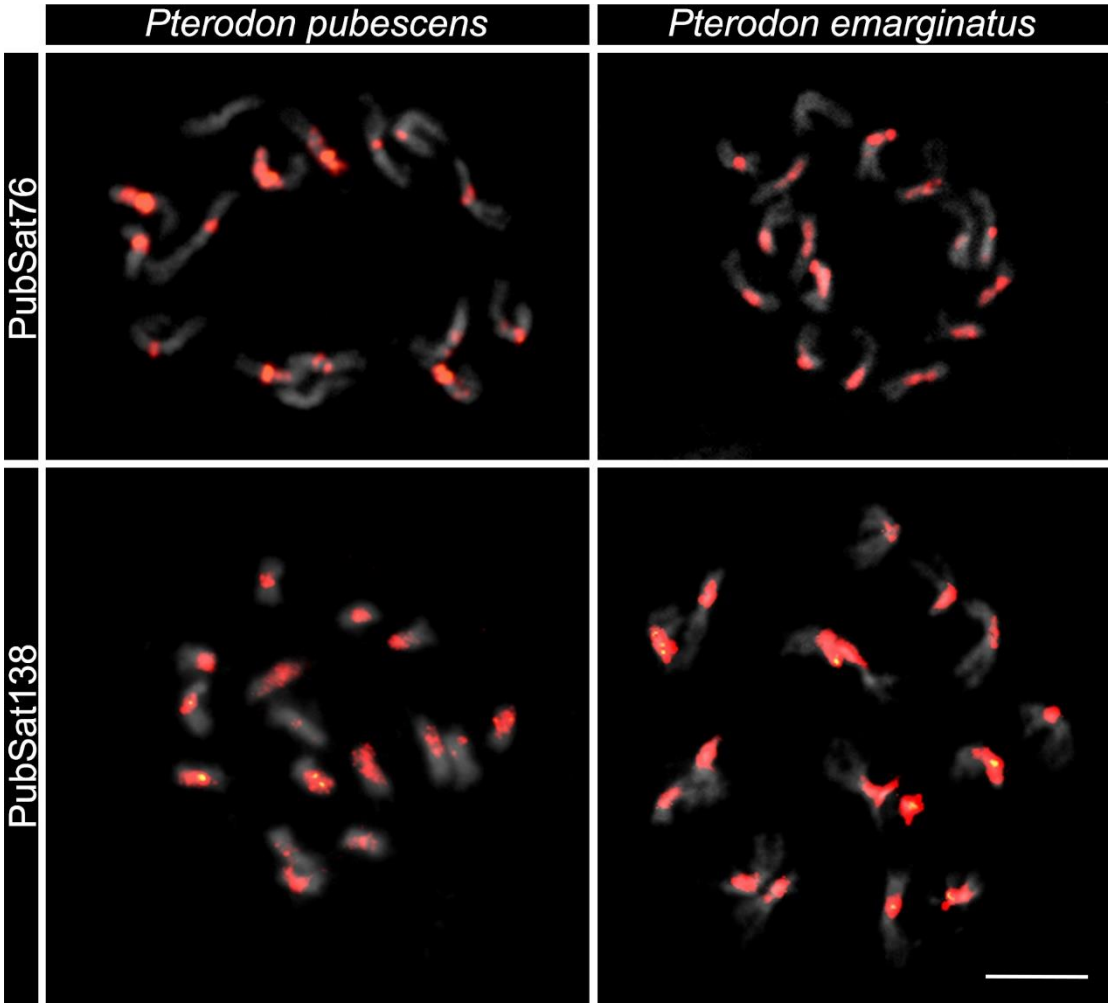

Figure 3: Distribution patterns of DNA satellite PubSat2-76 and PubSat4-138 on *P. pubescens* and *P. emarginatus*. The satellite DNA sequences were labeled with Cy3 (red signal) and the chromosomes were counterstained with DAPI. Bar represents 5 μm

Estimating the age of Pterodon

Because we were interested in having an estimation of the time of diversification of P. pubescens and P. emarginatus. We performed molecular dating with BEAST using ITS

and plastid trnL–trnF regions, with an estimed time divergence at approx.. in 0.905 Mya (0.519 - 1.593 Mya) (Online Resource 5).

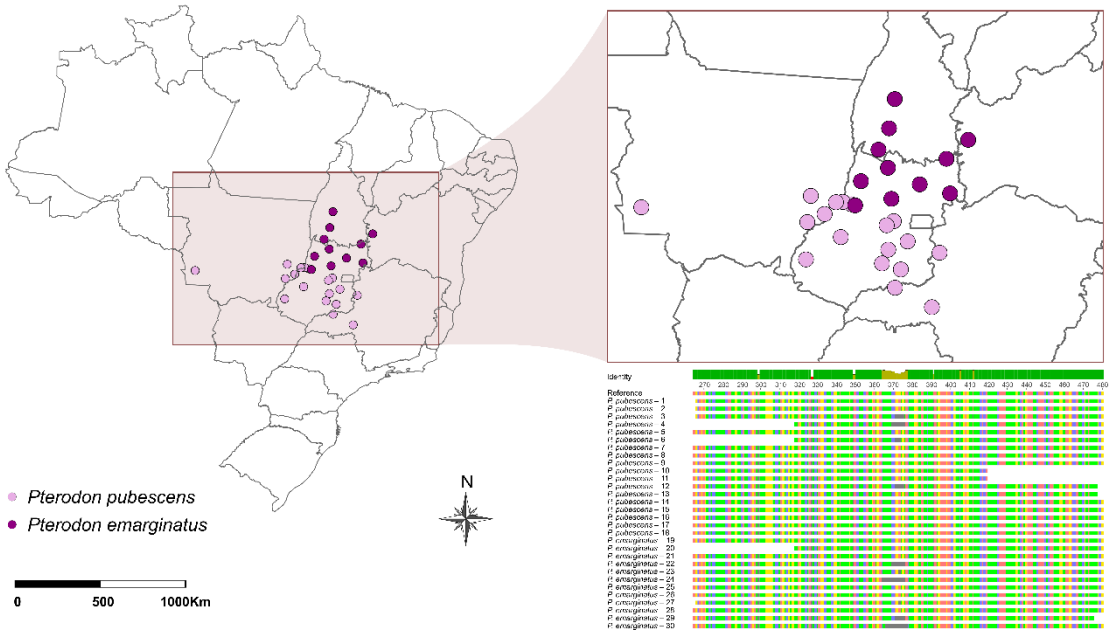

Figure 4 Sanger sequences of integrase region of retrotransposon Ty1/*copia*-Ale from native populations of *Pterodon pubescens* and *P. emarginatus*. Distribution map of the populations of *P. pubescens* and *P. emarginatus*

Discussion

Karyotypic characterization of the clade Dipterygeae

Our results revealed a high karyotype similarity between the analyzed species of the genus *Pterodon*. The previously reported karyotype 2*n* = 16, with small and undifferentiated chromosomes (Bandel 1974; Coleman and DeMenezes 1980) was confirmed here. This same chromosome number was reported also for *Dipteryx alata* Vogel and *D. magnifica* Ducke (Taquary 2017; Madrigal 2018), which together with *Pterodon*, *Monopteryx* Spruce ex Benth. and *Taralea* Aubl. compose the clade Dipterygeae (Cardoso et al. 2013). The genome sizes reported here for *P. pubescens* and *P. emarginatus* (1C = 0.665 pg and 1C = 0.620 pg, respectively) is about half of the

genome size of *P. abruptus* (1C = 1.300 pg, see Madrigal 2018) and can be also an indicative of phylogenetic proximity. This similarity could be also related to the recent diversification of this species, estimated here (Online Resource 5). In fact, *P. pubescens* and *P. emarginatus* seems to present the smallest genome size within the tribe so far, since the genera *Monopteryx* (1C ~ 1.125 pg), *Taralea* (1C ~ 1.255 pg) and *Dipteryx* (1C ~ 1.5605) presented higher values reported (Madrigal 2018). This difference suggests that *P. pubescens* and *P. emarginatus* suffered a strong genomic size reduction through evolutionary process.

The usual cytomolecular parameters do not differentiate* P. pubescens *and* P. emarginatus *karyotypes

Although chromosomal banding and *in situ* hybridization with rDNA probes have proved to be useful in differentiating plant karyotypes (de Souza Almeida et al. 2007; Barros and Silva et al. 2010; Van-Lume et al. 2017), our results do not allow a differentiation between *P. pubescens* and *P. emarginatus* with those markers. This may be related to the phylogenetic proximity of these species (Cardoso et al. 2013). The stability in legume karyotypes can be associated with the timing of diversification, reproduction strategies, and other factors related to the evolutionary history of each group (Soltis and Soltis 2009; Gu et al. 2016; Franco et al. 2019). Genome comparisons have shown conserved syntenic blocks between papilionoid genomes, especially among phylogenetically closely related species (Young and Bharti 2012). Groups of species with a recent diversification usually show evidences of strongly conserved karyotypes (Pinto et al. 2016). This stability of karyotypes found here was also observed in some groups of recent divergence was also identified in other papilionoid groups (Naganowska et al. 2005; Pinto et al. 2016; Susek et al. 2016; Zheng et al. 1993;

Almeida and Pedrosa-Harand 2013; Fonsêca and Pedrosa-Harand 2017; Franco et al. 2019).

***Retrotransposons and satellite DNA are enriched in the proximal heterochromatin of* P. pubescens *and* P. emarginatus**

The advent of NGS technologies has revolutionized cytogenetics, with much impact on the characterization of the heterochromatin (Liu et al. 2019; Bolsheva et al. 2019). The classic view that heterochromatic bands are composed of satellite DNA (Barnes et al. 1985; Pich et al. 1996; Barros and Silva et al. 2010; Kirov et al. 2017) has changed with cytogenomic data, which has revealed that heterochromatin is actually much more complex and heterogeneous in terms of composition (Peška et al. 2019; Hufnagel et al. 2019; Van-Lume et al. 2019). The literature indicates that retrotransposons generally accumulate in blocks or can be found dispersed across plant chromosomes, unlike satellite DNAs that form more defined blocks (Heslop-Harrison and Schmidt 2012; Santos et al. 2015; Ribeiro et al. 2017; Zhang et al. 2017; de Souza et al. 2018). We identified here by FISH the presence of three RTs (Ty3/*gypsy*-Tekay, Ty3/*gypsy*-Athila and Ty1/*copia*-Ale), two satellite DNA and 5S rDNA in the proximal heterochromatin of *P. pubescens* and *P. emarginatus*.

Concerning the RTs, the occurrence of Ty3/*gypsy* elements in the centromeric and pericentromeric regions of plant chromosomes has often been reported, particularly from those containing chromodomains (Neumann et al. 2011, 2019). A recent phylogenetic analysis of the protein-coding regions of LTR retrotransposons showed that such chromovirus-type Ty3/*gypsy* elements could be subdivided into six clades named Chlamyvir, Tcn1, Tekay, Reina, CRM and Galadriel, each one distinct from the remaining Ty3/*gypsy* retrotransposons that lacked chromodomains (e.g. Ogre, Tat and

Athila elements) (Hřibová et al. 2010; Neumann et al. 2019). Comprehensive analysis of the chromovirus elements has shown that genuinely centromeric-targeting retrotransposons actually represent only a fraction of the CRM lineage and are characterized by a distinctive type of chromodomain (CHDCR, Neumann et al. 2011, 2019). In contrast, Ty3/*gypsy*-Tekay elements have been shown to contain the more widespread CRD-type chromodomains previously classified as Type II by Neumann et al. (2011) and Gao et al. (2008). The presence of the chromodomain likely plays a role in targeting the insertion of Ty3/*gypsy* retrotransposons predominantly into heterochromatin since it can recognize and bind to methylated histone H3K9 (Jacobs and Khorasanizadeh 2002; Nielsen et al. 2002) and H3K27 (Fischle et al. 2003; Min et al. 2003) residues which are common in heterochromatic regions of the DNA (Lin et al. 2008; Gao et al. 2008). Certainly, our data show that Ty3/*gypsy*-Tekay retrotransposon are enriched in the pericentromeric region associated with a $CMA^{+/}DAPI^{-}$ heterochromatin bands, as observed in other legumes such as Caesalpinia group species (Van-Lume et al. 2019).

Previous studies have shown that heterochromatic bands can be composed of different types of repeats, although typically satellite DNA repeats are the most common (Barros and Silva et al. 2010; Samoluk et al. 2017; Zakrzewski and Schmidt 2017). Indeed, studies have shown centromeric and pericentromeric heterochromatin composed by combination of satellite DNA and retrotransposon, as reported here (Cheng et al. 2002; Mizuno et al. 2018; Van-Lume et al. 2019; Báez et al. 2019; Ribeiro et al. 2019). The results reported herein, reinforce the association between retrotransposons and heterochromatic CMA^{+} bands visible on *Pterodon* chromosomes.

Cytogenomic mapping of RTs Ty1/copia-Ale allows differentiation of* P. pubescens *and* P. emarginatus *genomes

The only repetitive element that presented clearly divergent chromosomal distribution between *P. pubescens* and *P. emarginatus* was Ty1/*copia*-Ale. Given the conserved distribution of all repeats analyzed in the proximal CMA^+ heterochromatin, our data suggest that there was an expansion of the Ty1/*copia*-Ale elements, which became dispersed in some chromosomal pairs of *P. pubescens*.

The remarkable stability at integrase sequence level in our comparative analysis of natural populations suggests that this expansion of the Ty1/*copia*-Ale elements in the *P. pubescens* genome was a recent event, considering that the genus *Pterodon* originated 4.21 Mya (Särkinen et al. 2012) (see Online Resource 5). Alternatively, the sequencing strategy used here (direct sequencing of PCR products) may be omitting a putative variability in these elements (see alignment of the sequences of retrotransposon Ty1/*copia*-Ale on Fig. 4). TEs expansion, detectable at chromosome level, has also been reported for *copia* and *gypsy* LTR-RT in species of the genus *Eleocharis* R. Br. (Cyperaceae) (de Souza et al. 2018). Herein, retrotransposons proliferated throughout the chromatin of only a few chromosomal pairs in *P. pubescens*. Probably, this difference between species is caused by such factors as the potential independent activity of each RT family in each genome, epigenetic controls in different genomes or chromosomal regions and the influence of neighboring sequences of other natures (de Souza et al. 2018). Our FISH results reinforce the idea that different families of LTR-RTs have independent activity on genomes and chromosomes, with different evolutionary histories and fates, being a genomic mark that differentiates karyotypes from *P. pubescens* and *P. emarginatus*.

Conclusions

The cytomolecular similarity observed here suggests that the genomes of *P. pubescens* and *P. emarginatus* have highly similar distribution of abundant repeats, which corroborates their phylogenetic proximity. *In situ* hybridization revealed that all repeats (RT and satellite DNA) were present in the proximal heterochromatin of the two species. The exception was the Ty1/*copia*-Ale retroelement, which was scattered across some chromosomal pairs of *P. pubescens*. The Ty1/*copia*-Ale results reinforce the idea that different families of LTR-RTs have independent activity on genomes and chromosomes, with different evolutionary histories and fates, being a genomic mark that differentiates karyotypes from *P. pubescens* and *P. emarginatus* and corroborate the delimitation of both species.

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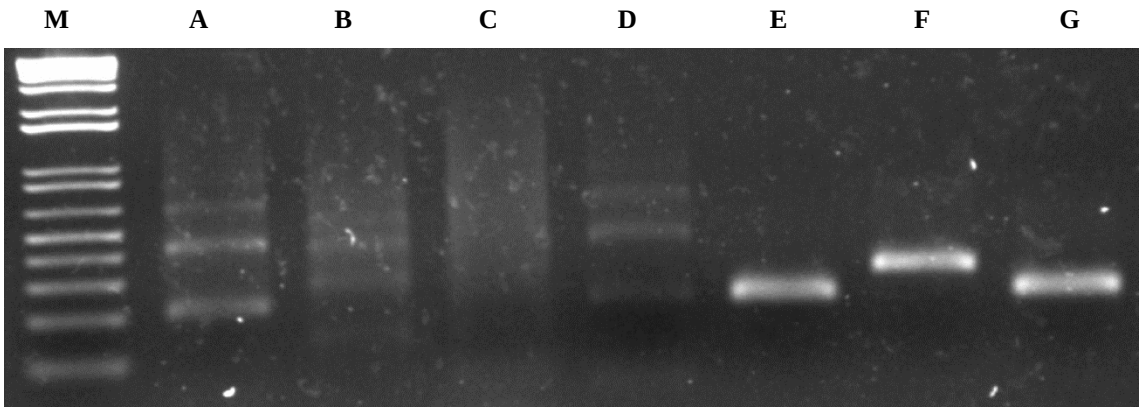
Supplementary material

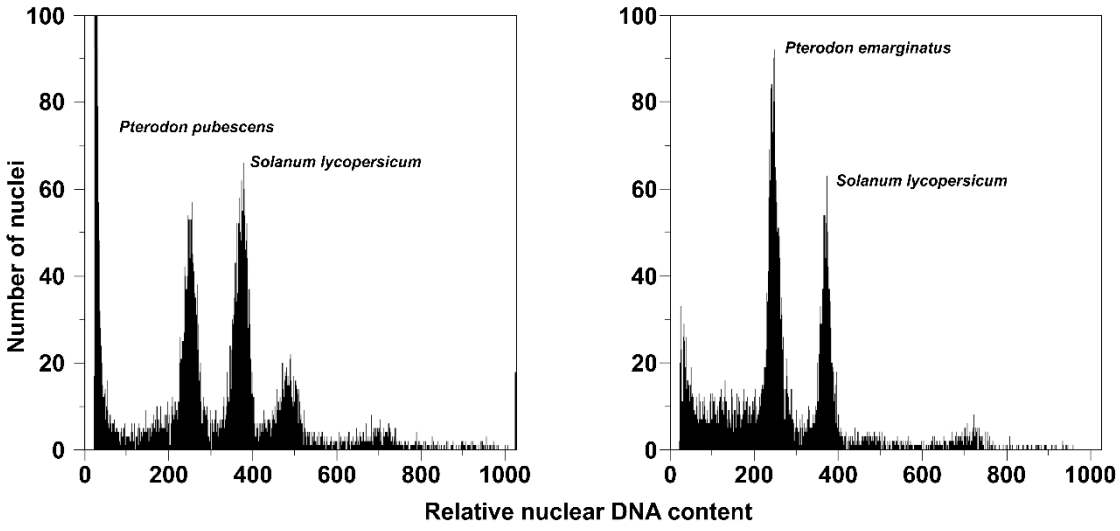


Online Resource 1 1% agarose gel showing the amplification of repetitive regions in *P. pubescens* (A) PubSat1-254, (B) PubSat4-138, (C) PubSat2-76, (K) PubSat3-216, (K) Ty1-Ale, (D) Ty3-Athila and (E) Ty3-Tekay

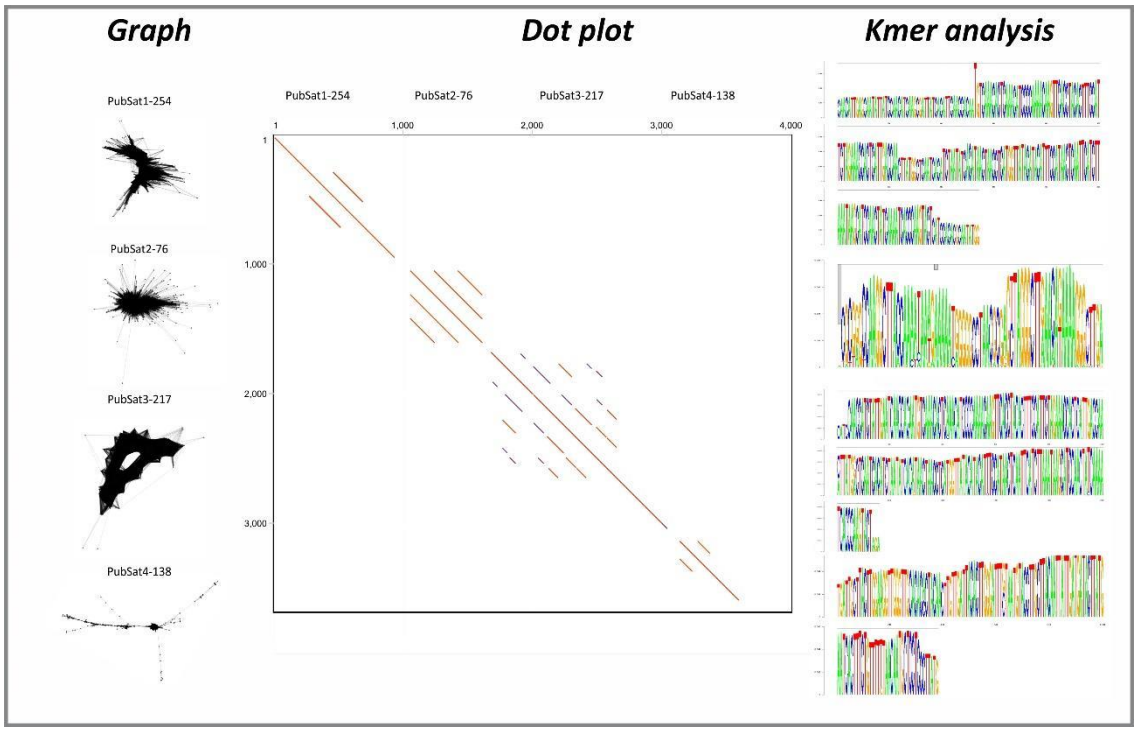
Online Resource 2 Information about the collected populations of *Pterodon pubescens* and *P. emarginatus* for Sanger sequences of integrase region of retrotransposon Ty1/*copia*-Ale, with geographical coordinates

Nº	Longitude	Latitude	Locality	Population Code	Species
1	-46,609800	-14,586420	Alvorada do Norte-GO	PeANOGO	*P. emarginatus*
2	-47,874230	-14,206910	Alto Paraíso-GO	PeAPAGO	*P. emarginatus*
3	-50,562940	-15,087270	Araguapaz-GO	PeAPZGO	*P. emarginatus*
4	-49,589200	-12,754200	Araguaçu-TO	PeARATO	*P. emarginatus*
5	-49,151080	-11,859240	Cariri-TO	PeCARTO	*P. emarginatus*
6	-45,855530	-12,339770	Luiz Eduardo-BA	PeLEMBA	*P. emarginatus*
7	-46,762120	-13,137970	Monte Alegre de Goiás-GO	PeMAGGO	*P. emarginatus*
8	-50,318970	-14,072610	Nova Crixás-GO	PeNCRGO	*P. emarginatus*
9	-48,911850	-10,634870	Nova Rosalândia-TO	PeNROTO	*P. emarginatus*
10	-49,195760	-13,514030	Porangatu-GO	PePORGO	*P. emarginatus*
11	-49,051850	-14,813040	Santa Rita do Novo Destino-GO	PeSRNGO	*P. emarginatus*
12	-50,721170	-15,040440	Araguapaz-GO	PpAPZGO	*P. pubescens*
13	-51,834090	-15,451030	Araguaiana-MT	PpARGMT	*P. pubescens*

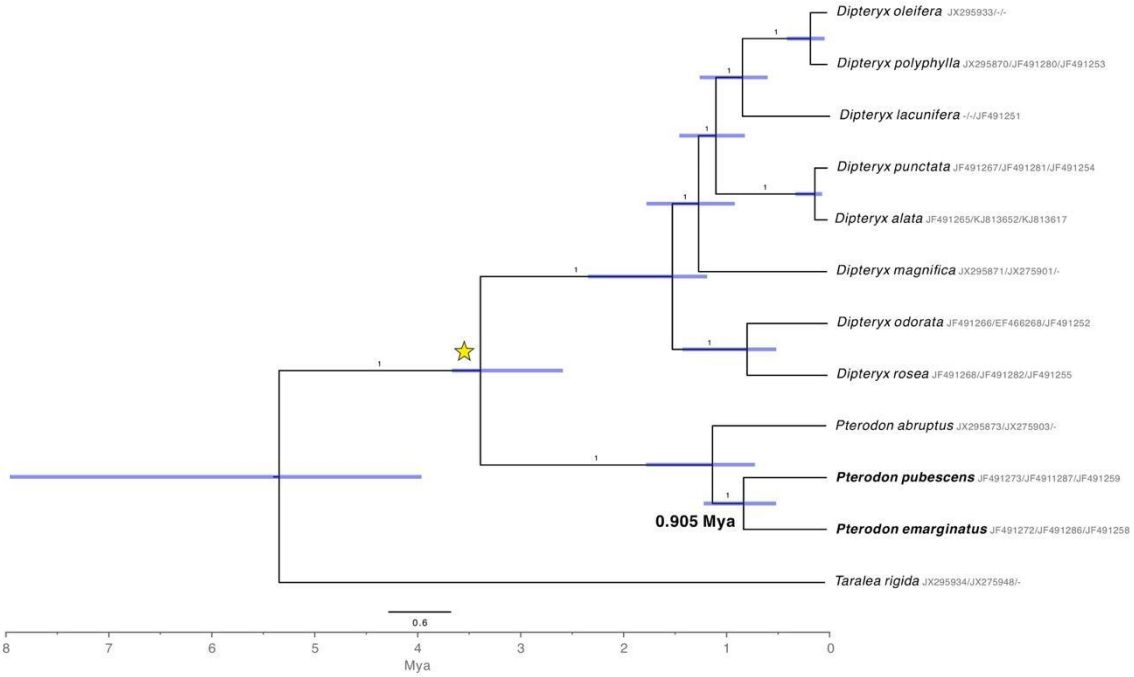
14	-51,078160	-14,940360	Aruanã-GO	PpARUGO	*P. pubescens*
15	-52,553110	-15,789990	Barra do Graças-MT	PpBAGMT	*P. pubescens*
16	-48.625278	-17.745278	Caldas Novas	PpCALGO	*P. pubescens*
17	-51,360290	-14,954970	Cocalinho-MT	PpCOCMT	*P. pubescens*
18	-49,183850	-16,94287	Hidrolândia-GO	PpHIDGO	*P. pubescens*
19	-51,157240	-16,416140	Ipora-GO	PpIPOGO	*P. pubescens*
20	-52,405612	-14,687747	Nova Xavantina-MT	PpNOXMT	*P. pubescens*
21	-47,366580	-19,350770	Perdizes-MG	PpPERMG	*P. pubescens*
22	-48,955280	-15,748170	Pirenópolis-GO	PpPIRGO	*P. pubescens*
23	-49,444200	-17,520200	Pontalina-GO	PpPONGO	*P. pubescens*
24	-52,607767	-17,350414	Portelândia-GO	PpPOTGO	*P. pubescens*
25	-47,045680	-17,069150	Paracatu-MG	PpPTUMG	*P. pubescens*
26	-48,660000	-17,772500	Serra de Caldas-GO	PpSECGO	*P. pubescens*
27	-49,251530	-15,920950	São Francisco de Goiás-GO	PpSFRGO	*P. pubescens*
28	-48,378230	-16,597670	Silvania-GO	PpSILGO	*P. pubescens*
29	-48,900820	-18,537490	Tupaciagura-MG	PpTUPMG	*P. pubescens*
30	-59,460050	-15,167570	Pontes e Lacerda-MT	PePOLMT	*P. pubescens*



Online Resource 3 Histograms of relative fluorescence intensities obtained by analyzing isolated nuclei from *Pterodon pubescens* — 1C = 0.665 pg DNA (left histogram) and, *Pterodon emarginatus* — 1C = 0.620 pg DNA (right histogram) and the internal reference standard *(Solanum lycopersicum* — 1C = 0.980 pg DNA)



Online Resource 4 Characterization of the putative satellite DNA repeats identified in *Pterodon pubescens* genome. (a) Graph layouts derived from RepeatExplorer output, (b) Dot-plot similarity comparison of all repeat sequences against themselves, showing that satellite DNA sequences from different clusters do not share similarity and (c) Monomer reconstruction using base frequency logo representation (WebLogo 3; http://weblogo.threeplusone.com)



Online Resource 5 Chronogram of Dipterygeae, with focus on the age of *Pterodon* species, based on BEAST analysis using nuclear and plastid consensus topology. Blue bars indicate 95 % highest posterior density intervals. GenBank accession numbers are given as grey letters on the side of each sample name in order: *mat*K/ *trn*L-*trn*F/ ITS

Considerações finais

- O tamanho do genoma de *P. pubescens* e *P. emarginatus* foram semelhantes.

- A fração repetitiva representou 26,4% do genoma obtido de *P. pubescens*, sendo as famílias Ty3-*gypsy* e Ty1-*copia* mais abundantes.

- As espécies analisadas apresentaram bandas heterocromáticas muito similares, concentrando-se principalmente na região pericentromérica de todos os cromossomos.

- As sondas de três retrotransposons do tipo LTR e de quatro DNA satélites foram desenvolvidas e transferidas para *P. emarginatus*. Porém apenas duas sondas de DNA satélites apresentaram sinal no experimento de FISH (PubSat2-76 e PubSat4-138).

- A hibridização *in situ* revelou que todas as sequências repetitivas analisadas estão colocalizadas com as bandas CMA^{+} pericentroméricas em ambas espécies, exceto o retroelemento Ty1-Ale, que também está disperso na eucromatina de quatro pares de cromossomos (I, II, III e VIII) de *P. pubescens*.

- A similaridade citomolecular observada aqui sugere que os genomas de *P. pubescens* e *P. emarginatus* possuem frações repetitivas altamente semelhantes.

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