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Julia Zappelini

Evolução molecular em Bambusoideae Luerss. (Poaceae Barnhart): taxonomia molecular e caracterização de genoma plastidial de espécies nativas.

Florianópolis
2022

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Orientador: Prof. Miguel Pedro Guerra, Dr.

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O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Prof. Miguel Pedro Guerra, Dr.
Orientador

Florianópolis, 2022.

Dedico este trabalho aos meus queridos pais, Aldo Cesar (*in memoriam*) e Norma, e às minhas parceiras de vida, Alessandra e Ana Paula.

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Eu dedico este trabalho, e agradeço à minha família de origem, meus pais, Aldo Cesar e Norma, e minhas irmãs, Alessandra e Ana Paula, pelo incentivo ao meu desenvolvimento ao longo da minha trajetória. Obrigada por acreditarem em todos os meus sonhos, sejam eles quais forem.

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“Precisamos, entretanto, dar sentido humano às nossas construções. E, quando o amor ao dinheiro, ao sucesso nos estiver deixando cegos, saibamos fazer pausas para olhar os lírios do campo e as aves do céu.” (Erico Veríssimo).

RESUMO EXPANDIDO

INTRODUÇÃO

A subfamília Bambusoideae (Poaceae) são gramíneas perenes de rápido crescimento, e reconhecidas como a única linhagem da família com diversificação em hábitats florestais, com mais de 1680 espécies distribuídas entre as tribos Olyreae (herbáceos), Arundinarieae (lignificados de clima temperado) e Bambuseae (lignificados de clima tropical). Os bambus desempenham papéis socioeconômicos em escala mundial, e que acompanhou o desenvolvimento de civilizações ancestrais como relevantes recursos florestais não-madeireiros. Através dos efeitos de manutenção do solo e interação com outras espécies de organismos, os bambus são essenciais componentes para a modulação e estruturação florestal. O Brasil abriga uma das maiores taxas de diversidade de espécies de bambus, cuja ocorrência preferencial acontece nos ameaçados domínios Mata Atlântica e Amazônia. A identificação de espécies, que acompanha a taxonomia clássica, é um dos principais desafios no estudo dos bambus, especialmente devido às raras descrições de eventos de floração e variabilidade morfológica numa mesma espécie. Ainda, considerando a diversidade específica dos bambus no Brasil e o grau de ameaça aos seus principais domínios de ocorrência, a identificação de regiões genômicas altamente polimórficas são ferramentas essenciais para a inferência evolutiva deste grupo pouco estudado.

OBJETIVOS

A presente tese tem como objetivo caracterizar padrões evolutivos na subfamília Bambusoideae em larga escala, com ênfase na identificação de espécies por DNA *barcoding*, bem como pela obtenção de sequências completas de genomas plastidiais e identificação de regiões genômicas altamente informativas.

METODOLOGIA

No Capítulo 1 da presente tese, foram testadas cinco regiões do genoma plastidial candidatas à barcode em Bambusoideae – *rbcL*, *matK*, *psbK-psbI*, *rpl32-trnL* e *trnH-psbA*. O grau de variabilidade nucleotídica e poder discriminatório a nível de espécie foi testado em larga escala pela abordagem filogenética, foi avaliado individualmente e em conjunto. No Capítulo 2, foram obtidas as sequências completas do genoma plastidial de três espécies neotropicais – *Guadua trinii*, *Parodiolyra micrantha* e *Apoclada simplex* – cujos padrões evolutivos e regiões polimórficas foram identificados dentre os respectivos grupos.

RESULTADOS e DISCUSSÃO

No Capítulo I, foram geradas 365 novas sequências com 100% de sucesso de amplificação e alta qualidade de sequenciamento, atendendo aos princípios de minimalismo e escalabilidade. As regiões *psbK-psbI* e *rpl32-trnL* apresentaram maiores taxas de variabilidade nucleotídica e, consequentemente, potencial de discriminação. As topologias Bayesianas obtidas não demonstraram alto grau de resolução em nível de espécie para Bambusoideae. Esta abordagem de avaliação

clássica, considerando o critério de monofilia recíproca, não considera a complexidade evolutiva do grupo estudado. No capítulo II, foram geradas as sequências completas e inéditas de três plastomas de bambus, os quais se mostraram os menores já descritos para a subfamília. Foram identificadas regiões de alta variabilidade nucleotídica, as quais podem ser empregadas na identificação de marcadores moleculares e como ferramentas de inferências evolutivas e para conservação. A conservada ordem e conteúdo gênico, em comparação aos representantes das suas respectivas subtribos, sugerem que as variações no tamanho genômico sejam decorrentes de eventos de inserção/deleção de nucleotídeos nas regiões intergênicas. Regiões de alta taxa de variabilidade nucleotídica foram identificadas nas sequências inéditas dos plastomas das três espécies, localizadas especialmente em regiões intergênicas. As informações obtidas se mostram importantes ferramentas para aplicação em análises filogenéticas e inferências evolutivas para a pouco estudada linhagem neotropical da subfamília Bambusoideae.

CONSIDERAÇÕES FINAIS

A presente tese é resultado do projeto multidisciplinar e interinstitucional Tecnologias para o Desenvolvimento Sustentável da Cadeia Produtiva do Bambu no Sul do Brasil" (Chamada MCTI/AÇÃO TRANSVERSAL/CNPq N.º 66/2013). A avaliação de regiões candidatas a barcode em Bambusoideae traz a luz a aplicação desta técnica em uma ampla escala amostral, devendo considerar, essencialmente, o padrão evolutivo do grupo estudado, trazendo frágeis aplicabilidades em grupos taxonômicos complexos como Bambusoideae. Dada a grande contribuição do Brasil na distribuição dos bambus, o sequenciamento e a montagem do genoma plastidial de três espécies neotropicais nativas do Brasil permitiu a identificação de padrões evolutivos conservados, como ordem e número gênico, apesar da variação de tamanho total observada, bem como de regiões altamente polimórficas e informativas. A execução das atividades referentes à presente tese proporcionou o fortalecimento do tema de pesquisa no Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal (LFDGV, UFSC), e desenvolvimento de trabalhos futuros para a compreensão dos processos evolutivos da subfamília Bambusoideae.

Palavras-chave: Bambus, cpDNA, diversidade genética, DNA *barcoding*, plastoma.

ABSTRACT

Bambusoideae subfamily represents one of the most diverse among Poeaceae family, with remarkable diversification in forest habitat and worldwide distribution. More than 1680 bamboo species are currently distributed in Olyreae (herbaceous bamboos), Arundinarieae (temperate woody bamboos) and Bambuseae (tropical woody bamboos), which socioeconomic and ecological functions are undoubtably recognized. Bamboo diversification, reflected at complex life cycle and morphological patterns, is considered as result of evolutionary events as polyploidy and hybridization preceding the radiation of current lineages. Considering the evolutionary complexity which encounters the subfamily, classical taxonomy is one of the most challenging tasks in bamboos, especially due to the rare description of flowering events. Then it's proposed, at chapter 1 on the present thesis, the test of five candidate plastidial genome regions as DNA barcode, rapid sequencing technique of informative genomic regions for species discrimination. However, the classical evaluation approach, which considers reciprocal monophyly as taxonomic criteria, don't consider the evolutionary complexity of the group, weakening the discriminatory power of the technique applied to bamboos in a large scale. Regarding the distribution of global diversity, Brazil harbors the greatest diversity and endemism degree of neotropical species (woody and herbaceous), occurring preferentially at Atlantic Forest and Amazonia domains. Sequencing of plastidial genomes is an important tool for evolutionary inferences, especially for the less studies ones as neotropical bamboos. Chapter 2 of the present document addresses sequencing data of three bamboo species native to Brazil – *Apoclada simplex*, *Guadua trinii* and *Parodiolyra micrantha*, along with comparative analysis with available plastomes of the corresponding subtribes. Despite being the smallest sequenced plastomes for bamboos, the preserved gene order and content suggests that genome size variation is due to insertion/deletion of bases in intergenic regions. Hypervariable regions were identified in the three new sequenced plastomes, which may contribute to increase resolution of phylogenetic inferences and to species identification of Neotropical bamboos.

Keywords: Bamboos, cpDNA, genetic diversity, DNA barcoding, plastome.

LISTA DE FIGURAS

Capítulo 1.

Guadua trinii. Red peaks represent nucleotide diversity per 500 bp. (S) Total segregating sites. Numbered peaks indicates (1) *ndhF–rpl32–trnL(UAG)* (π = 0.01181; S = 29); (2) *ndhK–trnV(UAC)* intergenic region (π = 0.01086; S = 16), (3) *psbK–psbI* IGS (π = 0.0099; S = 12), (4) *rbcL–psaI* (π = 0.00971; S = 10), (5) *trnG(UCC-trnT(GGU)* IGS (π = 0.00933; S = 10); (6) *rpl19–psbA* (π = 0.000857; S = 6). 95

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LISTA DE ABREVEATURAS E SIGLAS

BI – Inferência Bayesiana, do inglês "*Bayesian inference*"

cpDNA – genoma plastidial, do inglês "chloroplast DNA"

CRL – do inglês "*contiguous read length*"

dNTP – Desoxirribonucleotídeos fosfatados, do inglês "*deoxynucleotide triphosphates*"

EDTA – do inglês "*ethylenediamine tetraacetic acid*"

ILS – do ingles *"incomplete lineage sorting*"

IR – regiões invertidas do inglês "*inverted repeats*"

K2P – modelo de evolução molecular "*Kimura-2-parameters*"

LCB – blocos localmente colineares, do inglês "*locally collinear blocks*"

LSC – do inglês "*large single copy*"

MCMC – Cadeias de Markov e Monte Carlo, do inglês "*Monte Carlo Markov chains*"

NJ – do inglês, "*Neighbor-Joining*"

PCR – Reação em cadeia de polymerase, do inglês "*polymerase chain reaction*"

PEG8000 – polietilenoglicol, do inglês "*polyethylene glycol*"

PIC – Sítios parcimoniosamente informativos

PP – Probabilidade posterior, do inglês "*Posterior probabilities*"

QV – do inglês "*quality value*"

SSC – do inglês "*small Ssingle copy*"

TS – do inglês "*trace score*"

SUMÁRIO

1. ANTECEDENTES E JUSTIFICATIVA

A presente tese é um produto do projeto multidisciplinar e interinstitucional denominado "Tecnologias para o Desenvolvimento Sustentável da Cadeia Produtiva do Bambu no Sul do Brasil" (Chamada MCTI/AÇÃO TRANSVERSAL/CNPq N.º 66/2013)", com o objetivo de identificar e superar gargalos científicos e tecnológicos, sob a óptica de biotecnologias, limitantes ao desenvolvimento sustentável da cadeia produtiva do bambu no sul do Brasil. Abordando questões evolutivas de espécies nativas de bambus, as análises foram realizadas no Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal (LFDGV/UFSC) sob orientação do Prof. Dr. Miguel Pedro Guerra, com a colaboração do Prof. Dr. Valdir Stefenon (LFDGV/UFSC), do Prof. Dr. Emanuel Maltempi de Souza e da Profª Drª Leila do Nascimento da Universidade Federal do Paraná. Ainda, a execução das coletas de materiais nativos contou com a colaboração da Associação Catarinense do Bambu – BambuSC.

Visando a identificação de padrões evolutivos, a tese contempla duas abordagens distintas, sendo elas: *i*) Avaliação da aplicabilidade de regiões genômicas para taxonomia molecular (DNA *barcoding*) em Bambusoideae, e; *ii*) Estrutura e análise comparativa do genoma plastidial de espécies nativas de bambu. Portanto, pelas análises comparativas de evolução molecular, a presente tese buscou avançar no conhecimento científico associado à compreensão e distribuição da diversidade genética dos bambus.

1.1 A subfamília Bambusoideae: descrição, ocorrência e história evolutiva

Os bambus (Bambusoideae, Poaceae) são gramíneas perenes e de rápido crescimento, reconhecidos como a única linhagem da família com ampla diversificação em hábitats florestais (BPG 2012; Clark et al. 2015). Formando o clado BEP da família Poaceae (Bambusoideae, Ehrhartoideae e Pooideae), a subfamília Bambusoideae representa a terceira mais diversa dentre as gramíneas, agrupando 127 gêneros e 1680 espécies descritas distribuídas numa reconhecida linhagem monofilética (GPWG II 2012; Soreng et al. 2017; Clark & Oliveira 2018). Além de dados moleculares, sua monofilia é sustentada por sinapomorfias morfológicas, como a presença de células braciformes fortemente invaginadas e assimétricas no mesófilo foliar (Kelchner & BPG 2013; Leandro et al. 2016).

Essa subfamília está distribuída em três tribos monofiléticas, representando as linhagens dos bambus herbáceos (tribo Olyreae), lignificados de clima temperado (tribo Arundinarieae) e lignificados de clima tropical (tribo Bambuseae) (Sungkaew et al. 2009; Kelchner & BPG 2013). Com ocorrência natural em todos os continentes, exceto Europa e Antártica, os bambus ocorrem numa ampla gama de hábitats, incluindo campos graminosos e sub-bosques de florestas tropicais e temperadas de até 4.000 m de altitude, onde tendem a formar estandes florestais dominantes (Figura 1) (Judziewicz et al. 1999; BPG 2012; Clark et al. 2015).

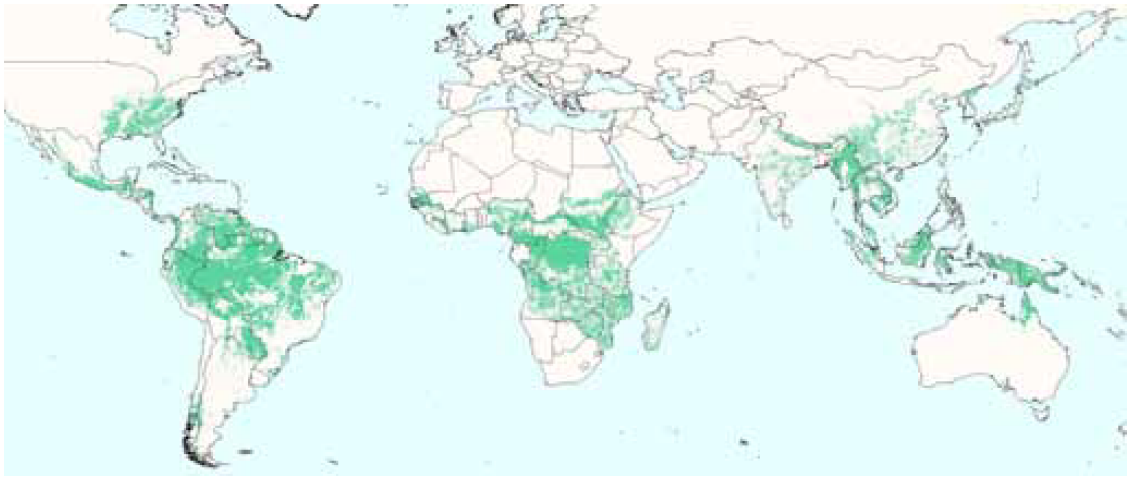

Figura 1. Potencial distribuição geográfica da subfamília Bambusoideae (Poaceae) dentro da cobertura florestal mundial remanescente (Adaptado de INBAR 2014).

Conhecidos como o "ouro verde" ou a "madeira dos pobres", os bambus acompanharam o desenvolvimento da humanidade, sendo considerados um dos mais antigos materiais estruturais utilizados pelas civilizações (Vorontsova et al. 2016; Emamverdian et al. 2020). Mais de 1.500 usos são atribuídos aos bambus, seja de maneira direta, como alimentação, artesanatos e construção civil, ou indireta, por serviços ecossistêmicos associados (Akinlabi 2017; Kumar et al. 2017; Singh et al. 2020). Como importante produto florestal não-madeireiro, processos e tecnologias industriais vêm sendo desenvolvidas para o uso dos bambus como substitutos a madeira das mais diversas formas, como fonte de fibras e celulose, na produção de biocompósitos e bioenergia (Sharma et al. 2015; van Dam et al. 2018; Muhammad et al. 2019; El-Sayed et al. 2020; Emamverdian et al. 2020).

A variação nas características morfológicas e ecofisiológicas observadas dentro da subfamília se reflete na plasticidade de adaptação em diferentes ambientes, reforçando

o potencial ecológico do grupo, especialmente na modulação da estrutura, composição e funções florestais (Ely et al. 2019; Canavan et al. 2019; Fradique et al. 2020). Em geral, os bambus atuam na manutenção da qualidade do solo (amplo sistema rizomatoso), rápido crescimento (eficiente captura de carbono) e restauração de matrizes vegetais degradadas. Portanto, em ambas as escalas ecológicas e sociais, os bambus tornam-se recursos estratégicos para mitigação da pressão sobre os recursos florestais tradicionais, e sobre os efeitos emergentes das mudanças climáticas (INBAR 2014; Yuen et al. 2017; Terefe et al. 2019; Padgurschi et al. 2021).

Os bambus são amplamente distribuídos nos ecossistemas neotropicais, ocorrendo numa ampla variação de gradientes de umidade e altitude, e onde destacam-se regiões de notória riqueza e endemismo de espécies (Clark & Oliveira 2018; Ruiz-Sanchez et al. 2021). O Brasil apresenta a maior diversidade e grau de endemismo de bambus lignificados neotropicais e herbáceos, com, atualmente, 168 espécies distribuídas em 17 gêneros e 90 espécies agrupadas em 18 gêneros, respectivamente, descritos – com continuamente novas descrições de espécies e gêneros (Santos-Gonçalves et al. 2012; Fisher et al. 2014; Mota et al. 2014; Carvalho et al. 2019; Oliveira et al. 2020a). Dentre o total de 258 espécies e 35 gêneros de bambus no Brasil, 175 espécies e 12 gêneros são considerados endêmicos, com ocorrência preferencial nos ameaçados domínios Mata Atlântica e Amazônia (Filgueiras & Viana 2017).

Característicos componentes de sub-bosques de florestas tropicais, os bambus herbáceos se diferem dos lignificados pela ocorrência de colmos fracamente lignificados, ausência de folhas do colmo, ramificação vegetativa limitada, ausência de lígula externa nas folhas de ramos, flores unissexuais e geralmente dimórficas, com floração anual e raramente monocárpica (Figura 2A) (Clark & Oliveira 2018; Lima et al. 2020). A tribo Olyreae apresenta origem monofilética, embora nenhuma sinapomorfia morfológica tenha sido identificada para o grupo (Oliveira et al. 2014; Ruiz-Sanchez et al. 2019).

As tribos de bambus lignificados, Bambuseae e Arundinarieae, apresentam características em comum que fazem referência à denominada 'síndrome de lignificação' (*woody syndrome*) – como a presença de um complexo sistema de rizomas, colmos lignificados, geralmente ocos e segmentados, folhas do colmo diferenciadas das folhas dos ramos, ramificação vegetativa complexa, presença de lígulas externas nas lâminas foliares e flores bissexuais (Figura 2B-C) (Clark et al. 2015; Chalopin et al. 2021; Ruiz-Sanchez et al. 2021). Ainda, ambas as tribos compartilham longos períodos vegetativos, de 30-40 anos para Bambuseae e 50-60 anos para Arundinarieae, seguidos de eventos de

floração geralmente gregária e monocárpica (Zheng et al. 2020; Wang et al. 2020a; Guerreiro et al. 2020). As tribos Arundinarieae e Bambuseae diferem entre si pelas características de desenvolvimento dos ramos, morfologia dos rizomas e nível de ploidia (BPG 2012; Soreng et al. 2017).

Figura 2. Morfologia e hábito das três tribos da subfamília Bambusoideae (Poaceae). (**A**) *Parodiolyra micrantha*, representante da tribo Olyreae; (**B**) *Dendrocalamus asper*, representante da tribo Bambuseae; (**C**) *Phyllostachys edulis*, representante da tribo Arundinarieae.

Estima-se que a subfamília Bambusoideae tenha se originado no final do período Eoceno (há 35-50 milhões de anos), com dispersão e radiação dependentes dos macroeventos climáticos do Mioceno, iniciando-se a partir da conexão entre placas tectônicas Índica e Euroasiática (Zhang et al. 2016; Srivastava et al. 2019; Ruiz-Sanchez et al. 2019). A partir de um ancestral diplóide extinto, as tribos lignificadas teriam se originado por eventos de hibridização independentes há cerca de 22 milhões de anos, gerando as linhagens tetraploides (Bambuseae neotropical e Arundinarieae) e hexaploides (Bambuseae paleotropical) (Peng et al. 2013; Triplett et al. 2104; Guo et al. 2019). Os bambus sustentam uma história evolutiva complexa, exibindo uma combinação de características únicas que permitiram eventos de radiação especialmente em florestas temperadas e tropicais (BPG 2012; Wysocki et al. 2016).

A poliploidização, considerada como um dos principais mecanismos evolutivos em plantas, teria, portanto, papel fundamental na diversificação dos bambus, resultando num mecanismo ativo de geração de matéria-prima para plasticidade genotípica e evolução adaptativa (Peng et al. 2013; Triplett et al. 2014; Soltis et al. 2015; Guo et al.

2019). Especialmente na família Poaceae, estima-se que todos seus representantes compartilhem, ao menos, dois eventos de duplicação total do genoma (Jiao et al. 2011; Paterson et al. 2012). Em Bambusoideae, estudos genômicos sugerem que a poliploidização teria atuado na evolução da fenologia associada a biossíntese de lignina e em longos ciclos de floração (Peng et al. 2013; Guo et al. 2019).

Apesar da notável discrepância entre as características morfológicas e fenológicas entre as linhagens dos bambus herbáceos e lignificados, as relações evolutivas entre e dentro as tribos ainda se mostram incertas, especialmente em relação à origem de características únicas, como a síndrome de lignificação e à delimitação dos limites de gêneros/espécies (Clark et al. 2015; Zhou et al. 2020; Chalopin et al. 2021). Inferências evolutivas baseadas em dados genéticos plastidiais indicam a origem parafilética da característica de lignificação, sugerindo um padrão de evolução paralela nas duas tribos lignificadas (Sungkaew et al. 2009; Triplett et al. 2014; Wysocki et al. 2015). No entanto, topologias baseadas em dados nucleares e de transcriptomas mostram a monofilia desta característica, indicando conflitos de sinais filogenéticos de origens distintas e associados aos complexos padrões evolutivos da subfamília (Zhang et al. 2012; Wysocki et al. 2016; Guo et al. 2019) (Figura 3). Tais discrepâncias filogenéticas são recorrentes em plantas, podendo indicar os reflexos de eventos de hibridização, poliploidização e segregação incompleta de linhagens (ILS, *incomplete lineage sorting*) (Guo et al. 2018; Meng et al. 2021; Nge et al. 2021; Triplett & Clark 2021).

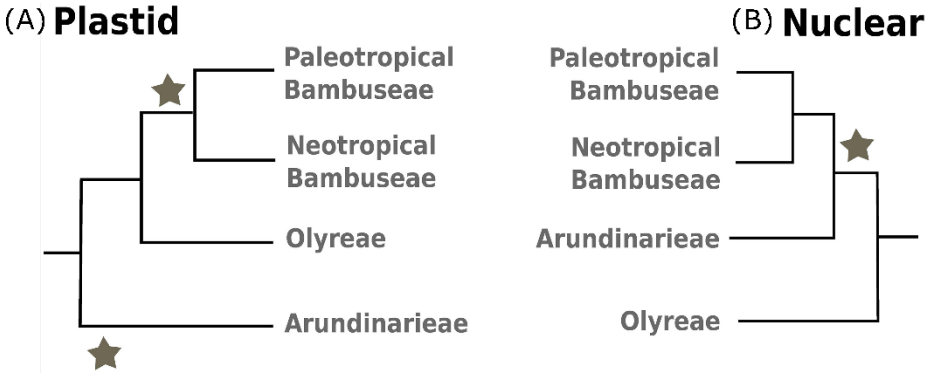

Figura 3. Relações filogenéticas entre as linhagens reconhecidas para Bambusoideae. Os dois painéis representam topologias baseadas em (**A**) dados plastidiais – indicando parafilia da síndrome de lignificação; e, (**B**) dados nucleares – indicando a monofilia dos bambus lignificados. A marcação em estrela demonstra o surgimento das características de lignificação nas linhagens (Adaptado de Chalopin et al. 2021).

Além da discrepância filogenética relacionada a origem da lignificação, o comportamento de floração diferenciado entre os bambus herbáceos e lignificados influencia profundamente as inferências evolutivas dentro da subfamília (Wysocki et al. 2015; Zheng et al. 2020a). O intervalo entre florações parece estar associado à distribuição geográfica das linhagens, onde as tribos tropicais (Bambuseae e Olyreae), com menor intervalo entre florações, apresentam maior taxa evolutiva em relação à tribo de distribuição em clima temperado (Arundinarieae), e, consequentemente, refletindo em variadas taxas de substituição nucleotídica entre as tribos e na resolução de inferências evolutivas amplas para a subfamília (Saarela et al. 2018; Wang et al. 2020a).

A delimitação taxonômica dentro e entre as tribos é afetada pela complexidade evolutiva dos bambus (poliploidia e hibridização), comportamento fenológico (raros eventos de floração), plasticidade fenotípica e caracteres morfológicos diagnósticos rasos (Yang et al. 2011; Sijimol et al. 2014; Zhou et al. 2017; Zhou et al. 2020; Triplett & Clark 2021). Diversos gêneros são descritos como parafiléticos/polifiléticos, constantemente trazendo novas delimitações/circunscrições taxonômicas tanto em nível de gênero quanto espécies, especialmente com base em resoluções filogenéticas e estudos moleculares (Jesus-Costa et al. 2018; Tyrrell et al. 2018; Oliveira et al. 2020a, 2020b; Carvalho et al. 2021; Ruiz-Sanchez et al. 2021).

Considerando a relevância ecológica e potencial socioeconômico da subfamília Bambusoideae, a correta classificação taxonômica e inferências de mecanismos evolutivos se tornam cruciais para a conservação do grupo, especialmente num cenário de constante ameaça de seus hábitats naturais e de mudanças climáticas globais (Clark et al. 2015; Canavan et al. 2019; Fradique et al. 2020). Constantes revisões em nível de gênero e subtribos vem sendo propostas com base em filogenias moleculares, enfatizando-se a necessidade de estudos amplos sobre evolução molecular, também complementados com aspectos ecológicos e de biologia reprodutiva, com propósitos de conservação deste grupo de ampla distribuição global (Clark & Oliveira 2018; Ruiz-Sanchez et al. 2021; Padgurschi et al. 2021).

1.2 DNA *barcoding*: a nova era da sistemática molecular

A rápida identificação de espécimes ou fragmentos de origem biológica, sejam amostras individuais ou coletivas/ambiental, sempre foi uma tarefa desejável para cientistas dos campos de sistemática, ecologia e evolução, porém limitada pelo

substancial desafio de especificidade técnica entre especialistas (Chase & Fay 2009; Kress 2017). A taxonomia clássica, que abrange a descoberta, descrição e classificação dos organismos vivos, demanda uma grande quantidade de dados morfo-anatômicos, fisiológicos, geográficos, comportamentais e ecológicos, o que podem representar fatores complicadores para grupos pouco documentados ou com história evolutiva complexa (Collins & Cruickshank 2013; Sheth & Thaker 2017, Tyrrell et al. 2018).

Este chamado 'inconveniente ou impedimento taxonômico' proporcionou o desenvolvimento de um sistema único e universal de identificação, em diferentes níveis taxonômicos, com base em sequências de DNA de uma região genômica padrão em todos os organismos, denominado de DNA *barcode* (Hebert et al. 2003a; Wiemers & Fiedler 2007; Hollingsworth et al. 2011). Dentre as principais conveniências desta abordagem destacam-se a identificação de uma espécie já descrita, ou a descrição de uma nova espécie, possibilitando a avaliação da variabilidade genética entre espécies e suas relações, e a compreensão da diversidade específica dentro de ecossistemas (Casiraghi et al. 2010; Kress 2017). A aplicação desta técnica incitou o ambicioso objetivo de criação de um inventário de diversidade global, considerando a geração de informação genética numa frequência que sustente um cenário de monitoramento e escalas apropriadas de esforços para conservação da biodiversidade (Lahaye et al. 2008; Valentini et al. 2008; Hajibabaei et al. 2016).

A identificação de espécies por DNA *barcode* envolve a seleção de uma (ou mais) região genômica, que atenda os princípios de padronização, minimalismo e escalabilidade (Hollingsworth et al. 2011). Em outras palavras, o segmento utilizado deve ser apresentar ocorrência e aplicação universal, com potencial de sequenciamento bidirecional rotineiro e de alta qualidade (com mínimo de esforço para correção de bases ambíguas), e suficiente variação nucleotídica que permita a distinção entre espécies (CBOL 2009; Hollingsworth et al. 2009). Atendendo aos critérios propostos, regiões *barcode* foram padronizadas para animais (gene mitocondrial da subunidade I da enzima citocromo oxidase; COI), fungos (espaçador interno transcrito, ITS, e a grande subunidade, LSU, do RNA ribossomal) e protistas (gene 16S do RNA ribossomal) (Hebert et al. 2003a; 2003b; Pawlowski et al 2012; Eberhardt 2012; Schoch et al. 2012; Xu 2016; Takayasu et al. 2019).

Apesar de ser considerado o barcode universal para a maioria dos grupos de animais (Hebert et al. 2003a, 2003b), o poder discriminatório individual do marcador COI é bastante variável, especialmente em grupos com fluxo gênico complexo e eventos de radiação recentes (van Velzen et al. 2012; Liu et al. 2017). Em plantas, o emprego do

segmento COI como barcode é, em geral, ineficiente para acurada discriminação de espécies, tendo em vista a baixa taxa de substituição nucleotídica do genoma mitocondrial (Mower et al. 2007; Chase et al. 2005; Kress & Erickson 2007; Chase & Fay 2009). A região ITS, apesar de se mostrar informativa em muitos grupos, pode não ser inadequado como barcode universal em plantas, uma vez que pode estar sujeito a processos evolutivos complicadores, como evolução em concerto, paralogia e homoplasia (Álvarez & Wendel 2003; Hollingsworth et al. 2009).

Portanto, o genoma cloroplastidial (cpDNA) vem sendo testado como fonte de regiões barcode de DNA em plantas, por apresentar características de abundância em organismos fotossintetizantes, herança uniparental e regiões genômicas altamente variáveis (*hotspots*) (Pennisi 2007; Shaw et al. 2007; Dong et al. 2012; Sawarkar et al. 2021). Diversas regiões do cpDNA foram testadas quanto à universalidade de amplificação de *primers* universais e poder de discriminação à nível de espécies em diferentes grupos de plantas, onde cada um dos marcadores candidatos apresentam vantagens ou desvantagens (Fazekas et al. 2012; Shneyer & Rodionov 2019). Apesar de muitos esforços iniciais, um marcador único para identificação de plantas ainda é desconhecido, onde a performance de diferentes *loci* é bastante variável entre os grupos de plantas (Pettengil & Neel 2010; Kress 2017; Stallmand et al. 2019).

Para construção de um banco de dados de barcode de DNA de plantas, o *core* proposto é composto por duas regiões codificantes do cpDNA, *rbcL* (ribulose-1,5-bisfosfato) e *matK* (maturase K) (CBOL 2009). A escolha destas regiões se faz com base nas suas propriedades combinadas, evidenciando a conservação e alta qualidade de sequenciamento de *rbcL*, e a rápida taxa evolutiva de *matK* (Newmaster et al. 2006; Hollingsworth et al. 2011). No entanto, a região altamente variável *matK* pode apresentar menor sucesso de amplificação e qualidade de sequenciamento em comparação à região *rbcL*, podendo demandar o estabelecimento de *primers* específicos para determinados grupos de plantas (Lahaye et al. 2008; Dunning & Savolainen 2010).

A adoção do core *rbcL* + *matK* como barcode para Angiospermas vem acompanhada do reconhecimento do potencial limitador de rastreio dos limites de espécies por parte das regiões plastidiais, a despeito dos acúmulos de caracteres variáveis em regiões altamente polimórficas (Hollingsworth et al. 2011). Diversos marcadores complementares vêm sendo testados em diferentes grupos de plantas a fim de se alcançar o nível desejado de discriminação de espécies, especialmente região não-codificantes do genoma plastidial, as regiões altamente variáveis *trnH-psbA*, *ndhF-rpl32*, *rpl32-trnL*,

atpF-atpH, *psbK-psbI*, entre outras (Kress et al. 2005; Lahaye et al. 2008; Pennisi 2007; Fazekas et al. 2009; Burgess et al. 2011; Pang et al. 2012; Shaw et al. 2014; Peterson et al. 2014; Bolson et al. 2015; Sawarkar et al. 2021). As regiões não-codificantes e de rápida evolução são propostas como eficientes para estudos em grupos de divergência recente, como a nível de espécies, enquanto que genes de evolução mais lenta, como regiões codificantes, são sugeridos como mais eficientes em eventos filogenéticos mais profundos (Wolf 2012; Peterson et al. 2014).

Os espaçadores intergênicos, como *trnH-psbA* e *rpl32-trnL*, são descritos como regiões de alta taxa evolutiva e divergência intraespecífica (Kress & Erickson 2007; Shaw et al. 2014; Loera-Sánchez et al. 2020). No entanto, os recorrentes eventos de inserções/deleções (*indels*), regiões de inversões ou repetições mononucleotídicas podem superestimar a divergência genética entre grupos, e consequentemente comprometendo a acurada discriminação de espécies (Chase et al. 2007; Whitlock et al. 2010; Liu et al. 2014). Portanto, a performance de cada marcador, ou conjunto de marcadores, pode ser idiossincrática em diferentes grupos de plantas, sugerindo uma relação de dependência com a história evolutiva, do nível taxonômico na aplicação das regiões barcode de DNA para distinção entre espécies próximas ou de evolução recente (Fazekas et al. 2008, Ali et al. 2014).

Considerando a desuniformidade das taxas de evolutivas e a descontinuidade dos processos de especiação nas principais linhagens de organismos, o poder de discriminação de espécies por DNA barcode pode ser dependente do variado conceito, e, muitas vezes, imperfeito limite de espécies (Casiraghi et al. 2010; Kress et al. 2015). Em outras palavras, o denominado "problema das espécies" se depara com a definição de espécie como unidade taxonômica discreta (limites nítidos/definidos) *vs*. unidade evolutiva contínua (limites vagos), e que participa ativamente do sistema hierárquico de classificação biológica (Zachos 2016; Freudenstein et al. 2017). Portanto, em plantas, considera-se menos definido o limite de espécies, dificultando o uso de sequências de DNA (*barcoding loci*) para discriminação de espécimes (Fazekas et al. 2009; Luo et al. 2018).

Como técnica, DNA *barcoding* se encontra com a sistemática ao nível da alfa taxonomia, no que se refere à descoberta, diagnóstico e descrição de novas espécies (DeSalle & Goldstein 2019). Diferentemente de inferências filogenéticas baseadas em sequências de DNA, onde o foco da análise é, majoritariamente, resolução de relações evolutivas, DNA *barcoding* se baseia na designação a nível de espécie de um espécime

não conhecido (Hajibabaei et al. 2016; Hebert et al. 2016). No entanto, numa abordagem integrativa, vem se tornando uma importante técnica para resolução de questões ecológicas e evolutivas em diferentes grupos de plantas, considerando espécies como unidades fundamentais para descrição da biodiversidade (Kress & Erickson 2012; Stanton et al. 2019; DeSalle & Goldstein 2019).

A grande vantagem do uso de regiões genômicas informativas para a identificação de organismos à nível de espécies (catalogada ou não) se concentra nos princípios de padronização (uso de sequências comuns para diferentes grupos), controle de qualidade (confiabilidade de bibliotecas referência de sequências de DNA), e minimalismo (uso de uma ou poucas regiões para garantir escalabilidade) (Kress & Erickson 2008; Coissac et al. 2016). No entanto, o sucesso de discriminação é totalmente dependente da taxa evolutiva da região barcode empregada no específico grupo avaliado, e, consequentemente, a identificação de indivíduos conspecíficos com base numa variabilidade nucleotídica pré-determinada, especialmente em grupos taxonomicamente e evolutivamente complexos (Hollingsworth et al. 2011; Ali et al. 2014; Hebert et al. 2016).

Com propósito de identificação de espécies, DNA *barcoding* envolve a construção de uma biblioteca de sequências de *loci* potenciais de espécies conhecidas e a correspondência de uma amostra desconhecida contra esta biblioteca (Kress 2017; Phillips et al. 2019). A construção de uma matriz de sequências de DNA pode ser utilizada diretamente, como uma série de estados de caracteres, ou convertida em matrizes de distância genética, e que podem ser representados graficamente como árvores filogenéticas ou por padrões de distribuição de distâncias (Goldstein & DeSalle 2010). Matrizes de distância genética, representando a variabilidade nucleotídica por sítio, com aplicação em três abordagens distintas, *i.e.*, por testes de similaridade, *barcoding gap*, ou fenogramas de agrupamento hierárquico (*Neighbor-Joining*; NJ) (Meier et al. 2006; Collins et al. 2012).

O método analítico inicialmente proposto, e mais amplamente empregado, é baseado na presunção de existência de um limiar fixo de separação (*threshold*) entre a distribuição da máxima distância interespecífica e mínima distância intraespecífica, denominado de *barcoding gap* (Figura 4) (Hebert et al. 2003; Meyer & Paulay 2005). A avaliação de um barcode exclusivamente por *barcoding gap* é extensivamente discutida, uma vez que assume um *threshold* arbitrário (usualmente 1-10%), desconsiderando mecanismos evolutivos, muitas vezes, intrínsecos de cada linhagem de planta (Fazekas et

al. 2009; Mallo & Posada 2016; Stallmand et al. 2019). A ausência do *barcoding gap* (sobreposição entre as distribuições) pode não indicar, necessariamente, a conspecificidade entre duas amostras (Wiemers & Fiedler 2007), mas a sensibilidade da avaliação referente à suficiente divergência genética intraespecífica, os limites claros entre espécies e ao tempo de coalescência do grupo estudado (Goldstein & DeSalle 2010; Naciri & Linder 2015).

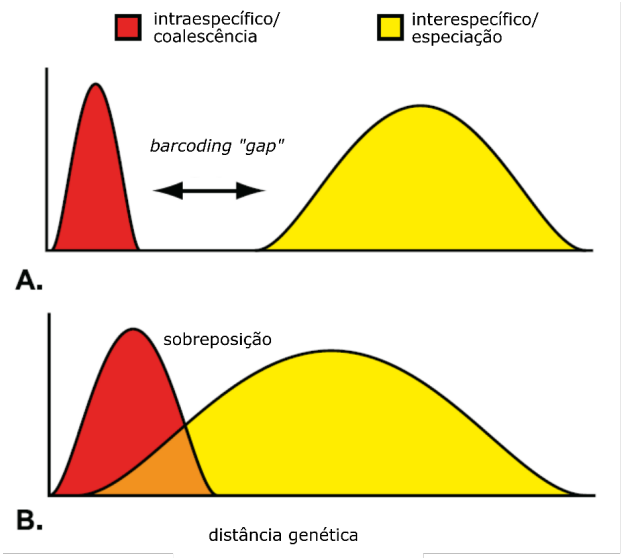

Figura 4. Distribuição da variação intraespecífica considerando um cenário de (**A**) discriminação entre espécies e de (**B**) sobreposição das distâncias genéticas. (Adaptado de Meyer & Paulay 2005).

Testes de similaridade, como BLAST, podem resultar em designações taxonômicas incorretas, uma vez que são altamente dependentes de banco de dados robustos, e não fornecem avaliações estatísticas para casos de ausência ou múltiplas correspondências (Casiraghi et al. 2010; Kress 2017). Ainda, o resultado apresentado é referente à similaridade mais próxima disponível, também assumindo o *barcoding gap*, ou seja, a similaridade intraespecífica é maior que a interespecífica (Mallo & Posada 2016).

A representação gráfica da distância genética por agrupamento hierárquico NJ é o método de avaliação mais amplamente empregado, geralmente utilizando-se do modelo de evolução molecular Kimura-2-parameters (K2P) (Hebert et al. 2003; DeSalle & Goldstein 2019). A aplicação de ambas as abordagens vem sendo discutida, tanto pela

resolução das árvores geradas pelo algoritmo NJ, quanto pela universalidade do modelo K2P para sequências intimamente relacionadas (Srivathsana & Meier 2012; Collins et al. 2012). Assim como os demais métodos baseados em árvores, a avaliação de sucesso de discriminação por NJ baseia-se no conceito monofilético de espécies, ou seja, a conspecificidade de duas amostras é confirmada pelo agrupamento monofilético (Casiraghi et al. 2010).

Além do método analítico empregado, a acurácia na discriminação de espécies com base em sequências de DNA é, essencialmente, dependente de uma definição *à priori* das unidades taxonômicas, considerando-as como unidades naturais fixas, e constantemente impactada pelo conceito de espécie previamente estipulado (Goldstein & DeSalle 2010; Mallo & Posada 2016; Luo et al. 2018; Padial & De la Riva 2021). Dentre os recorrentes conceitos de espécies, como o conceito biológico (Mayr 1942) e o conceito evolutivo (Wiley 1978), métodos de avaliação de DNA *barcoding* baseados em árvores assumem o pressuposto da monofilia recíproca do conceito filogenético de espécies (Rosen 1979). Em grupos onde hibridização é recorrente, ou eventos evolutivos complexos, como *incomplete lineage sorting* (ILS) e radiação recente, ao assumir este critério considera-se que a árvore do gene (*gene tree*) seja a representação exata da árvore de espécie (*species tree*), podendo resultar em identificação ambígua ou incorreta (Roy et al 2010; Federici et al 2013; Walker et al. 2019; Guo et al. 2019).

1.2.1 DNA *barcoding* em Bambusoideae

Os bambus são plantas desafiadoras quanto à identificação e classificação sistemática de espécies, onde, assim como em Poaceae em geral, a taxonomia do grupo é dependente de especialistas com anos de experiência, o que não ocorre na ampla extensão de ocorrência da subfamília (Vorontzova et al. 2016; Hodkinson 2018). A notória complexidade de identificação de espécies em Bambusoideae é decorrente do longo intervalo entre florações (em linhagens lignificadas), e diversificada morfologia de caracteres vegetativos dentro das subtribos, dificultando o estabelecimento de sinapomorfias e autapomorfias morfológicas (Yang et al. 2011; Sijimol et al. 2014; Zhou et al. 2017, 2020; Sawarkar et al. 2021).

Apesar da confirmada monofilia das subtribos, as relações filogenéticas e delimitação sistemática ao nível de gênero são ainda pouco resolvidas (Yang et al. 2010; Clark et al. 2015; Sijimol et al. 2020). Dentre as subtribos, alguns gêneros são resolvidos por parafilia ou polifilia, o que vem trazendo, recorrentemente, a descrição de novos taxa

(Tripllet & Clark 2010; Attigala et al. 2016; Jesus-Costa et al. 2018; Tyrrell et al. 2018; Zhou et al. 2019; Oliveira et al. 2020). Ainda, a evolução reticulada em decorrência de poliploidização e hibridização, combinada com a evolução convergente de caracteres vegetativos e variadas taxas de diversificação e radiação entre as tribos, dificultam ainda mais as inferências filogenéticas e delimitação de espécies em larga escala em Bambusoideae (Clark et al. 2019; Guo et al. 2019; Zhang et al. 2019; Zhou et al. 2020; Ye et al. 2021; Triplett & Clark 2021).

Considerando os marcantes obstáculos para classificação sistemática em Bambusoideae, influenciada pela inconstância de características florais e variação de caracteres vegetativos, o uso de dados moleculares para a identificação de espécies de bambus vem sendo testado, incluindo potenciais *loci* de DNA *barcode* (Cai et al. 2019; Liu et al. 2020; Sawarkar et al. 2021). No entanto, apesar da reconhecida contribuição para resolução de inferências filogenéticas do grupo, a discriminação de espécies de bambus por DNA *barcode* ainda se mostra inconclusiva, cujos resultados discrepantes são descritos para as diferentes linhagens/níveis taxonômicos e *loci* empregados (Horn & Häser 2016; Sijimol et al. 2014).

Diversos *loci* plastidiais, incluindo regiões codificantes e regiões intergênicas, vem sendo testados como barcode potencial para Bambusoideae, especialmente nas tribos Arundinarieae e Bambuseae paleotropical (Cai et al. 2012; Sosa et al. 2013; Meena et al. 2020; Sijimol et al. 2020). O core *rbcL* + *matK*, proposto como barcode em plantas terrestres (CBOL 2009), apresenta poder discriminatório insuficiente para Bambusoideae, e, portanto, propõe-se a adoção de *loci* complementares para aumentar o poder de resolução em níveis taxonômicos mais baixos (Cai et al. 2012; Zhang et al. 2013; Peterson et al. 2014; Horn & Häser 2016). No entanto, apesar de alguns *loci* apresentarem alta taxa de polimorfismo, como *psbK-psbI* e *trnH-psbA*, ainda não há consenso de aplicação de um core único para o grupo (Sosa et al. 2013; Horn & Häser 2016; Dev et al. 2020).

Os métodos de avaliação do potencial barcode dos *loci* candidatos se baseiam, essencialmente, na comparação entre distância genética intra- e interespecífica, e suposição de monofilia recíproca entre espécies (Hollingsworth et al. 2011; Collins & Cruickshank 2013). Porém, em grupos complexos, como Bambusoideae, os limites específicos podem ser confundidos pela ocorrência de eventos de hibridização, introgressão e evolução reticulada (Zhou et al. 2020; Liu et al. 2020; Ye et al. 2021; Triplett & Clark 2021). Portanto, a notória falta de resolução de *loci* plastidiais na discriminação de espécies de bambus pode ser atribuída aos complexos padrões

evolutivos da subfamília, como ocorrência de gêneros parafiléticos e grupos de morfoespécies (Das et al. 2013; Liu et al. 2020; Sijimol et al. 2020; Carvalho et al. 2021).

O potencial de resolução de sequências de DNA para discriminação de espécies de bambus tem se baseado, especialmente, em indivíduos das tribos Arundinarieae e Bambuseae paleotropical (Cai et al. 2012; Zhou et al. 2013; Sijimol et al. 2020; Dev et al. 2020; Meena et al. 2020). Dentre as linhagens neotropicais, a avaliação do poder discriminatório de sequências de DNA na abordagem de barcode é limitada, com o teste de somente algumas espécies de Bambuseae e Olyreae (Sosa et al. 2013; Horn & Häser 2016). No entanto, a avaliação de regiões altamente variáveis do genoma plastidial como barcode de DNA ainda se apresenta como alternativa metodológica a ser empregada em larga escala (Shaw et al. 2014), especialmente em grupos complexos como Bambusoideae.

1.3 O genoma plastidial de plantas

Plastídios constituem uma importante família de organelas, que atuam como centros metabólicos para a biossíntese de compostos essenciais para a fisiologia e desenvolvimento de plantas (Daniell et al. 2016). A existência de plastídios é uma das principais características distintivas das células vegetais de outras eucarióticas, cujos diferentes tipos (protoplastos, amiloplastos, cromoplastos, cloroplastos) apresentam funções metabólicas específicas essenciais para a viabilidade celular (Ruhlman & Jansen 2014; Rogalski et al. 2015). Contendo o pigmento clorofila, a função central dos cloroplastos é a realização da fotossíntese, processo de fixação de CO_2 em carboidratos elaborados por mecanismos fotoquímicos, e com liberação de oxigênio atmosférico (Green 2011). O estabelecimento da fotossíntese oxigênica realizada por cianobactérias primitivas permitiu o Grande Evento de Oxigenação (há 2,4-2,7 bilhões de anos), onde o drástico aumento nos níveis de oxigênio atmosférico moldaram a evolução e o estabelecimento de organismos multicelulares na Terra (Kump 2008; Junge 2019).

Assim como ocorreu para as mitocôndrias, a origem dos plastídios remete à Teoria Endossimbiótica (Keeling 2010; Zimorski et al 2014). Cloroplastos teriam se originado por um evento de endossimbiose primário, cuja incorporação de uma cianobactéria fotossintética de vida-livre à uma célula eucariótica permitiu a transição da heterotrofia para autotrofia (Keeling 2010; Wicke et al. 2011). Evidências importantes que corroboram a Teoria Endossimbiótica é a presença de um genoma único particular em cloroplastos (referido, no presente contexto, como genoma plastidial, plastoma ou

cpDNA), e que exemplifica a forma mais completa de transferência horizontal de genes (Timmis et al. 2004; Bock 2017).

Durante sua história evolutiva, plastomas endossimbiontes de plantas mantiveram algumas características ancestrais, incluindo a estrutura circular, compartimentalização em nucleoides, organização gênica em operons e a maquinaria de expressão gênica típica de procariotos (Bock 2007). Grande parte dos genes foi transferido para o genoma nuclear, retendo não mais que 10% dos genes hipoteticamente existentes no genoma da cianobactéria primitiva, especialmente aqueles envolvidos na regulação e expressão do sistema fotossintético (Martin 2002; Gould et al. 2008; Greiner & Bock 2013). A semiautonomia dos plastomas é representada pela dependência da expressão de mais de 90% das proteínas funcionais nos cloroplastos, sendo estas sujeitas a regulação no genoma nuclear, tradução e transporte no citosol (Timmis et al. 2004; Daniell et al. 2016; Zoschke & Bock 2018).

Assim como as mitocôndrias, o padrão de herança dos plastomas é, em geral, não-Mendeliano e uniparental em todos os eucariotos, mais frequentemente de origem materna para Angiospermas (Greiner et al. 2014). No entanto, desvios do padrão de herança materna são observados em diferentes linhagens, como a predominância de herança paterna na maioria das Gimnospermas, e herança bipaternal em algumas Angiospermas (Bock 2007; Matsushima et al. 2008; Adams 2019). A transmissão uniparental implica na baixa taxa de recombinação gênica e na dependência de coevolução com o genoma nuclear, podendo refletir em processos de incompatibilidade citoplasmática (Birky et al. 2001; Greiner et al. 2014).

O cpDNA de plantas terrestres apresenta estrutura e organização altamente conservadas, compartimentalizando cerca de 120 genes em 120-170 kilobases (kB) (Jansen & Ruhlman 2012). Compreendendo uma molécula circular única quadripartida, os plastomas estão organizados em duas regiões de cópias únicas, a grande (LSC; *large single copy*) e a pequena (SSC; *small single copy*), separadas por duas regiões invertidas (IR_A e IR_B; *Inverted Repeats*) (Figura 5) (Greiner et al. 2014; Daniell et al. 2016). As sequências nucleotídicas das duas IRs são idênticas, diferenciando somente nas orientações relativas, indicando a duplicação do conteúdo de gênico nessas regiões (Bock 2007). Embora a estrutura circular seja predominante, uma fração de diferentes conformações, como formas circulares ou multiramificadas, podem ser observadas em plastomas de diferentes linhagens ou tecidos de plantas (Bendich 2004; Shaver et al. 2008; Oldenburg & Bendich 2016).

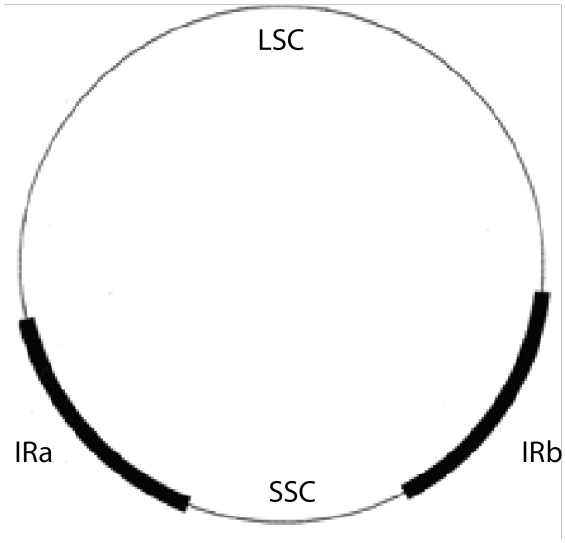

Figura 5. Esquema geral do mapa circular de um genoma plastidial de plantas, ilustrando a organização estrutural quadripartida das regiões de cópia única **LSC** (large single-copy) e **SSC** (small single-copy), e as duas regiões invertidas repetidas, **IRa** e **IRb** (*inverted repeat* A e B).

Apesar das características conservativas aparentes dos cpDNA, rearranjos estruturais em larga escala são observados nas principais linhagens de plantas terrestres, envolvendo, principalmente, mecanismos de variação no tamanho genômico (Wicke et al. 2011). De fato, tais rearranjos não são observados em plastomas da maioria das linhagens de plantas não-vasculares, sugerindo um padrão de ancestralidade estrutural anterior à colonização do ambiente terrestre pelas algas verdes ancestrais (Qiu et al. 2006; Turmel et al. 2006; Mower & Vickrey 2018). Responsáveis pela variação plastidial geral em Angiospermas, diversos fenômenos de expansão e contração das IRs, inclusão ou perda de genes e/ou íntrons, variações nos espaçadores intergênicos, e variações nas sequências repetitivas são relatados como padrões evolutivos independentes em todos os níveis taxonômicos (Lin et al. 2010; Wu & Chaw 2014; Schwarz et al. 2015; Chen et al. 2018; Wang et al. 2018a; Choi et al. 2019).

Desde os primeiros plastomas de plantas sequenciados, a obtenção de sequências completas de cpDNA se tornou efetivo em termos de custo e tempo com o advento das técnicas de alto rendimento (Ohyama et al. 1986; Shinozaki et al. 1986; Wicke & Schneeweiss 2015). Características inerentes dos plastomas em comparação ao genoma nuclear, como o reduzido tamanho, grande proporção do genoma total celular, presença de regiões altamente conservadas intercaladas a regiões com elevadas taxas evolutivas,

tornaram estes genomas pontos de partida para estudos comparativos de relações filogenéticas em diferentes níveis taxonômicos, padrões de especiação e dispersão, indicação do limite de espécies, variações ecológicas e interpopulacionais, juntamente com a observação cada vez mais comum de plastomas não-padronizados em monocotiledôneas e dicotiledôneas (Fazekas et al. 2009; Scarcelli et al. 2011; Ruhlman & Jansen 2014; Shaw et al. 2014; Zhang et al. 2017; Tonti-Fillipini et al. 2017; Gitzendanner et al. 2018; Zhou et al. 2019; Wang et al. 2020b; Roy et al. 2020). Portanto, sequências plastidiais são essenciais para aprimorar o conhecimento sobre a biologia fundamental de plantas, além de facilitar o desenvolvimento de aplicações biotecnológicas (como o '*plastid engineering*') e eficiência de sistemas de cruzamentos (Diekmann et al. 2008; Bock 2014; Daniell et al. 2021).

Devido a sua importância econômica, amplitude ecológica e dominância nos principais biomas terrestres, a família Poaceae se tornou ponto focal para estudos evolutivos, tendo duas espécies dentre os primeiros plastomas sequenciados, *Oryza sativa* (Hiratsuka et al. 1989) e *Zea mays* (Maier et al. 1995). Desde então, um grande número de espécies de gramíneas vem tendo seus genomas plastidiais sequenciados, cujas análises comparativas revelam variações organizacionais incomuns em relação a outros grupos de Angiospermas, representando um modelo geral para estudos de evolução genômica de plastídios (Guisinger et al. 2010; Saarela et al. 2018; Duvall et al. 2020).

Além de alto grau de conservação na ordem e conteúdo gênico em genomas plastidiais, rearranjos estruturais no cpDNA característicos das gramíneas teriam atuado como gatilho para adaptação evolutiva e, consequentemente, no pronunciado sucesso de radiação e diversificação da família (Cosner et al. 2004; Zhong et al. 2009). Tais rearranjos, alguns indicando sinapomorfias para a família, envolvem eventos de inversões em regiões genicas (como *trnT*, *trnG-UCC*, *rps14*), pseudogeneização (*rpl23*), inserções nucleotídicas em regiões codificantes (*rpl16*, *rpoC2*), perdas de íntrons em regiões específicas (como nos genes *clpP* e *rpoC1*), perdas de genes com degradações progressivas das sequências (p. ex. os genes *accD*, *ycf1* e *ycf2*), e alterações nas regiões limites entre IR/SSC e IR/LSC (Morton & Clegg 1993; Michelangeli et al. 2003; Davis & Soreng 2010; Morris & Duvall 2010; Guisinger et al. 2010; Wu & Ge 2012; Duvall et al. 2016; Burke et al. 2016; Saarela et al. 2018; Zhou et al. 2019; Liu et al. 2021).

Diferentemente da proposição primária, a presença das IRs não assegura a estabilidade genômica, uma vez que diversos eventos de contração e expansão são frequentes, tornando-os *hostpots* de rearranjos estruturais propensos a variação no

tamanho do genoma plastidial (Maréchal & Brisson 2010; Chen et al. 2018; Choi et al. 2019). No entanto, estas regiões apresentam reduzida taxa de substituição nucleotídica e baixa frequência de mudanças microestruturais, como inserções e deleções (indels), sendo estes mais concentrados nas regiões de cópia única, especialmente em espaçadores intergênicos (SSC e LSC) (Zhu et al. 2016; Mower & Vickrey 2018). Consideradas umas das mais pronunciadas forças na evolução de sequências em Poaceae, esta recorrente heterogeneidade implica na seleção de marcadores com taxas evolutivas apropriados para resolução de filogenias em diferentes níveis taxonômicos (Yamane et al. 2006; Zhong et al. 2009; Wolf 2012; He et al. 2019; Liu et al. 2021).

1.3.1 Evolução de genoma plastidial de bambus

Os bambus são plantas que abrigam uma história evolutiva complexa, envolvendo eventos hibridização e diferentes níveis de ploidia, o que dificulta a identificação de genes ortólogos de cópia única em genomas nucleares para a resolução de filogenias em níveis específicos (Triplett et al. 2014; Clark et al. 2015). Portanto, considerando que todo o genoma abriga um forte sinal filogenético num reduzido número de loci, sequências de genomas plastidiais são ferramentas indispensáveis para a compreensão mais profunda das relações evolutivas em grupos grandes, de ampla distribuição global, e com espécies intimamente relacionadas, como Bambusoideae (Parks et al. 2009; Wu et al. 2015; Zhou et al. 2017).

Atualmente, o banco de dados GenBank conta com 162 plastomas de Bambusoideae, representando menos de 10% das espécies atualmente descritas, incluindo 74 da tribo Bambuseae, 79 da tribo Arundinarieae e 9 da tribo Olyreae (https://www.ncbi.nlm.nih.gov/genome; acesso em 17 de maio de 2022). Análises comparativas de cpDNA da subfamília revelam um certo grau de conservação estrutural, especialmente em relação à ordem e de conteúdo gênico, e conteúdo de íntrons e %GC (Zhang et al. 2011; Wu et al. 2015; Attigala et al. 2016; Zhou et al. 2019). No entanto, a evolução de cpDNA de bambus acompanha os padrões evolutivos propostos para Poaceae em geral, incluindo mecanismos de expansão e contração das IRs, ocorrência de características únicas no limite das regiões IR/SSC, e perdas de genes e introns, cujos rearranjos podem estar acompanhados de processos importantes de especiação e evolução adaptativa (Cosner et al. 2004; Zhang et al. 2011; Vieira et al. 2015; Zhou et al. 2019). Ainda, as taxas de divergência de sequências se mostram heterogêneas em diferentes regiões genômicas e entre as diferentes linhagens, como esperado, tendo as regiões

codificantes mais conservadas que as não-codificantes, e as IRs mais conservadas que a LSC e SSC, respectivamente (Zhang et al. 2011; Ma et al. 2014; Wang et al. 2018b).

No entanto, estima-se que espécies de distribuição neotropical teriam acumulado e mantido características únicas em seus plastomas, devido ao isolamento geográfico evolutivo, especificamente no que se trata de variação no tamanho total de genomas, características nos limites IR/SSC e diferencial taxa evolutiva (Burke et al. 2012, 2014; Wu et al. 2015; Wang et al. 2020a). Plastomas de espécies de bambu neotropicais apresentam, tipicamente, tamanho menor em relação à bambus paleotropicais, cujas diferenças estão atribuídas, especialmente, a eventos de inserção e deleção em espaçadores intergênicos nas regiões IR e LSC (Burke et al. 2012; Vieira et al. 2015; Wu et al. 2015; Wysocki et al. 2015). Ainda, estima-se que a taxa de substituição molecular seja mais elevada na tribo Olyreae, com diminuição gradual entre Bambuseae e Arundinarieae, indicando um importante padrão de evolução e diversificação nos trópicos e decorrente do menor tempo entre gerações em Olyreae (Wu et al. 2015; Wysocki et al. 2015; Saarela et al. 2018; Wang et al. 2018b, 2020a).

Algumas características únicas de evolução do genoma plastidial de espécies herbáceas são consideradas sinapomorfias para a tribo Olyreae, ou numa escala linhagem-específica, especialmente decorrentes de expansão e contração de regiões IRs, com consequências na variação no número de genes (pseudogeneização) e no tamanho genômico (Burke et al. 2012; Wang et al. 2018b). Adicionalmente, ainda que sejam eventos raros em Angiospermas, observa-se a ocorrência de transferência horizontal de fragmentos de origem do genoma mitocondrial para o cpDNA de espécies da subtribo Parianinae (Olyreae) – eventos únicos em Bambusoideae, mas descritos em outras linhagens de Poaceae (Ma et al. 2014; Wysocki et al. 2015; Burke et al. 2018; Mower & Vickrey 2018; Wang et al. 2018b).

Dados os padrões estruturais que acompanham a evolução dos plastomas de bambus, e as diferenças específicas entre linhagens, mais dados são requeridos para a compreensão ampla da evolução de cpDNA em Bambusoideae (Wu et al. 2015; Wang et al. 2020a). Ainda, a compreensão sobre as diferentes taxas evolutivas pode trazer informações sobre os padrões de radiação e inferências sobre eventos morfofisiológicos maiores (como floração) das diferentes tribos (Ma et al. 2017). Portanto, considerando a abundância de espécies de bambus neotropicais no Brasil, e a constante ameaça de seus domínios preferenciais de ocorrência, o sequenciamento dos genomas plastidiais de espécies nativas se torna uma importante ferramenta para a compreensão dos padrões de

riqueza, endemismo, distribuição e relações filogenéticas dos bambus neotropicais, contribuindo para a massiva conservação deste grupo (Filgueiras & Viana 2017; Zhou et al 2019; Wang et al. 2020a; Ruiz-Sanchez et al. 2021).

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3. OBJETIVOS

3.1 OBJETIVO GERAL

3.1.1 Capítulo 1. DNA barcoding in Bambusoideae (Poaceae): are we getting close?

Testar a aplicabilidade de cinco regiões do genoma plastidial candidatas à barcode, visando a resolução em ampla escala de questões taxonômicas em Bambusoideae.

3.1.2 Capítulo 2. Sequenciamento e análise comparativa do genoma plastidial de espécies de bambus neotropicais (Bambusoideae, Poaceae) nativas do Brasil

Realizar a análise comparativa de genomas plastidiais de espécies endêmicas e ameaçadas de bambus nativos do Brasil, visando a caracterização de padrões evolutivos e identificação de regiões polimórficas.

3.2 OBJETIVOS ESPECÍFICOS

3.2.1 Capítulo 1.

i) Selecionar regiões candidatas à *barcode* de DNA para Bambusoideae;

ii) Sequenciar as regiões selecionadas para espécies abrangentes das três tribos de Bambusoideae;

iii) Avaliar as características moleculares das regiões sequenciadas, comparando os níveis de conservação e variabilidade nucleotídica;

iv) Testar o poder discriminatório a nível de espécie marcadores aplicados com base na abordagem filogenética;

v) Selecionar a região *barcode*, a combinação destas, com maior poder discriminatório a nível de espécie para subfamília Bambusoideae.

3.2.2 Capítulo 2.

i) Identificar populações naturais e coletar amostras de *Guadua trinii*, *Apoclada simplex*, *Parodiolyra micrantha*, espécies de Bambusoideae nativas do Brasil;

ii) Isolar e obter a sequência completa de cpDNA das três espécies selecionadas;

iii) Realizar a montagem e anotação dos genomas plastidiais destas três espécies;

iv) Comparar o conteúdo e estrutura gênica dos novos genomas plastidiais sequenciados com relação às suas respectivas subtribos;

v) Identificar regiões altamente polimórficas e com aplicabilidade em DNA *barcoding* e resolução filogenética em Bambusoideae neotropical.

4. CAPÍTULO I.

Manuscrito submetido ao periódico Molecular Phylogenetics and Evolution.

DNA barcoding in Bambusoideae (Poaceae): are we getting close?

ABSTRACT

Specimens' identification has been performed by using DNA barcoding technique, which employs the rapid and standard sequencing of a short DNA region variable enough to distinguish between species. Despite many efforts, there is no DNA barcode standardized for all land plants, including the worldwide distributed group of bamboos. Bambusoideae subfamily (Poaceae) encompasses more than 1680 species of forest grasses with greatest taxonomic challenge, mainly posed by its rare flowering events and morphological similarity. However, given their greatest ecological and economic importance, accurate bamboo species classification is crucial for their evolutionary inference and conservation programs. Based on the three principals of DNA barcoding technique (standardization, minimalism, and scalability), the present work tested five chloroplast genomic regions proposed as DNA barcode candidates (*rbcL*, *matK*, *psbK-psbI*, *rpl32-trnL* and *trnH-psbA*) in a widely sampling approach for Bambusoideae subfamily, regarding their nucleotide variability and phylogenetic signal for species discrimination. All samples were successfully amplified, and 365 newly generated sequenced were obtained in high sequencing quality. Among the tested cpDNA regions, rpl32-trnL showed the greatest nucleotide variability. However, none of them, either single-tested or combined, resulted in satisfied species discrimination. Considering the phylogenetic approach of species discrimination, meaning the reciprocal monophyly criteria, application of DNA barcoding technique in Bambusoideae should strongly consider the subfamily life cycle and recurrence of hybridization events, which influence the accuracy of DNA barcoding in evolutionary complex groups.

Keywords: Bamboo, chloroplast genome. molecular taxonomy, systematics.

INTRODUCTION

Rapid identification of biological specimens has always been a challenge, since it relies on the rare natural history specialists, and the use of molecular data has been tested to overcome such challenges (Chase & Fay 2009). DNA barcoding is a proposed technique for characterizing species of organisms using rapid sequencing of a short and DNA region from a standard genome position, which may show significant nucleotide variation capable of distinguishing between species (Hebert et al. 2003). Some DNA regions have been proposed as barcode for animals, bacteria, and fungi, but although great effort the standardization of DNA barcodes for all land plants has not yet been achieved (Peterson et al. 2014). The barcode core composed by chloroplast genome sequences, *rbcL* and *matK*, are proposed as the initial point of start for land plants (CBOL 2009; Hollingsworth et al. 2011). Those molecular markers can show differential performance of species discrimination between different groups of plants, including Bambusoideae, in which supplementary markers with higher evolutionary rates are recommended aiming to increase barcode performance in low taxonomic level (Dong et al. 2012; Peterson et al. 2014; Horn & Häser 2016).

Bambusoideae (Poaceae) subfamily encompasses perennial grasses with a remarkable worldwide distribution, representing the only major grass lineage with forest diversification (BPG 2012; Clark et al. 2015). Currently, 127 genera/1680+ species are distributed in three monophyletic tribes, the herbaceous (Olyreae) and woody bamboos (Bambuseae and Arundinarieae (Sungkaew et al. 2009; Soreng et al. 2017; Clark & Oliveira 2018). Additionally, the remarkable growing number of genera and species described reinforces the taxonomic and evolutionary complexity of the group (Tyrell et al. 2018; Ruiz-Sanchez et al. 2019; Clark et al. 2019; Oliveira et al. 2020).

Bamboos are a taxonomically challenging group, presenting a particularly scientific problem, especially for non-specialists in diverse tropical plant groups (Vorontzova et al. 2016; Hodkinson 2018). Systematic classification based on floral and vegetative characters is mostly unreliable for bamboos, considering their unusual floral behavior and life cycle (Sawarkar et al. 2021; Triplett & Clark 2021). The underpinned shallow morphological variation between species and the rare and infrequent flowering events in woody lineages propose the main challenge of species identification in bamboos, hindering the establishment of morphological autapomorphies and/or synapomorphies in (low) generic levels (Yang et al. 2011; Sijimol et al. 2014, 2020; Zhou

et al. 2017). However, given their greatest ecological and economic importance, correct systematic classification is crucial to conservation programs and development of robust phylogenetic inferences (Clark et al. 2015; Ruiz-Sanchez et al. 2019; Triplett & Clark 2021).

Many attempts have been made to establish an ideal genomic region for bamboo species discrimination, including both coding and non-coding regions of chloroplast genomes (cpDNA), especially in temperate (Arundinarieae) e paleotropical (Bambuseae) lineages (Cai et al. 2012; Meena et al. 2020; Sijimol et al. 2020; Dev et al. 2020). However, those efforts present unequal and inconstant results, indicating differential discriminatory power of cpDNA regions within and among tribes (Sosa et al. 2013; Sawarkar et al. 2021). Along with that, most analytical evaluation methods rely on sufficient genetic distances between species and on reciprocal monophyly, which can be unsuitable for DNA barcoding application in complex evolutionary groups such as Bambusoideae (Hollingsworth et al. 2011; Collins & Cruickshank 2013).

Based on the proposed principles of DNA barcoding technique – standardization, minimalism, and scalability (Hollingsworth et al., 2011) – we evaluated five selected plastid genome loci to access the criteria of universality of amplification and sequence quality parameters, nucleotide variability and discriminatory power in a wide sampling approach for Bambusoideae. In the present work, we aimed to address the following questions: *i*) The selected genomic loci and primers are widely suitable to generate high quality sequences; *ii*) the selected barcode regions present sufficient nucleotide diversity to increase species discrimination power? *iii*) phylogenetic approaches are informative in a large-scale for Bambusoideae?

MATERIAL AND METHODS

Sampling and DNA extraction

In total, 73 samples were collected in 11 distinct locations, mainly in personal collections or native populations in private properties, aiming to avoid primary identification errors. Sample collected in natural populations were identified by floral or vegetative diagnostic characteristics. All samples represented 14 species (19 specimens) of Arundinarieae tribe, 15 species (16 specimens) of neotropical Bambuseae tribe, 27 species (33 specimens) of paleotropical Bambuseae tribe, and 3 species (3 specimens) of

Olyreae tribe, totalizing 19 genera and 59 species (table S1). Young leaves of each sample were collected, dried in silica gel, and stored at -20°C for posterior DNA extraction§.

§ Specimens of each sample were collected according to Soderstrom & Young (1983) and deposited at herbarium FLOR (Federal University of Santa Catarina).

DNA extraction of each sample was performed by CTAB 2% protocol (Doyle & Doyle, 1990), with minor modifications for Bambusoideae. Quality and concentration of isolated DNA were evaluated by visual estimation in 0.8% agarose electrophoresis (Sambrook & Russel, 2001), and by Nanodrop 1000® spectrophotometry (Thermo Scientific). Afterwards, DNA were diluted for a final concentration of 10 ng/µl for posterior amplification reactions.

Amplification and sequencing of selected barcode regions

Five plastidial regions were selected and sequenced as DNA barcode candidate, based on previously reports for Angiosperms and Bambusoideae: the recommended two-locus core, *rbcL* and *matK*, and supplementary intergenic spacers, *psbK-psbI*, *rpl32-trnL* and *trnH-psbA*. Primers and their respective PCR conditions are summarized in table 1.

PCR conditions (Polymerase Chain Reactions) were standardized, aiming the amplification of the greatest number of samples with similar parameters. The amplification reactions were performed in a final volume of 20 µl, containing 0.2 mg/ml BSA, 1x PCR buffer, 1 mM (for *rbcL*, *trnH-psbA* and *rpl32-trnL*) to 1.5 mM $MgCl_2$ (for *matK* and *psbK-psbI*), 0.15 mM of each dNTP, 0.15 µM of each primer (10 µM), 0.8 U Platinum Taq DNA polymerase and from 10 ng (for *rpl32-trnL*) to 20 ng (for *rbcL*, *matK*, *psbK-psbI* and *trnH-psbA*) of DNA. PCR products were purified by differential precipitation with PEG8000 solution (20% PEG8000 and 2,5M NaCl; Lis & Schleif, 1975), and verified in 1.5% agarose electrophoresis.

Table 1. Universal primers selected as DNA barcode in Bambusoideae and PCR amplification cycles.

Locus [1]	**Primers: 5' - 3' sequences**	**PCR** [2]	**Refs.** [3]
rbcL	109-F: TGGCAGCATTCCGAGTAASTCCT 926-R: CATACGCAATGCTTTAGCTAATACACG	94°C 4 min; 30x (94°C 30 seg, 60°C 30 seg, 72°C 1 min); 72°C 7 min	1
matK	454-F: CATATAGARATACCYTAYCCTATC 1315-R: GCTAAAGTTCTAGCRCATGAAAG	94°C 4 min; 30x (94°C 30 seg, 54°C	1, 2

		45 seg, 72°C 1 min); 72°C 7 min	
trnH-psbA	trnH2-F: CGCGCATGGTGGATTCACAATCC psbA-R: GTTATGCATGAACGTAATGCTC	94°C 4 min; 30x (94°C 30 seg, 56°C 45 seg, 72°C 1 min); 72°C 7 min	3, 4, 5, 9
psbK-psbI	psbK-F: TTAGCCTTTGTTTGGCAAG psbI-R: AGAGTTTGAGAGTAAGCAT	94°C 4 min; 30x (94°C 30 seg, 58°C 45 seg, 72°C 1 min); 72°C 7 min	6, 7, 9
rpl32-trnL	rpl32-F: CAGTTCCAAAAAAACGTACTTC trnL (UAG)-R: CTGCTTCCTAAGAGCAGCGT	94°C 4 min; 30x (94°C 30 seg, 56°C 30 seg, 72°C 1 min); 72°C 7 min	1, 8

[1] Expected amplicon size: *rbcL*, 824 pb; *matK*, 832 pb; *trnH-psbA*, 645 pb (variable); *psbK-psbI*, 443 pb; *rpl32-trnL*, 686 pb (variable). [2] PCR conditions; [3] References: 1. Peterson et al., 2014; 2. GPWG, 2011; 3. Cai et al., 2012; 4. Tate & Simpson, 2003; 5. Sang et al., 1997; 6. Sosa et al., 2013; 7. Lahaye et al., 2008; 8. Shaw et al., 2007; 9. Fazekas et al., 2008.

Bidirectional sequencing reactions were performed in both directions, forward and reverse, using BigDye® Terminator V3.1 cycle kit, according to the manufacturer's protocol. (Applied Biosystems, 2002), in an automate ABI 35000xl Genetic Analyzer (Applied Biosystems, Foster City, CA) at Laboratory of Developmental Physiology and Genetics (LFDGV, UFSC). Each reaction contained 1 µl of purified amplification product, 1 µl of BigDye Terminator Ready Reaction Mix, 4 µl of 5x Sequencing buffer and 1 µl of 3,2 µM primer (each), for a final volume of 20 µl. PCR sequencing amplifications were performed according to the following conditions: initial denaturation at 96°C for 2 min; 35 cycles of denaturation (96°C for 10 sec), primer annealing (temperature of each primer) for 5 sec, and extension at 60°C for 4 min, followed by a final step at 4°C. Afterwards, PCR products were precipitated with ethanol/EDTA/5M ammonium acetate to remove excesses of dye terminators, and resuspended in 10 µl of HiDi formamide.

After visual inspection in both ends of the sequences, electropherograms were subjected to basecalling using Sequence Analysis v1.0 software, considering as minimum quality parameter an average QV of 20. Consensus sequences for each sample and locus were obtained by assembling forward and reverse trace data using CLC Main Workbench v.8.0.1 (CLC Bio, Aarhus, Denmark), and manually checked for quality scores and edited, as necessary. All newly generated sequences will be deposited in GenBank database (www.ncbi.nlm.nih.gov).

Alignment and dataset reconstruction

To enhance analysis robustness, sequences from GenBank were carefully selected and compared to our newly generated sequences, regarding length and coverage of the genomic region, and doubtful sequences were ignored. Aiming to avoid species misidentification, 39 accessions of complete plastid genomes were prioritized, of which sequences of each locus individually retrieved (table S2). Also, to increase intraspecific sampling, unique GenBank accessions were selected by equal voucher, when available, excluding shortest and doubtful sequences (table S3).

For each locus, multiple sequence alignments (MSA) were performed using MAFFT online service (Katoh et al. 2019) with default parameters, and subsequent manual optimization retaining common start and end lengths. Alignment gaps or ambiguous sites were rechecked on trace files of newly generated sequences, in order to confirm variable sites, and those present in at least two sequences were kept for analysis. Afterwards, individual MSA were concatenated, using MEGA platform, generating 5 combined datasets: (1) *rbcL* + *matK*; (2) *rpL32-trnL* + *psbK-psbI*; (3) *rpL32-trnL* + *psbK-psbI* + *trnH-psbA*; (4) *rpL32-trnL* + *psbK-psbI* + *matK*; and (5) *rbcL* + *matK* + *rpL32-trnL* + *psbK-psbI* + *trnH-psbA*.

Data analysis and evaluation of DNA barcode potential of selected regions

The candidate barcode regions were evaluated to attend the principles of standardization, minimalism, and scalability (Hollingsworth et al., 2011), *i.e.*, accessing the criteria of universality of amplification and sequence quality parameters, nucleotide variability and species discrimination power.

Amplification success was estimated, individually, by the number of amplified and sequenced samples following the PCR conditions predominant for each individual locus. Sequencing quality was assessed by mean values of sequence length, CRL (Contiguous Read Length), QV20+ and TS (Trace Score) for each region, obtained through Sequence Scanner v2 software (Applied Biosystems 2012).

Nucleotide variability of each locus was estimated based on MSA of each enriched database, using DnaSP v.6 software (Rozas et al., 2017). Several parameters were estimated, including mean alignment length (pb), number of sites excluding gaps, number of monomorphic (invariable) sites, total number of mutations (Eta), number of segregating sites (S), number of parsimony informative sites (PIC), number (h) and haplotype diversity (Hd), and nucleotide diversity (π).

In each locus individually aligned, best-fit models of molecular evolution were determined using 'jModelTest2 on XSEDE' (Darriba et al. 2012), implemented in CIPRES Science Gateway (Miller et al. 2010; https://www.phylo.org), according to hierarchical likelihood ratio tests based on the Akaike information criteria (AIC; Akaike 1974). After preliminary evaluation of nucleotide diversity of individual locus, the following combinations were constructed and analyzed with phylogenetic inference: *i*) *rbcL* + *matK*, according to CBOL (2009) recommendations; *ii*) *rpl32-trnL* + *psbK-psbI*, harboring the two most variable loci; *iii*) *rpl32-trnL* + *psbK-psbI* + *trnH-psbA*, encompassing the tested intergenic spacers; *iv*) *rpl32-trnL* + *psbK-psbI* + *matK*, with the three most variable loci, and; *v*) *rbcL* + *matK* + *rpl32-trnL* + *psbK-psbI* + *trnH-psbA*, including all the five tested loci combined.

Aiming to evaluate species discriminatory power of the five selected barcode loci and their combinations using reciprocal monophyly as criteria, phylogenetic analysis was performed in the total 10 datasets. Bayesian inferences (BI) analysis was carried out using MrBayes 3.2.7 (Ronquist et al. 2012) in the XSEDE platform in the CIPRES Science Gateway (Miller et al. 2010; https://www.phylo.org). Analysis consisted in 1 million generations in two runs, each with four Monte Carlo Markov chains (MCMC, with one cold and three heated), and sampling trees every 1000 generations and 25% of burn-in. Coverage of the runs were assessed by checking effective sample sizes of all parameters greater than 200 using Tracer v1.6 (Rambaut et al. 2014). The remaining trees were summarized in a majority rule consensus including the posterior probabilities (PP) as branch support estimates, considering PP ≥ 0.9 as strongly supported nodes. Afterwards, the best trees were visualized using FigTree v.1.4.4 (Rambaut, 2019) and edited with InkScape 1.0.1 software (Inkscape Project 2020), with labeled nodes with posterior probabilities.

RESULTS

Sequencing success and molecular nucleotide diversity

The five selected cpDNA barcode regions were remarkably efficient for amplification and bidirectional sequencing success (table 2). All the selected primers were able to amplify 100% of bamboo samples (73 each), providing a total of 365 newly generated sequences. This is the first inclusion reported of neotropical bamboo species (Bambuseae and neotropical) in a DNA barcoding approach.

Evaluation of sequencing quality (table 2) showed greatest mean values of CRL, Trace Score and QV20+. CRL represents the longest contiguous sequence length of bases with Phred score above a specified threshold (QV > 20) within a window size. Also, trace score means basecalling Phred scores after sequence trimming, and QV20+ represents the total number of bases within the electropherogram with basecalling values above 20.

Table 2. Evaluation of sequence quality parameters. All the selected primers successfully amplified 100% of samples, representing 73 sequences, each.

Locus	Mean seq. length, pb (SD)	Sequence length range, pb (SD)	CRL[1] (SD)	TS[2] (SD)	QV20+ (SD)
rbcL	770.01 (3.0)	765 – 783	784.40 (11.61)	50.91 (3.79)	775.08 (38.55)
matK	800.37 (1.85)	798 – 808	801.96 (37.47)	49.71 (5.96)	786.41 (43.58)
psbK-psbI	386.78 (4.09)	376 – 399	422.76 (60.61)	48.37 (6.08)	427.76 (74.33)
rpl32-trnL	844.51 (2.68)	747 – 836	845.82 (63.81)	47.08 (3.83)	831.48 (48.37)
trnH-psbA	590.67 (6.27)	575 – 600	610.22 (18.95)	44.35 (9.61)	603.49 (45.01)

[1]CRL, Contiguous read length. [2]TS, Trace Score. Numbers within parenthesis represents standard deviations.

After database enrichment with NCBI accessions, sequence variability analysis was performed in a final matrix composed of 144 sequences of *rbcL*, 143 of *matK*, 128 of *psbK-psbI*, 135 of *rpl32-trnL* and 141 of *trnH-psbA*. Assessment of nucleotide diversity within individual alignments are summarized in table 3. *Pariana campestris* (NCBI cpGenome; NC02749.1) was removed from *trnH-psbA* datasets due to the lack of one *rps19* gene copy, resulting in ambiguous alignments.

Sequence alignment length was longer in *rpl32-trnL* locus (994 pb), influenced by the recurrence of sites with gaps (400 sites). Sequence conservation, by means of monomorphic sites, was higher in *rbcL* alignment (90.84%), followed by *matK* (82.64%) and intergenic spacers *trnH-psbA* (77.72%), *psbK-psbI* (61.42%) and *rpl32-trnL* (42.45%). Following conservation patterns, sequence alignment of *psbK-psbI* and *rpl32-trnL* showed greater nucleotide diversity considering the number of segregating/polymorphic sites, parsimony informative sites (PICs) and nucleotide diversity (π), when compared to the three remaining analyzed loci (table 3). Despite medium conservation rate, *trnH-psbA* sequences recovered the lowest percentage of parsimony informative sites (7.34%), followed by *rbcL* (most conserved loci) and *matK* (increasing order).

Table 3. Assessment of nucleotide diversity of individual alignments of the five selected barcoding loci in Bambusoideae datasets.

	rbcL	*matK*	*psbK-psbI*	*rpl32-trnL*	*trnH-psbA*
N. of sequences	144	143	128	135	141
Alignment length, pb	767	801	407	994	615
Sites without gaps (n. of gaps)	767 (0)	798 (3)	352 (55)	594 (400)	545 (70)
N. of invariable/monomorphic sites[1]	694 (90.48%)	662 (82.64%)	250 (61.42%)	422 (42.45%)	478 (77.72%)
Total n. mutations, Eta	80	155	116	201	77
N. segregating/polymorphic sites, S[1]	73 (9.51%)	136 (16.97%)	102 (25.06%)	172 (17.31%)	67 (10.89%)
PIC (%)[2]	57 (7.43)	97 (12.16)	78 (22.16)	126 (21.22)	40 (7.34)
N. haplotypes, h	40	43	43	48	37
Haplotype diversity, Hd (SD)	0.92 (0.014)	0.917 (0.012)	0.933 (0.011)	0.939 (0.011)	0.879 (0.015)
Nucleotide diversity, π (SD)[3]	0.01387 (0.00068)	0.0169 (0.00134)	0.03469 (0.00217)	0.02719 (0.00198)	0.01105 (0.00066)
Evolutionary model[4]	TIM1+I+G	TVM+I+G	TPM1uf+G	TPM3uf+I+G	TPM3uf+I+G

[1] % of monomorphic and polymorphic sites estimated based on total alignment length; [2] % of PIC (Parsimony informative sites) estimated based on the number of sites excluding gaps; [3] Nucleotide diversity (π) estimated with Jukes & Cantor correction. [4] Evolutionary model according to Akaike information criteria (AIC).

Bayesian inferences and species discrimination power of the selected loci, individually and combined

Species discriminatory power of each locus was assessed through Bayesian inferences, taking into consideration the best individual evolutionary model. The obtained phylogenetic trees for the five individual locus displayed incongruent topologies between each other when considered the expected placement of the main bamboo lineages (Figure 1A-E). Also, none of them were sufficiently informative to resolve among species and genera in a wide basis, mainly in the ingroups of woody bamboo lineages expressing short branch lengths in closely related taxa. However, it's remarkably stated that ingroup of Olyreae tribe showed long branch lengths and congruent topology among the five generated trees.

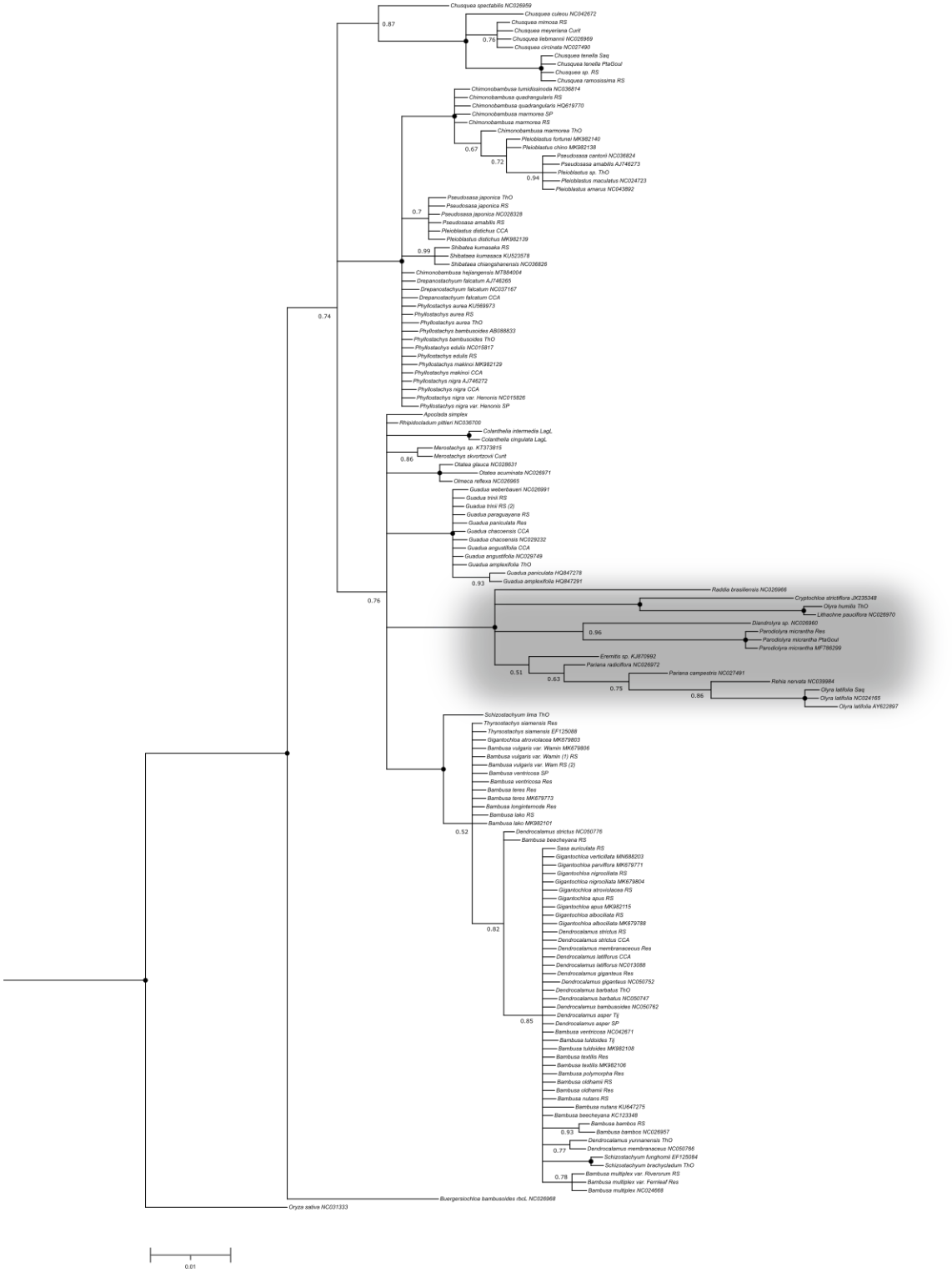

Figure 1A. Bayesian inferences of *rbcL* sequence alignment of Bambusoideae clades. Numbers of nodes indicate posterior probabilities (PP), and those marked with round black circles represent PP=1. Highlighted clade indicates the Olyreae tribe.

Figure 1B. Bayesian inferences of *matK* sequence alignment of Bambusoideae clades. Numbers of nodes indicate posterior probabilities (PP), and those marked with round black circles represent PP=1. Highlighted clade indicates the Olyreae tribe.

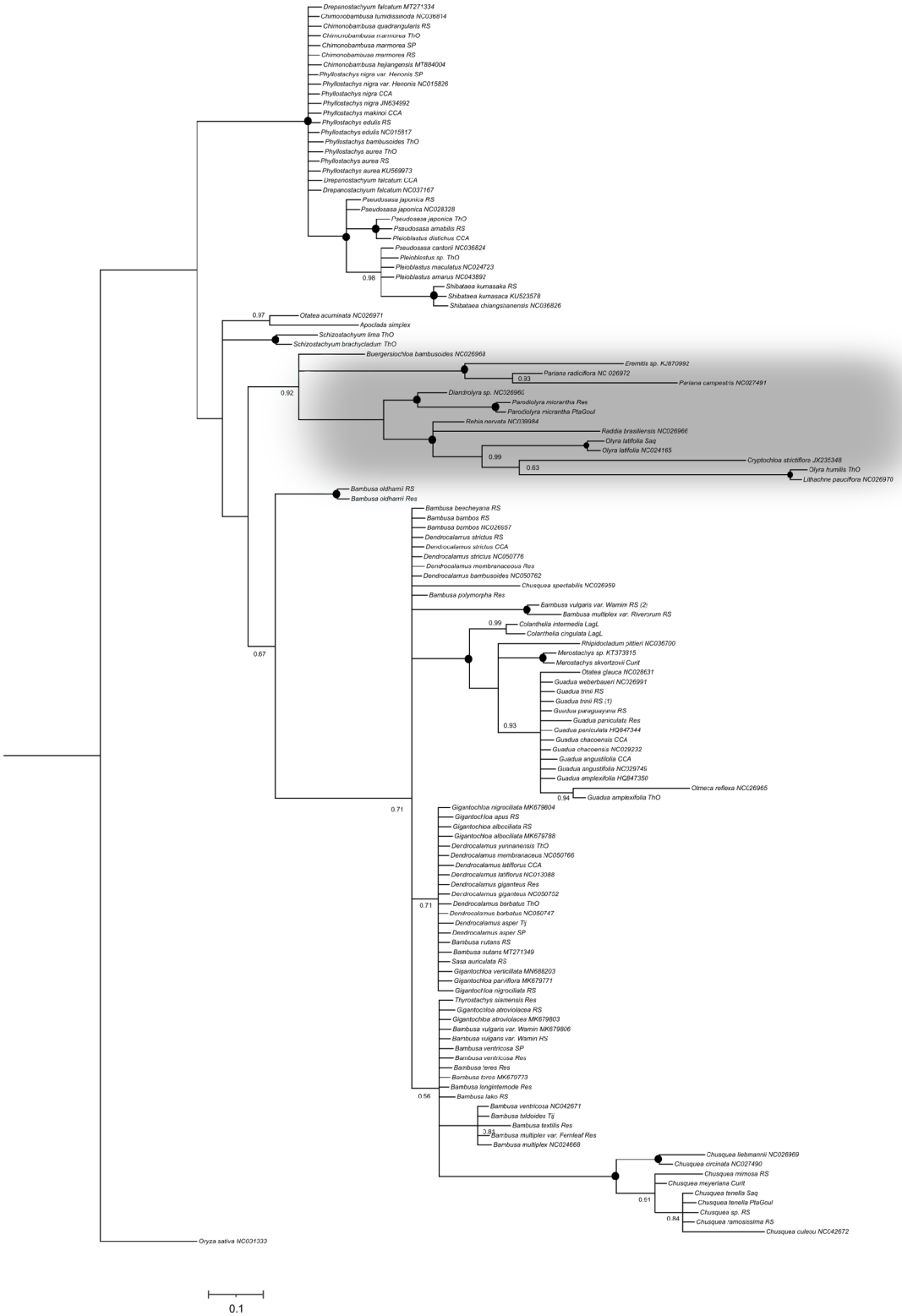

Figure 1C. Bayesian inferences of *psbK-psbI* sequence alignment of Bambusoideae clades. Numbers of nodes indicate posterior probabilities (PP), and those marked with round black circles represent PP=1. Highlighted clade indicates the Olyreae tribe.

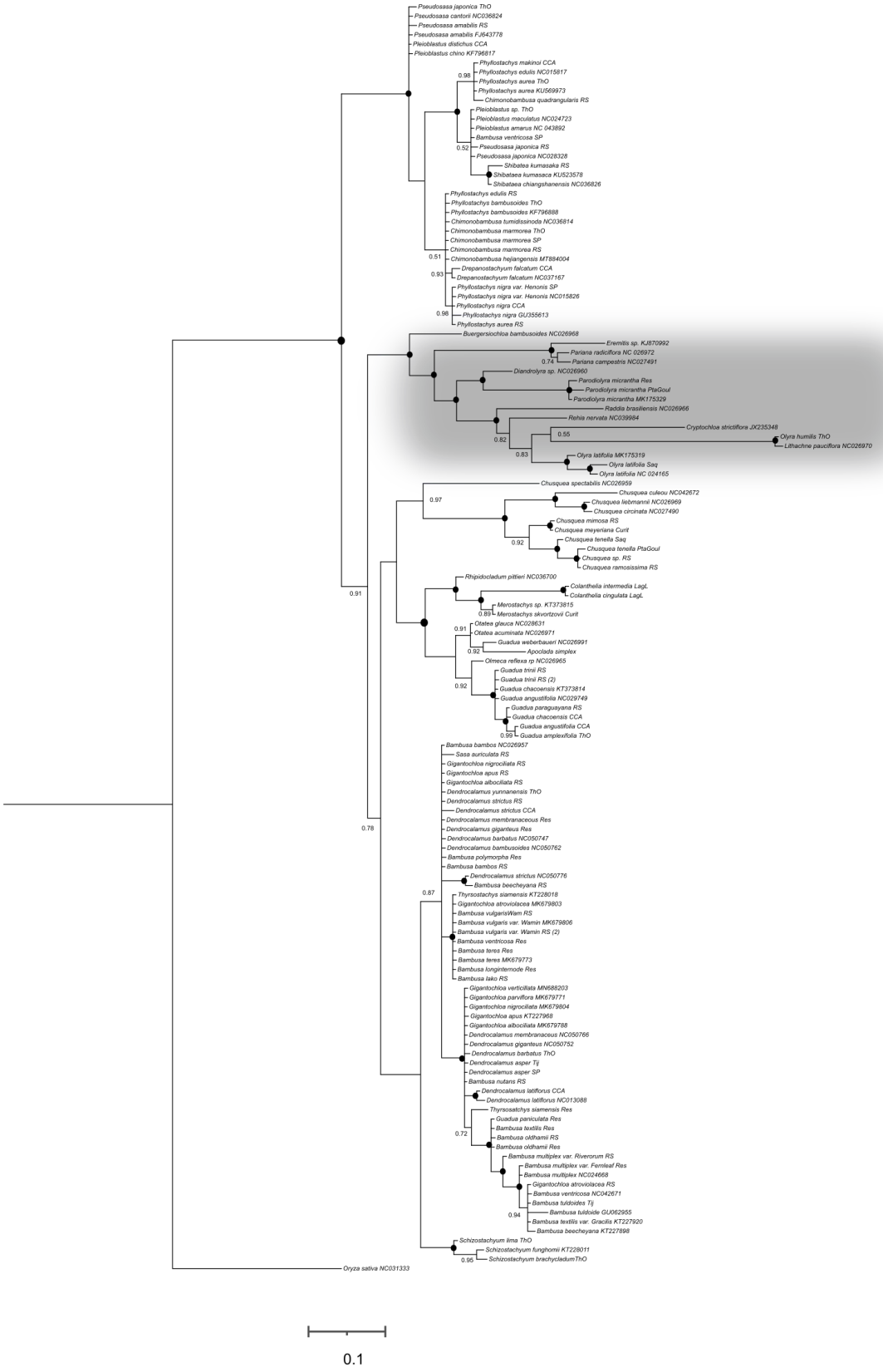

Figure 1D. Bayesian inferences of *rpl32-trnL* sequence alignment of Bambusoideae clades. Numbers of nodes indicated posterior probabilities, and those marked with round black circles represent PP=1. Highlighted clade indicates the Olyreae tribe.

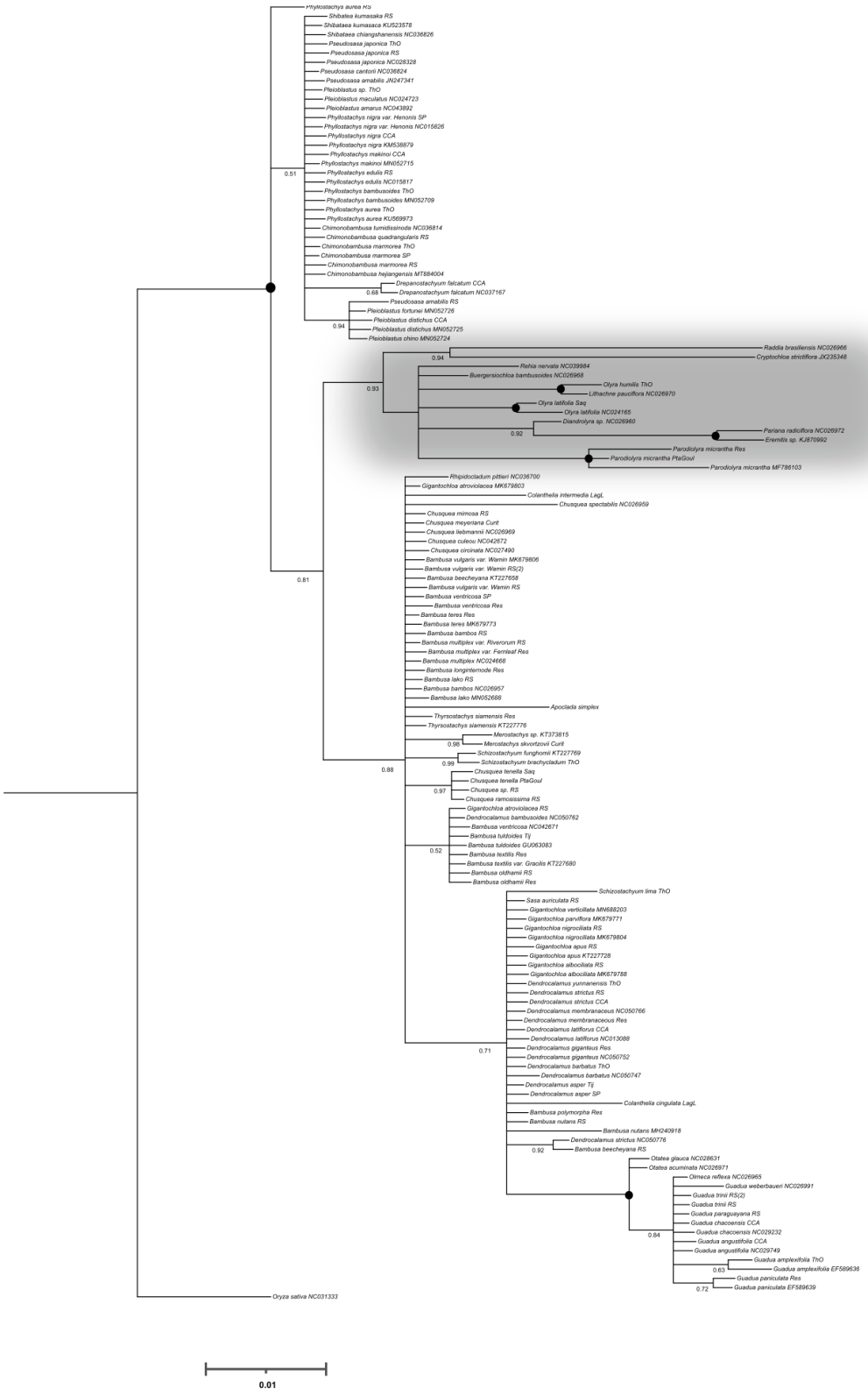

Figure 1E. Bayesian inferences of *trnH-psbA* sequence alignment of Bambusoideae clades. Numbers of nodes indicate posterior probabilities (PP), and those marked with round black circles represent PP=1. Highlighted clade indicates the Olyreae tribe.

Based on such observed nucleotide diversity, along with the recommendation of CBOL (2009), the following dataset were constructed to access their potential discriminatory power of wide-broad Bambusoideae species: *i*) *rbcL* + *matK*; *ii*) *rpl32-trnL* + *psbK-psbI*; *iii*) *rpl32-trnL* + *psbK-psbI* + *trnH-psbA*; *iv*) *rpl32-trnL* + *psbK-psbI* + *matK*; and *v*) *rbcL* + *matK* + *rpl32-trnL* + *psbK-psbI* + *trnH-psbA*. For that, five combined datasets were employed in phylogenetic reconstruction and species identification through reciprocal monophyly BI approach (Figure 2). shows phylogenetic topologies of combined datasets.

Bayesian analysis of combined datasets shows some remarkable features, as follow:

i) *rpl32-trnL* + *psbK-psbI*, *rpl32-trnL* + *psbK-psbI* + *trnH-psbA* and *rpl32-trnL* + *psbK-psbI* + *matK* showing topological congruence with phylogenies based on nuclear data, instead of cpDNA, *i.e.*, closest relationship among woody bamboos clades after herbaceous bamboos.

ii) Long branches at the ingroup regarding Olyreae tribe;

iii) Nodes harboring paleotropical lineage undistinguishing *Bambuseae*, *Dendrocalamus* and *Gigantochloa* genera;

iv) Chusqueinae subtribe showing close relationship with Arundinarieae tribe rather than others from Bambuseae neotropical lineage, as it should be expected;

v) Topology of *rbcL* + *matK* + *rpl32-trnL* + *psbK-psbI* + *trnH-psbA* congruent with cpDNA phylogenetic signal previously reported, *i.e.*, sister relationship among {Bambuseae + Olyreae} + Arundinarieae.

vi) Achievement of generic discrimination in neotropical lineages, meaning *Chusquea*, *Guadua*, *Colanthelia* and *Merostachys* genera.

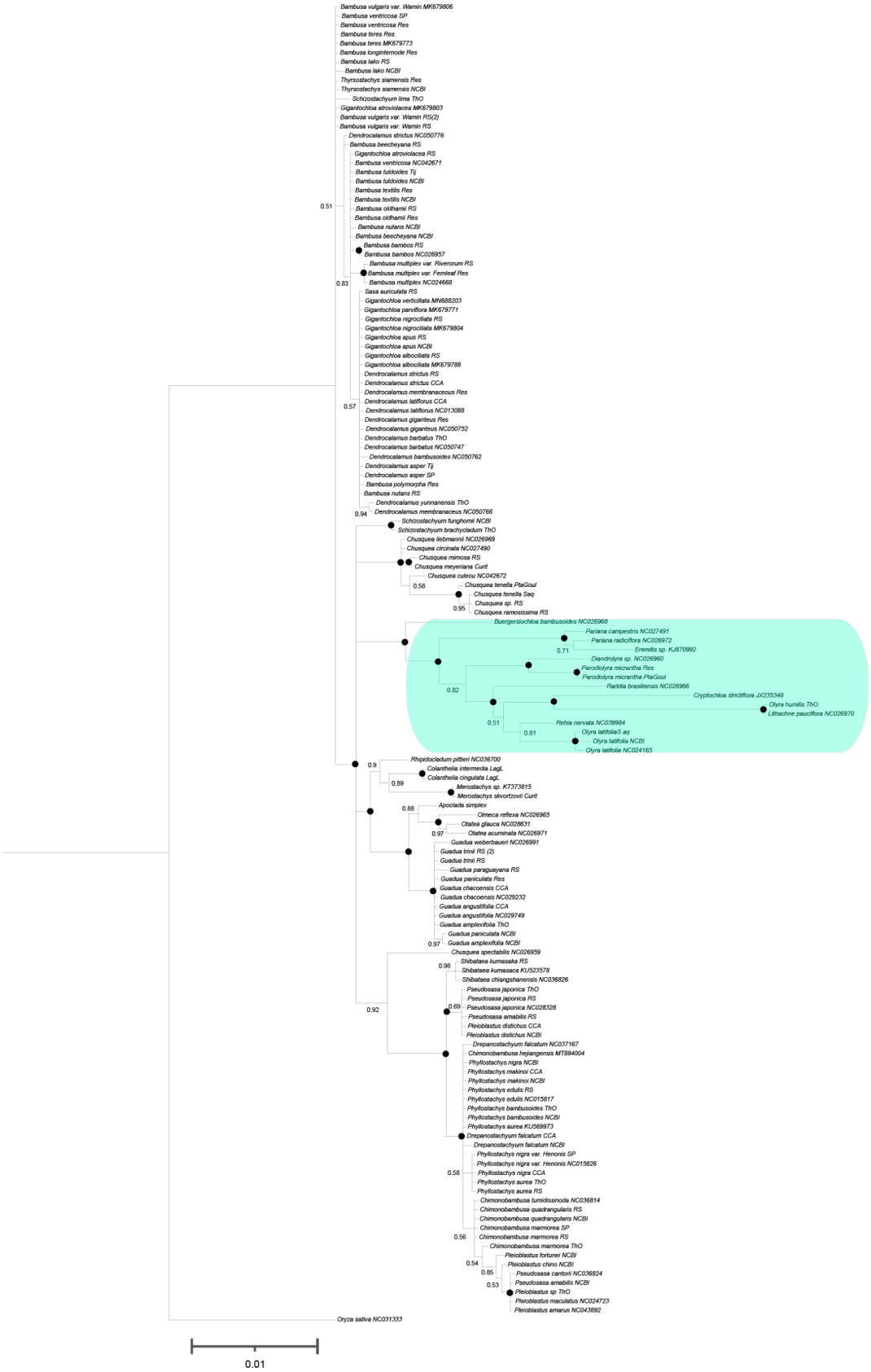

Figure 2A. Bayesian inferences of combined datasets, using *rbcL* + *matK* alignments of Bambusoideae. Numbers in nodes indicate posterior probabilities (PP), and those marked with round black circles represent PP=1. Highlighted clade indicates the Olyreae tribe.

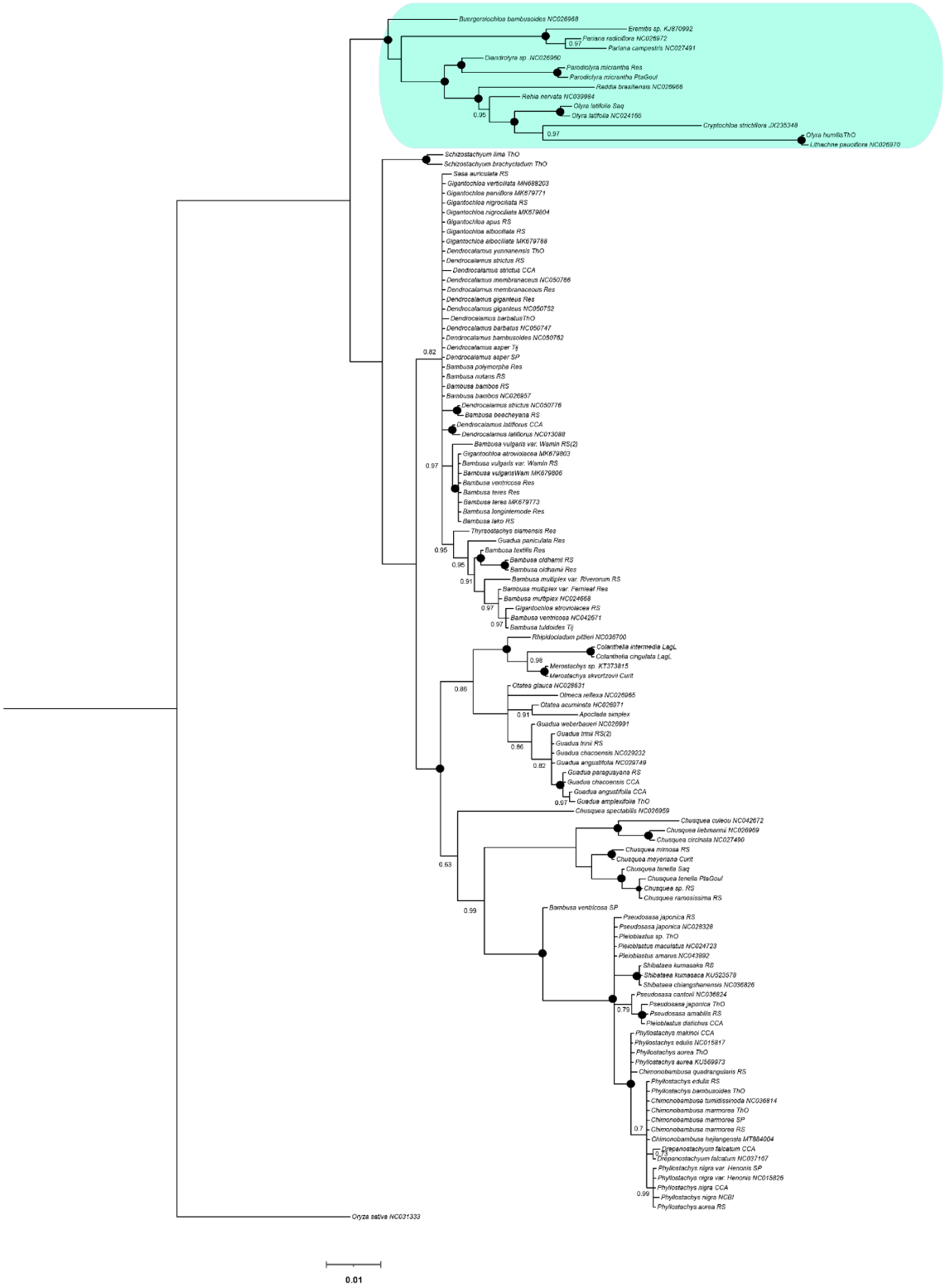

Figure 2B. Bayesian inferences of combined datasets, using *rpl32-trnL* + *psbK-psbI* alignments of Bambusoideae. Numbers in nodes indicate posterior probabilities (PP), and those marked with round black circles represent PP=1. Highlighted clade indicates the Olyreae tribe.

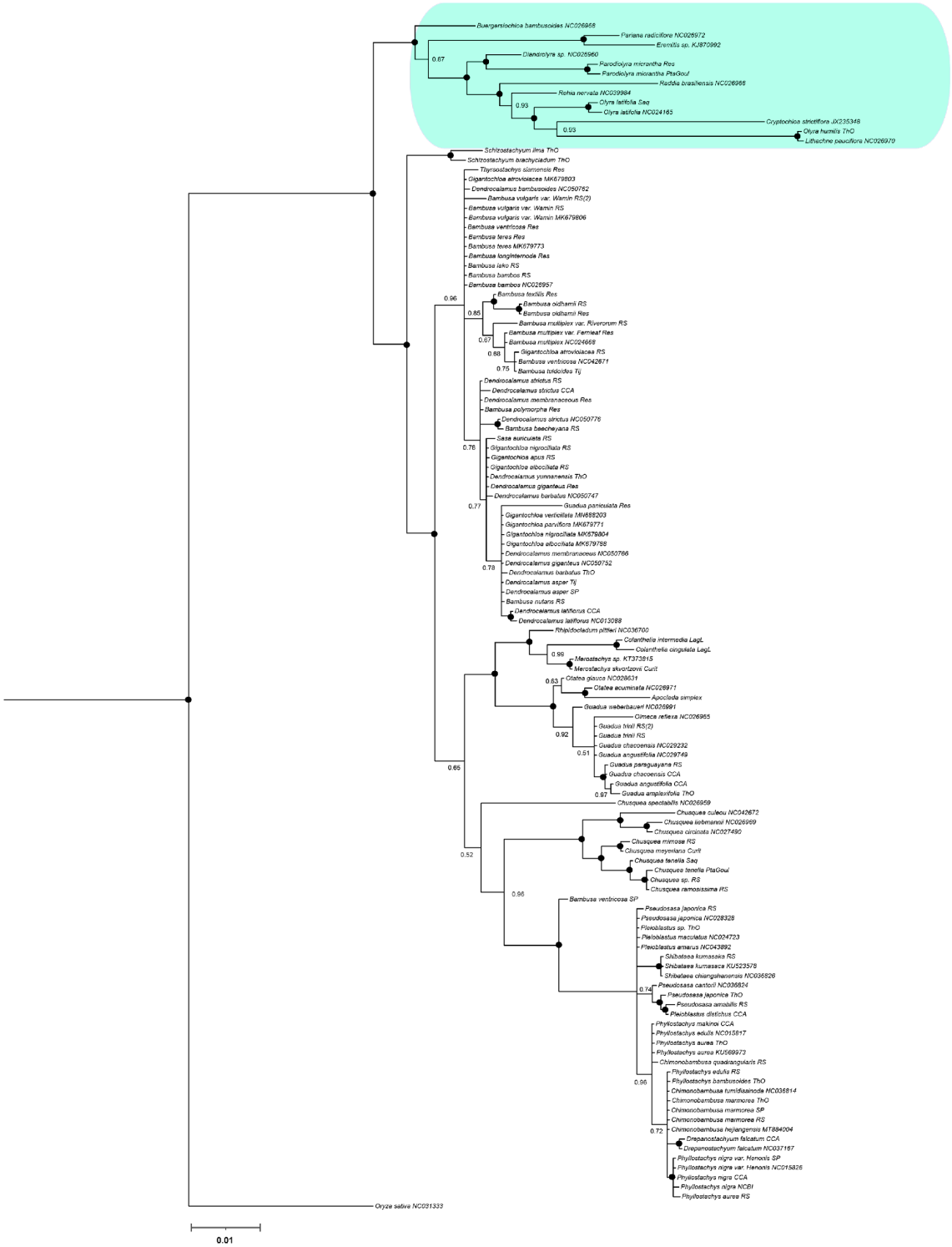

Figure 2C. Bayesian inferences of combined datasets, using *rpl32-trnL + psbK-psbI + trnH-psbA* alignments of Bambusoideae. Numbers in nodes indicate posterior probabilities (PP), and those marked with round black circles represent PP=1. Highlighted clade indicates the Olyreae tribe.

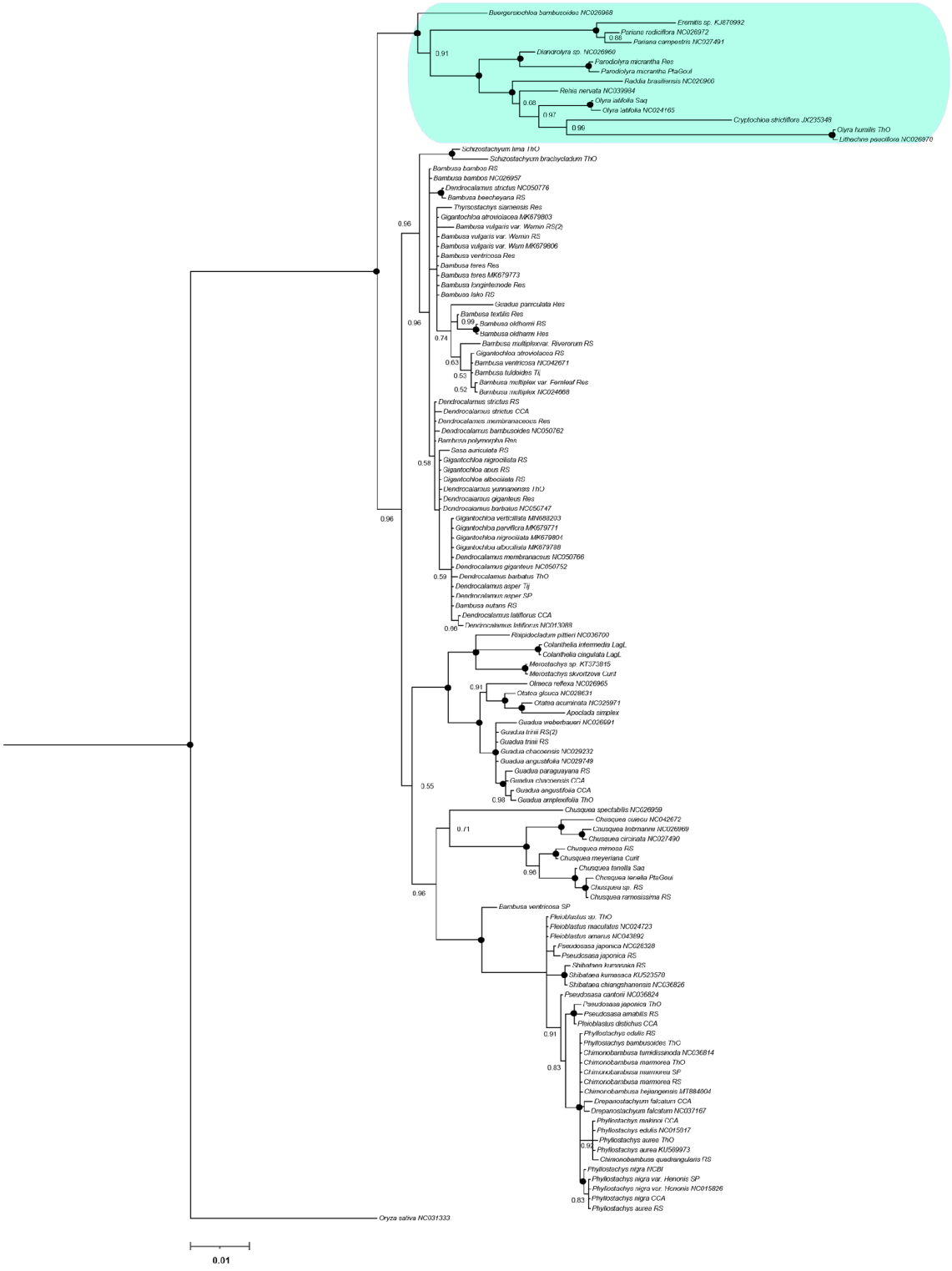

Figure 2D. Bayesian inferences of combined datasets, using *rpl32-trnL* + *psbK-psbI* + *matK* alignments of Bambusoideae. Numbers in nodes indicate posterior probabilities (PP), and those marked with round black circles represent PP=1. Highlighted clade indicates the Olyreae tribe.

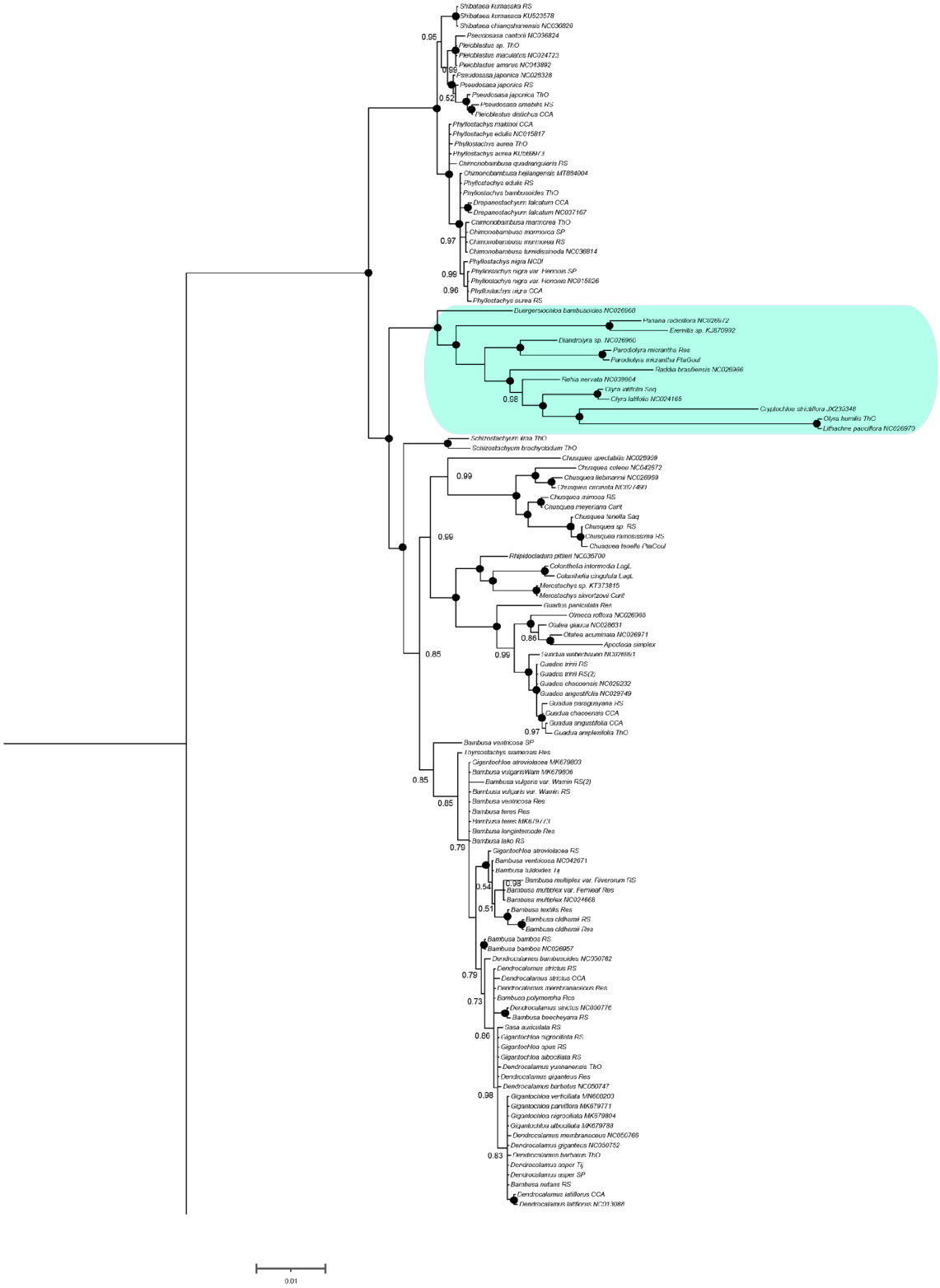

Figure 2E. Bayesian inferences of combined datasets, using *rbcL* + *matK* + *rpl32-trnL* + *psbK-psbI* + *trnH-psbA* alignments of Bambusoideae. Numbers in nodes indicate posterior probabilities (PP), and those marked with round black circles represent PP=1. Highlighted clade indicates the Olyreae tribe.

DISCUSSION

DNA barcoding is a promising technique to discriminate species, encountering a single or multilocus approach, suggested to overcome problems related to classical systematic classification (Sawarkar et al. 2021). The selected regions should present conserved flanking regions enabling the design of universal primers, and sufficient nucleotide variation to distinguish between species (Pettengil & Neel 2010). Thus, due to low rates of nucleotide substitutions of plant mitochondrial genome and extensive gene duplication in nuclear genome, cpDNA have been suggested as the most suitable for candidate barcodes for plants (Kress & Erickson 2007; Lahaye et al. 2008).

The five selected potential DNA barcode for Bambusoideae showed greatest efficiency regarding amplification success (100%) and sequencing quality, reflecting on routinely and reliable sequencing criteria. Such property contributes to one of the main goals of DNA barcoding technique, by means of stablishing a shared community of DNA sequences for taxonomic assignments purposes, based on universality (ease of amplification and sequencing), sequence quality and discriminatory power (CBOL 2009; Hollingsworth et al. 2011; Cai et al. 2012). However, the greatest challenge to construct a robust DNA barcoding assay is to capture the proper evolutionary relationship and nucleotide variation among both highly divergent and closely related species, enabling the achievement of sufficient discriminatory power (Kress et al. 2015).

Plastidial genomes harbors differential nucleotide substitution rates among distinct regions, in which some hyper variable sites suggest the successful application of cpDNA to discriminate species at lowest taxonomic levels (Shaw et al. 2014, Qin et al. 2020). Those candidate regions both coding regions (e.g., *rbcL* and *matK*), and rapidly evolving loci (e.g., *trnH-psbA*, *psbK-psbI*, *rpl32-trnL*), having their efficiency subjected to low nucleotide variation in coding regions, and difficulties related to sequence alignment of distantly related species and homoplasy in non-coding regions (Shaw et al. 2007; Pettengil & Neel 2010; Dong et al. 2012).

The five selected regions showed differential nucleotide diversity, in which the most conserved ones, *rbcL* and *matK* (90.48 and 82.64% of monomorphic sites, respectively) comprises gene coding regions of cpDNA (Kress & Erickson 2007; Hollingsworth et al. 2011). Despite being proposed as core barcode for land plants, *rbcL* + *matK* showed low discriminatory in the analyzed database, also reported for other bamboo assay (Sosa et al. 2013; Zhang et al. 2013; Dev et al. 2020). However, the

proposed combination of the barcode core with more variable cpDNA loci in order to increase species discrimination has been still controversial, where no universal loci has been stated as standard DNA barcode for Bambusoideae.

Considered one of the most promising and used plastid barcode (Kress & Erickson 2007; Shaw et al. 2007; Liu et al. 2014), *trnH-psbA* recovered the lowest percentage of parsimony informative sites (7.34%) among the five analyzed locus, being equivalent to *rbcL* (7.43%) and lower than *matK* (12.16%). In most bamboo species, this intergenic spacer flaking a coding *rps19* gene at LSC/IR border, is encountering a higher conservation rate and variable barcode performance according to the plant group (Shaw et al. 2014; Dev et a. 2020; Sijimol et al. 2020). Another promising cpDNA loci is the variable *psbK-psbI* intergenic spacer, showing the greatest potential of phylogenetic signal followed by its percentage of polymorphic sites (25.06%) and PIC's (22.16%). However, Bayesian inference of the referred region didn't reflect differential topologies compared to the other four analyzed cpDNA regions.

One of the most variable loci among non-coding cpDNA in bamboos, *rpl32-trnL* recovered a remarkable distinct topology among the five analyzed loci, both individually or combined with other IGS (*psbK-psbI* and *trnH-psbA*), *i.e.*, closely relationship among woody bamboo lineages compatible with phylogenies based on nuclear DNA data (Wysocki et al. 2015; Guo et al. 2019; Carvalho et al. 2021). However, all the analyzed database recovered distinguished topologies, highlighting the importance of species concept and analytical methods adopted to evaluate DNA barcode efficiency (Collins & Cruickshank 2013).

Several analytical methods are proposed to evaluate barcode discrimination power, which are primarily based on genetic distances and phylogenetic approaches (Hajibabaei et al. 2016). Both approaches, in a certain manner, rely on the assumption that intraspecific variability exceeds interspecific diversity, and that species are independent evolutionary units (Mallo & Posada 2016). Still, in some complex evolutionary groups, such as Bambusoideae, species limit might not be clear, along with different levels of sequence divergence among lineages (Naciri & Linder 2015; Walker et al. 2019).

In fact, bamboo diversity is described to be a product of several independent hybridization events, and relationships among main lineages are not well defined (Peng et al. 2013; Triplett et al. 2014; Guo et al. 2019; Wang et al. 2020). Although herbaceous bamboos display high nucleotide evolutionary rate, due to its short generation time, woody bamboos are recognized by rapid radiation, intrageneric hybridization and

convergent evolution (Tyrrell et al. 2018; Zhou et al. 2020; Ye et al. 2021). Thus, low resolution of DNA barcode assays, regarding the selection of one specific and universal loci for Bambusoideae, must be the result of evolutionary features including hybridization, convergence, and occurrence of poly/paraphyletic genera (Goh et al. 2010; Ruiz-Sanchez et al. 2019; Liu et al. 2020; Sijimol et al. 2020; Triplett e Clark 2021).

Even though the advantage of using DNA barcoding for specimen identification is clearly stated, the usefulness regarding Bambusoideae subfamily is still in its early stages. However, sequencing analysis of highly diverging cpDNA regions should be continuously addressed to explore many evolutionary inquiries (Shaw et al. 2014). For evolutionary complex groups, such as bamboos, the poorly resolved phylogenies are still a challenge to be overcome, in which the attempts should consider differential evolutionary rates among distinct genome regions, especially when DNA barcoding technique is applied in a wide-broad taxonomic range (Dong et al. 2012; Wang et al. 2018). Some molecular techniques should be considered in such cases, for instance the highly polymorphic SSR markers, which can be resolutive in polyploid and diverse groups as Bambusoideae (Cai et al. 2019; Meena et al. 2020).

CONCLUSIONS

Application of DNA barcoding technique has been tested in a widely broad sampling approach for Bambusoideae, in which 365 cpDNA sequences were newly generated. Although the selected loci showed nucleotide diversity, species discrimination power has been insufficient for the subfamily. Due to its life cycle and evolutionary story, species discrimination based on cpDNA barcoding should strongly consider intergeneric and hybridization events.

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SUPPLEMENTARY MATERIAL

Table S1. Samples collected for the present study and their respective collection sites.

	Tribe[1]	Genera	Species	Collection[2]
1	A	*Chimonobambusa*	*Chimonobambusa marmorea*	RS
2	A	*Chimonobambusa*	*Chimonobambusa marmorea*	ThO
3	A	*Chimonobambusa*	*Chimonobambusa marmorea*	SP
4	A	*Chimonobambusa*	*Chimonobambusa quadrangularis*	RS
5	A	*Drepanostachyum*	*Drepanostachyum falcatum*	CCA
6	A	*Phyllostachys*	*Phyllostachys aurea*	ThO
7	A	*Phyllostachys*	*Phyllostachys aurea*	RS
8	A	*Phyllostachys*	*Phyllostachys bambusoides*	ThO
9	A	*Phyllostachys*	*Phyllostachys edulis*	RS
10	A	*Phyllostachys*	*Phyllostachys makinoi*	CCA
11	A	*Phyllostachys*	*Phyllostachys nigra*	CCA
12	A	*Phyllostachys*	*Phyllostachys nigra var. henonis*	SP
13	A	*Pleioblastus*	*Pleioblastus distichus*	CCA
14	A	*Pleioblastus*	*Pleioblastus sp.*	ThO
15	A	*Pseudosasa*	*Pseudosasa amabilis*	RS
16	A	*Pseudosasa*	*Pseudosasa japonica*	ThO
17	A	*Pseudosasa*	*Pseudosasa japonica*	RS
18	A	*Sasa*	*Sasa auriculata*	RS
19	A	*Shibataea*	*Shibataea kumasaka*	RS
20	B (N)	*Apoclada*	*Apoclada simplex*	BR470
21	B (N)	*Chusquea*	*Chusquea meyeriana*	Curit
22	B (N)	*Chusquea*	*Chusquea mimosa*	RS
23	B (N)	*Chusquea*	*Chusquea ramosissima*	RS
24	B (N)	*Chusquea*	*Chusquea sp.*	RS
25	B (N)	*Chusquea*	*Chusquea tenella*	Pta. Goul
26	B (N)	*Chusquea*	*Chusquea tenella*	Saq
27	B (N)	*Colanthelia*	*Colanthelia cingulata*	Lag. Leste
28	B (N)	*Colanthelia*	*Colanthelia intermedia*	Lag. Leste
29	B (N)	*Guadua*	*Guadua amplexifolia*	ThO

30	B (N)	*Guadua*	*Guadua angustifolia*	CCA
31	B (N)	*Guadua*	*Guadua chacoensis*	CCA
32	B (N)	*Guadua*	*Guadua paniculata*	Res
33	B (N)	*Guadua*	*Guadua paraguayana*	RS
34	B (N)	*Guadua*	*Guadua trinii*	RS
35	B (N)	*Guadua*	*Guadua trinii*	RS
36	B (N)	*Merostachys*	*Merostachys skvortzovii*	Curit
37	B (P)	*Bambusa*	*Bambusa bambos*	RS
38	B (P)	*Bambusa*	*Bambusa beecheyana*	RS
39	B (P)	*Bambusa*	*Bambusa lako*	RS
40	B (P)	*Bambusa*	*Bambusa longinternode*	Res
41	B (P)	*Bambusa*	*Bambusa multiplex* "fernleaf"	Res
42	B (P)	*Bambusa*	*Bambusa multiplex* "Riverorum"	RS
43	B (P)	*Bambusa*	*Bambusa nutans*	RS
44	B (P)	*Bambusa*	*Bambusa oldhamii*	Res
45	B (P)	*Bambusa*	*Bambusa oldhamii*	RS
46	B (P)	*Bambusa*	*Bambusa polymorpha*	Res
47	B (P)	*Bambusa*	*Bambusa teres*	Res
48	B (P)	*Bambusa*	*Bambusa textilis*	Res
49	B (P)	*Bambusa*	*Bambusa tuldoides*	Tij
50	B (P)	*Bambusa*	*Bambusa ventricosa*	Res
51	B (P)	*Bambusa*	*Bambusa ventricosa*	SP
52	B (P)	*Bambusa*	*Bambusa vulgaris* "Wamin" (2)	RS
53	B (P)	*Bambusa*	*Bambusa vulgaris* "Wam" (3)	RS
54	B (P)	*Dendrocalamus*	*Dendrocalamus asper*	Tij
55	B (P)	*Dendrocalamus*	*Dendrocalamus asper*	SP
56	B (P)	*Dendrocalamus*	*Dendrocalamus barbatus*	ThO
57	B (P)	*Dendrocalamus*	*Dendrocalamus giganteus*	Res
58	B (P)	*Dendrocalamus*	*Dendrocalamus latiflorus*	CCA
59	B (P)	*Dendrocalamus*	*Dendrocalamus membranaceus*	Res
60	B (P)	*Dendrocalamus*	*Dendrocalamus strictus*	CCA
61	B (P)	*Dendrocalamus*	*Dendrocalamus strictus*	RS
62	B (P)	*Dendrocalamus*	*Dendrocalamus yunnanensis*	ThO
63	B (P)	*Gigantochloa*	*Gigantochloa albociliata*	RS
64	B (P)	*Gigantochloa*	*Gigantochloa apus*	RS
65	B (P)	*Gigantochloa*	*Gigantochloa atroviolacea*	RS
66	B (P)	*Gigantochloa*	*Gigantochloa nigrociliata*	RS
67	B (P)	*Schizostachyum*	*Schizostachyum brachycladum*	ThO
68	B (P)	*Schizostachyum*	*Schizostachyum lima*	ThO
69	B (P)	*Thyrsostachys*	*Thyrsostachys siamensis*	Res
70	O	*Olyra*	*Olyra humilis*	ThO
71	O	*Olyra*	*Olyra latifolia*	Saq
72	O	*Parodiolyra*	*Parodiolyra micrantha*	Pta. Goul
73	O	*Parodiolyra*	*Parodiolyra micrantha*	Res

[1] **Tribes** Arundinarieae (**A**), neotropical [**B (N)**] and paleotropical Bambuseae [**B (P)**], and Olyreae (**O**). [2] **Collection places**; Private properties: BambooPlatz Garten, Eldorado do Sul, RS (**RS**); Bambuset of Thiago S. Ornellas, Florianópolis, SC (**ThO**); Instituto Jatobás, Pardinho, SP (**SP**), and Sítio Tabaetê, Tijucas, SC (**Tij**); Matriz plants in 'Centro de Ciências Agrárias/UFSC (**CCA**)' and 'Fazenda Experimental da Ressacada (**Res**)', Florianópilis, SC; Native populations in particular properties, Brunópolis, SC (**BR470**) and 'Ponta do Goulart', Florianópolis, SC (**Pta. Goul**); UFSC, campus Curitibanos (**Curit**), and hiking ways of 'Saquinho' (**Saq**) and 'Lagoinha do Leste' (**Lag. Leste**), Florianópolis, SC.

Table S2. Sequences retrieved from GenBank (NCBI) based on complete chloroplast genomes and used for database enrichment.

	Tribe [1]	**Species**	**GenBank accesion**
1	B (P)	*Bambusa bambos*	NC_026957.1
2	B (P)	*Bambusa multiplex*	NC_024668.1
3	B (P)	*Bambusa teres*	MK679773.1
4	B (P)	*Bambusa ventricosa*	NC_042671.1
5	B (P)	*Bambusa vulgaris* Wam	MK679806.1
6	O	*Buergersiochloa bambusoides*	NC_026968.1
7	A	*Chimonobambusa hejiangensis*	MT884004.1
8	A	*Chimonobambusa tumidissinoda*	NC_036814.1
9	B (N)	*Chusquea circinata*	NC_027490.1
10	B (N)	*Chusquea culeou*	NC_042672.1
11	B (N)	*Chusquea liebmannii*	NC_026969.1
12	B (N)	*Chusquea spectabilis*	NC_026959.1
13	O	*Cryptochloa strictiflora*	JX235348.1
14	B (P)	*Dendrocalamus bambusoides*	NC_050762.1
15	B (P)	*Dendrocalamus barbatus*	NC_050747.1
16	B (P)	*Dendrocalamus giganteus*	NC_050752.1
17	B (P)	*Dendrocalamus latiflorus*	NC_013088.1
18	B (P)	*Dendrocalamus membranaceus*	NC_050766.1
19	B (P)	*Dendrocalamus strictus*	NC_050776.1
20	O	*Diandrolyra* sp.	NC_026960.1
21	A	*Drepanostachyum falcatum*	NC_037167.1
22	O	*Eremitis* sp.	KJ870992.1
23	B (P)	*Gigantochloa albociliata*	MK679788.1
24	B (P)	*Gigantochloa atroviolacea*	MK679803.1
25	B (P)	*Gigantochloa nigrociliata*	MK679804.1
26	B (P)	*Gigantochloa parviflora*	MK679771.1
27	B (P)	*Gigantochloa verticillata*	MN688203.1
28	B (N)	*Guadua angustifolia*	NC_029749.1
29	B (N)	*Guadua chacoensis*	NC_029232.1
30	B (N)	*Guadua weberbaueri*	NC_026991.1
31	O	*Lithachne pauciflora*	NC_026970.1
32	B (N)	*Merostachys* sp.	KT373815.1
33	B (N)	*Olmeca reflexa*	NC_026965.1

34	O	*Olyra latifolia*	NC_024165.1
35	B (N)	*Otatea acuminata*	NC_026971.1
36	B (N)	*Otatea glauca*	NC_028631.1
37	O	*Pariana campestris*	NC_027491.1
38	O	*Pariana radiciflora*	NC_026972.1
39	A	*Phyllostachys aurea*	KU569973.1
40	A	*Phyllostachys edulis*	NC_015817.1
41	A	*Phyllostachys nigra var. henonis*	NC_015826.1
42	A	*Pleioblastus amarus*	NC_043892.1
43	A	*Pleioblastus maculatus*	NC_024723.1
44	A	*Pseudosasa cantorii*	NC_036824.1
45	A	*Pseudosasa japonica*	NC_028328.1
46	O	*Raddia brasiliensis*	NC_026966.1
47	O	*Rehia nervata*	NC_039984.1
48	B (N)	*Rhipidocladum pittieri*	NC_036700.1
49	A	*Shibataea chiangshanensis*	NC_036826.1
50	A	*Shibataea kumasaka*	KU523578.1

[1] Tribe: Arundinarieae (**A**), neotropical [**B (N)**] and paleotropical [**B (P)**] Bambuseae, and Olyreae (**O**).

Table S3. GenBank accessions retrieved individually, corresponding to each barcode locus evaluated, and used for database enrichment.

Tribe [1]	**Species**	***rbcL***	***matK***	***psbK-psbI***	***rpL32-trnL***	***trnH-psbA***
B (P)	*Bambusa beecheyana*	KC123348.1	EU434244.1	N/A	KT227898.1	KT227658.1
B (P)	*Bambusa lako*	MK982101.1	MK612105.1	N/A	N/A	MN052688.1
B (P)	*Bambusa nutans*	KU647275.1	MT271295.1	MT271349.1	N/A	MH240918.1
B (P)	*Bambusa textilis*	MK982106.1	KT226141.1	N/A	KT227920.1	KT227680.1
B (P)	*Bambusa tuldoides*	MK982108.1	MN043621.1	N/A	GU062955.1	GU063083.1
A	*Chimonobambusa quadrangularis*	HQ619770.1	HQ619826.1	N/A	N/A	N/A
A	*Drepanostachyum falcatum*	AJ746265.1	EF125170.1	MT271334.1	N/A	N/A
B (P)	*Gigantochloa apus*	MK982115.1	KT226189.1	N/A	KT227968.1	KT227728.1
B (N)	*Guadua amplexifolia*	HQ847291.1	HQ847224.1	HQ847350.1	N/A	EF589636.1
B (N)	*Guadua paniculata*	HQ847278.1	HQ847211.1	HQ847344.1	N/A	EF589639.1
O	*Olyra latifolia*	AY622897.1	AF164386.1	N/A	MK175319	N/A
O	*Parodiolyra micrantha*	MF786299.1	N/A	N/A	MK175329.1	MF786103.1
A	*Phyllostachys bambusoides*	AB088833.1	MN043636.1	N/A	KF796888.1	MN052709.1
A	*Phyllostachys makinoi*	MK982129.1	MN043642.1	N/A	N/A	MN052715.1
A	*Phyllostachys nigra*	AJ746272.1	EU434241.1	JN634992.1	GU355613.1	KM538879.1
A	*Pleioblastus chino*	MK982138.1	MN043651.1	N/A	KF796817.1	MN052724.1
A	*Pleioblastus distichus*	MK982139.1	MN043652.1	N/A	N/A	MN052725.1
A	*Pleioblastus fortunei*	MK982140.1	MN043653.1	N/A	N/A	MN052726.1
A	*Pseudosasa amabilis*	AJ746273.1	JN247151.1	N/A	FJ643778.1	JN247341.1

B (P)	*Schizostachyum funghomii*	EF125084.1	KT226230.1	N/A	KT228011.1	KT227769.1
B (P)	*Thyrsostachys siamensis*	EU434261.1	KT226237.1	N/A	KT228018.1	KT227776.1

1 Tribe: Arundinarieae (**A**), neotropical [**B (N)**] and paleotropical [**B (P)**] Bambuseae, and Olyreae (**O**).
N/A: unavailable sequences.

5. Capítulo II

Manuscrito formatado de acordo com a normativa do periódico Plant Molecular Biology.

New plastid genomes of three Brazilian native bamboo species – *Parodiolyra micrantha* (Kunth) Davidse & Zuloaga, *Guadua trinii* Nees ex Rupr. and *Apoclada simplex* McClure & L.B. Sm. – and comparative analysis of structure and genetic diversity of Neotropical bamboos (Bambusoideae, Poaceae)

ABSTRACT

The bamboos, representatives of Bambusoideae subfamily (Poaceae), comprise 1680 species of wide global distribution, being Brazil the country with highest diversity and endemism of neotropical species (woody Bambuseae and herbaceous Olyreae tribes). These species occur preferentially in humid forest habitats, such as the threatened Atlantic Forest and Amazonia. Bamboo diversity is a consequence of independent polyploidization and hybridization events, in which plastidial genome sequences become important resources for evolutionary and phylogeographic studies. In the present study we sequenced and assembled the plastomes of three neotropical bamboo species (*Parodiolyra micrantha*, *Apoclada simplex* and *Guadua trinii*) aiming to perform a comparative analysis among their respective groups and to encounter evolutionary patterns and sequence diversity hotspots. The three newly sequenced plastomes represent the smaller ones among Bambusoideae records (127,443 bp for *P. micrantha*; 134,624 bp for *G. trinii*; 134,771 bp and for *A. simplex*). The observed conserved gene order and structures suggests that genome size variation is due to insertion/deletion of sequences at intergenic spacers, gradative pseudogenization or copy loss of pseudogenes, leading to the slight differences in gene content. Comparative analysis within herbaceous bamboo representatives, including *P. micrantha*, reinforces the more frequent occurrence of expansion/contraction of IR borders in relation to neotropical woody representatives, including *G. trinii* and *A. simplex*. Sequence nucleotide polymorphism was accessed by Sliding Window analysis, comparing within Olyreae and Bambuseae representatives. Among the most variable genomic regions, both comparative analysis shares greatest nucleotide diversity at intergenic regions of *ndhF–rpl32–trnL*, *rbcL–psaI* and *trnG(UCC-trnT(GGU)*. We presented the first plastome sequences of *Apoclada* and *Parodiolyra* genera, along with the first chloroplast sequences *Guadua trinii*, providing informative tools for phylogenetic analysis and evolutionary statements for the poorly studied neotropical lineage of Bambusoideae.

Keywords: Bambuseae, evolution, next-generation sequencing, Olyreae, plastome.

INTRODUCTION

Plant genomes comprehend an integrated and coevolving genetic system, compartmentalized in three subcellular components – the nucleus, mitochondrion, and plastid (Choi et al. 2019). Origins of plastid genome genomes, just like mitochondrial, refers to the Theory of Endosymbiosis, whose presence is a distinctive feature of plant cells (Timmis et al. 2004; Ruhlman & Jansen 2014; Rogalski et al. 2015). Among the different types of plastids, chloroplast are active metabolic centers mainly evolved in photosynthetic processes, representing one of the most important cellular structures for sustaining life on Earth (Green et al. 2011; Daniell et al. 2016; Junge 2019).

Specially among land plants lineages, functional plastid genomes (hereby referred as plastomes or cpDNA), presents conserved gene arrangement and structure, encompassing about 120 genes in 120-170 kB, and with uniparental inheritance, mostly of maternal origin with nonrecombinant nature (Greiner et al. 2014; Jansen & Ruhlman 2012; Daniell et al. 2016). Plastomes are, generally, characterized as a single circular and quadripartite molecule, surrounding two single gene copy regions (small; SSC; and large, LSC) flanked by two inverted repeated regions (IRA and IRB) (Bock 2007; Keeling 2010). Along with its intrinsic nature and low level of substitution rate compared to nuclear genome, cpDNA sequences has been widely applied to evolutionary plant molecular studies, regarding species discrimination and phylogenetic inferences (Daniell et al. 2016; Fuentes-Pardo & Ruzzante 2017; Wang et al. 2018).

Bambusoideae subfamily (Poaceae) are perennial grasses of wordwide distribution, in which species diversity (127 genera/1680+ species) is based on evolutionary mecanisms as polyploidy and hybridization (Triplett et al. 2014; Clark & Oliveira et al. 2018; Guo et al. 2019). Currently, three monophyletic lineages are recognized as bamboo tribes, represented by herbaceous bamboos (Olyreae tribe), and temperate (Arundinarieae tribe) and tropical (Bambuseae tribe – paleotropical and neotropical lineages) woody bamboos (Sungkaew et al. 2009; Kelchner & BPG 2013). Brazil is one of the main centers of diversity and endemism of Neotropical bamboo species (Bambuseae and Olyreae), with preferential occurrence at endangered tropical forests, as Atlantic Forests, and high-elevation grasslands (Fisher et al. 2014; Filgueiras & Viana 2017).

Comparative analysis of Bambusoideae plastomes reveals similar evolutionary patterns as those proposed to Poaceae in general, including mechanisms of expansion and contraction of IRs, unique characteristics at IR/SSC borders, and gene loss or gradative

pseudogenization, and variations in intron sequences in specific regions (Zhang et al. 2011; Wu et al. 2015; Attigala et al. 2016; Burke et al. 2016; Saarela et al. 2018; Zhou et al. 2019). However, plastomes of Neotropical distribution species (Bambuseae and Olyreae tribes) presents unique characteristics compared to other lineages, which would have been results of geographic evolution isolation, such as smaller genome size, specific IR/SSC boundaries and differential evolutionary rates of cpDNA (Burke et al. 2012, 2014; Vieira et al. 2015; Wu et al. 2015; Wysocki et al. 2015; Wang et al. 2018, 2020a). Furthermore, rare horizontal transfer events from the mitochondrial genome to the plastid genome were observed in herbaceous bamboo species (Wysocki et al. 2015; Ma et al. 2015; Wang et al. 2018).

Bamboo evolution harbors complex evolutionary events, such as polyploidization and hybridization, which difficult the use of single copy nuclear regions in low level phylogenies and or in recent-radiated groups (Triplett et al. 2014; Clark et al. 2015). Thus, plastid genomes are widely employed for systematic and evolutionary inferences in large groups, and with low level of molecular divergence as Bambusoideae, as well as for inferences of rare evolutionary events such as genome structural rearrangements, intron/gene loss, gene horizontal transfer and phylogeography studies (Zeng et al. 2010; Wicke et al. 2011; Wu et al. 2015; Attigala et al. 2016; Zhou et al. 2017; Stefenon et al. 2019; Loeuille et al 2021).

Therefore, considering the abundance of Neotropical bamboos in Brazil, and the constant threat to their preferential occurrence domains, comparative analysis of plastid genomes of native species becomes an important tool for understanding patterns of richness, endemism, distribution, and phylogenetic relationships of Neotropical bamboos, contributing significantly to the conservation of this group (Filgueiras & Viana 2017; Zhou et al 2019; Wang et al. 2020; Ruiz-Sanchez et al. 2021). Aiming to compare evolutionary patterns of cpDNA, as to generate subsides to conservation programs of Neotropical bamboo species, we newly sequenced and analyzed three Brazilian native bamboos, encompassing both Neotropical lineages – Bambuseae and Olyreae tribes, with certain endangered and endemism degree, and selected based on innovation factor: *Parodiolyra micrantha* (Kunth) Davidse & Zuloaga, *Guadua trinii* (Nees) Nees ex Rupr., and *Apoclada simplex* McClure & L.B. Sm.

MATERIAL AND METHODS

Plant material and DNA isolation

Samples of three species were collected in private properties, in each of them with owner or administration permissions. *Parodiolyra micrantha* were collected at natural population of Fazenda da Ressacada, of Fererederal University of Santa Catarina (UFSC); *Guadua trinii* at matrices plants located at Centro de Ciências Agrárias of UFSC; and *Apoclada simplex* located in private property at Brunópolis/SC. The voucher specimens were deposited in Herbarium FLOR of UFSC.

Fresh leaves of each species (50 g) were collected from one single plant, except for *P. micrantha*, whose plant morphology and structure doesn't allow such initial material, and then samples were collected from five closest individuals of the same population (Figure 1). Samples were maintained at 4ºC for at least 10 days, aiming to reduce starch levels at the leaves (Vieira et al. 2014).

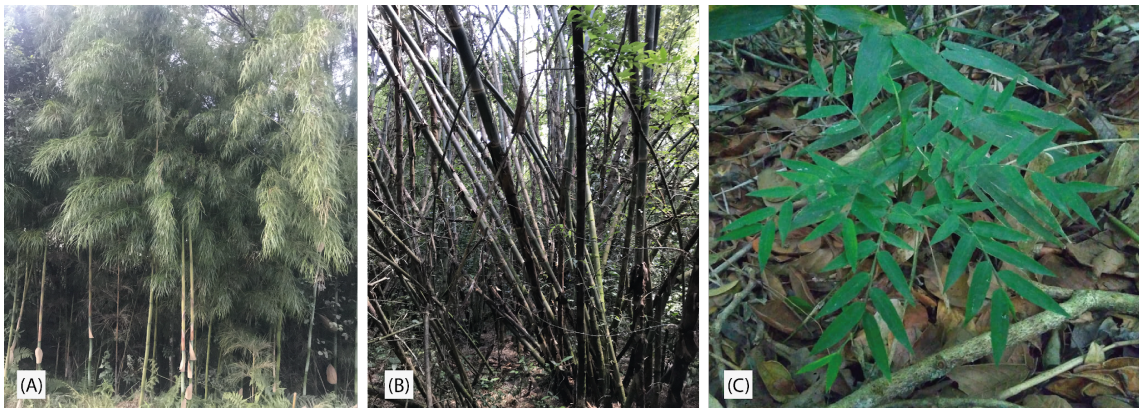

Figure 1. Morphology and habit of selected Neotropical bamboo species for plastid genome analysis. (**A**) *Apoclada simplex*; (**B**) *Guadua trinii*; (**C**) *Parodiolyra micrantha*.

Plastid enrichments were performed for three species, using "High salt plus saline Percoll gradient method" (Vieira et al. 2014) for *G. trinii*, and "Modified high salt method" (Shi et al. 2012) for *P. micrantha* and *A. simplex*. Isolation of cpDNA were performed by CTAB 2% method (Doyle & Doyle, 1990), with modifications (using 1mM of 1,4-dithiothreitol), followed by organic extraction phase with chloroform: isoamyl alcohol (24:1, v/v). Isolated cpDNA were evaluated by its purity and concentration using Nanodrop® ND-1000 UV-Vis (Thermo Scientific, Carlsbad, CA, USA) spectrophotometry, and Qubit® fluorometry (Thermo Scientific, Carlsbad, CA, USA), and stored at -20ºC for further cpDNA sequencing.

Neotropical bamboo plastomes sequencing

Plastomes of Guadua trinii and Parodiolyra micrantha were sequenced using Illumina sequencing platform (short-read sequencing), while Apoclada simplex plastome was sequenced by Oxford Nanopore MinION™ (long-read sequencing) technology.

Samples of P. micrantha and G. trinii were purified with DNA Clean and Concentrator kit (Zymo Research, Orange, CA). A total of 1 ng of DNA was used for library preparation using Nextera XT DNA Sample Prep kit (Illumina Inc., San Diego, CA, EUA), according to manufacturer's instructions. Sequencing was performed on Illumina MiSeq Sequencer (Illumina Inc., San Diego, California, USA) using MiSeq Reagent Kit v3 (600 cycles), obtaining paired-end reads (2 x 250 bp).

For *A. simplex* cpDNA sequencing, the library was prepared using a rapid sequencing kit of genome DNA (SQK-LSK109 (Oxford Nanopore Technologies), applied at MinION™ device with the flowcell R9.4.1, according to manufacturer's instructions. The raw long-reads were acquired through Minion 21.06.0 software in a 24 hours run experiment, followed by basecalling.

For the three cpDNA sequences, the paired reads were imported as FASTQ format in CLC Genomics Workbench v.8.0.1 (CLC Bio, Aarhus, Denmark), and trimmed with a 0.05 quality threshold (corresponding to Phred score of 15) and removal of one base in both terminal regions. General features of read size and quality are summarized in table S1.

Plastomes assembly and annotation

The trimmed reads were applied on a reference-guided assembly, using *Diandrolyra* sp. (NC026960.1) as reference for *P. micrantha*, and *Guadua chacoensis* (NC029232.1) as reference for both *G. trinii* and *Apoclada simplex*, using CLC Genomics software (Qiagen, Hilden, Germany). The same software was used to estimate reference-guided assembly coverage statistics and circularization (total consensus length, %GC, fraction of reference covered, average cpDNA coverage).

Preliminary annotation was performed in GeSeq platform (Tillich et al. 2017; https://chlorobox.mpimp-golm.mpg.de/geseq.html), and manually checked using ExPASy (Gasteiger et al. 2003) for protein-coding regions and tRNAscan-SE v2.0.7 (Lowe & Chan 2016) for tRNA. Afterwards, the graphic representation of circular maps

of each sequenced plastomes were reconstructed using OrganellarGenomeDRAW (OGDRAW) tool (Greiner et al. 2019) implemented in the GeSeq platform.

Comparative analysis of plastomes structures

The three new plastomes sequenced were compared with representatives of their respective subtribes (table S2) regarding total sequence length and of each subunit (LSC/SSC and IRs), and gene order and content. Multiple genome alignment, using Mauve v.2.4.0 software (Darling et al. 2004) were employed to identify locally collinear blocks (LCBs), allowing to evaluate large structural rearrangements and sequence divergence. Furthermore, the junctions of Inverted Repeated regions (IRs) were compared using IRscope online tool (Amiryousefi et al. 2018).

Whole-plastome alignment for each dataset (table S2) was conducted by MAFFT online service (Katoh et al. 2019), with default parameters. Sliding Window analysis, using DnaSP v.6 software (Rozas et al. 2017), was performed to access sequence polymorphisms (window size = 500 bp; step size = 500 bp) comparing among representatives of Olyrinae subtribe (OLY) and Guaduinae subtribe (GUA). For each dataset, average nucleotide diversity (π) was calculated, and 5-fold number used as π threshold for sequence comparison, corresponding to $\pi = 0.05$ to OLY, and $\pi = 0.01$ to GUA.

RESULTS AND DISCUSSION

In the present work, plastomes sequences of three Neotropical bamboo species were newly generated – *Parodiolyra micrantha*, *Guadua trinii* and *Apoclada simplex*. *Parodiolyra micrantha* is representative of the Olyreae tribe (herbaceous bamboos) with wide distribution in Neotropics (Oliveira et al. 2020). Both representing Neotropical lineage of Bambuseae tribe, *G. trinii* and *A. simplex* occur in Atlantic Forest remnants (Guerreiro 2014; Kellermann & Lacerda 2019). The three selected species are important components of forest regeneration and strongly associated with different animal community dynamics (Bodrati & Cockle 2006; Kellermann & Lacerda 2019; Ziccardi et al. 2020; Corahua-Espinoza et al. 2022)

Sequencing data employed in genome assembly resulted in a high fraction of reference genome covered, and high average base covered for all three species (Table 1).

Although the percentage of plastomes reads was inferior in *A. simplex*, the total number of mapped reads was sufficient to recover 100% of the reference genome. The superior number of total reads in *A. simplex* sequencing data should be the result of the used sequencing platform, in which Nanopore technology produces long reads and in high quantities, different from Illumina sequencing platform.

Table 1. Plastid genome sequencing and assembly statistics of *Parodiolyra micrantha*, *Guadua trinii* and *Apoclada simplex*. The two formers' data were obtained by Illumina sequencing platform, and A. simplex by MinION technique.

	P. micrantha	***G. trinii***	***A. simplex***
Total read number	664,587	280,854	1,340,869
nº of mapped plastid reads (%)	35,383 (5,32%)	9,869 (3,51%)	15,903 (1,19%)
Reference genome length (bp) *	137,469	135,403	135,403
Fraction of reference covered	93%	99%	100%
Average coverage (SD)	47.66 (58.67)	16.94 (9.55)	93.18 (21.98)

* Genome of *Guadua chacoensis* (NC029232.1) was used as reference to *G. trinii* and *A. simplex*; and *Diandrolyra* sp. (NC026960.1) as reference to *P. micrantha*.

The three new plastome sequences present typical Angiosperms' structure, comprehending a quadripartite molecule with large and small single copy regions (LSC and SSC, respectively) flanked by two inverted repeated regions (IR_A and IR_B). Physical maps of each plastome are illustrated in figures 2-4, and general genomes structures are described in table 2.

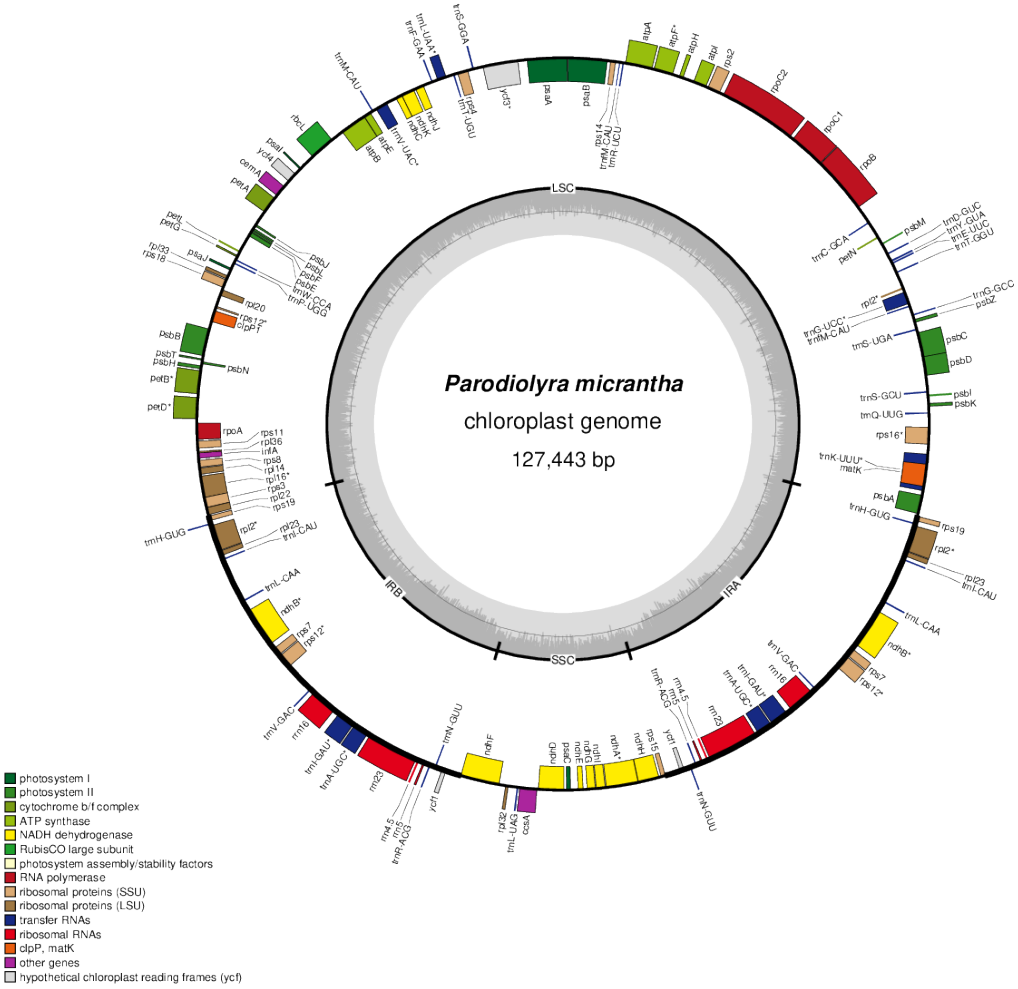

Figure 2. Circular map of the plastidial genome of *Parodiolyra micrantha*. Genes of different functional groups are colored-coded. Genes represented inside the circle are transcribed counterclockwise, and those outside the circle are transcribed clockwise. The dark gray inner-circle corresponds to GC content, and light gray, to the AT content.

Figure 3. Circular map of plastidial genome of *Guadua trinii*. Genes of different functional groups are colored-coded. Genes represented inside the circle are transcribed counterclockwise, and those outside the circle are transcribed clockwise. The dark grey inner-circle corresponds to GC content, and the light gray, to the AT content.

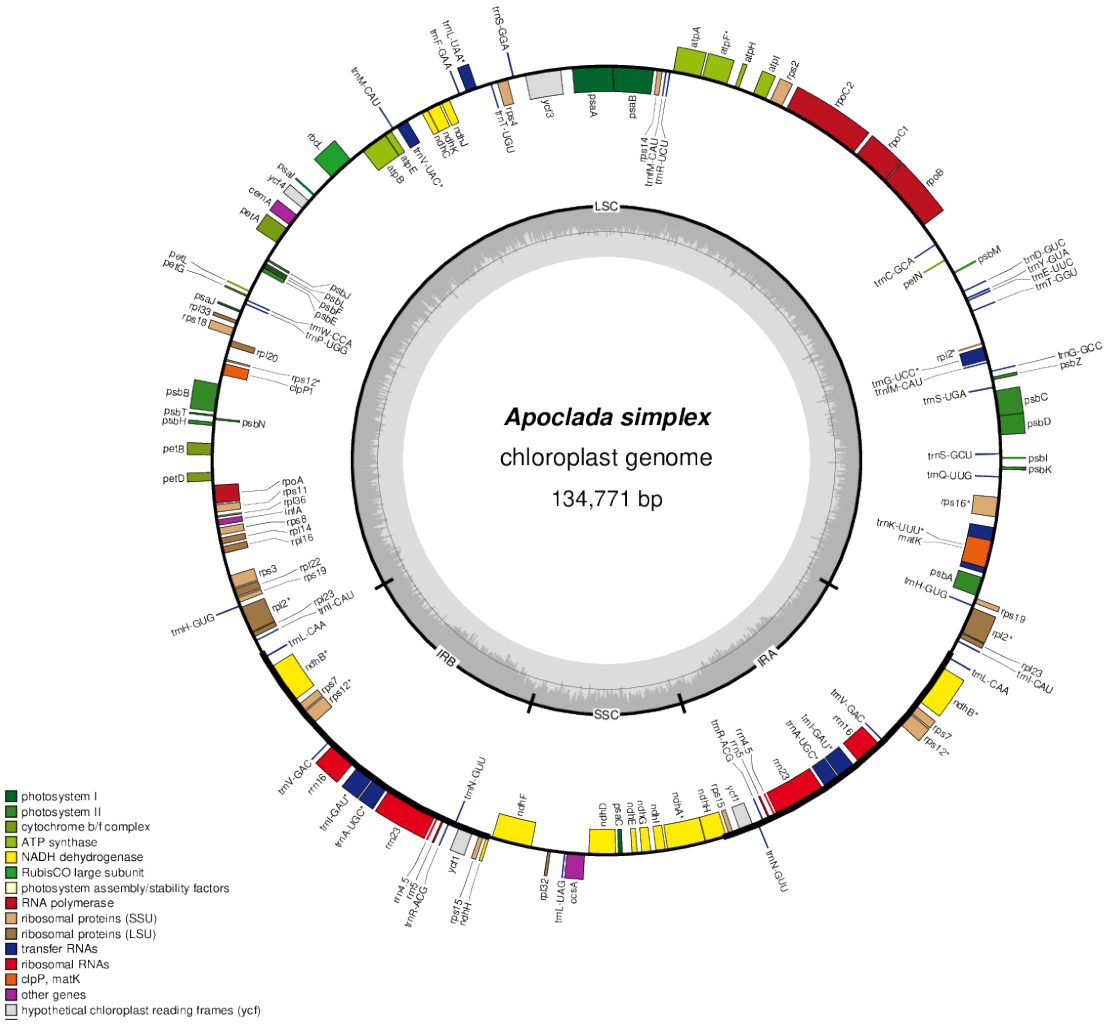

Figure 4. Circular map of plastidial genome of *Apoclada simplex*. Genes of different functional groups are colored-coded. Genes represented inside the circle are transcribed counterclockwise, and those outside the circle are transcribed clockwise. The dark gray inner-circle corresponds to GC content, and light gray, to the AT content.

The three sequenced plastomes recovers the smallest ones recorded to Bambusoideae, in which *Parodiolyra micrantha* showed 127,443 bp length (Figure 2), followed by *Guadua trinii* (134,624 bp; Figure 3) and *Apoclada simplex* (134,771 bp; Figure 4). The size of SSC did not vary among the three species, representing around 9% of total genomes (Table 2). However, LSC was substantially larger in *A. simplex* (66% of the genome), and IRs smaller (24.28% of the genome) compared to *P. micrantha* (58% and 33%, respectively) and *G. trinii* (61% and 28.86%, respectively).

Although Bambusoideae plastidial genomes are considered one of the most conserved among Poaceae family, due to its rare flowering events of woody tribes, they harbor a recognized heterogeneity of nucleotide evolutionary rates between and among

tribes (Burke et al. 2012; Ma et al. 2017; Wang et al. 2020). Neotropical lineages are associated with a major structural variation of plastomes, with notable size reduction compared to Paleotropical Bambuseae tribe and Arundinarieae tribe (Ma et al. 2015; Wu et al. 2015).

The overall GC content was similar in the three newly sequenced plastomes, ranging from 38.75 – 39.90%. Coding regions represent 44 to 45% of plastid genomes, with GC content varying among 38 to 39%. As expected, cpDNA base composition seems to be asymmetrical in genomic regions, showing greatest relevance in repeated regions (44 – 45% of GC content) compared to single copy regions, mainly due to exclusive allocation of four rRNA genes (table 2) (Zhou et al. 2019; Vu et al. 2020; Liu et al. 2021). Furthermore, predominant characteristics of grass plastomes are observed in the three species, as gene loss or gradative pseudogenization (*accD*, *ycf1* and *ycf2*), and intron loss at *rpoC1* and *clpP* genes (Guisinger et al. 2010; Attigala et al. 2016; Burke et al. 2016; Duvall et al. 2020; Liu et al. 2021).

Table 2. Plastome structure and region sizes of *Parodiolyra micrantha*, *Guadua trinii* and *Apoclada simplex*.

	P. micrantha	***G. trinii***	***A. simplex***
Plastome length (pb)	127,443	134,624	134,771
GC content (%)	39.90	38.84	38.75
LSC length (bp) / GC (%)	73,945 / 37.99	82,846 / 36.96	89,129 / 37.17
SSC length (bp) / GC (%)	11,428 / 34.71	12,902 / 33.28	12,902 / 33.09
IR length (bp) / GC (%)	21,035 / 44.66	19,438 / 44.69	16,370 / 45.29
N. of genes (unique)	129 (110)	132 (112)	132 (111)
N. of pseudogenes (unique)	3 (2)	4 (3)	4 (3)
N. of CDS (unique)	82 (77)	85 (78)	83 (77)
Coding region length, pb (GC%)	58,229 (45.69%)	60,436 (44.89%)	59,392 (44.06%)

* CDS (protein-coding sequences), excluding tRNA and rRNA genic regions.

The newly sequenced bamboo plastomes share the greatest part of gene content, including genes involved in photosynthesis apparatus (45), genomic autoregulation (59), other (4) or unknown function (2), and pseudogenes (2). Among the 22 introns, 20 of them occur individually in 10 single copy genes, and 5 occur in duplicated genes (table 3). The three species differ in gene content by the absent copy of *rps15* gene and *ndhH* pseudogene in *P. micrantha*, and by the absence of two copies of *ycf68* gene in both *A. simplex* and *P. micrantha* (table 4).

Table 3. Gene list identified in the sequenced plastomes of *Parodiolyra micrantha*, *Guadua trinii* and *Apoclada simplex*.

Gene category	Group of genes	Name of genes			
Self-replication	Ribosomal RNA	*rrn4.5*[a]	*rrn5*[a]	*rrn16*[a]	*rrn23*[a]
	Transfer RNA	*trnA-UGC*[a,b]	*trnC-GCA*	*trnD-GUC*	*trnE-UUC*
		trnF-GAA	*trnfM-CAU*[a]	*trnG-GCC*	*trnG-UCC*[b]
		trnH-GUG[a]	*trnI-CAU*[a]	*trnI-GAU*[a,b]	*trnK-UUU*[b]
		trnL-CAA[a]	*trnL-UAA*[b]	*trnL-UAG*	*trnM-CAU*
		trnN-GUU[a]	*trnP-UGG*	*trnQ-UUG*	*trnR-ACG*[a]
		trnR-UCU[a]	*trnS-GCU*	*trnS-GGA*	*trnS-UGA*
		trnT-GGU	*trnT-UGU*	*trnV-GAC*	*trnV-UAC*[b]
		trnW-CCA	*trnY-GUA*		
	Small subunit of ribosome	*rps2*	*rps3*	*rps4*	*rps7*[a]
		rps8	*rps11*	*rps12*[a,b]	*rps14*
		rps15[a,*]	*rps16*[b]	*rps18*	*rps19*[a]
	Large subunit of ribosome	*rpl2*[a,b]	*rpl14*	*rpl16*[b]	*rpl20*
		rpl22	*rpl23*[a]	*rpl32*	*rpl33*
		rpl36			
	RNA polymerase subunits	*rpoA*	*rpoB*	*rpoC1*	*rpoC2*
	Translational initiation factor	*infA*			
Photosynthesis	Subunits of NADH dehydrogenase	*ndhA*[b]	*ndhB*[a,b]	*ndhC*	*ndhD*
		ndhE	*ndhF*	*ndhG*	*ndhH*
		ndhI	*ndhJ*	*ndhK*	
	Photosystem I	*psaA*	*psaB*	*psaC*	*psaI*
		psaJ			
	Photosystem II	*psbA*	*psbB*	*psbC*	*psbD*
		psbE	*psbF*	*psbH*	*psbI*
		psbJ	*psbK*	*psbL*	*psbN*
		psbM	*psbT*	*psbZ*	
	Cytochrome b/f complex	*petA*	*petB*[b]	*petD*[b]	*petG*
		petL	*petN*		
	ATP synthase	*atpA*	*atpB*	*atpE*	*atpF*[b]
		atpH	*atpI*		
	Large subunit of Rubisco	*rbcL*			
Other genes	Maturase	*matK*			
	Envelope membrane protein	*cemA*			
	C-type cytochrome synthesis gene	*ccsA*			
	Protease	*clpP1*			
Unknown function		*ycf3*[b]	*ycf4*	*ycf68*[a,**]	
Pseudogenes		*ycf1*[a]	*rpl2*	*ndhH*[*]	

[a] Duplicated gene; [b] Genes containing introns; [*]Absent copy in *P. micrantha*; [**] Absent copies in both *P. micrantha* and *A. simplex*.

In order to evaluate the occurrence of major genomic rearrangements, multiple genome alignment was performed to compare local collinear blocks within the analyzed bamboo groups. Comprehending Neotropical lineage of Bambuseae tribe, *G. trinii* and *A. simplex* were compared with 5 representatives of the same group, and *P. micrantha* was compared to other 6 representatives of Olyreae tribe (table S2). *Guadua trinii* and *Apoclada simplex* showed conserved gene order and content when compared to other close-relatives, highlighted by the presence of one local collinear block (LCB) (Figure 5).

The conserved gene order and absence of structural rearrangements after sequence alignments comparisons (Figure 5; Figure 6) suggest that genome size variation is due to insertion/deletion in intergenic regions or shifts in IR/single copy boundaries (Wu et al. 2015; Zhu et al. 2016; Chen et al. 2018; Choi et al. 2019). Mechanisms of expansion/contraction of IRs are frequently reported in Angiosperms, which can occur independently and in different taxonomic levels (Davis & Soreng 2010; Mower & Vickrey 2018; He et al. 2019; Li et al. 2021).

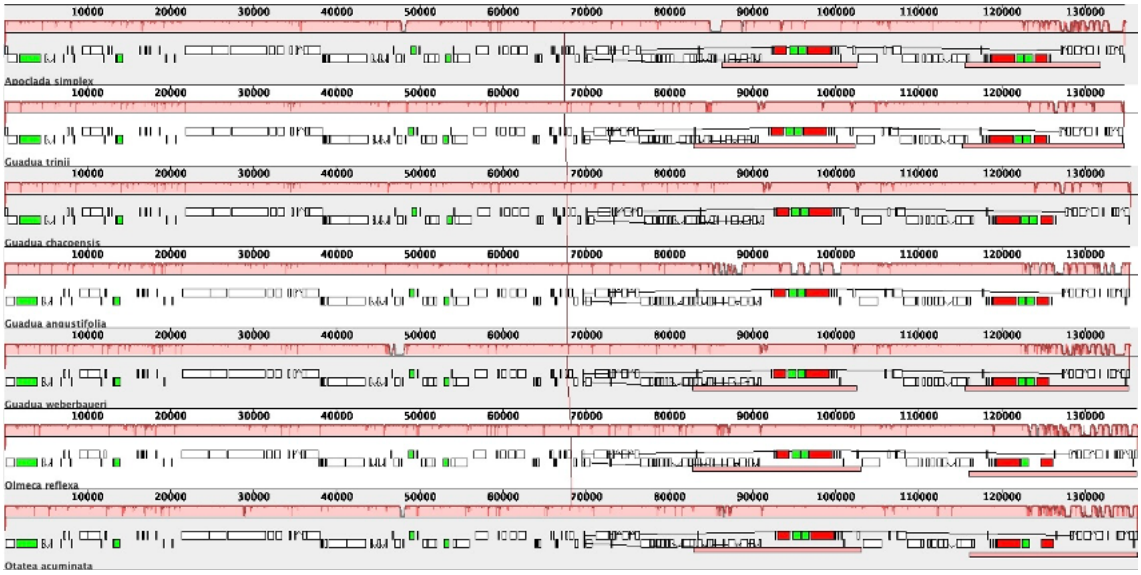

Figure 5. Multiple plastome alignment within species of Bambuseae tribe, subtribe Guaduinae, including the two new sequences of *Apoclada simplex* and *Guadua trinii*. The presence of only one local collinear block (LCB) indicated homologous regions without major structural rearrangement or sequence divergence among the compared plastomes.

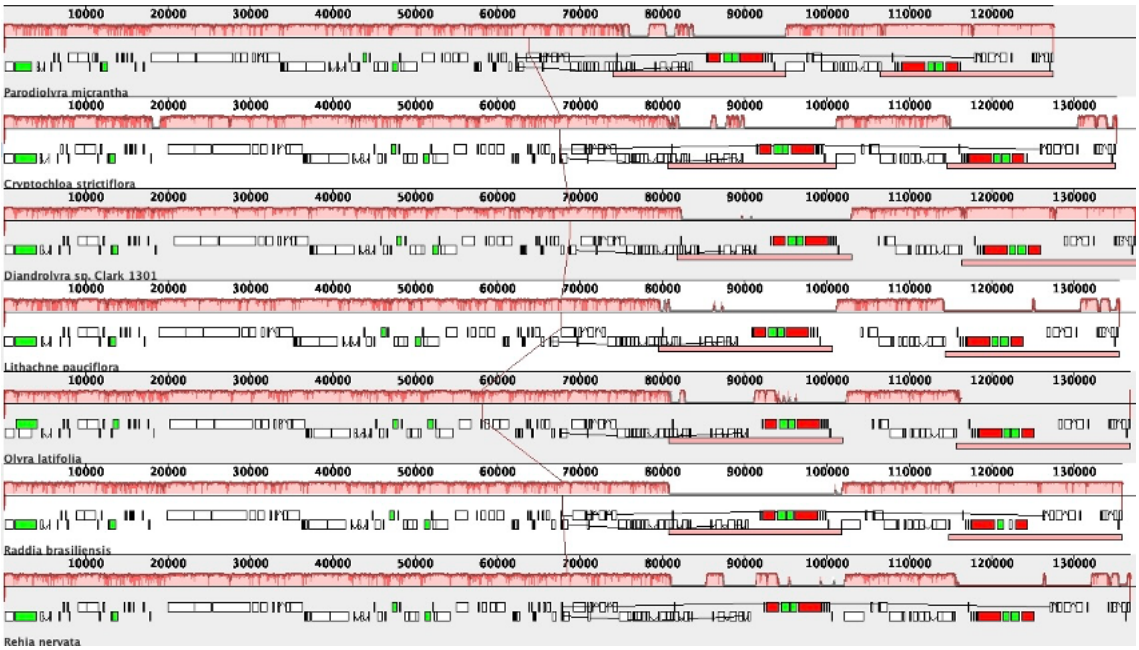

Figure 6. Multiple plastome alignment within species of Olyreae tribe, subtribe Olyrinae, including the new sequence of *Parodiolyra micrantha*. The presence of local collinear blocks (LCB) indicated homologous regions without major structural rearrangement or sequence divergence among the compared plastomes.

The comparison between representatives of Bambuseae and Olyreae tribes was independently performed, including *A. simplex*, *G. trinii* and *P. micrantha*, respectively (Figure 7-8). Among Guaduinae comparison, the junctions of SSC/IR_A (JSA) and SSC/IR_B (JSB) show conserved characteristics, with similar features to those reported to Bambusoideae species and other grasses (Burke et al. 2012; Wu et al. 2015; Vieira et al. 2015; Wang et al. 2018; Xiong et al. 2020; Liu et al. 2021), such as the proximity of the intact open reading frame (ORF) of *ndhF* gene to the JSB (Figure 7). The JSA intersects the coding region of *ndhH* gene, creating a short fragment in IR_A, and an additional copy identical to 3' portion of its functional copy in IR_B (ψ ndhH). *Guadua chacoensis* and *G. trinii* shared the same characteristics at JSA and JSB, including the shifting of rps19 gene position to the LSC, which is common in bamboos, but rarely reported to Olyreae tribe (Wang et al. 2008; Burke et al. 2016; Wang et al. 2018).

Apoclada simplex recovered different JLA and JLB features, compared to other Guaduinae species (Figure 7). The *rpl2* gene is placed just within the border of LSC and IR_A/IR_B, resulting in a short fragment of the gene in the IR regions. The presence of *rpl2* gene in IR regions is reported to some ancestral Angiosperms, and can be observed in a few bamboo species, as *Froesiochloa boutelouoides* and *Cryptochloa strictiflora* (Zhu et

al. 2016; Wang et al. 2018). Such feature is probably due to IR contraction events, resulting in IR shifting boundaries and reduced length in *A. simplex*.

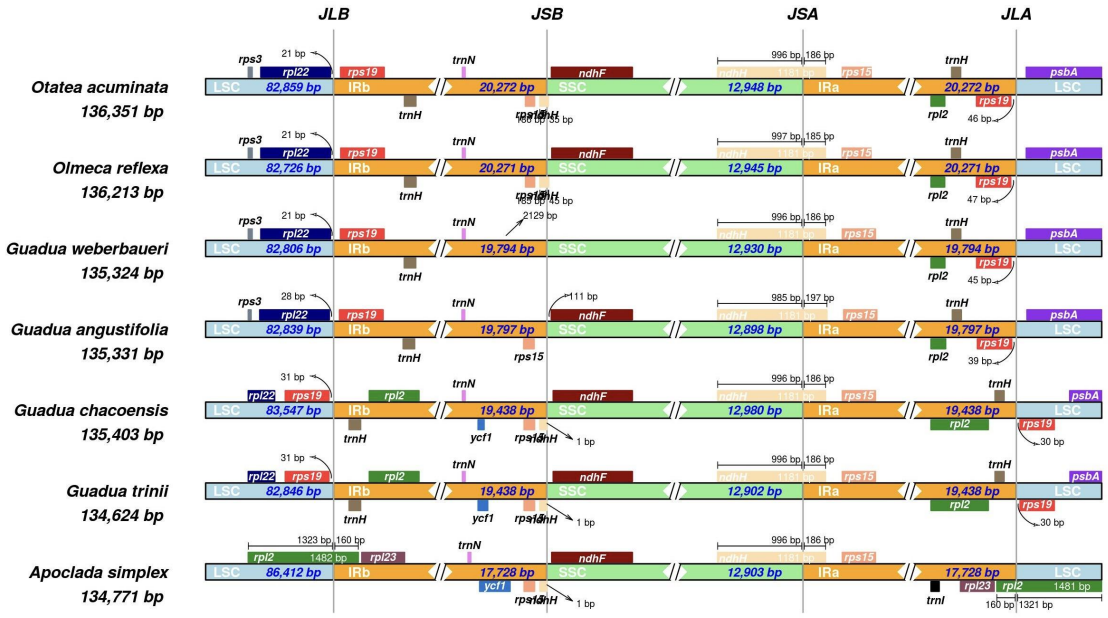

Figure 7. Comparison of the boundaries of LSC, SSC, and IR regions of the plastomes of *A. simplex* and *G. trinii*, compared to Guaduinae subtribe representatives. JLB (IRb /LSC), JSB (IRb/SSC), JSA (SSC/IRa) and JLA (IRa/LSC) denote the junctions between each correspondent region in the genome.

Unlike woody bamboos, independent evolutionary events of expansion/contraction of IR regions results in highly variable genomic organization in IR/single copy borders of herbaceous bamboos plastomes (Wang et al. 2018). *Parodiolyra micrantha* shows typical IR_B features, resulting in the JSB within the limit of *ndhF* gene, which is considered a synapomorphy for the Olyreae tribe (Figure 8) (Burke et al. 2012; 2014). In addition to the absence *ycf68* gene (duplicated copies), shifting in JSA and disassociation between the second copy of *rps15* and ψ*ndhH* results in the differential gene/pseudogene numbers reported to *P. micrantha* (Wu et al. 2015; Wysocki et al. 2015; Wang et al. 2018).

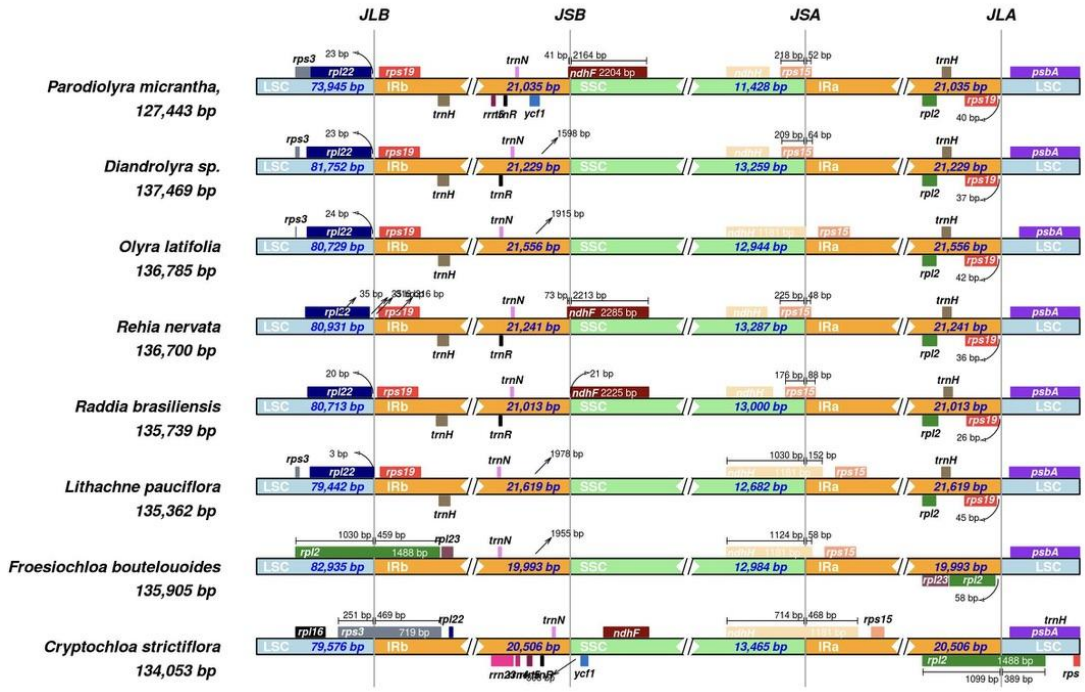

Figure 8. Comparison of the boundaries of LSC, SSC, and IR regions of the plastome of *P. micrantha* compared to Olyrinae subtribe representatives. JLB (IRb /LSC), JSB (IRb/SSC), JSA (SSC/IRa) and JLA (IRa/LSC) denote the junctions between each correspondent region in the genome.

Hotspots of polymorphism were accessed using Sliding Window Analysis, by comparing levels of nucleotide diversity within subtribes. In general, Olyrinae dataset recover high mean value of nucleotide diversity (π = 0.01889) and total number of segregating sites (S = 6,973) compared to Guaduinae representatives (π = 0.00239; S = 930), highlighting the most conservative nature of woody bamboo plastomes (Wang et al. 2020). Figures 9 and 10 represent nucleotide diversity peaks of each analyzed dataset.

In Olyrinae comparison, the highest peak, representing the greatest nucleotide polymorphism, occurred within *trnG(UCC)–trnT(GGU)* intergenic spacer (π = 0.05371), followed by flaking sequences between *trnS(GCU)–psbD* genes (π = 0.05633), *atpB–rbcL–psaI* region (π = 0.05714), *ndhF–rpl32–trnL*(UAG) and rpl16 gene (π = 0.04114) (Figure 9). Although IR are frequently more conserved than single copy regions (Wang et al. 2018), Olyrinae species showed a variable site at LSC/IR boundaries represented by *trnL(CAA)–trnI(CAU)* intergenic spacer (π = 0.02095).

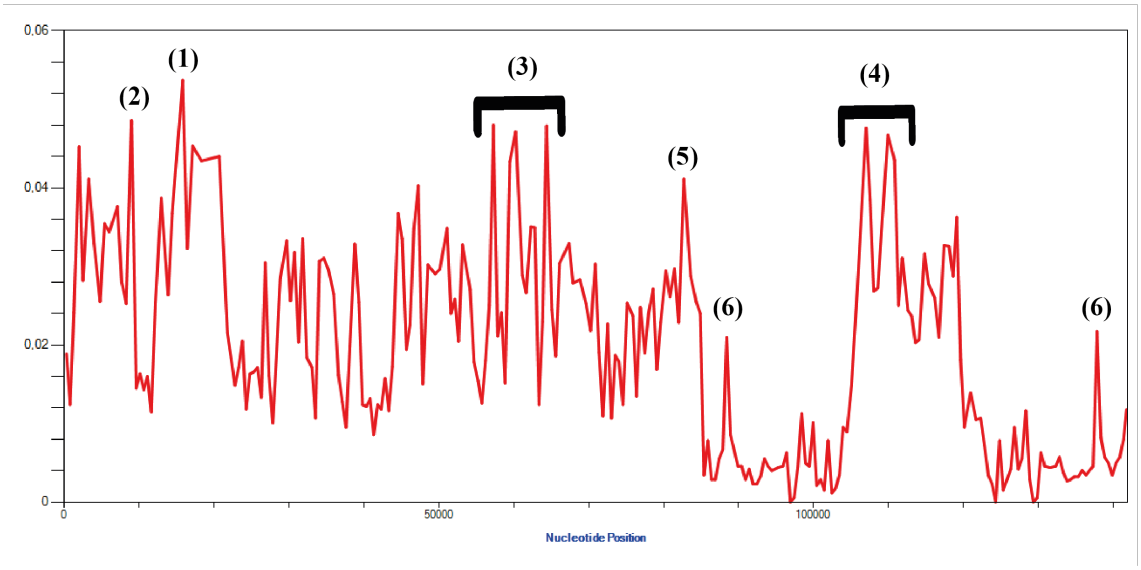

Figure 9. Nucleotide diversity (π) of whole-plastome comparison among 7 species of Olyrinae subtribe, including the newly sequenced plastome of *Parodiolyra micrantha.* Red peaks represent nucleotide diversity per 500 bp (S) Total segregating sites. Numbered peaks indicates (**1**) *trnG(UCC)–trnT(GGU)* intergenic spacer (π = 0.05371; S = 80), (**2**) *trnS(GCU)*–psbD genes (π = 0.05633; S = 69), (**3**) *atpB–rbcL–psaI* region (π = 0.05714; S = 70), (**4**) *ndhF–rpl32–trnL(UAG)* (π = 0.04762; S = 70), (**5**) *rpl16* (π = 0.04114; S = 56) and (**6**) *trnL(CAA)–trnI(CAU)* (π = 0.02095; S = 36).

Comparison between plastomes of Guaduinae species, including *A. simplex* and *G. trinii*, revealed greatest nucleotide diversity at *ndhF–rpl32–trnL(UAG)* region (π = 0.01181), followed by *ndhK–trnV(UAC)* intergenic region (π = 0.01086), *psbK–psbI* IGS (π = 0.0099), *rbcL–psaI* (π = 0.00971) and *trnG(UCC-trnT(GGU)* IGS (π = 0.00933) (Figure 10). Also, *rpl19–psbA* recovered high nucleotide diversity at LSC (π = 0.000857).

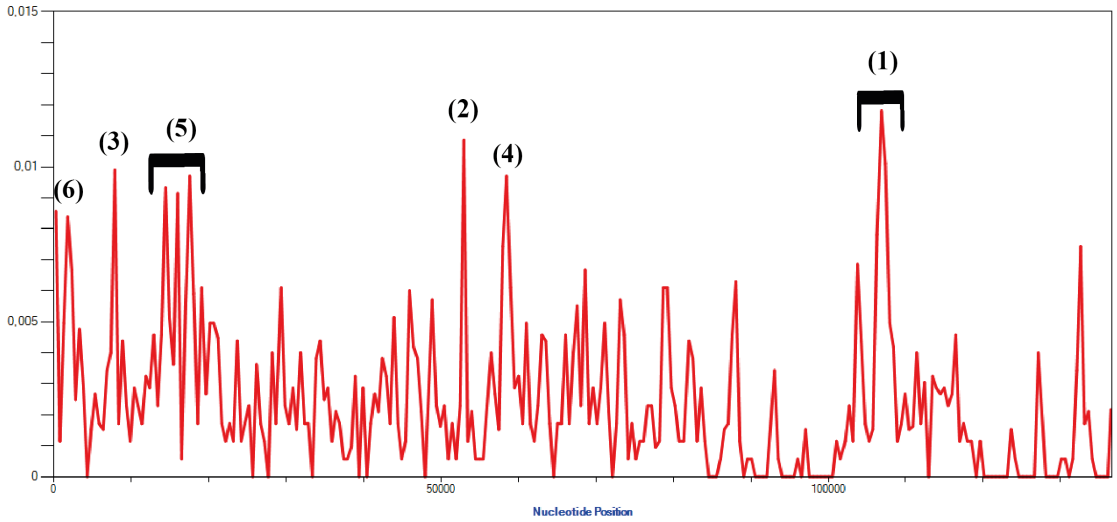

Figure 10. Nucleotide diversity (π) of whole-plastome comparison among 7 species of Guaduinae subtribe, including the newly sequenced plastome of *Apoclada simplex* and *Guadua trinii*. Red peaks represent nucleotide diversity per 500 bp. (S) Total segregating sites. Numbered peaks indicates (**1**) *ndhF–rpl32–trnL(UAG)* (π = 0.01181; S = 29); (**2**) *ndhK–trnV(UAC)* intergenic region (π = 0.01086; S = 16), (**3**) *psbK–psbI* IGS (π = 0.0099; S = 12), (**4**) *rbcL–psaI* (π = 0.00971; S = 10), (**5**) *trnG(UCC-trnT(GGU)* IGS (π = 0.00933; S = 10); (**6**) *rpl19–psbA* (π = 0.000857; S = 6).

Although often regarded as highly conserved, the chloroplast genomes are subjected to evolutionary events that could be identified at different taxonomic levels (Zhu et al. 2015; Li et al. 2021). Comparative analysis of the three newly sequenced genomes of Neotropical bamboos revealed conserved organization within its respective subtribes. However, some structural features observed in *P. micrantha*, *G. trinii* and *A. simplex*, especially in IR/single copy junctions, should be investigated to inquire about evolutionary tendencies in each species. Also, the identified hypervariable regions could be explored in taxonomic and phylogenetic resolution.

CONCLUSION

In the present work, three newly sequenced plastomes of Neotropical bamboo species were reported – *Apoclada simplex*, *Guadua trinii* and *Parodiolyra micrantha*. Gene order and genomic organization were relatively conserved among the compared representatives of each subtribe, with some unique characteristics mainly in *A. simplex* and *P. micrantha* IR/single copy boundaries. Comparative analysis highlighted

hypervariable sites, which should be informative tools for species discrimination and inferences of phylogenetic relationship within Neotropical bamboo lineages.

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SUPPLEMENTARY MATERIAL

Table S1. General features of reads obtained by short-read and long-read sequencing of the plastomes of *Parodiolyra micrantha*, *Guadua trinii* and *Apoclada simplex*.

	P. micrantha [1]	***G. trinii*** [1]	***A. simplex*** [2]
Total number of reads	666,614	280,966	1,344,307
Average length (pb)	190.34	228.21	948.61
Number of reads after trimming	664,587	280,854	1,340,869
% of trimmed reads	99.7 %	99.96 %	99.74 %
Average length of trimmed reads (pb)	189.87	227.02	348.61

[1]Paired-end reads obtained by Illumina MiSeq sequence platform; [2]Single-end reads obtained by Oxford Nanopore Technology (MinION).

Table S2. Reference genomes used in plastomes comparative analysis of *Guadua trinii*, *Apoclada simplex* (Bambuseae tribe) and *Parodiolyra micrantha* (Olyreae tribe).

Species	Accession GenBank n.	Plastome size (bp)	GC (%)[b]	LSC (bp)[c]	SSC (bp)[d]	IR (bp)[e]	CDS (bp)[f]
Bambuseae, Guaduinae subtribe							
G. angustifolia	NC_029749.1	135,331	38.73	82,839	12,898	19,797	60,405
G. chacoensis	NC_029232.1	135,403	38.76	82,877	12,980	19,773	60,414
G. weberbaueri	NC_026991.1	135,324	38.75	82,806	12,930	19,794	57,012
Otatea acuminata	NC_026971.1	136,351	38.78	82,859	12,948	20,272	59,610
Olmeca reflexa	NC_026965.1	136,213	38.77	82,726	12,945	20,271	59,403
Olyreae, Olyrinae subtribe							
Cryptochloa strictiflora	JX235348.1[a]	135,033	38.25	80,556	13,465	20,506	59,442
Diandrolyra sp.	NC_026960.1	137,469	38.81	81,752	13,259	21,229	56,784
Lithachne pauciflora	NC_026970.1	135,385	38.62	79,465	13,676	21,122	56,607
Olyra latifolia	NC_024165.1	136,785	38.87	80,729	12,944	21,556	56,724
Raddia brasiliensis	NC_026966.1	135,739	38.85	80,713	13,000	21,013	59,709
Rehia nervata	NC_039984.1	136,700	38.82	80,931	13,273	21,248	60,234

[a]Accession unavailable at https://www.ncbi.nlm.nih.gov/genome platform; [b]GC content; [c]total length of large (LSC) and [d]small single copy regions (SSC); [e]inverted repeats (IR); [f]coding sequences (CDS).

6. CONSIDERAÇÕES FINAIS

Os bambus são gramíneas de ampla distribuição global e de grande relevância socioeconômica e ecológica. O Brasil engloba um dos maiores níveis de diversidade e endemismo de espécies, porém com constante grau de ameaça decorrente da fragmentação dos seus habitats de ocorrência, como a Mata Atlântica e a Amazônia. Estudos sobre a diversidade e evolução das espécies são, portanto, importantes ferramentas para o conhecimento aprofundado deste grupo pouco estudado.

A avaliação de regiões candidatas a *barcode* em Bambusoideae traz a luz a aplicação desta técnica em uma ampla escala amostral. Considerando os padrões evolutivos da subfamília, como hibridização, poliploidia e ocorrência de cruzamento intergenérico, o uso de marcadores moleculares para identificação de espécies com base no conceito de monofilia recíproca, ou seja, na presunção de espécie como unidade evolutiva definida, pode tornar-se pouco informativo quando avaliado em um grupo com taxas de evolução molecular tão diversa, como os bambus. Portanto, estudos complementares se fazem necessários, adicionando-se um maior número de marcadores, a fim de aumentar a resolução analítica de DNA barcoding em Bambusoideae.

A execução das atividades referentes a este capítulo proporcionou a inserção deste tema no Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal (LFDGV-UFSC), bem como o estabelecimento de parcerias em outros trabalhos correlatos, com ênfase em DNA *barcode*, no LFDGV. Estas atividades possibilitarão a expansão do conhecimento e o poder de resolução da aplicação da técnica em diferentes grupos de organismos, assim como o fortalecimento desta linha de pesquisa no âmbito do LFDGV e do PPGRGV.

O sequenciamento e a montagem do genoma plastidial de três espécies neotropicais, nativas do Brasil – *Parodiolyra micrantha*, *Guadua trinii* e *Apoclada simplex* – tornou-se possível a partir de parcerias previamente estabelecidas entre o Núcleo de Fixação de Nitrogênio (UFPR), Profª Leila do Nascimento (UFPR), e Profº Valdir Stefenon (UFSC). A análise comparativa destes novos plastomas sequenciados permitiu a identificação de padrões evolutivos conservados, como ordem e número gênico, apesar da variação de tamanho total observada. Ainda a identificação de regiões altamente polimórficas pode contribuir para o desenvolvimento de marcadores moleculares aplicados à discriminação de espécies e inferências filogenéticas do grupo de bambus neotropical, bem como no desenvolviento de trabalhos futuros para a compreensão do estado de conservação de populações nativas.