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AMANDA DE SOUZA SANTOS

ANÁLISE FILOGENÉTICA E EVOLUÇÃO CARIOTÍPICA DE *Ameroglossum* Eb.Fisch., S.Vogel & A.V.Lopes (LINDERNIACEAE) E GÊNEROS RELACIONADOS

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Tese apresentada ao Programa de Pós-Graduação em Biologia Vegetal da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutora em Biologia Vegetal.

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Orientador: Dr. Marcelo dos Santos Guerra Filho (Depto. Botânica, UFPE)

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RESUMO

Ameroglossum é um gênero endêmico do Nordeste do Brasil, composto por nove espécies e ecologicamente restrito a ambientes de afloramento rochoso ou *inselbergs*. Esses ambientes são considerados sistemas semelhantes a ilhas, e o isolamento geográfico desempenha um papel importante na restrição do fluxo gênico desses ambientes, criando oportunidades para divergência genética e fenotípica entre as populações. O gênero *Ameroglossum* tem um posicionamento taxonômico incerto, ainda não testado em uma perspectiva molecular. Inicialmente alocado em Scrophulariaceae, morfologicamente *Ameroglossum* tem sido associado aos gêneros *Catimbaua*, *Cubitanthus* e *Isabelcristinia*, que pertencem a Linderniaceae. Cariologicamente, esta família possui poucas contagens cromossômicas, mas bastante variáveis, variando de 2*n* = 14 a 60 cromossomos, sendo esse maior número encontrado apenas em *Ameroglossum*, *Catimbaua* e *Isabelcristinia*. O objetivo desse estudo foi investigar a evolução cariotípica e relações filogenéticas de *Ameroglossum* e gêneros relacionados. No primeiro capítulo, foram investigados os números cromossômicos de 14 espécies de Linderniaceae, analisando a distribuição de heterocromatina e sítios de DNAr 5S e 35S. As análises cariotípicas mostraram que *Ameroglossum*, *Catimbaua* e *Isabelcristinia* possuem 2*n* = 60, com exceção de *A. genaroanum* com 2*n* = 64, e *Cubitanthus alatus* com 2*n* = 50, todas associadas a ambientes de afloramento rochoso. Enquanto isso, as espécies de ambientes úmidos *Torenia thouarsii* e *Vandellia diffusa* têm ambas 2*n* = 28. Contudo, todas elas possuem cromossomos pequenos e sítios de rDNA 5S e 35S sobrepostos à bandas CMA^{+}. As semelhanças cariotípicas entre as espécies de *Ameroglossum*, *Catimbaua*, *Cubitanthus* e *Isabelcristinia* sugerem uma estreita relação entre representantes de Linderniaceae típicos de *inselbergs*. No segundo capítulo, foram avaliadas as relações filogenéticas de *Ameroglossum*, além de reconstrução de áreas ancestrais, além do papel da radiação adaptativa associada a ambientes de afloramentos rochosos na diversificação de *Ameroglossum*. As análises mostraram que *Ameroglossum* é um gênero pertencente Linderniaceae, formando um clado fortemente suportado com *Cubitanthus* e *Stemodiopsis*. Porém, *Ameroglossum* não é monofilético devido ao posicionamento incerto de *Catimbaua* e *Isabelcristinia* entre suas espécies. Foi identificado também um aumento na taxa de diversificação no clado *Ameroglossum* incluindo *Catimbaua* e *Isabelcristinia* (~19 Ma) que pode estar associado à mudança antiga para habitat de afloramentos rochosos. Essa mudança foi relacionada com a colonização da América do

Sul por dispersão à longa distância, dada a distribuição ancestral de Linderniaceae na África/Ásia, e a estreita relação de *Ameroglossum* + *Cubitanthus* com *Stemodiopsis*, que é endêmico da África.

Palavras-chave: Afloramentos Rochosos, Biogeografia, *Cubitanthus*, Filogenia, Heterocromatina, Número Cromossômico.

ABSTRACT

Ameroglossum is an endemic genus of Northeastern Brazil, composed of nine species and ecologically restricted to rocky outcrop environments or *inselbergs*. These environments are considered island-like systems, and its geographic isolation plays an important role in restricting gene flow in these environments, creating opportunities for genetic and phenotypic divergence between populations. *Ameroglossum* has an uncertain family placement which has not yet been tested in a molecular structure. Initially allocated in Scrophulariaceae, morphologically *Ameroglossum* has been associated with the genera *Catimbaua*, *Cubitanthus* and *Isabelcristinia*, which belong to Linderniaceae. Kariologically, Linderniaceae has few chromosome counts, but quite variable, ranging from 2*n* = 14 to 60 chromosomes, with this higher number found only in *Ameroglossum*, *Catimbaua* and *Isabelcristinia*. The objective of this study was to investigate the karyotypic evolution and phylogenetic relationships of *Ameroglossum* and related genera. In the first chapter, the chromosome numbers of 14 species of Linderniaceae were investigated, analyzing the distribution of heterochromatin and sites of 5S and 35S rDNA. Karyotypic analyses showed that *Ameroglossum, Catimbaua* and *Isabelcristinia* have 2*n* = 60, with the exception of *A. genaroanum* with 2*n* = 64, and *Cubitanthus alatus* with 2*n* = 50, all associated with rocky outcrop environments. Meanwhile the wetland species *Torenia thouarsii* and *Vandellia diffusa* both have 2*n* = 28. However, they all have small chromosomes and overlapping 5S and 35S rDNA sites with CMA^+ bands. The karyotypic similarities between the species of *Ameroglossum, Catimbaua, Cubitanthus* and *Isabelcristinia* suggest a close relationship between representatives of Linderniaceae typical of *inselbergs*. In the second chapter, the phylogenetic relationships of *Ameroglossum* were evaluated, in addition to the reconstruction of ancestral areas, and the role of adaptive radiation associated with environments of rocky outcrops in the diversification of *Ameroglossum*. The analyses showed that *Ameroglossum* was positioned in Linderniaceae, forming a strongly supported clade with *Cubitanthus* and *Stemodiopsis.* However, *Ameroglossum* is not monophyletic due to the uncertain placement of *Catimbaua* and *Isabelcristinia* among its species. An increase in the rate of diversification in the *Ameroglossum* clade (~19 Ma) was also identified, which may be associated with the ancient shift to rocky outcrop habitat. This change was related to the colonization of South America by long-distance dispersal, given the ancestral distribution

of Linderniaceae in Africa/Asia, and the close relationship of *Ameroglossum* + *Cubitanthus* with *Stemodiopsis*, which is endemic to Africa.

Palavras-chave: Heterochromatin, Chromossome Number, Phylogeny, Biogeography, Rocky Outcrops, *Cubitanthus.*

SUMÁRIO

1 INTRODUÇÃO

O gênero *Ameroglossum* Eb.Fisch., S.Vogel & A.V.Lopes (Linderniaceae) é constituído por subarbustos que crescem em rochas graníticas, endêmicas do Nordeste brasileiro, em enclaves de Mata Atlântica chamados de "Brejos de Altitude" *sensu* Andrade-Lima (FISCHER; VOGEL; LOPES, 1999) ou totalmente imersos na Caatinga (ALMEIDA et al., 2016). Muitos desses afloramentos rochosos constituem *inselbergs*, formações graníticas ou gnáissicas naturais, similares a ilhas rochosas em ambientes terrestres planos ou suavemente ondulados (POREMBSKI; BARTHLOTT, 2012). O grau de isolamento geográfico dos *inselbergs* é bastante variado, podendo ocorrer isolados ou aglomerados, elevando-se abruptamente do restante da paisagem, separados por alguns ou muitos quilômetros de distância (POREMBSKI; BARTHLOTT, 2012).

Inselbergs, assim como lagos, montanhas e cavernas por vezes podem ser tratados como parte de um sistema tipo ilha (ILS). Nesses ambientes ILS, o isolamento geográfico desempenha um papel importante na restrição do fluxo gênico, criando oportunidade para divergências genéticas e fenotípicas entre populações por meio da deriva genética e, consequentemente, especiação (ITESCU, 2019; NACIRI; LINDER, 2020; MENDEZ-CASTRO et al., 2021). Aparentemente as radiações adaptativas são muito frequentes em ilhas oceânicas ou ambientes similarmente isolados (GIVNISH, 1997; ITESCU, 2019).

Atualmente, *Ameroglossum* possui nove espécies (ALMEIDA et al., 2021), que podem ser caraterizadas morfologicamente por terem corola tubular vermelha a laranja-amarelada, lábio inferior da corola amarelo, bilobado ou levemente bilobado, estaminódio filiforme e sementes castanhas com estrias longitudinais. Na ocasião de sua descrição, o gênero era monotípico e foi incluído inicialmente em Scrophulariaceae, por compartilhar algumas similaridades morfológicas, como o tipo de inflorescência e hábito arbustivo (FISCHER; VOGEL; LOPES, 1999). Com a mais recente circunscrição de Scrophulariaceae (OLMSTEAD et al., 2001; OXELMAN et al., 2005), *Ameroglossum* ficou com um posicionamento incerto dentro da ordem Lamiales. Dentre as novas famílias botânicas desmembradas a partir de Scrophulariaceae, Linderniaceae foi uma delas (RAHMANZADEH et al., 2005), caracterizada principalmente por apresentar filetes anteriores geniculados, geralmente com edema basal. A inclusão provisória de *Ameroglossum* em Linderniaceae foi proposta por Souza e Lorenzi (2012) com base em dados morfológicos e moleculares não publicados. Esse posicionamento foi seguido por

Christenhusz, Fay, Chase (2017) e Almeida et al. (2019), com base na posição dos estames e na presença de sementes com ranhuras.

Foram incluídos também em Linderniaceae dois gêneros monotípicos recentemente descritos: *Catimbaua* L.P.Felix, Christenh. & E.M.Almeida e *Isabelcristinia* L.P.Felix, Christenh. & E.M.Almeida (ALMEIDA et al., 2019). Assim como *Ameroglossum*, os gêneros *Catimbaua* e *Isabelcristinia* possuem distribuição geográfica restrita a afloramentos rochosos do Nordeste brasileiro (ALMEIDA et al., 2019). Além de compartilharem algumas características morfológicas e distribuição geográfica restrita, Almeida et al. (2019) perceberam que as espécies desses três gêneros possuíam o mesmo número cromossômico, $2n = 60$, bastante distinto de outras Linderniaceae (RICE et al., 2015). Citogeneticamente, Linderniaceae é pouco estudada, com dados de contagens cromossômicas para apenas 33 de suas espécies, sendo $2n = 18$ e 16 os números cromossômicos mais frequentes (RICE et al., 2015).

Em uma revisão taxonômica e filogenética mais abrangente realizada por Fischer, Schäferhoff, Müller (2013) foi utilizado a região *trnK/matK* para 48 táxons, onde foram reconhecidos 17 clados para Linderniaceae. Posteriormente, Biffin et al. (2018) usando sequências *matK*, adicionaram espécies australianas nas análises filogenéticas da família e reconheceram três clados principais para Linderniaceae. O primeiro deles formado pelos gêneros *Stemodiopsis* Engl., e *Cubitanthus* Barringer., que compõem as primeiras linhagens divergentes da família. Os outros dois clados incluem gêneros mais antigos taxonomicamente como *Torenia* L., *Artanema* D. Don, *Lindernia* All. e *Craterostigma* Hochst., que já existiam antes da família emergir de Scrophulariaceae (BIFFIN et al., 2018), cujas as relações filogenéticas ainda precisam ser melhores estudadas, uma vez que alguns gêneros emergem não monofiléticos.

Geograficamente, não é só a distribuição de *Ameroglossum* que chama atenção em Linderniaceae. A família possui distribuição Pantropical, com suas espécies encontradas em regiões tropicais e temperadas, mas com centro de diversidade no continente africano e no sudeste asiático (CHRISTENHUSZ; FAY; CHASE, 2017). As espécies africanas ocorrem geralmente em ambientes especializados, como poços rochosos preenchidos sazonalmente com água e *inselbergs*, enquanto as espécies asiáticas ocorrem principalmente em áreas de floresta tropical e solos alagados (LEWIS, 2000; RAHMANZADEH et al., 2005; CHRISTENHUSZ; FAY; CHASE, 2017). Geralmente a distribuição geográfica das espécies se dá por meio de endemismo, atingindo o nível de gêneros inteiros endêmicos em determinados continentes como é o caso de *Cubitanthus*

e *Micranthemum* Desf., endêmicos do continente americano (BARRINGER 1984; FISHER, 2004), *Stemodiopsis* e *Crepidorhopalon* Eb. Fischer, endêmicos da África (FISHER, 1997). Também é possível encontrar espécies de Linderniaceae mais amplamente distribuídas como *Torenia crustacea* (L.) Cham. & Schltdl., e *Lindernia procubens* (Krock.) Borbás (FISHER, 2004; FISCHER; SCHÄFERHOFF; MÜLLER, 2013).

Nenhuma análise filogenética anterior incluiu amostras de *Ameroglossum* e gêneros relacionados, portanto, seu monofiletismo e posicionamento filogenético em Linderniaceae permanecem incertos. Além disso, análises filogenéticas moleculares, baseadas em regiões nucleares e plastidiais, em associação com dados cariotípicos, tem se mostrado importantes ferramentas para esclarecer relações taxonômicas entre alguns gêneros de plantas (e.g. SCHUBERT; LYSAK, 2011; SOUZA et al., 2016). Diante disso, os objetivos principais desse trabalho são: obter um panorama da evolução cariotípica de *Ameroglossum*, conhecer as relações filogenéticas de *Ameroglossum* com gêneros relacionados como *Catimbaua*, *Cubitanthus* e *Isabelcristinia*, assim como seu pocionamento em Linderniaceae.

2 REVISÃO DE LITERATURA

2.1 O Gênero *Ameroglossum*

Quando foi descrito em 1999, *Ameroglossum* E. Fischer e A.V. Lopes, foi posicionado em Scrophulariaceae e poderia estar relacionado a gêneros como *Russelia* Jaqc., *Hemichaena* Benth., ou *Eremogeton* Standl. & L.O.Williams, por compartilhar caracteres como inflorescência do tipo tirso e hábito arbustivo, apesar de diferir em vários outros aspectos morfológicos. Na ocasião, *Ameroglossum pernambucense* Eb.Fisch., S.Vogel & A.V.Lopes (Figura 1A), espécie tipo do gênero, foi coletada no município de Brejo da Madre de Deus, Pernambuco, Brasil (FISCHER; VOGEL; LOPES, 1999). A espécie foi descrita como apresentando um longo estaminódio, lábio inferior da corola indiviso e semelhante à uma língua, mas que era na verdade trilobulado no seu ápice e apontada como endêmica do nordeste do Brasil (FISCHER; VOGEL; LOPES, 1999). Em uma revisão taxonômica para Scrophulariaceae (FISCHER, 2004), o gênero *Ameroglossum* foi incluído na tribo Russelieae. Com base em dados morfológicos e moleculares não publicados, Souza e Lorenzi (2012) propuseram informalmente a inclusão provisória de *Ameroglossum* em Linderniaceae, junto dos gêneros *Cubitanthus*, *Lindernia*, *Stemodiopsis* e *Torenia*. Esse posicionamento foi seguido por Christenhusz, Fay, Chase (2017) e Almeida et al. (2019) com base na posição dos estames e na presença de sementes com ranhuras.

Ameroglossum permaneceu monotípico por muitos anos, até que em 2016 foi descrita uma nova espécie para o gênero, *Ameroglossum manoelfelixii* L.P.Felix & E.M.Almeida (ALMEIDA et al., 2016). Essa nova espécie foi coletada no município de Esperança, Paraíba, Brasil, embora outras populações tenham sido encontradas em outros municípios do estado como em Areia, Alagoa Nova, Algodão de Jandaíra, Bananeiras, Puxinanã e Remígio. Apesar de pertencer claramente ao gênero *Ameroglossum*, *A. manoelfelixii* pode ser diferenciada de *A. pernambucense* por ter flores tubulares com corola de cor vinho a púrpura (Figura 1B), caules e folhas arroxeados, caule quadrangular, alado e glabro, folhas opostas com margens revolutas e ápices atenuados e recurvados, venação foliar fortemente impressa na face abaxial, além de tricomas arroxeados no lábio inferior da corola e inflorescência em dicásio composto. Em contrapartida, *A. pernambucense* não possui caule alado, tem venação levemente impressa nas folhas, folhas verticiladas com ápice acuminado e não recurvado, caules e folhas verdes,

inflorescência em dicásio simples e principalmente flores tubulares com corola escarlate e tricomas esbranquiçados no lábio inferior (ALMEIDA et al., 2016, 2021).

Figura 1. Flores de Diferenças florais entre *Ameroglossum pernambucense* (A) com flores laranjadas e partes vegetativas verdes e *A. manoelfelixii* (B) com flores vermelhas e partes vegetativas arrocheadas. Fonte: Almeida, EM (2016).

As espécies do gênero foram encontradas exclusivamente em afloramentos rochosos da região Nordeste, em ambientes de mata úmida, vegetação de Agreste e na Caatinga, porém as diferentes populações apresentavam diferenças marcantes em suas estruturas vegetativas e reprodutivas (FISCHER; VOGEL; LOPES, 1999; ALMEIDA et al., 2016). *Ameroglossum pernambucense* ocorre em terras altas (1000-1200 m) do Planalto da Borborema, em floresta tropical atlântica, elevadas acima da floresta tropical seca da Caatinga. Outras populações da espécie foram encontradas nos municípios de Brejo da Madre de Deus e São Caetano, no estado de Pernambuco, enquanto *Ameroglossum manoelfelixii* ocorre em floresta de altitude média (500-600m) em arredores de Caatinga.

Para Almeida (2016) as espécies de *Ameroglossum* estavam reunidas em dois agrupamentos taxonômicos: o primeiro formadao por populações de *A. pernambucense* que se caracterizam por apresentar lobo mediano do lábio inferior da corola involuto, e ocorrem a altitudes superiores a 1.000 metros nos estados da Paraíba e Pernambuco. Já o segundo grupo seria formado por populações de *A. manoelfelixii* que se distingue, entre outros caracteres, por apresentar lobo mediano do lábio inferior da corola revoluto, ocupando afloramentos graníticos com diferentes condições climáticas e de três diferentes

microrregiões do estado da Paraíba. Essas espécies de *Ameroglossum* são plantas de polinização ornitófila e sementes pequenas de dispersão barocórica (WANDERLEY; LOPES; MACHADO, 2014a; ALMEIDA, 2016). As duas espécies reconhecidas até aquele momento para o gênero foram consideradas ameaçadas de extinção, de acordo com os critérios da IUCN, por apresentarem populações fragmentadas, distribuição geográfica restrita a afloramentos rochosos e diminuição na qualidade do habitat devido à atividade humana (WANDERLEY; ALMEIDA; FELIX, 2014b).

Em uma extensa análise populacional das duas espécies descritas até aquele momento para o gênero, Wanderley et al. (2018) analisaram os componentes climáticos e geográficos da variação genética em 54 alelos de microssatélites convertidos em 48 caracteres e a variação fenotípica em 11 características de folhas e flores, abordando os dados para cada espécie separadamente e combinados. Os resultados revelaram que a variação genética e fenotípica intra- e interespecífica em *Ameroglossum* estão associadas de forma semelhante ao isolamento geográfico e ambiental. Além disso, foi possível identificar que as populações de *A. pernambucense* apresentavam uma mistura genética formada por dois conjuntos genéticos. Porém, a maioria das populações de *A. pernambucense* e *A. manoelfelixii* analisadas por Wanderley et al. (2018) constituem hoje populações de diferentes espécies que só foram descritas recentemente.

Desde a descrição de *A. manoelfelixii* (ALMEIDA et al., 2016), os autores tinham conhecimento da existência de outras populações de *Ameroglossum* que abrigavam morfotipos diferentes das duas espécies descritas para o gênero (ALMEIDA, 2016). Recentemente, os morfotipos dessas populações foram descritos (Tabela 1), surgindo então sete novas espécies para o gênero (ALMEIDA et al., 2021), sendo elas: *A. alatum* E.M.Almeida, A.M.Wanderley & L.P.Felix, *A. asperifolium* E.M.Almeida, J.M.P.Cordeiro & L.P.Felix, *A. bicolor* E.M.Almeida, A.M.Wanderley & L.P.Felix, *A. fulniorum* E.M.Almeida, A.M.Wanderley & L.P.Felix, *A. genaroanum* E.M.Almeida, J.M.P.Cordeiro & L.P.Felix, *A. intermedium* E.M.Almeida, A.M.Wanderley & L.P.Felix e *A. xukuruorum* E.M.Almeida, Christenh. & L.P.Felix (ALMEIDA et al., 2021).

Com a descrição de novas espécies do gênero, é possível um novo olhar sobre os resultados de Wanderley et al. (2018). Em seu trabalho foi apontada a existência de atribuições genéticas em três grupos genéticos distintos, e mais um grupo não atribuído, por apresentar mistura genética interespecífica, na época apontadas entre populações de *A. pernambucense* e *A. manoelfelixii*. Hoje sabe-se que um dos grupos genéticos é formado por populações de *A. manoelfelixii* + *A. genaroanum* (populações RN, TAC,

SER, AJ, ESP, AN), outro grupo formado apenas por populações de *A. pernambucense* (populações CAT, P, PC), o terceiro grupo formado por populações de *A. asperifolium* + *A. bicolor* + *A. fulniorum* + *A. intermedium* + *A. xukuruorum* (populações SJTP, REI, PG, LB, AB, SPE, QA), e por fim, o grupo que apresentou mistura genética interespecífica, representando a população de *A. alatum* (WANDERLEY et al., 2018; ALMEIDA et al., 2021).

2.2 Posicionamento taxonômico e filogenético de *Ameroglossum*

Como já foi apontado, *Ameroglossum* foi incluído na família Scrophulariaceae quando descrito (FISCHER; VOGEL; LOPES, 1999). Ainda hoje é possível encontrar bases de dados e trabalhos que apontam esse mesmo posicionamento para o gênero (POWO, 2021; STEVENS, 2021; WFO. 2021). Sendo assim, fez-se necessário apresentar o histórico taxonômico e filogenético esclarecendo seu nessas duas famílias.

2.2.1 Sistemática da Família Scrophulariaceae

Por muito tempo Scrophulariaceae foi considerada a maior da ordem Lamiales por possuir cerca 35 gêneros e 1.500 espécies de distribuição cosmopolita (TANK et al., 2006; SOUZA; FERREIRA; GIULIETTI, 2009; SOUZA; LORENZI, 2012). Ao contrário da maioria das famílias botânicas que são caracterizadas pela presença de caracteres exclusivos que permitem suas identificações, Scrophulariaceae era caracterizada pela ausência de sinapomorfias (TANK et al., 2006).

Scrophulariaceae foi apontada como polifilética na primeira análise filogenética molecular baseada em sequências *rbcL* e *ndhF* (OLMSTEAD; REEVES, 1995), nesse trabalho foi possível identificar dois clados distintos e bem suportados: ‘Scroph I’ com os gêneros *Verbascum* L., *Celsia* L., *Scrophularia* L., *Selago* L., *Buddleja* L. e *Nicodemia* Tenore; e ‘Scroph II’ com *Antirrhinum* L., *Digitalis* L., *Veronica* L., *Plantago* L., *Hippuris* L. e *Callitriche* L. Porém, os gêneros *Schlegelia* Miq., e *Paulownia* Siebold & Zucc., que tradicionalmente estavam em Scrophulariaceae, não pertenciam a nenhum dos dois clados encontrados. Um estudo subsequente (OLMSTEAD et al., 2001), usando as regiões *rbcL*, *ndhF* e *rps2*, sugeriu que a família estaria posicionada na base da ordem Lamiales. Além disso, dividia Scrophulariaceae em cinco linhagens monofiléticas distintas, contendo os principais táxons anteriormente colocados em Scrophulariaceae *s.l.*

como: Calceolariaceae, Orobanchaceae, Scrophulariaceae *s.s.* (Scroph I), Stilbaceae e Veronicaceae (Scroph II) (OLMSTEAD et al., 2001), ainda nesse trabalho, alguns gêneros não se agruparam em nenhuma destas linhagens, como *Schlegelia* e *Paulownia*, novamente, além de *Mimulus* L. Scrophulariaceae estava então composta por um clado de sete tribos: Scrophularieae, Manuleeae, Buddlejeae, Myoporeae, Leucophylleae, Aptosimeae e Hemimerideae (OLMSTEAD et al., 2001; APG II, 2003).

Em uma revisão taxonômica que reuniu diversos dados disponíveis para Scrophulariaceae, Fischer (2004) listou as tribos a serem reconhecidas a partir daquele momento, além de reestabelecer famílias informalmente e seus respectivos gêneros a partir de Scrophulariaceae. Para Scrophulariaceae, foram reconhecidas 10 tribos que reuniam 53 gêneros, sendo a maior delas a tribo Manuleae com 25 gêneros. Neste ponto, destaca-se a tribo Russelieae, composta por três gêneros, dentre eles *Ameroglossum*, que havia sido descrito em 1999. Entre as famílias reestabelecidas por Fischer (2004) foram listadas Schlegeliaceae, Calceolariaceae, Paulowniaceae, Veronicaceae, Phrymaceae, Orobanchaceae e Stilbaceae. Dessas famílias, a maior foi Veronicaceae, com três subfamílias, 13 tribos e 118 gêneros. Entre as tribos de Veronicaceae, destacam-se a tribo Stemodieae e a tribo Lindernieae que abrigavam gêneros que hoje pertencem as famílias Linderniaceae e Plantaginaceae.

Um estudo filogenético utilizando sequências de DNA plastidial (*ndhF*, *trnL/F* e *rps16*) realizado por Oxelman et al. (2005), voltado para as relações de grupos previamente designados como Scrophulariaceae, confirmou o monofiletismo da família apresentada por Olmstead et al. (2001) e APG II (2003). Oxelman et al. (2005) também encontraram dados que suportam Plantaginaceae como um clado separado de Scrophulariaceae e composto por subgrupos bem suportados como Gratioleae, Antirrhineae, Globularieae, Digitalideae, Cheloneae e Russelieae. Plantaginaceae já havia sido reconhecida por Olmstead e Reeves (1995) dentro do clado 'Scroph I', e complementada com mais taxa nos estudos de Olmstead et al. (2001). Quanto à composição de Scrophulariaceae, Oxelman et al. (2005) confirmaram as tribos Scrophularieae, Manuleeae, Buddlejeae, Myoporeae, Leucophylleae, Aptosimeae e Hemimerideae. A tribo Lindernieae emergiu como um clado fortemente suportado, separado de Scrophulariaceae pela primeira vez, e composta por gêneros como *Stemodiopsis*, *Torenia*, *Micranthemum* e *Picria* Lour. Esse clado representando a tribo Lindernieae foi então caracterizado por apresentar filamentos anteriores geniculados (Oxelman et al., 2005).

Atualmente, Scrophulariaceae possui 59 gêneros e cerca de 1.880 espécies distribuídas pelos cinco continentes, sendo dividida em sete subfamílias: Hemimerideae, Aptosimieae, Myoporeae, Leucophylleae, Buddlejeae, Limoselleae e Scrophularieae (STEVENS, 2021). Das famílias citadas anteriormente por Fischer (2004), apenas Veronicaceae foi sinonimizada e hoje compõe Plantaginaceae.

2.2.2 Sistemática da Família Linderniaceae

Ao longo dos anos, Linderniaceae e os 17 gêneros que hoje a compõem já foram modificados diversas vezes. Os gêneros *Lindernia* e *Craterostigma* Hochstetter, por exemplo, foram inicialmente incluídos na tribo Gratioleae, em Scrophulariaceae (TANK et al., 2006); e posteriormente, Fisher (1992) tratou Linderniaeae como uma tribo distinta de Gratioleae. Como apontado anteriormente, Oxelman et al. (2005) apontou que a tribo Linderniaeae formava um clado bem suportado, separada de Scrophulariaceae.

Ainda em 2005, um estudo molecular (RAHMANZADEH et al., 2005) baseado em dados de sequências plastidiais *trnK/matK*, contendo 53 táxons, elevou a tribo Lindernieae para a categoria de família. Este trabalho incluiu cinco dos 13 gêneros da tribo (*Artanema* D. Don, *Craterostigma*, *Crepidorhopalon*, *Lindernia* e *Torenia*). Dentro de Linderniaceae, o gênero *Lindernia* como o maior em número de espécies, emergiu como não monofilético. O estudo também mostrou evidências de que Linderniaceae é uma linhagem separada de Scrophulariaceae *s.s*.

Em uma revisão filogenética mais abrangente, Fischer, Schäferhoff, Müller (2013) utilizaram sequências completas de DNA plastidiais *trnK/matK* de 48 taxa, incluindo os grupos estudados por Rahmanzadeh et al. (2005) além dos gêneros *Bryodes*, *Micranthemum, Stemodiopsis, Psammetes* e *Chamaegigas*. Foram reconhecidos 17 gêneros para Linderniaceae, adotando a abordagem de dividir a família em vários gêneros monofiléticos, ao invés de criar seções dentro de *Lindernia*. Gêneros amplamente reconhecidos como *Stemodiopsis*, *Torenia*, *Artanema*, *Craterostigma* e *Crepidorhopalon* foram mantidos, enquanto *Bonnaya* Link & Otto e *Vandellia* L. foram novamente separados de *Lindernia* s.l., (FISCHER; SCHÄFERHOFF; MÜLLER, 2013). O gênero *Micranthemum* foi considerado próximo de *Lindernia*, por compartilhar caracteres morfológicos como estames abaxiais com geniculações e endosperma não-alveolado, porém com posicionamento incerto, devido à baixa amostragem.

O trabalho de Biffin et al. (2018) utilizou sequências de DNA plastidiais *matK* de espécies australianas em uma análise filogenética conjunta com os dados de Fischer, Schäferhoff, Müller (2013) a fim de testar os conceitos genéricos de Linderniaceae. Em seus resultados, os autores encontraram três clados distintos para a família, no primeiro deles sendo posicionados *Cubitanthus* e *Stemodiopsis* como um clado mais externo na família, e nos outros dois clados ficaram gêneros como: *Torenia*, *Artanema*, *Lindernia*, *Craterostigma*, entre outros. Biffin et al. (2018) ainda reconheceram três subgêneros monofiléticos dentro de *Lindernia* (*L*. subg. *Lindernia*, *L*. subg. *Ilysanthes* Rafinesque e *L*. subg. *Didymadenia*) e, resolveram também o não monofiletismo de *Vandellia*, com a alteração de nomes de algumas espécies e estreitamento da circunscrição de Fischer, Schäferhoff, Müller (2013). Entretanto o posicionamento de *Micranthemum* em relação à *Lindernia* continua incerto, principalmente por suas espécies pouco conhecidas, tendo em vista que apenas *Micranthemum umbrosum* (Walt. Ex J.F.Gmel.) foi estudada (FISCHER; SCHÄFERHOFF; MÜLLER, 2013; BIFFIN et al., 2018).

Espécies de muitos gêneros que pertenciam à Scrophulariaceae ainda não foram estudadas geneticamente, como em *Ameroglossum*, *Bampsia* Lisowski e Mielcarek, *Bythophyton* Hooker, *Dintera* Stapf em Stapf & Schinz e *Encopella* Pennell, *Catimbaua* e *Isabelcristinia* que podem vir a pertencer à Linderniaceae, embora outras posições dentro de Lamiales não possam ser descartadas (ALMEIDA et al., 2019).

Atualmente Linderniaceae abrange 17 gêneros e cerca de 255 espécies, de acordo com Stevens (2021). Tem sua diversidade concentrada principalmente na África tropical, Madagascar, Ásia Tropical, com poucas espécies na América tropical, geralmente em ambientes especializados como poços rochosos sazonais e *inselberg*s (RAHMANZADEH et al., 2005; ALMEIDA et al., 2019). Suas espécies são terrestres ou saxícolas, ervas aquáticas ou pequenos arbustos, algumas espécies têm a adaptação para o crescimento em áreas sazonalmente secas (ALMEIDA et al., 2019).

2.3 Gêneros Relacionados a *Ameroglossum*

2.3.1 *Cubitanthus* Barringer

O gênero monotípico *Cubitanthus* foi descrito por Barringer (1984) a partir da ilustração de descrição original do táxon, e colocado em Gesneriaceae por apresentar placentação parietal, um caráter diagnóstico frequentemente usado para essa família

(PERRET et al., 2013). Estudos posteriores analisaram o fruto fresco de *Cubitanthus alatus* (Cham. & Schltdl.) Barringer e mostraram que a placentação parietal está restrita a porção superior do fruto, enquanto a parte inferior apresenta placentação axilar. O padrão de placentação mista está presente em várias linhagens que antes pertenciam a Scrophulariaceae s.l. (PERRET et al., 2013).

Cubitanthus alatus é uma espécie endêmica do estado da Bahia, encontrada nos municípios de Almadina, Ilhéus, Itabuna, Itacaré, Itajuípe, Itamarajú, Ubaitaba, Una e Wenceslau Guimarães, e ocorre no domínio da Mata Atlântica, em ambientes de floresta ombrófila (FLORA E FUNGA DO BRASIL, 2023; SOUZA, 2015). O gênero pode ser caracterizado por apresentar caule alado, uma flor axilar, corola bilabiada com lábio superior inteiro e lábio inferior trilobado (Tabela 1), quatro estames geniculados e base da corola adnata (BARRINGER, 1984). Essas são algumas das características morfológicas que caracterizam Linderniaceae, corroborando os dados filogenéticos de Rahmanzadeh et al. (2005) que o incluiu na família desde então.

2.3.2 *Catimbaua* L.P.Felix, Christenh. & E.M.Almeida

O gênero *Catimbaua* foi encontrado exclusivamente no Parque Nacional do Catimbau, Buíque, Pernambuco. Trata-se de um gênero monotípico, com a espécie *Catimbaua pendula* L.P.Felix & E.M.Almeida crescendo em abismos e fendas em escarpas íngremes e areníticas em vegetação de caatinga. A espécie se caracteriza por apresentar caule quadrado com indumento simples, aracnoide, folhas com margens revolutas e corola laranja com lábio superior bipartido (ALMEIDA et al., 2019).

O gênero possui semelhanças morfológicas com *Ameroglossum* e *Cubitanthus* (Tabela 1), por apresentar a presença de tricomas no lobo inferior da corola e nas sementes e estames sulcados com filetes abaxiais curvos. Ainda possui brácteas, flores tubulares, laranja-avermelhadas dispostas em um dicásio e pedicelos recurvados na maturidade como *Ameroglossum*, além do lábio inferior com lobos bem definidos e pólen branco como *Cubitanthus*. O compartilhamento de caracteres com esses gêneros, sugeriu a inclusão de *Catimbaua* em Linderniaceae (ALMEIDA et al., 2019).

2.3.3 *Isabelcristinia* L.P.Felix, Christenh. & E.M.Almeida

O gênero *Isabelcristinia* ocorre em afloramentos rochosos de granito (*inselberg*s) nos municípios de Belo Jardim, Brejo da Madre de Deus e Jataúba, Pernambuco, em

vegetação de caatinga semiárida com altitudes que variam de 502 m em uma das populações do Brejo da Madre de Deus a 926 m no município de Jataúba (ALMEIDA et al., 2019).

Também é um gênero monotípico, com *Isabelcristinia aromatica* L.P.Felix & E.M.Almeida, que caracteriza-se por apresentar corola lilás com lábio inferior amarelo, tricomas glandulares aromáticos nos ramos e folhas. Assemelha-se a *Ameroglossum* e *Cubitanthus* por ter bractéolas e tricomas no lobo inferior da corola e sementes sulcadas (Tabela 1). Com *Ameroglossum* ainda compartilha o hábito ereto, flores dispostas em dicásio, pólen azul e filetes maduros recurvados. Também apresenta filamentos geniculados, principal sinapomorfia de Linderniaceae. A espécie possui indumento constituído por tricomas glandulares que produzem óleo essencial em grande quantidade, sendo a única do grupo com flores aromáticas abertas, bicolores (violeta e amarela) que produzem néctar (ALMEIDA et al., 2019).

2.4 Distribuição geográfica de Linderniaceae

Linderniaceae apresenta uma distribuição Pantropical, com suas espécies encontradas em regiões tropicais e temperadas (CHRISTENHUSZ; FAY; CHASE, 2017), com maior diversidade no continente africano (~ 89 spp) e no sudeste asiático (~ 84 spp). As espécies africanas ocorrem em sua maioria em ambientes especializados como poços rochosos preenchidos sazonalmente com água, *inselbergs*, fendas de rochas e solos de metais pesados (FISHER, 1997, 2004; LEWIS, 2000; POREMBSKI, 2007; DARBYSHIRE et al., 2019; BALKWILL, 2021). As espécies da Ásia e Oceania ocorrem principalmente em áreas de florestas tropicais, áreas perturbadas, margens de rios, áreas úmidas, inundadas e/ou abertas, e arrozais (LEWIS, 2000; FISHER, 2004; LIANG; WANG, 2014). Também é possível encontrar espécies de Linderniaceae no continente americano (~ 37 spp), em ambientes bem variáveis como bordas de florestas, áreas úmidas, alagáveis, abertas e/ou perturbadas (FISHER, 2004; SOUZA; LORENZI, 2012; SOUZA; GIULIETTI, 2009), afloramentos rochosos (ALMEIDA et al., 2019, 2021), e até espécies aquáticas, como é o caso de *Micranthemum umbrosum* (CHRISTENHUSZ; FAY; CHASE, 2017).

Tabela 1. Alguns caracteres morfológicos diagnósticos para as espécies de *Ameroglossum* + *Catimbaua* + *Isabelcristinia* + *Cubitanthus*.

Espécies	Formato do caule	Tricomas caulinares e foliares	Cor dos órgãos vegetativos	Disposição das folhas	Cor da corola	Tipo de inflorescência	Cor dos tricomas da corola	Lábio inferior da corola
Ameroglossum								
A. alatum	Quadrangular alado	Ausente	Esverdiada	Opostas decussadas	Laranjada	Dicásio simples	Brancos	Levemente bilobado, revoluto
A. asperifolium	Quadrangular não alado	Presente	Avermelhada	Opostas decussadas	Vermelha	Dicásio simples	Violeta	Levemente bilobado, revoluto
A. bicolor	Quadrangular não alado	Ausente	Avermelhada ou esverdiada	Opostas decussadas	Laranjada ou vermelha	Dicásio composto	Brancos	Levemente bilobado, revoluto
A. fulniorum	Quadrangular não alado	Ausente	Avermelhada	Opostas decussadas	Vermelha	Dicásio simples	Brancos	Levemente bilobado, revoluto
A. genaroanum	Quadrangular não alado	Ausente	Esverdiada	Opostas decussadas	Laranjada	Dicásio simples	Violeta	Levemente bilobado, revoluto
A. intermedium	Quadrangular ou hexagonal levemente alado	Presente	Esverdiada	Opostas decussadas	Laranjada	Dicásio composto	Brancos	Levemente bilobado, revoluto
A. manoelfelixii	Quadrangular alado	Ausente	Avermelhada	Opostas decussadas	Vermelha	Dicásio composto	Violeta	Levemente bilobado, revoluto
A. pernambucense	Cilíndrico não alado	Presente	Esverdiada	Verticiladas	Laranjada com amarelo	Dicásio simples	Brancos amarelados	Bilobado, involuto
A. xukuruorum	Cilíndrico levemente alado	Ausente	Esverdiada	Verticiladas	Laranjada com amarelo	Dicásio simples	Brancos amarelados	Bilobado, involuto

Catimbaua *C. pendula*	Cilíndrico não alado	Presente	Esverdiada	Opostas decussadas	Laranjada	Dicásio simples	Brancos	Trilobado, revoluto
Isabelcritinia *I. aromatica*	Quadrangular levemente alado	Presente	Esverdiada	Opostas decussadas	Branca e roxo	Dicásio simples	Brancos	Trilobado involuto
Cubitanthus *C. alatus*	Quadrangular alado	Presente	Esverdiada	Opostas	Branca e roxo	Flor simples	Brancos	Trilobado, involuto

A maioria dos gêneros de Linderniaceae apresenta distribuição geográfica concentradas a determinados ambientes, é possível encontrar gêneros inteiros endêmicos de um mesmo continente, com poucos exemplos de distribuição mais ampla (POWO, 2021; WFO. 2021). No continente africano são listados os gêneros endêmicos *Bampsia* (~ 2 spp.), *Crepidorhopalon* (~ 33 spp.), *Hartliella* (~ 4 spp.), *Linderniella* (~ 16 spp.) e *Stemodiopsis* (~ 7 spp.), além de *Craterostigma* (~ 26 spp.), que não é totalmente endêmico, pois possui três espécies ocorrendo na Ásia, embora seu centro de diversidade seja a África (FISHER, 2004; FISCHER; SCHÄFERHOFF; MÜLLER, 2013). Ainda é possível encontrar poucas espécies de *Artanema*, *Lindernia*, *Torenia* e *Vandellia* na África, embora o centro de diversidade desses gêneros sejam a Ásia. No continente Asiático, são encontrados também alguns gêneros endêmicos, como *Pierranthus* (1 sp.), *Schizotenia* (2 spp.) e *Scolophyllum* (3 spp.), porém são gêneros poucos conhecidos, e não há informações sobre seu posicionamento filogenético na família (FISHER, 2004; FISCHER; SCHÄFERHOFF; MÜLLER, 2013; POWO, 2021). Na Oceania, mais especificamente na Austrália, são encontrados dois gêneros endêmicos, *Hemiarrhena* e *Picria* ambos com uma espécie apenas (FISHER, 2004; FISCHER; SCHÄFERHOFF; MÜLLER, 2013). Esses dois continentes Asia e Oceania, reúnem um número significativo de espécies da família, uma vez que é possível encontrar alguns gêneros com centro de diversidade compartilhado entre os dois, como é o caso de *Artanema* (3 spp.), *Bonnaya* (~16 spp., com apenas uma endêmica da África), *Lindernia* (~ 65 spp.), *Torenia* (~ 68 spp.), *Vandellia* (~ 53 spp.) e *Yamazakia* (2 spp.) (FISHER, 2004; FISCHER; SCHÄFERHOFF; MÜLLER, 2013; BIFFIN et al., 2018).

Neste ponto é necessário chamar atenção para os três maiores gêneros da família, *Lindernia*, *Torenia* e *Vandellia*. São os gêneros mais antigos de Linderniaceae, já passaram por diversas modificações ao longo do tempo (veja sessão 2.2.2), e é provável que suas espécies ainda precisem de mais revisões do ponto de vista filogenético (BIFFIN et al., 2018; ver capítulo 1). Ao observar a distribuição geográfica desses gêneros, fica claro que a maior riqueza de espécies está de fato concentrada entre os continentes Ásia + Oceania, porém é possível encontrar algumas espécies também em outros continentes, inclusive espécies endêmicas (FISHER, 2004; FISCHER; SCHÄFERHOFF; MÜLLER, 2013; BIFFIN et al., 2018; POWO, 2021). O melhor exemplo de gênero mais bem distribuído pelos trópicos, é o gênero *Lindernia*. Ele possui espécies que são encontradas em praticamente todos os continentes, como é o caso de *Lindernia procubens* (Krock.) Borbás, única espécie que pode ser encontrada inclusive pela Europa, e *Lindernia*

rotundifolia (L.) Alston, encontrada pela América, África e Asia, além de espécies endêmicas dos continentes africano e americano (FISHER, 2004; FISCHER; SCHÄFERHOFF; MÜLLER, 2013; BIFFIN et al., 2018; POWO, 2021). O gênero *Torenia*, de fato possui pouquíssimas espécies fora da Ásia, apenas quatro são endêmicas da África (*T. daubyi* Eb.Fisch. & O.Lachenaud, *T. dinklagei* Engl., *T. mannii* Skan, e *T. silvícola* A.Raynal, e não possuem dados moleculares disponíveis), apenas seis também ocorrem na Oceania, e apenas uma espécie *T. crustacea* (L.) Cham. & Schltdl., encontra-se atualmente espalhada pelos trópicos, provavelmente fruto de introdução (FISHER, 2004; FISCHER; SCHÄFERHOFF; MÜLLER, 2013; BIFFIN et al., 2018; POWO, 2021).

No que se refere ao continente americano, a família também possui alguns gêneros endêmicos, como *Ameroglossum* (~ 9 spp), *Catimbaua* (1 sp), *Cubitanthus* (1 sp), *Isabelcristinia* (1 sp) e *Micranthemum* (~ 13 spp).O gênero *Micranthemum* é o menos conhecido, com a maioria de suas espécies sendo apontadas como endêmicas de Cuba, e a única espécie que possui mais dados disponíveis, é a aquática: *M. umbrosum* (BIFFIN et al., 2018; POWO, 2021), que é normalmente utilizada como planta ornamental em aquários.. Ainda é possível encontrar na América outras espécies, como no caso do gênero *Lindernia* que possui nove endêmicas no continente, e o gênero *Vandellia*, com uma espécie endêmica, além das bem distribuídas *L. procubens* e *T. crustacea* (LEWIS, 2000; FISHER, 2004; FISCHER; SCHÄFERHOFF; MÜLLER, 2013).

2.5 Dados Citogenéticos

A interpretação de dados citogenéticos tem sido utilizada ao longo dos anos na identificação de características citológicas de grupos de plantas que não possuem dados moleculares disponíveis. Muitas vezes os dados cariotípicos auxiliam os dados morfológicos, e se tornam capazes de elucidar da origem e diversificação evolutiva de um grupo.

2.5.1 Variação de número cromossômico

O número cromossômico é a característica citológica mais investigada em análises citogenéticas, além de ser o parâmetro de que mais se tem dados disponíveis. Os números cromossômicos não são influenciados pelas condições externas do organismo, fases de crescimentos ou idade (GUERRA, 2008). Dentre as variações numéricas cromossômicas

com efeitos relacionados à filogenia e evolução cariotípica de um grupo se destacam a poliploidia (multiplicação de todo o conjunto haploide), e a disploidia (aumento ou diminuição gradual do número original resultantes de rearranjos cromossômicos). Embora haja outras variações numéricas como haploidias, aneuploidias e cromossomos B, que não têm implicações claras para a evolução das espécies (GUERRA, 2000a; BEDINI; GARBARI; PERUZZI, 2012).

A variação de número cromossômico nas angiospermas é acentuada. O menor registro numérico é de 2*n* = 4 para as espécies *Rhynchospora tenuis* Link., (Cyperaceae), *Ornithogalum tenuifolium* F.Delaroche (sin. *Albuca virens* (Lindl.) J.C.Manning & Goldblatt.) (Asparagaceae) e *Haplopappus gracilis* (Nutt.) A.Gray (sin. *Xanthisma gracile* (Nutt.) D.R.Morgan & R.L.Hartm.) (Asteraceae) (JACKSON; JORDAN, 1975; STEDJE, 1988; VANZELA; GUERRA; LUCEÑO, 1996). Por outro lado, o registro de maior número cromossômico é de 2*n* ≈ 606 para *Voanioala gerardii* J. Dransf. (Arecaceae), uma palmeira endêmica de Madagascar (RÖSER, 2015).

Algumas famílias botânicas apresentam notável variação numérica, como Commelinaceae com registros de 2*n* = 12 a 2*n* = 76 nos gêneros *Callisia* Loefl. e *Dichorisandra* J.C.Mikan, respectivamente (PITREZ et al., 2001). Na família Orchidaceae, o gênero *Epidendrum* é altamente variável, com números desde 2*n* = 24 em *E. fulgens* Brongn., a 2*n* = 240 para *E. cinnabarinum* Salzm. ex Lindl. (DE ASSIS et al., 2013; MEDEIROS-NETO et al., 2017). No entanto, outros grupos são bastante conservados, como *Jacaranda* Juss. (Bignoniaceae) com 2*n* = 36 (CORDEIRO et al., 2016), grupo Caesalpinia (Fabaceae) com 2*n* = 24 (VAN-LUME et al., 2017), *Senna* Mill. (Fabaceae) com 2*n* = 28 (CORDEIRO; FELIX, 2018) e diversos gêneros de Lorantaceae que mantêm o mesmo número cromossômico para suas diferentes espécies (ANDRADE; GIULIETTI; GUERRA, 2005).

Em Linderniaceae, a variação de número cromossômico encontrada é bastante acentuada, pois há contagens de 2*n* = 14, 16, 18, 20, 24, 26, 28, 30, 32, 34, 36, 38, 40 e 42, 60, 112, porém 2*n* = 18 aparece com mais frequência, e de acordo com a base de dados Chromosome Counts DataBase - CCDB (RICE et al., 2015), há registros de contagens cromossômicas para apenas 33 espécies, o que corresponde a cerca de 12% das espécies, entre *Artanema*, *Bonnaya*, *Craterostigma*, *Lindernia*, *Linderniella*, *Torenia*, *Vandellia* e *Yamazakia*. Os novos gêneros *Isabelcristinia* e *Catimbaua* foram descritos como pertencentes a Linderniaceae e apresentam 2*n* = 60 (ALMEIDA et al., 2019). As espécies de *Ameroglossum* também apresentaram 2*n* = 60 cromossomos (ALMEIDA et

al., 2016), número esse bastante distinto do restante das Linderniaceae conhecidas cariologicamente.

Por serem espécies endêmicas de *inselbergs* do nordeste do Brasil, poderia esse número cromossômico distinto de *Ameroglossum*, *Isabelcristinia* e *Catimbaua* ser um indício de sua afinidade entre si, e representar a diferenciação do restante das Linderniaceae? Esse é um dado curioso, porém com o baixo número cromossômico conhecido para o restante das Linderniaceae, e a total ausência de dados citogenéticos para outras espécies endêmicas de *inselbergs* da África como é o caso de *Stemodiopsis*, estudos adicionais comparativos são necessários, a fim de preencher essas lacunas existentes para o gênero.

2.5.2 Heterocromatina em *Ameroglossum*

A heterocromatina pode ser definida como a porção do cromossomo que permanece condensada durante a maior parte do ciclo celular e se caracteriza por mostrar pouca ou nenhuma atividade gênica, além de replicação tardia do DNA (EISSENBERG; ELGIN, 2005). Porém, a heterocromatina não é homogênea, podendo variar em número, tamanho, distribuição e conteúdo de DNA entre e dentro de espécies (GUERRA, 2000b).

Os métodos de coloração de heterocromatina visam principalmente a identificação de sítios cromossômicos, investigação da variação heterocromática, identificação de cromossomos, utilização do heteromorfismo da heterocromatina como marcador para distinguir homólogos, além da evolução cromossômica, sendo esses métodos essenciais na caraterização do cariótipo de uma espécie (GUERRA, 1988; SILJAK-YAKOVLEV; PERUZZI, 2012). A heterocromatina tem como funções reprimir a transcrição e a recombinação em elementos de DNA repetitivos, e interfere na segregação adequada dos cromossomos e nas interações de longo alcance da cromatina (GREWAL; JIA, 2007).

Um método para distinção de heterocromatina é a coloração com fluorocromos com afinidade preferencial para DNA rico em AT ou GC. Esse tipo de coloração permite diferenciar a heterocromatina em diferentes grupos, facilitando sua observação (GUERRA, 2000b). Os fluorocromos quinacrina, Hoechst 33258 e 4',6'-diamino-2-fenil-indol coram preferencialmente regiões do DNA ricas em AT, formando as chamadas bandas Q, H, e DAPI, respectivamente. Já os fluorocromos mitramicina e cromomicina A3 coram as regiões ricas em GC formando as bandas MM e CMA, respectivamente (GUERRA, 2000b; SILJAK-YAKOVLEV; PERUZZI, 2012). A cromomicina A3

também pode ser usada para a detecção das regiões organizadoras de nucléolos (RONs), visto que estas regiões são normalmente ricas em pares de bases GC (SCALDAFERRO; MOSCONE, 2019). Porém, há uma técnica específica para a detecção dos sítios de DNA ribossomais, a hibridização *in situ* fluorescente (FISH).

Entre as angiospermas, há uma notável variabilidade nos padrões de distribuição da heterocromatina, mas são raras as mudanças extremas ou descontínuas dentro de um grupo de espécies relacionadas (GUERRA, 2000b). Em grupos como Bignoniaceae (CORDEIRO et al., 2017) e Caesalpinia - Fabaceae (VAN-LUME et al., 2017), o número de bandas e a quantidade de heterocromatina variaram, mas o padrão geral é relativamente constante. Por outro lado, em *Capsicum* L. (Solanaceae)foram encontrados quatro tipos de heterocromatina constitutiva, com padrões de coloração ricos em GC e AT variáveis. Já as regiões organizadoras de nucléolo foram encontradas associadas à heterocromatina CMA^+ (SCALDAFERRO; MOSCONE, 2019).

No que se refere a *Ameroglossum*, as duas espécies com cariótipos descritos apresentam $2n = 60$, com padrões de bandas CMA/DAPI variáveis. *Ameroglossum pernambucense* apresentou duas bandas CMA^+ terminais formando satélites e dois pares de bandas CMA^+ proximais, enquanto *A. manoelfelixii* apresentou apenas um par de bandas CMA^+ proximais formando satélites cromossômicos (ALMEIDA, 2016). Outra informação disponível para o gênero é o conteúdo de DNA, que parece ser um caráter taxonômico importante por permitir distinguir claramente as duas espécies. Quanto ao tamanho do genoma as espécies diferem de 2C = 1.312 pg DNA em *A. pernambucense* e 2C = 0.916 pg DNA em *A. manoel-felixii*. Esse tipo de variação cariológica corroborou a descrição desta última espécie (ALMEIDA et al., 2016).

2.5.3 Hibridização *in situ* fluorescente – FISH

A FISH tem sido usada para responder questões relacionadas à estrutura e evolução de cromossomos individuais até genomas inteiros, além de servir como ferramenta para identificação de cromossomos em muitas espécies de plantas (JIANG, 2019). A técnica consiste no pareamento de um segmento de DNA ou RNA com uma sequência de nucleotídeos complementar na célula, a fim de verificar se a célula em questão possui essa sequência e qual sua localização. Esse segmento de DNA é marcado com uma molécula de fácil identificação, funcionando como uma sonda. Para tanto, a fita de DNA é desnaturada e renaturada em seguida, voltando ao estado de fita dupla durante

a renaturação. A sonda que estará disponível no meio irá competir com as fitas de DNA, podendo ser hibridizada *in situ* no ponto exato onde a sequência ocorre naturalmente. Os fluoróforos utilizados na marcação de sondas para FISH geralmente são o Cy3 (Cianinas), FITC (Isotiocianato de fluoresceína), vermelho-Texas e Rodamina (GUERRA, 2004), de forma direta ou indireta, através do reconhecimento de biotina ou digoxigenina por anticorpos conjugados a estes haptenos.

O DNAr é formado por duas unidades principais, o DNAr 5S e 35S. Normalmente, o sítio de DNAr 35S está associado às regiões organizadoras do nucléolo (RONs) dos cromossomos, enquanto o DNAr 5S pode ser encontrado em qualquer região nucleolar não ligada ao nucléolo (SHAW; BROWN, 2012; ROA; GUERRA, 2015). Os genes ribossomais são conhecidos por sua organização em tandem, que podem chegar a milhares de cópias seguidas umas das outras em um genoma (ROA; GUERRA, 2015). Esses sítios de DNAr podem ser detectados por meio da técnica de hibridização *in situ* fluorescente (FISH), independentemente de estarem ativos ou não, através de sondas que contêm sequências de DNAr 5S e 35S (PEDROSA et al., 2002).

Nas angiospermas, é mais comum encontrarmos apenas um ou dois pares de sítios de DNAr em seus cariótipos, com cerca de 54,1% e 25,8%, respectivamente, do que as espécies que apresentam um maior número de sítios (ROA; GUERRA, 2012; 2015), como em *Spondias* L. (ALMEIDA et al., 2007), *Daucus* L. (IOVENE et al., 2008), e *Coffea* L. (HAMON et al., 2009). Mesmo sendo um pouco mais raro, não é difícil encontrar diferentes espécies de um mesmo gênero ou família com diferentes quantidades de sítios de DNAr. A exemplo disso, temos o gênero *Cuscuta* L., com espécies que vão de dois sítios de DNAr 5S e 35S (*Cuscuta denticulata* Engelm.) a impressionantes 36 e 30 sítios de DNAr 5S e 35S, respectivamente, em *Cuscuta monogyna* Vahl. (IBIAPINO et al., 2019; 2020).

Em Linderniaceae, há apenas um trabalho que investigou o DNAr de duas espécies do gênero *Torenia*. A espécie *T. fournieri* Linden ex Fourn. possui apenas um par de sítios de DNAr 5S e 35S, enquanto *T. flava* Buch.-Ham. ex Benth. (sin. *T. baillonii* Godefroy ex André) possui um par de 35S e três pares de 5S (KIKUCHI *et al*., 2006). Até mesmo a ordem Lamiales é pouco investigada quanto aos DNAr 5S e 35S, havendo poucos trabalhos relacionados à algumas espécies das famílias Byblidaceae (FUKUSHIMA; NAGANO; HOSHI, 2008; FUKUSHIMA et al., 2010), Lamiaceae (DIAO et al., 2009; KITTLER et al., 2015; JANG et al., 2016), Plantaginaceae (DHAR

et al., 2002; 2006; ZHANG et al., 2008; WONG et al., 2014) e Scrophulariaceae (DATSON; MURRAY, 2006).

2.6 Radiação adaptativa e sistemas semelhantes a ilhas

Radiação adaptativa refere-se ao surgimento de uma grande diversidade de espécies a partir de uma única linhagem, em um curto espaço de tempo, e dotadas de grande diversidade de estratégias ecológicas (GIVNISH, 1997; LOSOS; MAHLER, 2010; BOUCHENAK-KHELLADI et al., 2015). Aparentemente, as radiações adaptativas são muito frequentes em ilhas oceânicas ou ambientes similarmente isolados, como lagos, montanhas, cavernas ou afloramentos rochosos (GIVNISH, 1997; ITESCU, 2019). Nesses ambientes, conhecidos como sistemas tipo ilha (ILS), o isolamento geográfico desempenha um papel importante na restrição do fluxo gênico, criando oportunidade para divergências genéticas e fenotípicas entre populações por meio da deriva genética e, consequentemente, especiação (ITESCU, 2019; NACIRI; LINDER, 2020; MENDEZ-CASTRO et al., 2021).

As radiações adaptativas são comumente associadas ao isolamento reprodutivo, à especiação alopátrica (SIMÕES et al., 2015; NACIRI; LINDER, 2020; SCHENK, 2021), à capacidade de dispersão bem-sucedida das espécies envolvidas (CARLQUIST 1966; SCHENK; STEPPAN, 2018), assim como à colonização geográfica e ausência de competição (STROUD, LOSOS, 2016). Embora pareça haver a redução das síndromes de dispersão que favorecem a dispersão a longa distância ao fim de uma radiação adaptativa (GIVNISH et al. 2009; SCHENK, 2021), esse papel da dispersão se mostra semelhante para ilhas verdadeiras e sistemas ILS (ITESCU, 2019; MENDEZ-CASTRO et al., 2021).

O surgimento de novas linhagens pode ser promovido pela seleção natural, atuando sobre novas características fenotípicas, em um curto período de tempo (LAMICHHANEY et al., 2017). De fato, as radiações adaptativas são altamente frequentes em sistemas insulares e fornecem vislumbres interessantes sobre como os processos macroevolutivos operam (GIVNISH, 1997). Grupos que sofreram radiações adaptativas costumam apresentar alta taxa de diferenciação morfológica (com ou sem forte divergência filogenética), o que torna desafiadora a interpretação de sua sistemática e evolução (GLOR, 2010; KNOPE et al., 2020).

Casos de radiação adaptativa em ilhas oceânicas são largamente discutidos e investigados na literatura, como os diversos casos nas ilhas Havaianas (Lobélias, GIVNISH; MONTGOMERY; GOLDSTEIN, 2004; GIVNISH et al., 2009; *Plantago* L., DUNBAR-CO; WIECZOREK; MORDEN, 2008; *Bidens* L., KNOPE et al., 2012, 2020; aliança Silverword, LANDIS; FREYMAN; BALDWIN, 2018), na Oceania (*Pachycladon* Hook.f., HEENAN; MITCHELL, 2003; *Diospyros* L., TURNER et al., 2013; PAUN et al., 2016; *Geissois* Labill., PILLON et al., 2014; *Nicotiana* L., DODSWORTH et al., 2020) e entre outros. Mas também é possível encontrar casos de radiação adaptativa em ambientes ILS, como nos Andes (*Lupinus* L., HUGHES; EASTWOOD, 2009, HUGHES; ATCHISON, 2015), no Mediterrâneo (*Cistus* L., GUZMAN; LLEDO; VARGAS, 2009), ou ilhas edáficas pelos continentes (Bromeliaceae, GIVNISH et al., 2011, 2014; *Pentameris* P.Beauv. e *Rytidosperma* Steud., LINDER; BOUCHENAK-KHELADI, 2017).

3 RESULTADOS

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Island-like radiation of *Ameroglossum* (Linderniaceae) triggered by rocky outcrop environments in the Northeast of Brazil

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ABSTRACT

Ameroglossum is endemic to the Northeast of Brazil and ecologically restricted to rocky inselberg habitats that function as island-like systems (ILS) with spatial fragmentation, limited area, environmental heterogeneity, temporal isolation and low connectivity. Here we use a phylogenetic perspective to test the hypothesis that diversification in *Ameroglossum* is the result of evolutionary trends in *inselbergs*. Our results support the monophyly of *Ameroglossum*, perhaps with the inclusion of the related genera *Catimbaua* and *Isabelcristinia*, a group well-supported as successive sisters to *Cubitanthus* and *Stemodiopsis*, respectively. We identified an increase in the diversification rate in *Ameroglossum* clade (~19 million years ago) which might be associated with an ancient shift to rocky outcrop habitats. This change is correlated with the colonization of South America by long distance dispersal, given the ancestral distribution of Linderniaceae in Africa/Asia. We also discuss micro- and macroevolutionary trends in this ILS lineage, suggesting that in more general terms such rocky outcrops environments are important environments for plant diversification.

Keywords: *Cubitanthus*, *inselbergs*, long-distance dispersal, South American biogeography, *Stemodiopsis*.

1. Introduction

Island biogeographic studies focus on processes that drive lineage diversification in isolated geographic areas across space and time, and island systems have typically been used as models for such biogeographic, ecological and evolutionary studies (MacArthur & Wilson, 1967). However, a biological definition for these is complex because spatial fragmentation, limited area, temporal isolation and low connectivity are also features of other environments such as mountains, lakes, caves and rocky outcrops such as inselbergs (Wanderley et al., 2018; Itescu, 2019; Mendez-Castro et al., 2021). In these environments, known as island-like systems (ILS), geographic isolation plays an important role in constraining gene flow and creating opportunities for both genetic and phenotypic divergence among populations mainly through genetic drift and, consequently, speciation (Givnish et al., 2014; Hughes and Atchison, 2015; Dodsworth et al., 2020; Mendez-Castro et al., 2021).

Although genetic drift plays an important role in ILS, emergence of new lineages may be promoted by natural selection, acting rapidly on new phenotypic traits (Lamichhaney et al., 2017). In fact, adaptive radiations are abundant in island systems and provide important insights into how macroevolutionary processes operate (Givnish & Systma, 1997). Adaptive radiations in ILSs are particularly valuable as natural experiments, in which island clades serve as evolutionary replicates for the study of outcomes through ecological adaptation and morphological differentiation (Givnish et al., 2009; Baldwin & Wagner, 2010; Knope et al., 2012, 2020; Turner et al., 2013). In this regard, groups that have undergone adaptive radiation usually show a high rate of morphological differentiation (with or without obvious phylogenetic divergence), which makes interpretation of their phylogenetics and evolution challenging (Glor, 2010; Knope et al., 2020). The roles of genetic, geographic and environmental isolation, alone and variously combined, in promoting divergence of ILS plant clades have often been recognized (Hughes & Eastwood, 2006; Givnish et al., 2009; Gaudeul et al., 2012; Turner et al., 2013; Wanderley et al., 2018), but few studies have explicitly attempted to discuss their taxonomic impact.

In this sense, endemic ILS species can be excellent models to test whether this environment provides evolutionary trends similar to those described for insular clades. *Ameroglossum* Eb.Fisch., S.Vogel & A.V.Lopes comprises nine perennial chamaephytes, endemic to rocky outcrops in Northeast Brazil (Fischer et al., 1999; Almeida et al., 2016,

2021). *Ameroglossum* species occur in ecologically heterogeneous inselbergs varying in elevation (500–1200 m) and neighboring areas (Atlantic Forest or Caatinga). The genus exhibits phenotypical and genetic variation within and among populations, which are associated with geographic distance and environmental traits in rocky outcrops environments (Wanderley et al., 2018). The taxonomy of *Ameroglossum* is complex and may include *Catimbaua* L.P.Felix, Christenh. & E.M.Almeida and *Isabelcristinia* L.P.Felix, Christenh. & E.M.Almeida (Almeida et al., 2016; 2019; 2021). Besides minimal morphological differentiation, *Ameroglossum*, *Catimbaua* and *Isabelcristinia* exhibit a consistent chromosome number of $2n = 60$, except for *Ameroglossum genaroanum* E.M.Almeida, J.M.P.Cordeiro & L.P.Felix with $2n = 64$ (Almeida et al., 2019; Santos et al., 2021). All species have similar small chromosomes (~ 1.5 μm) and number/distribution of ribosomal DNA (rDNA) sites, although additional variable, small heterochromatic bands were identified in number/position (Santos et al., 2021).

The familial position of *Ameroglossum* is still unresolved. The genus was initially included in Scrophulariaceae (Fisher et al., 1999) and later in Linderniaceae based on morphological data (Souza & Lorenzi, 2012; Almeida et al., 2021). Christenhusz et al. (2017) and Almeida et al. (2019) corroborated the latter placement based on the stamen position and morphology and seeds with grooved testas. Linderniaceae comprise about 20 genera and 260 species with a wide global distribution and high diversity in Africa (~ 89 spp.) and Southeast Asia (~ 84 spp.; Christenhusz et al., 2017). African species occur in specialized environments such as seasonal pools, rocky outcrops and heavy metal soils, whereas Asian species occur mainly in rainforests (Lewis, 2000; Rahmanzadeh et al., 2005). Given the paucity of South American species (~37 spp.) with only a few strictly Neotropical genera, the biogeographic events that explain the presence of *Ameroglossum* in the Brazilian Northeast are still unknown. In general, the geographic distribution of the genera seems to be concentrated in certain types of environments, and in addition to *Ameroglossum*, other inselberg-dwelling genera include *Cubitanthus* Barringer, *Stemodiopsis* Engl., *Chamaegigas* Dinter ex Heil, *Craterostigma* Hochst., *Crepidorhopalon* Eb.Fisch., and *Linderniella* Eb.Fisch., Schäferh. & Kai Müll. (Fischer, 1997, 2004; Balkwill, 2021).

Although the characteristic features of adaptive radiation can be controversial (Soulebeul et al., 2015), three key features have been proposed to easily distinguish it from other evolutionary events in a phylogenetic context (Glor, 2010): 1) multiplication of species from a common ancestor; 2) adaptation via natural selection; and 3)

extraordinary species diversification. We presented here the first molecular phylogenetic hypothesis for *Ameroglossum* and used it to address these key features to test the hypothesis that the diversification of this genus follows an island-like adaptive radiation contributing to its taxonomic uncertainty. In light of the phylogenetic tree, we interpret the taxonomy (Almeida et al., 2019; 2021) and biogeographic history of the *Ameroglossum* clade. We specifically addressed three topics: (1) phylogenetic relationships of *Ameroglossum* and related genera among Linderniaceae; (2) evidence of adaptive radiation associated with the occurrence of the *Ameroglossum* species in inselbergs; and (3) the biogeographic events explaining the presence of *Ameroglossum* in the Brazilian Northeast.

2. Material and Methods

2.1. Taxon sampling and DNA extraction

All nine species of *Ameroglossum* plus the single species each of *Catimbaua*, *Cubitanthus* and *Isabelcristinia* were analyzed in this study (Table 1). For some *Ameroglossum* species (Fig. 1), we analyzed samples from more than one population if available, for those with only one known population, we analyzed different individuals, and we also included one potentially new species of *Ameroglossum*. Voucher specimens were deposited at EAN and CCA-UFPB. Genomic DNA was extracted from 50–70 mg of silica-gel-dried leaves following the method described by Doyle & Doyle (1987) with the modifications in Ferreira & Grattapaglia (1995).

2.2. PCR amplification and sequencing

One nuclear, ribosomal ITS and two plastid loci, the *matK* exon and *rps16-trnQ* spacer, were amplified for all species using the primers in Sun et al. (1994), Sang et al. (1997) and Steele et al. (2010), respectively. The plastid amplifications were performed with a volume of 50 µL containing: 100 ng of gDNA, 1× PCR buffer, 1× TBT, 0.2 mM dNTP, 3 mM MgCl, 0.1 µM of each primer, 0.2µL of a homemade Taq polymerase, and water to the final volume. For nrITS, we used GoTaq® DNA Polymerase (Promega) kit according to the manufacturer's instructions, with final volume of 50 µL containing: 100 ng of genomic DNA, 10 µL Rapid Ligation buffer, 0.2 mM dNTP, 0.1 µM of each primer, 0.3 µL DNA polymerase, and water to the final volume.

Thermal cycling for *matK* included: 1 cycle of 4 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 50°C, and 2:30 min at 72°C, and a final extension of 8 min at 65°C; for the *rps16-trnQ* intron: one cycle of 1.5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min 72°C, and a final extension of 15 min at 72°C; and for nrITS: 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 at 60°C, and 1 min 72°C, and a final extension of 10 min at 72°C. Successfully amplified products were cleaned using isopropanol 75% and purified by precipitation.

Table 1. List of accessions and species included in the *Ameroglossum* clade in this study.

Species (author)	Provenance	Abrev.	Voucher
Ameroglossum alatum E.M.Almeida, A.M.Wanderley & L.P.Felix	Maravilha, Alagoas	SM9 + SM12 + 2.2	EMA 461
A. asperifolium E.M.Almeida, J.M.P.Cordeiro & L.P.Felix	Ibateguara, Alagoas	17982 a/b/c	LPF 17982
A. bicolor E.M.Almeida, A.M.Wanderley & L.P.Felix	Agrestina, Pernambuco	LB	LPF 17160
	Bonito, Pernambuco	ABO + ABN	LPF18664
	Camocim de São Felix, Pernambuco	CSF	LPF 18729
A. fulniorum E.M.Almeida, A.M.Wanderley & L.P.Felix	Águas Belas, Pernambuco	ABQ + AB	EMA 439
A. genaroanum E.M.Almeida, J.M.P.Cordeiro & L.P.Felix	Tacima, Paraiba	TAC	EMA 397
	Serrinha, Rio Grande do Norte	RN	Pitrez 365
	Serra da Raiz, Pernambuco	SR	LPF 17486
A. intermedium E.M.Almeida, A.M.Wanderley & L.P.Felix	Quebrangulo, Alagoas	QA	EMA 461
	Quebrangulo, Alagoas	18000	LPF 18000
	Lagoa do Ouro, Pernambuco	SPE	-
A. manoelfelixii L.P.Felix & E.M.Almeida	Esperança, Paraiba	ESP	EMA 785
	Serraria, Paraiba	SER	EMA 1598
	Areia, Paraiba	A	EMA 385
	Algodão de Jandaíra, Paraiba	AJ	EMA 799
	Alagoa Nova, Paraiba	AN	-

A. aff *manoelfelixii*	Pedra do Guariba, Caruaru, Pernambuco	PG	-
	Serra Negra, Pernambuco	SN + SN5	EMA 3428
A. pernambucense Eb.Fisch., S.Vogel & A.V.Lopes	Brejo da Madre de Deus, Pernambuco	P	LPF 13150
	São Caetano, Pernambuco	CA	LPF 15687
	Taquaritinga do Norte, Pernambuco	TN	-
	Pedra do caboclo, Belo Jardim, Pernambuco	PC	EMA 1695
A. xukuruorum E.M.Almeida, Christenh. & L.P.Felix	Pesqueira, Pernambuco	REI	LPF 15647
	São João do Tigre, Pernambuco	STJI	Nasc. 168
Ameroglossum sp.	Itabaiana, Sergipe	ASE	LPF 19333
Catimbaua pendula L.P.Felix & E.M.Almeida	Buíque, Pernambuco	1569	EMA 1569
	Buíque, Pernambuco	CPE	LPF 15673
Cubitanthus alatus (Cham &Schltdl) Barringer	Ubaitaba, Bahia	2614	EMA 2614
Isabelcristinia aromatica L.P.Felix & E.M.Almeida	Boa Vista, Belo Jardim, Pernambuco	BV	EMA 2794
	Jataúba, Pernambuco	JAT	EMA 1672

2.3. Phylogenetic analysis

To examine the taxonomic position of *Ameroglossum*, a broad sampling of sequences for species of Linderniaceae was obtained from GenBank (Table S1), in addition to an outgroup, *Nicotiana paniculata* L. (Solanaceae). Representatives of 22 of the 24 families that make up Lamiales (APG IV, 2016) were also included from GenBank (Table S1). For the interpretation of evolutionary relationships, the accessions of *Ameroglossum* were analyzed in three matrices: (i) families of Lamiales (ITS, *matK* and *rps16-trnQ*); (ii) all accessions available for Linderniaceae (*matK* and *rps16-trnQ*); and (iii) the *Ameroglossum* clade: *Ameroglossum*, *Catimbaua*, *Cubitanthus* and *Isabelcristinia* (ITS, *matK* and *rps16-trnQ*). *Nicotiana paniculata* was used as outgroup in the matrices i and ii, and *Cubitanthus* was specified as outgroup in matrix iii. To evaluate the position of Linderniaceae in Lamiales (i) and the position of *Ameroglossum* and related genera relative to Linderniaceae (ii) only a Bayesian inference (BI) was

performed. For matrix *Ameroglossum* (iii), maximum likelihood (ML) and BI analyses were performed with each marker separately and combined (Table 2).

Sequences were manually edited and aligned with MAFFT (Katoh & Standley, 2013) in Geneious v. 7.1.4 (http://www.geneious.com, Kearse et al., 2012). All sequences were submitted to GenBank (Table S1). The most appropriate model of sequence evolution for each region was determined using the Akaike information criterion (AIC) as implemented in the program jModelTest v.2.1.6 (Darriba et al., 2012), and the GTR+G model was selected as the most appropriate for all loci. Phylogenetic relationships were inferred using MrBayes v.3.2.7a (Ronquist et al., 2012) in CIPRES Science Gateway (Miller et al., 2010), and in BEAST v. 1.10.4 (Drummond & Rambaut 2007). All analyses were performed for each region separately and combined. Four independent runs with four Markov Chain Monte Carlo (MCMC) runs were conducted, sampling every 1,000 generations for 100.000.000 generations. Each run of BEAST was evaluated in TRACER v.1.6 (Rambaut et al., 2014) to determine a burn-in of 25%. The posterior probability (PP) tree was edited in FigTree v.1.4.2. (Rambaut, 2014). The ML analysis was performed using RAxML-HPC2 (8.2.12) in CIPRES Science Gateway, using the bootstrap algorithm with 1,000 replicates to assess support. Bootstrap percentages (BS) of 95–100% are described as highly supported. Posterior probabilities (PP) are described as high support (>0.95).

Table 2. Statistics of the alignments. For each marker, number of taxa analyzed, the number of aligned characters (bp) and percentage of variable characters are presented.

	ITS	*matK*	*rps16-trnQ*	All data combined
Matrix (i) - Lamiales				
No. of taxa	64	84	83	89
Aligned length (pb)	930	821	1275	3026
Variable characters	563 (60,5%)	530 (64,6%)	624 (48,9%)	1717 (56,7%)
Missing data (gaps)	93 (10%)	38 (4,6%)	288 (22,6)	419 (13,8)
Potentially informative sites	413 (44,4%)	339 (41,2%)	339 (26,5%)	1091 (36%)
Matrix (ii) - Linderniaceae				
No. of taxa	-	100	81	104
Aligned length (pb)	-	1946	943	3101

Variable characters	-	625 (32.1%)	338 (35.8%)	1072 (34.6%)
Missing data (gaps)	-	171 (8.8%)	80 (8.5%)	677 (21.8%)
Potentially informative sites	-	357 (18.3%)	203 (21.5%)	593 (19.1%)
matrix (iii) – *Ameroglossum*, *Catimbaua*, *Cubitanthus* and *Isabelcristinia*				
No. of taxa	38	37	37	38
Aligned length (pb)	724	782	873	2379
Variable characters	74 (10,2%)	22 (2,8%)	80 (9,2%)	176 (7,4%)
Missing data (Gaps)	5 (0,7%)	8 (1%)	54 (6,1%)	67 (2,8)
Potentially informative sites	35 (4,8%)	10 (1,3%)	13 (1,5%)	58 (2,4%)

2.4. Divergence time estimates

For divergence time estimates, we used BEAST v.1.10.4 (Drummond & Rambaut, 2007) with matrix (ii), consisting of one accession per species, that of the population of the holotype. An uncorrelated relaxed lognormal clock (Drummond & Rambaut, 2007) and a birth-death speciation model (Gernhard, 2008) were applied. Two independent runs of 100,000,000 generations were performed, sampling every 10,000 generations. After removing 25% of samples as burn-in, the independent runs were combined and a maximum clade credibility (MCC) tree was constructed using TreeAnnotator v.1.8.2 (Rambaut & Drummond, 2015). To verify the effective sampling of all parameters and assess convergence of independent chains, we examined their posterior distributions in TRACER. The MCMC sampling was considered sufficient at effective sampling sizes (ESS) equal to or higher than 200.

Because no fossils are available for Linderniaceae, we used a secondary calibration based on the fossil-calibrated asterid tree of Zhang et al. (2020), who used transcriptomic and genomic data to estimate the divergence of Linderniaceae and Scrophulariaceae at 77 million years ago (ma).

2.5. Ancestral range reconstruction

To investigate the historical biogeography of Linderniaceae, we employed a model-based likelihood approach implemented in the BioGeoBEARS (Matzke, 2013,

2014) using RASP 4 (Yu et al., 2020). The dated consensus tree from matrix (ii) with BEAST was used as the input tree for the biogeographic analysis. The Akaike weight index (AICc_wt) was used to select the best model among the six available BioGeoBEARS models. BAYAREALIKE+J achieved the best AIC index, which does not permit vicariance events (Landis et al., 2013) because the areas in which these species occur were already geographically separated at their time of origin. This model was also tested with the addition of the free parameter *j*, which treats jump dispersal as a cladogenetic event and has been shown to improve model likelihood (Matzke, 2014). We compared the results of models with and without parameter *j* using likelihood ratio tests and the AIC model weights.

Six biogeographic regions were defined for ancestral range analyses: A, North America + Central America + South America; B, Northeast of Brazil; C, Africa + Middle East; D, Madagascar; E, Asia; and F, Oceania. The areas were coded according to the species distribution in the *Global Biodiversity Information Facility* (gbif.org) and *Plants of the World Online* (powo.science.kew.org) (Table S2).

2.6. Phylogenetic comparative methods

We first evaluated whether *Ameroglossum* species have characteristics adaptive for specific ecological niches by assessing if floral tube length was correlated by bill size of the main pollinators for each species. For this, we used morphological data from Wanderley et al. (2018) and updated it to reflect the new species for the analyzed populations (*sensu* Almeida et al., 2021; Table S3). To account for phylogenetic relationships, we did a regression analysis using phylogenetic independent contrasts (PICs) for both traits using the R package *phytools* (Revell, 2012). Since we did not have morphological and pollinator data for all *Ameroglossum* species analyzed here, we used the *drop.tip* function, also implemented in *phytools*, to prune the tree to contain only the eight species for which we had data.

Secondly, we tested whether *Ameroglossum* exhibited shifts in species diversification using a speciation/extinction model in BAMM (Rabosky et al., 2014). Priors for the BAMM control file were generated using the dated Linderniaceae tree input into the function set BAMM priors in the package BAMMtools v. 2.5.0 implemented in R. The control file was set for 10,000,000 generations, and the analysis was run twice as recommended, returning similar results. Resulting MCMC log likelihoods were tested

against generation number using the CODA package (Plummer et al., 2006) implemented in R. All remaining outputs contained in the event file were analyzed using BAMMtools. BAMMtools was then used to generate a figure showing the best set of rate shift configurations as well as two graphics of diversification through time, one for just the *Ameroglossum* clade and another comprising all remaining Linderniaceae species. We also constructed a mean phylorate, in which each unique color section of a branch represents the mean of the marginal posterior density of speciation rates on a localized segment of a phylogenetic tree. We also collected data on which species of the family occur on inselbergs.

3. Results

3.1. Relationships of the *Ameroglossum* clade

In the analysis based on combined data (matrix ii), the *Ameroglossum* clade was well-supported as a member of Linderniaceae (PP 1.00). *Ameroglossum* (perhaps including *Catimbaua* and *Isabelcristinia*) was sister to *Cubitanthus* (PP 0.98), and this clade was sister to *Stemodiopsis* (PP 1.00; Fig. 2). The *Ameroglossum* clade + *Stemodiopsis* was sister to the rest of the Linderniaceae (PP 1.00; Fig. 2). The clade of Linderniaceae + Scrophulariaceae + Byblidaceae + Stilbaceae was sister to a clade (PP 1.00) comprised of well supported families: Pedaliaceae, Lentibulariaceae, Acanthaceae, Martyniaceae, Bignoniaceae, Verbenaceae, Orobanchaceae, Paulowniaceae, Lamiaceae, Phrymaceae and Schlegeliaceae, which are of unclear inter-relationships (Fig. 2).

3.2. Phylogenetic relationships in *Ameroglossum*

Individual analyses of ITS, *matK* and *rps16-trnQ* in matrix (iii) each resulted in poorly resolved topologies (Fig. S1 – S6), although nrITS presented the highest supports (Fig. S1). The combined matrix produced a strongly supported *Ameroglossum* clade (PP 1.00) including *Isabelcristinia* and *Catimbaua* with *Cubitanthus* as their sister (Fig. 3, Fig. S7). The ML and BI trees had similar topologies/support, and we show/discuss only that produced by BI (Fig. 3).

Ameroglossum comprised two clades (Fig. 3), A (PP 0.90) only moderately supported and including *A. pernambucense*, *A. xukuruorum*, *Isabelcristinia* and *Catimbaua* and B (PP 1.0) well supported and comprising the rest of the *Ameroglossum*

species, including a morphotype identified as *Ameroglossum*_sp_ASE. Support for the internal relationships of the second clade is variable, but mostly low. There is some evidence of incongruence around the position of *Catimbaua* and *Isabelcristinia*; in the nrITS tree, support was higher (PP 0.91 and 1.00, respectively) than in the combined tree (PP 0.90 for each), even though in the plastid results individually there is only weak support for these in alternative positions. From these results, it seems that we cannot exclude the possibility that *Catimbaua* and *Isabelcristinia* have some other relationships and are not embedded in *Ameroglossum*. However, that they are members of the *Ameroglossum* clade with their sister *Cubitanthus* seems clear. Their exact relationships do not need to be clear to support our conclusions in the other analyses we conduct in this study.

3.4. Divergence time and biogeography of *Ameroglossum*

Linderniaceae and most internal nodes were well supported (Fig. S8). According to our results, the crown node of Linderniaceae is 75 ma old (Fig 4; Fig. S9), with Africa as the most probable ancestral area (58.96% HPD), followed by Asia (12.40% HPD; Fig. 4, Fig. S10). The relationship between the African genus *Stemodiopsis* and the *Ameroglossum* clade indicates that the latter arrived in the Northeast of Brazil by long-distance dispersal 27–19 ma from Africa. (70.18% HPD; Fig. 4, Fig. S10, S11, nodes 119 and 116).

The remainder of Linderniaceae form two clades that diverged about 48.7 ma, with Africa alone having the highest probability as the ancestral range (55.2% HPD; Fig. S13 and S10, node 205). However, the common ancestor of the clade that includes *Artanema* D.Don, *Bonnaya* Link & Otto, *Chamaegigas*, *Craterostigma*, *Linderniella*, *Picria* Lour., *Torenia* L., and *Yamazakia* W.R.Barker, Y.S.Liang & Wannan was well-supported to originate in Asia around 38 ma (96.4% HPD, node 180), which suggests that there was a dispersal from Asia back to Africa around 20 ma (98.2%, HPD node 138) for the clade of *Craterostigma*, *Chamaegigas* and *Linderniella*, which are predominantly African. The clade comprising *Crepidorhopalon*, *Hemiarrhena* Benth., *Lindernia* and *Micranthemum* Michx., diverged around 37 ma in Africa (64.3% HPD, node 204). *Crepidorhopalon* is predominantly African. However, the other genera dispersed to America, Asia and Oceania from around 28 ma onwards. *Micranthemum* is endemic to the Americas. *Lindernia* has most species in Australia, but it is the only genus with species

on all continents, including Europe. With species in Africa, Asia and Oceania, *Vandellia* L. is polyphyletic in Linderniaceae (Fig. S8).

3.5. Adaptive radiation in *Ameroglossum*

We investigated whether *Ameroglossum* has the key features of an island-like adaptive radiation. We used linear regression of PICs to test the relationship between flower tube length and pollinator bill-length. The overall regression was statically significant ($R^2 = 0.88$, $F = 44.35$, $DF = 5$, $p < 0.01$), showing that pollinator bill length could accurately predict flower tube length ($\beta = 0.697$; Fig. 5), suggesting that *Ameroglossum* species on different inselbergs have evolved to accommodate characteristics of local pollinators.

To consider if the *Ameroglossum* clade could have undergone an extraordinary diversification, we employed a diversification rate shift analysis. The 95% credible set of rate shift configurations yielded by BAMM included five possible shift configurations, with the most probable scenario ($f = 0.41$) showing a steady diversification rate throughout the whole tree without a distinctive shift. It was, however, possible to identify a distinctive increase in diversification in the last 19 ma originating at the ancestral node of the *Ameroglossum* clade after it split from *Cubitanthus* (Fig. 6).

4. Discussion

4.1. *Ameroglossum* and the families of Lamiales

Our results clearly confirm the position of *Ameroglossum* in Linderniaceae, which is significant because no representatives of this genus had been included in previous phylogenetic studies. Various morphological traits have been used to justify this placement, such as geniculate filaments (curved in bud but elongate when the corolla expands) and furrowed seeds (Almeida et al., 2019). These characters are also shared with other genera of Linderniaceae and were identified as a potential familial synapomorphies (Rahmanzadeh et al., 2005). *Cubitanthus*, which is sister here to the rest of the *Ameroglossum* clade, was previously identified as sister of *Stemodiopsis* (Perret et al., 2013; Christenhusz et al., 2017; Biffin et al., 2018), and together they were sister to the rest of Linderniaceae.

Since their segregation from Scrophulariaceae (Rahmanzadeh et al., 2005), Linderniaceae have been ambiguously placed among the many families of Lamiales. Most studies point to Byblidaceae as the closest family and Scrophulariaceae as sister to these, but these results were either not well resolved or highly supported (Schäferhoff et al., 2010; Refulio-Rodriguez & Olmstead, 2014; Christenhusz et al., 2017; Li et al., 2021). Similarly, our results do not offer a clear picture of relationships in Lamiales. Recently, in a large phylogenomic analysis (transcriptome sequences) of 365 species in 102 asterid families, Zhang et al. (2020) demonstrated that Linderniaceae and Scrophulariaceae formed a moderately supported clade, but due to the low sampling (four and eight species, respectively), the authors did not rule out a relationship of this clade to Byblidaceae.

4.2. Molecular systematics of *Ameroglossum*

In its current circumscription (Almeida et al., 2021), *Ameroglossum* is potentially not monophyletic, although the embedded positions of *Catimbaua* and *Isabelcristinia* are not strongly supported. There is some evidence of incongruence between the nuclear ribosomal and plastid trees, so until the cause(s) of this are better understood, taxonomic changes are premature. When described, *Catimbaua* and *Isabelcristinia* were clearly members of Linderniaceae because of their geniculate filaments, the principal familial synapomorphy (Rahmanzadeh et al., 2005; Almeida et al., 2019). Their separate generic status was supported by distinctive morphologically and ecologically exclusive characters (Almeida et al., 2019). Our molecular results do not provide a clear picture of their phylogenetic relationships, but if they are embedded in *Ameroglossum* this suggests a rapid rate of morphological differentiation in *Catimbaua* and *Isabelcristinia* that might be associated with ecological specializations different from those of most species of *Ameroglossum*.

Although evolutionary principles predict a relationship between rates of species diversification and morphological divergence (Omland 1997; Adams et al., 2009), these hypotheses were focused on groups with abundant morphological change, which undoubtedly was underlaid by extensive genetic change. In contrast, with the finding that many changes in DNA sequences are neutral, Zuckerkandl & Pauling (1962, 1965) proposed that molecular and morphological evolution will be uncoupled, and many studies have shown that rates of speciation and morphological evolution are not always significantly correlated, such that rapid diversification can occur with little morphological

change and vice-versa (Omland 1997; Adams, 2009; Asar et al., 2022; Chase et al., 2021). Determining the link between phylogenetic and phenotypic evolution is a fundamental goal to propose classifications that reflect natural processes. However, it can be challenging in groups with heterogeneous morphological evolutionary rates, as observed here in the *Ameroglossum* clade. Assessing this linkage is also complicated by human perception of morphological change, which focuses on some features and completely ignores others. Of course, it is always possible that chemical or micromorphological features are the focus of evolutionary change in *Ameroglossum*, not the gross morphology upon which taxonomists generally focus their attention.

The split of *Ameroglossum* in two clades corroborates this scenario of heterogeneity in morphological diversification. In one clade there is greater morphological diversity since it includes species with atypical morphology, *Catimbaua* and *Isabelcristinia* with their features divergent from those of the species of *Ameroglossum* (Almeida et al., 2019). In contrast, the other clade with slightly more species presented a lower resolution and a greater morphological homogeneity (Almeida et al., 2021). Included in the latter is the new species, *Ameroglossum*.sp.ASE, which differs from the rest in its pendent habit and toothed leaves, making it impossible to confuse with other species of *Ameroglossum*. The three markers sequenced in this study putatively did not provide enough variation to discriminate relationships in the larger clade, e.g. between the accessions of *A. genaroanum* + *A. manoelfelixii* and *A. asperifolium* + *A. bicolor*. The combined tree does not support their distinct species status, but it also clearly does not refute these concepts (they are unresolved and poorly supported due to the low level of variation detected). This explanation rather than incomplete divergence, gene flow or inappropriate taxonomy is supported by the clear morphological (Almeida et al., 2021) and cytogenetic (Santos et al., 2021) differentiation of these taxa. Although the cytogenetic markers (CMA/DAPI banding and 5S/35S rDNA) characterized by Santos et al. (2021) are species specific if compared to the most similar species morphologically, they are overall homoplastic when plotted on the phylogenetic tree (Fig. S12). Wanderley et al. (2018) studied populations of two of these species (one from each of the two main clades) using six nuclear microsatellites and detected populations of both clades characterized by the same cluster (K), which might be indicative of contemporary gene flow or incomplete lineage sorting (retention of ancestral polymorphisms).

The remarkable morphological differentiation of the clade with *Catimbaua* and *Isabelcristinia* focuses on characters markedly different from the rest *Ameroglossum* (Almeida et al., 2019, 2021), and these are in general terms more similar to those of *Cubitanthus* and *Stemodiopsis* (Barringer, 1984; Fisher, 1997; Balkwill, 2020), e.g. pendent habits (*Catimbaua* and *Cubitanthus*) and an inflated corolla with a white outer tube, curved upwards and tinged with purple, and a trilobed lower lip (Barringer, 1984; Fisher, 1997; Almeida et al., 2019; 2021; Balkwill, 2020). If *Catimbaua* and *Isabelcristinia* are indeed embedded in *Ameroglossum*, then these features represent homoplasy (reversals) in these morphological traits, but until a clearer phylogenetic relationship emerges, this speculation is unwarranted. If *Ameroglossum* can be shown to exclude *Catimbaua* and *Isabelcristinia*, then these potentially homoplastic features would represent symplesiomorphies.

4.3. Occurrence of *Ameroglossum* in the Northeast of Brazil due to long-distance dispersal

We present here the first biogeographic hypothesis of Linderniaceae and their presence in the Americas. Corroborating previous analyses (Fischer et al., 2013; Biffin et al., 2018), we identified in Linderniaceae three main geographically well-structured clades. The genera missing from our analyses are unlikely to change the picture we obtained because they are morphologically similar to those we did include and fit into the general biogeographical patters we obtained. Clade A (*sensu* Biffin et al., 2018) is exclusively Brazilian (the *Ameroglossum* clade) and African (*Stemodiopsis*), making it the clade with the most restricted distribution in the family. They are clearly ecologically specialized for rocky outcrops (Fischer, 1997; Almeida et al., 2019; 2021; Balkwill, 2020). The divergence times found here suggest arrival of the *Ameroglossum* clade in the Northeast of Brazil by long-distance dispersal 27–19 ma (Miocene) from Africa, with a clear increase in the diversification rate of *Ameroglossum* following its split from *Cubitanthus*. This South American + African disjunction is reported for other plant groups (Givnish et al., 2004; Dick et al., 2007; Drummond, 2008; Cron et al., 2012; Christenhusz & Chase, 2013), although such dispersals are difficult to explain in plants without apparent dispersal mechanisms (e.g., thorns, fibers, floating fruits etc.), as is the case for *Ameroglossum*, which has small seeds dispersed by gravity (Wanderley et al., 2020). It has been proposed that such small seeds can become attached to the legs of

migratory non-sea birds, providing dispersal over long distances (Mummenhoff & Franzke, 2007; Givnish et al., 2009), but few birds are known to regularly make flights between these continents. Long-distance dispersal is a frequent phenomenon in other Lamiales (Roalson et al., 2008; Drew & Sytsma, 2012; Nylinder et al., 2012; Tripp & McDade, 2014; Roy & Lindqvist, 2015) and seems to have played an important role in the biogeography and evolutionary success of Linderniaceae.

4.4. Diversification in *Ameroglossum* reflects island-like evolutionary trends

Recent divergence (5 ma), small microendemic populations and geographic isolation on inselbergs makes *Ameroglossum* an excellent model for investigating adaptive radiation in ILSs (Almeida et al., 2021; Wanderley et al., 2018). Inselbergs are known to be centers of diversity for desiccation-tolerant vascular plants (Porembski, 2007), and such ecologically heterogeneous environments function as "insular" systems that have previously been demonstrated to serve as a stage for major radiations (Givnish et al., 2009; Drummond et al., 1012; Knope et al., 2020; Dodsworth et al., 2020; Naciri & Linder, 2020). Here, we assessed if the *Ameroglossum* clade exhibited the three key characteristics of adaptive radiation (listed in the Introduction). Our BAMM analysis shows that the *Ameroglossum* clade exhibited a steady increase in rate in comparison to other Linderniaceae. Small, isolated populations, subject to high rates of inbreeding, can quickly evolve through genetic drift with fixation of divergent morphologies, adaptive or not (Naciri & Linder, 2020). Each species of *Ameroglossum* comprises small, fragmented populations in different inselbergs, and for two species only the type population is known (Almeida et al., 2021). It has also been shown that there is limited pollen flow between nearby populations of *A. manoelfelixii* (Wanderley et al., 2020). Limited gene flow between populations can promote genetic differentiation and isolation, culminating in speciation (Naciri & Linder, 2020). *Ameroglossum* species have gravity-dispersed seeds (Wanderley et al., 2014; 2020), which also not promote long-distance gene flow. Development of self-compatibility may have prevented extinction of small populations and a corresponding decline of pollinators if these depended solely on these plants (Naciri & Linder, 2020), which does not appear to be the case for hummingbirds with *Ameroglossum*.

Although genetic drift in ISLs is a common driver of speciation, geographic isolation and diversity of ecological opportunities in these systems can also favor

evolution by natural selection (Barton, 1996). In this context, Wanderley et al. (2018) detected a role not only for geography (i.e., isolation by distance) but also environmentally correlated traits (especially pollination, temperature and precipitation), which may promote morphological and genetic divergence in species of the *Ameroglossum* clade. They observed that some populations, especially species of the *A. pernambucense* clade, exhibited 1.5 times greater isolation by environment than distance. Revisiting the pollination data of Wanderley et al. (2018) from the perspective of our new phylogenetic tree, we recovered a clear phylogenetic correlation between floral tube length and pollinator bill size, which suggests that morphological variation in *Ameroglossum*, although perhaps arising randomly by genetic drift, may nonetheless have developed adaptive value. In clear contrast, putatively punctuated change (if an embedded position turns out to be demonstrated) from ornithophily to melittophily occurred in *Isabelcristinia*.

We recovered a higher rate of diversification in the *Ameroglossum* clade than in other Linderniaceae genera, but *Ameroglossum* was the only genus with all of its species analyzed. In addition, both population genetic (Wanderley et al., 2018) and phylogenetic methods demonstrate a role for natural selection in the genetic and morphological divergence of *Ameroglossum* populations. Thus, we suggest that adaptive radiation in the Northeast of Brazil may have been associated with ecological specialization to both pollinators (e.g., hummingbirds and bees) and climate, leading to differences in floral and vegetative morphology among these species (Fig. 7). Studies of adaptation in populations of *Ameroglossum* thus suggest that the hummingbird syndrome permits local floral adaptation without affecting vegetative adaptation, even when populations occur in heterogeneous landscapes (Wanderley et al., 2016). Among the main pollinators of *Ameroglossum* are the hummingbirds *Chionomesa fimbriata* (Gmelin, 1788), *Eupetomena macroura* (Gmelin, 1788), *Phaethornis pretrei* (Lesson & Delattre, 1839) and *Chlorostilbon lucidus* (Shaw, 1812), but these have not been demonstrated in all populations of each species (Wanderley et al., 2014; 2016).

5. Conclusions

Our results showed a close relationship between the genera *Ameroglossum*, *Catimbaua* and *Isabelcristinia*, although it was not possible with these data to assess their

relationships with confidence. However, it is premature to integrate these two genera in *Ameroglossum* until a more definitive phylogenetic study of nuclear data can be completed. Internally, *Ameroglossum* is split in two clades, one with weak support and greater morphological diversity among its species, whereas the other is more strongly supported but has poor internal resolution among the species. Additionally, multiple accessions of some species are not uniquely clustered due putatively to the low levels of variation in the markers employed in our study. The small, isolated populations, endemic to rocky outcrops, with genetic structure associated with both geographical distance and environmental heterogeneity and constant rate increase in diversification suggests that *Ameroglossum* underwent an adaptive radiation subsequent to their arrival in northeastern South America. Their evolution appears to be associated with ecological specialization for pollinator relationships and climate traits that have led to the observed differences in floral and vegetative morphology. Furthermore, we demonstrated that the arrival of the *Ameroglossum* clade in South America was by long-distance dispersal from Africa, 27–19 ma, which was followed by a subsequent rate increase of diversification potentially linked to their preference for rocky outcrops. Additional genetic studies are required to resolve internal relationships among the species of *Ameroglossum* clade, but this study renders this Brazilian clade the best-explored of all Linderniaceae at both micro- and macroevolutionary scales.

CRediT authorship contribution statement

Amanda S. Santos: Conceptualization, investigation, data curation, methodology, software, formal analysis, writing – original draft, review & editing. **Erton M. Almeida:** methodology, review & editing. **Paulo Aecio:** methodology, review & editing. **Lucas Costa:** methodology, software, formal analysis, review & editing. **Artur Wanderley:** methodology, review. **Henrique Batalha-Filho:** review & editing. **Magdalena Vaio:** review & editing. **Mark W. Chase:** review & editing. **Maarten J. M. Christenhusz:** review & editing. **Leonardo P. Felix:** Conceptualization, funding acquisition, review & editing. **Gustavo Souza:** Conceptualization, funding acquisition, data curation, software, formal analysis, review & editing. **Marcelo Guerra:** Conceptualization, financing acquisition, review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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LEGENDS

Fig. 1. Map of the investigated populations of *Ameroglossum, Catimbaua*, *Cubitanthus* and *Isabelcristinia*. Abbreviations refer to populations (Table 1). Colors refer to the different genera: black – *Cubitanthus*, green – *Ameroglossum*, orange – *Isabelcristinia*, blue – *Catimbaua*.

Fig. 2. Bayesian inference (BI) consensus tree with the combination of nuclear (ITS) and plastid (*matK* and *rps16-trnQ*) markers of *Ameroglossum* and Lamiales families (i), with *Nicotiana paniculata* L. (Solanaceae) as outgroup.

Fig. 3. Reconstruction of the Bayesian Inference (BI) consensus tree with the combination of nuclear (ITS) and plastid (*matK* and *rps16-trnQ*) markers of the *Ameroglossum* clade (iii), with *Cubitanthus alatus* as an outgroup. The two branch colors highlight the main groups of the *Ameroglossum* clade: orange, clade A; green, clade B.

Fig. 4. Reconstruction of the ancestral distribution of Linderniaceae with a focus on *Ameroglossum*. Consensus tree reconstructed from Bayesian Inference (BI) with the combination of plastid markers (*matK* and *rps16-trnQ*). Divergence time based on secondary calibration based on the asterid tree of Zhang et al (2020). Pie charts on the nodes represent probabilities of potential ancestral ranges. Black, areas with less than 10% occurrence; Light blue, Asia + Oceania; Dark blue, Africa + Madagascar; Brown, America + Northeast Brazil.

Fig. 5. Scatterplot of phylogenetic independent contrasts (PICs) between *Ameroglossum* floral tubes and hummingbird bill lengths to illustrate the range from the minimum size in *A. xukuruorum* - *Chlorostilbon lucidus* to the maximum in *A. manoelfelixi* - *Phaethornis pretrei*.

Fig. 6. Mean phylorate of Linderniaceae (left) showing rate of diversification according to the legend on lower left. On the right, plots of diversification through time for *Ameroglossum* (upper right) and remaining Linderniaceae clades (lower right).

Fig. 7. Adaptive radiation scheme in the *Ameroglossum* clade (Linderniaceae). Phylogenetic relationships are based on the combined Bayesian inference tree. Illustrations from Fisher and Lopes (1999) and Almeida et al (2016; 2019; 2021).

Figure S1. Phylogenetic relationships of the *Ameroglossum* clade based on Bayesian inference of the nuclear ribosomal ITS dataset. With *Cubitanthus alatus* as outgroup.

Figure S2. Phylogenetic relationships of the *Ameroglossum* clade based on Bayesian inference of the plastid *matK* dataset. With *Cubitanthus alatus* as outgroup.

Figure S3. Phylogenetic relationships of the *Ameroglossum* clade based on Bayesian inference of the plastid *rps16-trnQ* dataset. With *Cubitanthus alatus* as outgroup.

Figure S4. Phylogenetic relationships of the *Ameroglossum* clade based on maximum likelihood of the nuclear ribosomal ITS dataset. With *Cubitanthus alatus* as outgroup.

Figure S5. Phylogenetic relationships of the *Ameroglossum* clade based on maximum likelihood of the plastid *matK* dataset. With *Cubitanthus alatus* as outgroup.

Figure S6. Phylogenetic relationships of the *Ameroglossum* clade based on maximum likelihood of the plastid *rps16-trnQ* dataset. With *Cubitanthus alatus* as outgroup.

Figure S7. Phylogenetic relationships of the *Ameroglossum* clade (iii) based on maximum likelihood with the combination of nuclear (ITS) and plastid (*matK* and *rps16-trnQ*) markers, with *Cubitanthus alatus* as an outgroup.

Figure S8. Phylogenetic relationships of Linderniaceae based on Bayesian inference of the combined plastid (*matK* and *rps16-trnQ*) dataset. This tree was for the dating analysis in Figure S9.

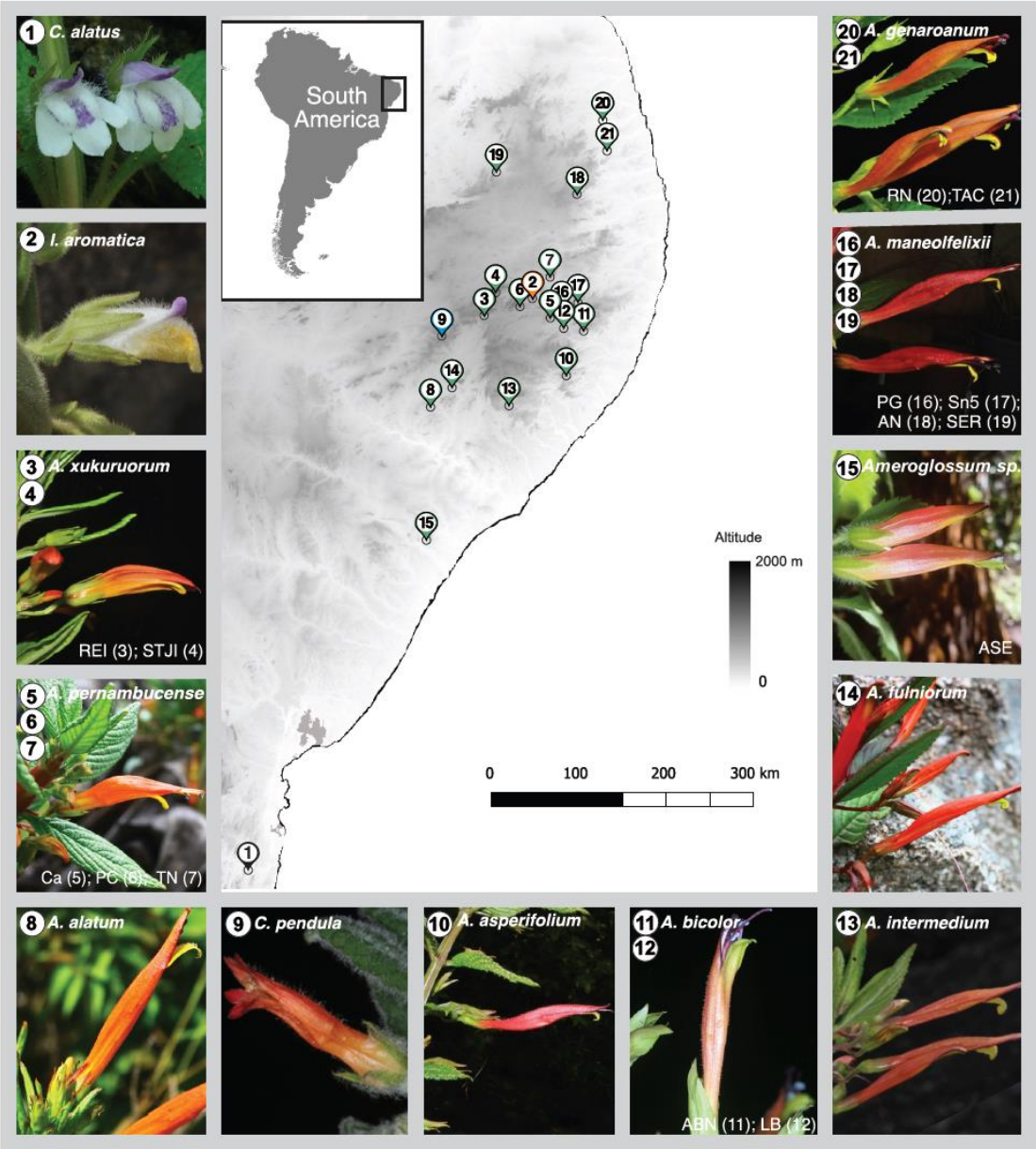
Figure S9. Phylogenetic relationships of Linderniaceae based on Bayesian Inference using the combined plastid dataset (*matK* and *rps16-trnQ*), with the date from Zhang *et al*. (2020). This tree was used as the basis for the biogeographical analysis in Figure S10.

Figure S10. Phylogenetic relationships of Linderniaceae based on Bayarealike + J in RASP. This tree was used as the basis for the biogeographical analysis in Figure 4.

Figure S11. Graph generated in RASP from the dated tree used for biogeographic analysis based on Bayarealike + J, which shows that the long-distance dispersal event has the highest probability for the Linderniaceae dataset.

Figure S12. Phylogenetic relationships of *Ameroglossum* (Bayesian inference of the combined matrix) compared to the cytogenetic results of the species. Idiograms of *Ameroglossum* chromosomes showing heterochromatic bands: yellow = CMA; purple = DAPI; red = 5S rDNA; green = 35S rDNA.

ILLUSTRATIONS



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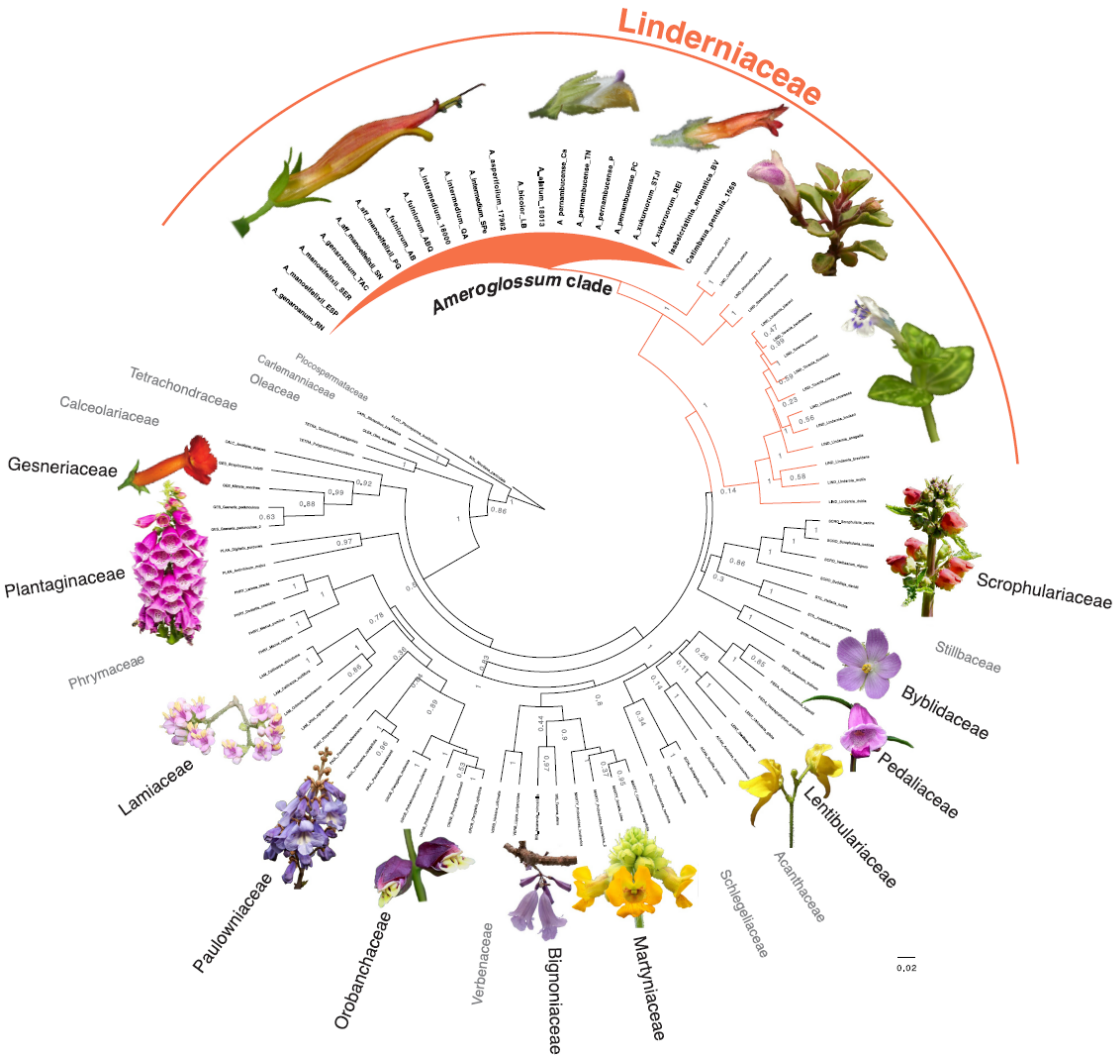

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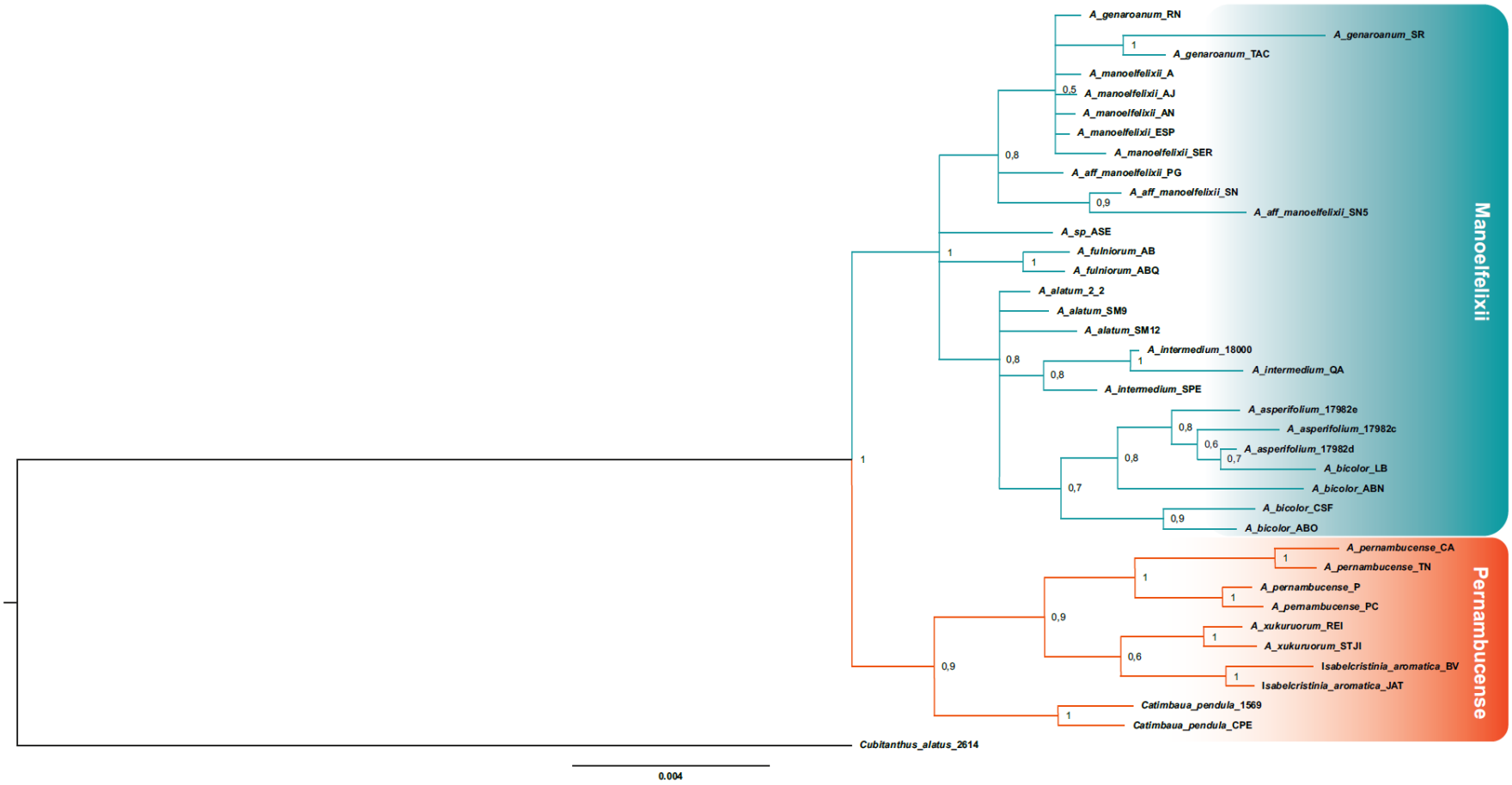


Fig. 3. Reconstruction of the Bayesian Inference (BI) consensus tree with the combination of nuclear (ITS) and plastid (*matK* and *rps16-trnQ*) markers of the *Ameroglossum* clade (iii), with *Cubitanthus alatus* as an outgroup. The two branch colors highlight the main groups of the *Ameroglossum* clade: orange, clade A; green, clade B.

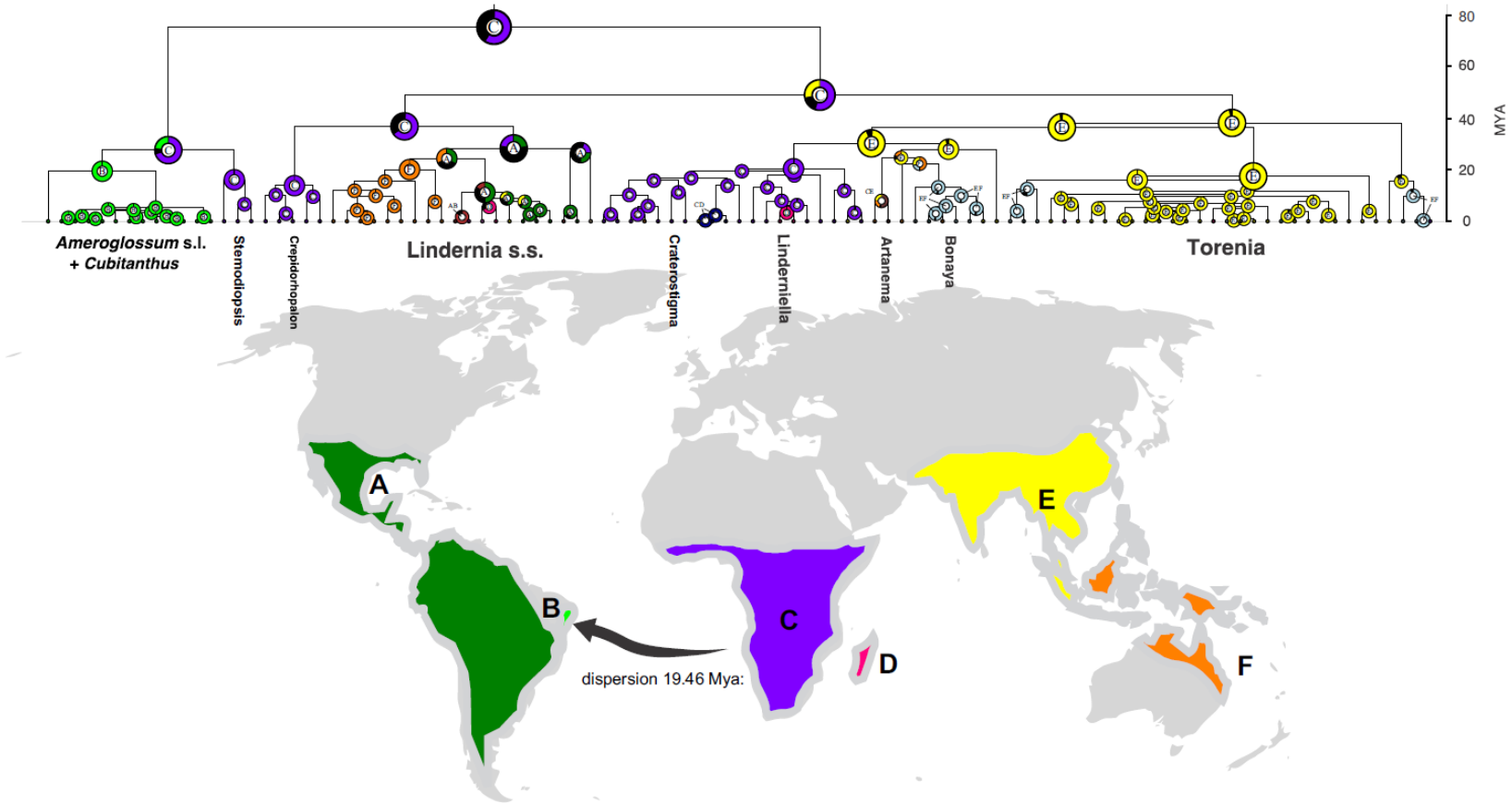
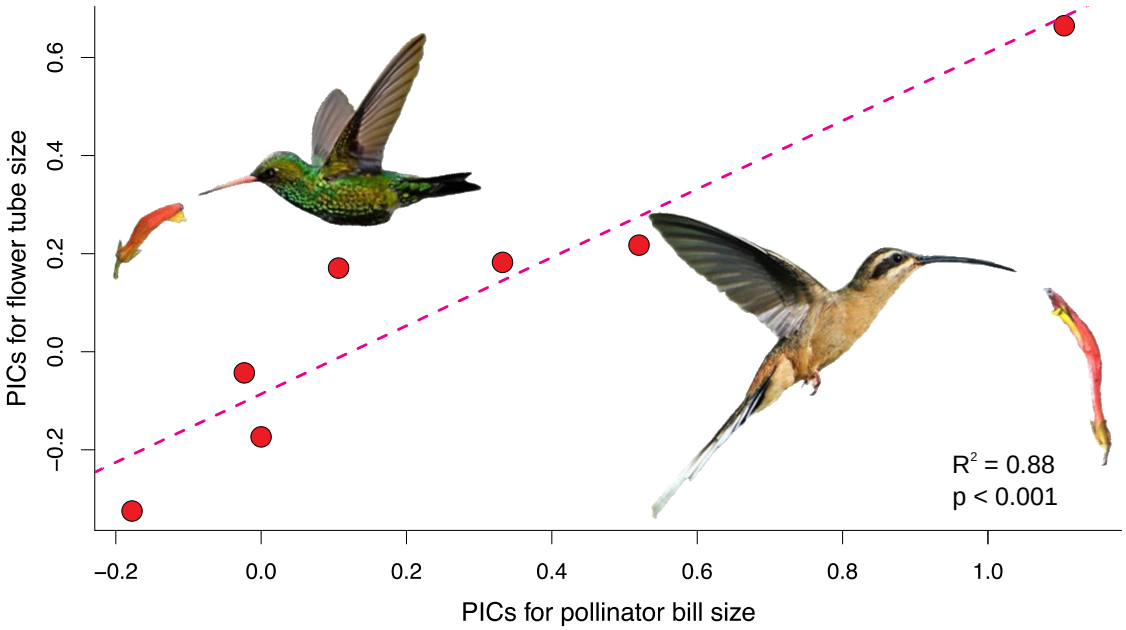


Fig. 4. Reconstruction of the ancestral distribution of Linderniaceae with a focus on *Ameroglossum*. Consensus tree reconstructed from Bayesian Inference (BI) with the combination of plastid markers (*matK* and *rps16-trnQ*). Divergence time based on secondary calibration based on the asterid tree of Zhang et al (2020). Pie charts on the nodes represent probabilities of potential ancestral ranges. Black, areas with less than 10% occurrence; Light blue, Asia + Oceania; Dark blue, Africa + Madagascar; Brown, America + Northeast Brazil.



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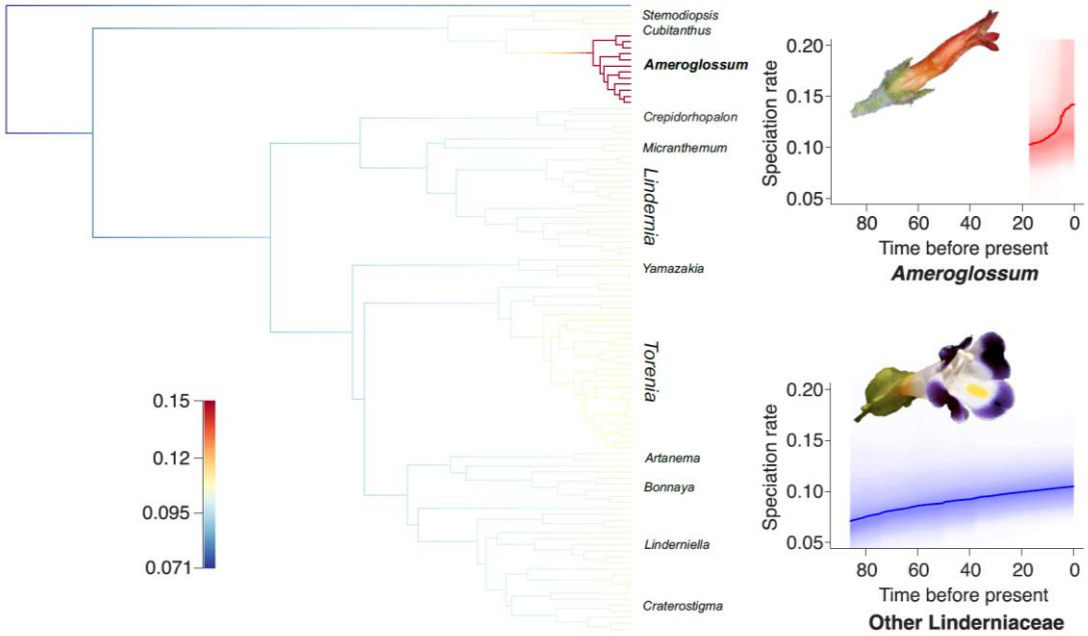

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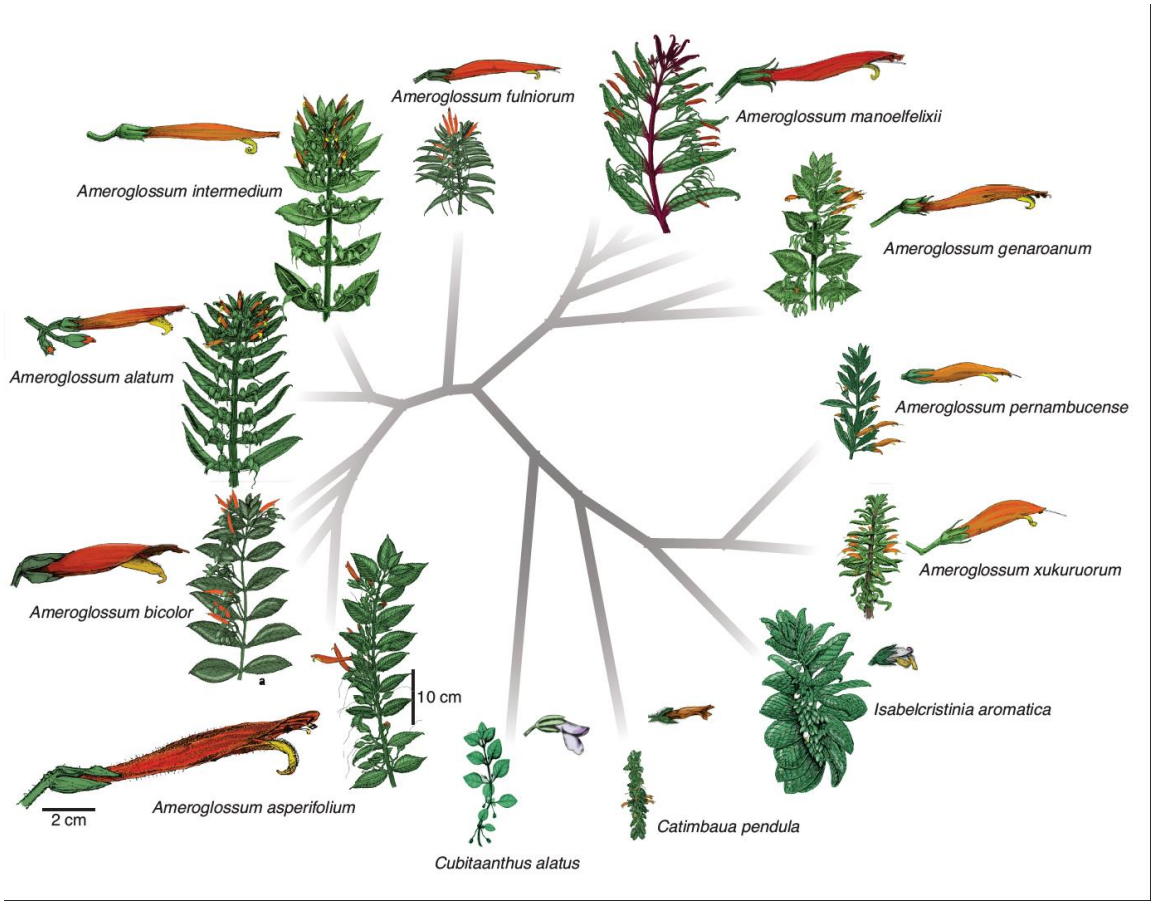


Fig. 7. Adaptive radiation scheme in the *Ameroglossum* clade (Linderniaceae). Phylogenetic relationships are based on the combined Bayesian inference tree. Illustrations from Fisher and Lopes (1999) and Almeida et al (2016; 2019; 2021).

SUPPLEMENTARY MATERIAL - ILLUSTRATIONS

Fig. S4

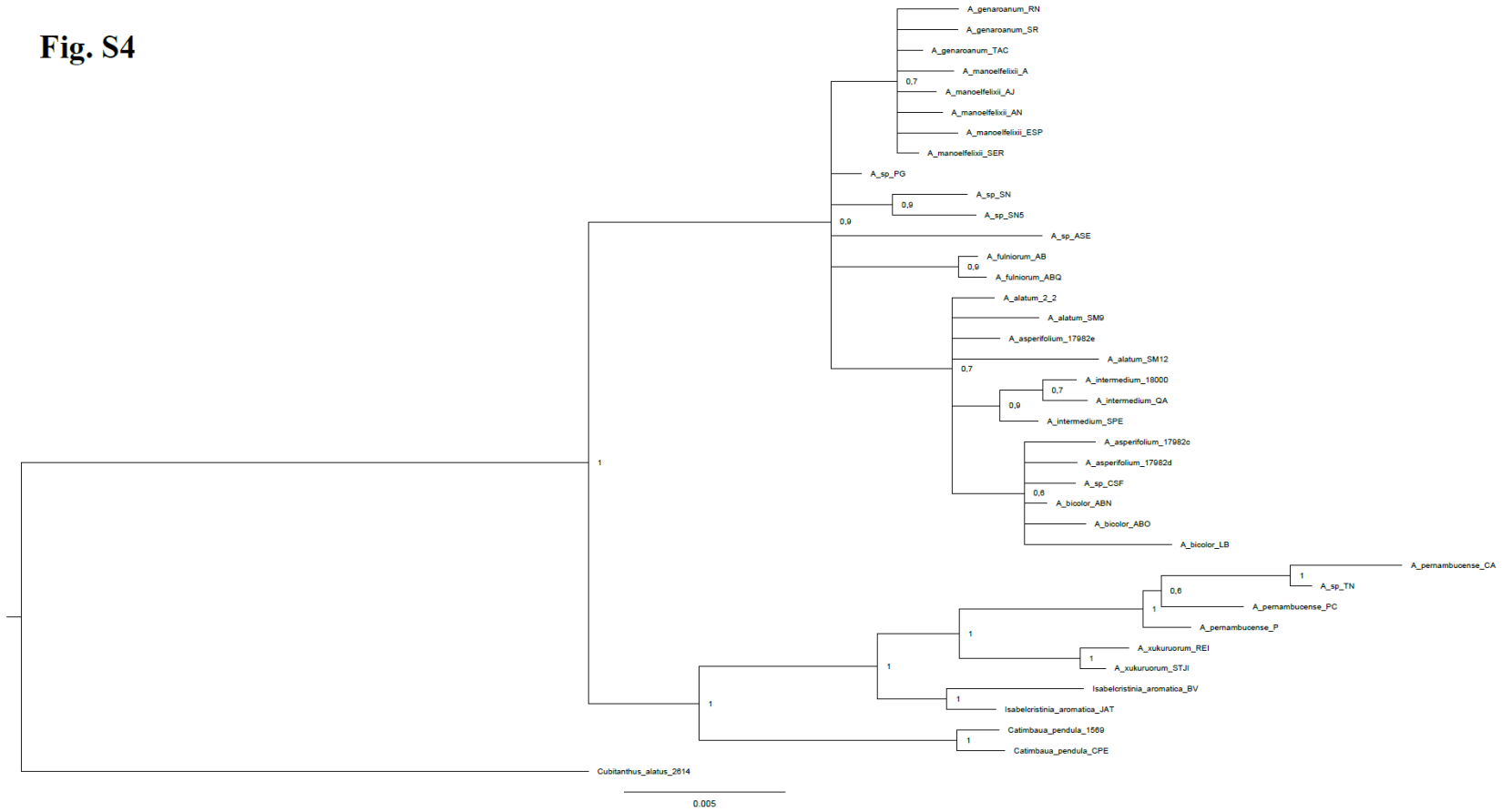


Figure S1. Phylogenetic relationships of the *Ameroglossum* clade based on Bayesian inference of the nuclear ribosomal ITS dataset. With *Cubitanthus alatus* as outgroup.

Fig. S5

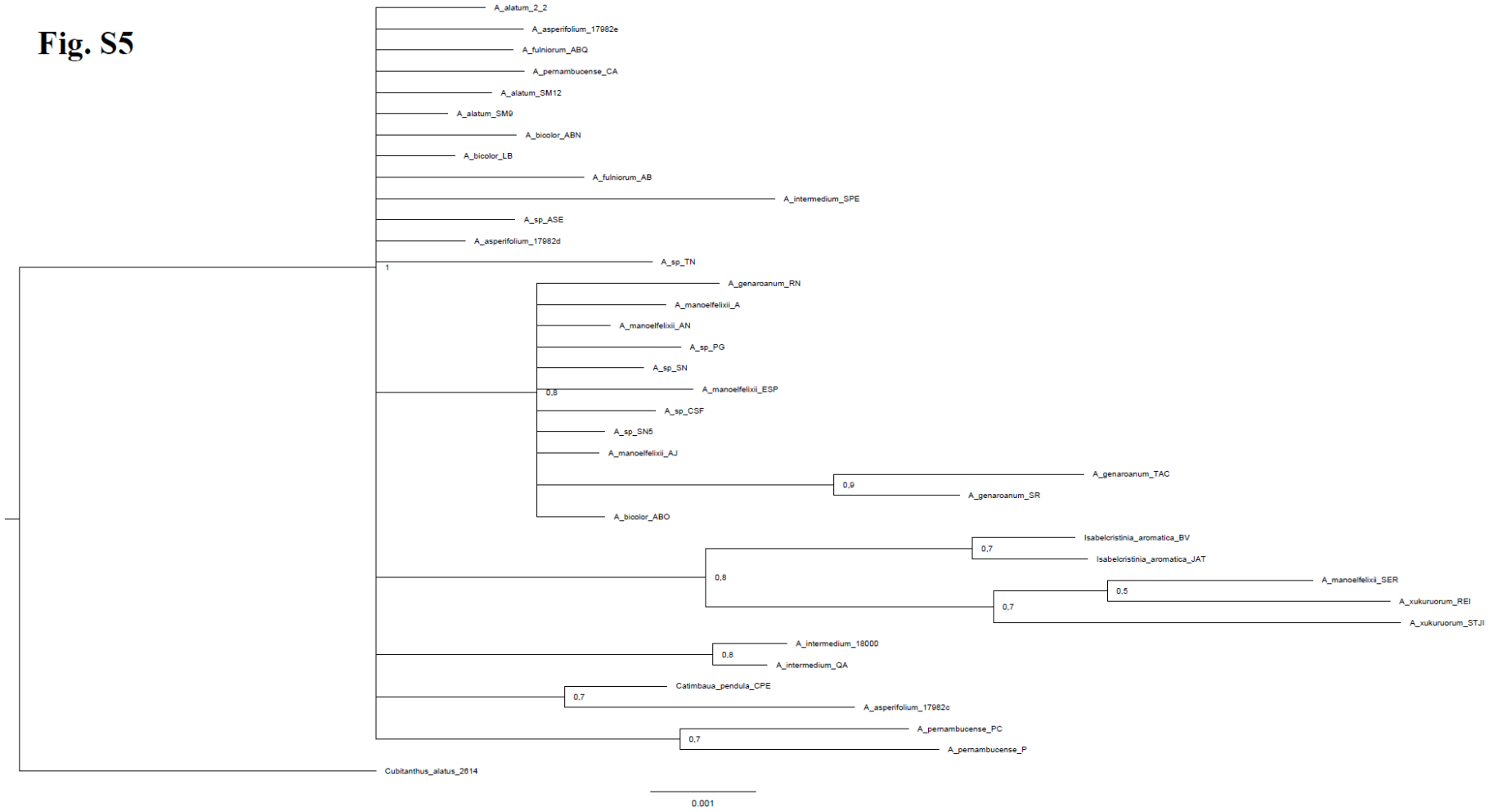


Figure S2. Phylogenetic relationships of the *Ameroglossum* clade based on Bayesian inference of the plastid *matK* dataset. With *Cubitanthus alatus* as outgroup.

Fig. S6

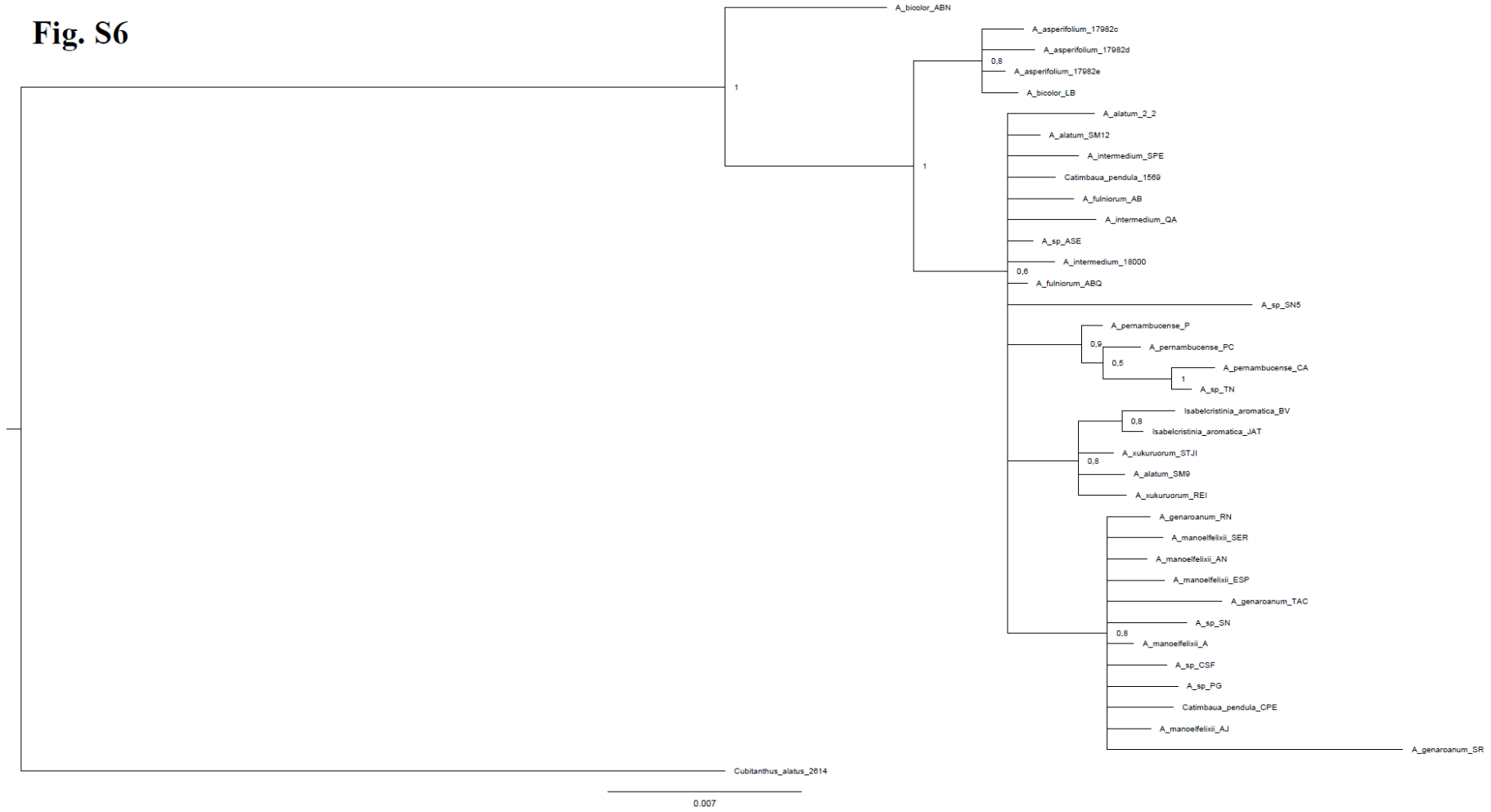


Figure S3. Phylogenetic relationships of the *Ameroglossum* clade based on Bayesian inference of the plastid *rps16-trnQ* dataset. With *Cubitanthus alatus* as outgroup.

Fig. S7

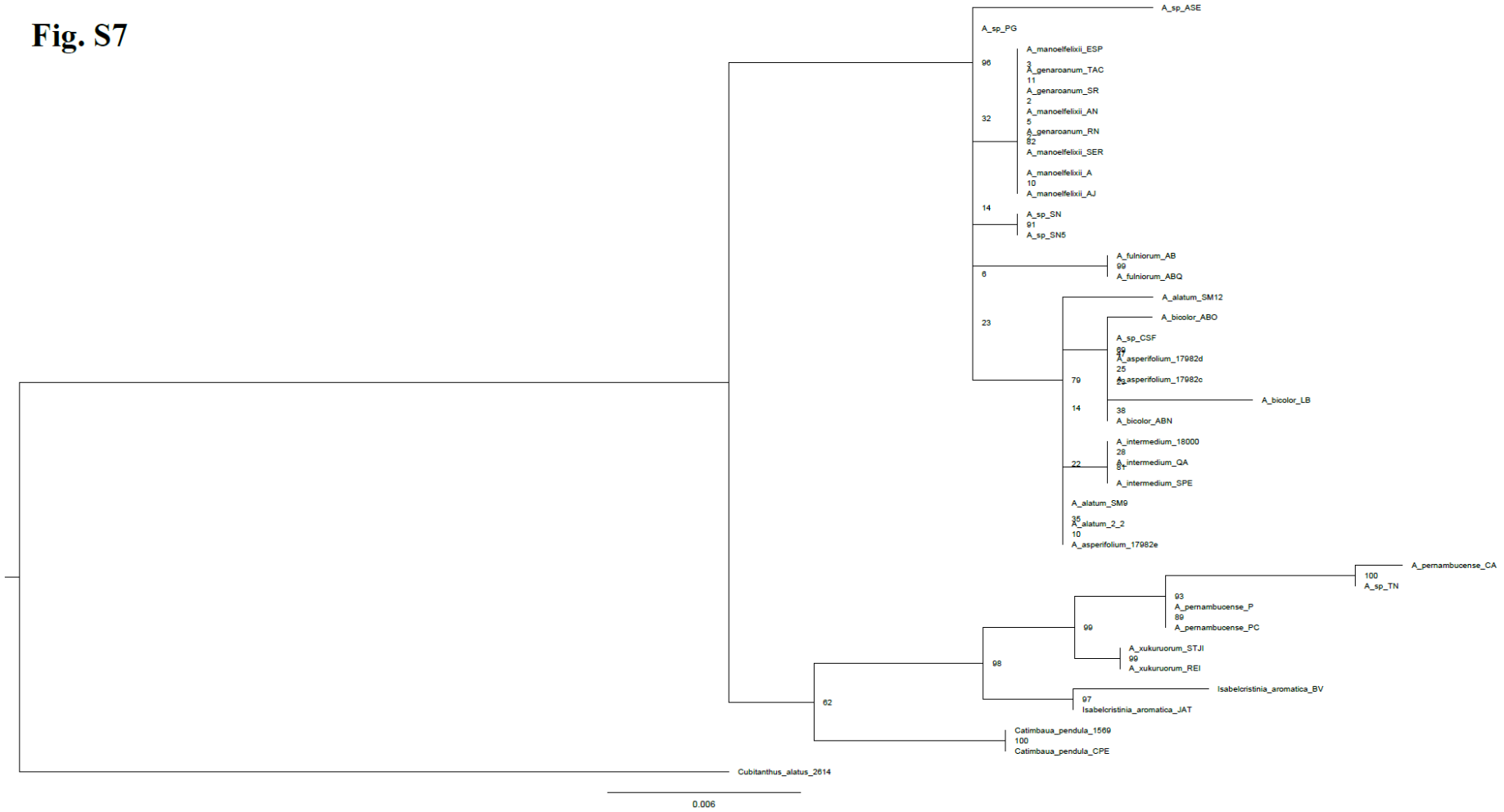


Figure S4. Phylogenetic relationships of the *Ameroglossum* clade based on maximum likelihood of the nuclear ribosomal ITS dataset. With *Cubitanthus alatus* as outgroup.

Fig. S8

Figure S5. Phylogenetic relationships of the *Ameroglossum* clade based on maximum likelihood of the plastid *matK* dataset. With *Cubitanthus alatus* as outgroup.

Fig. S9

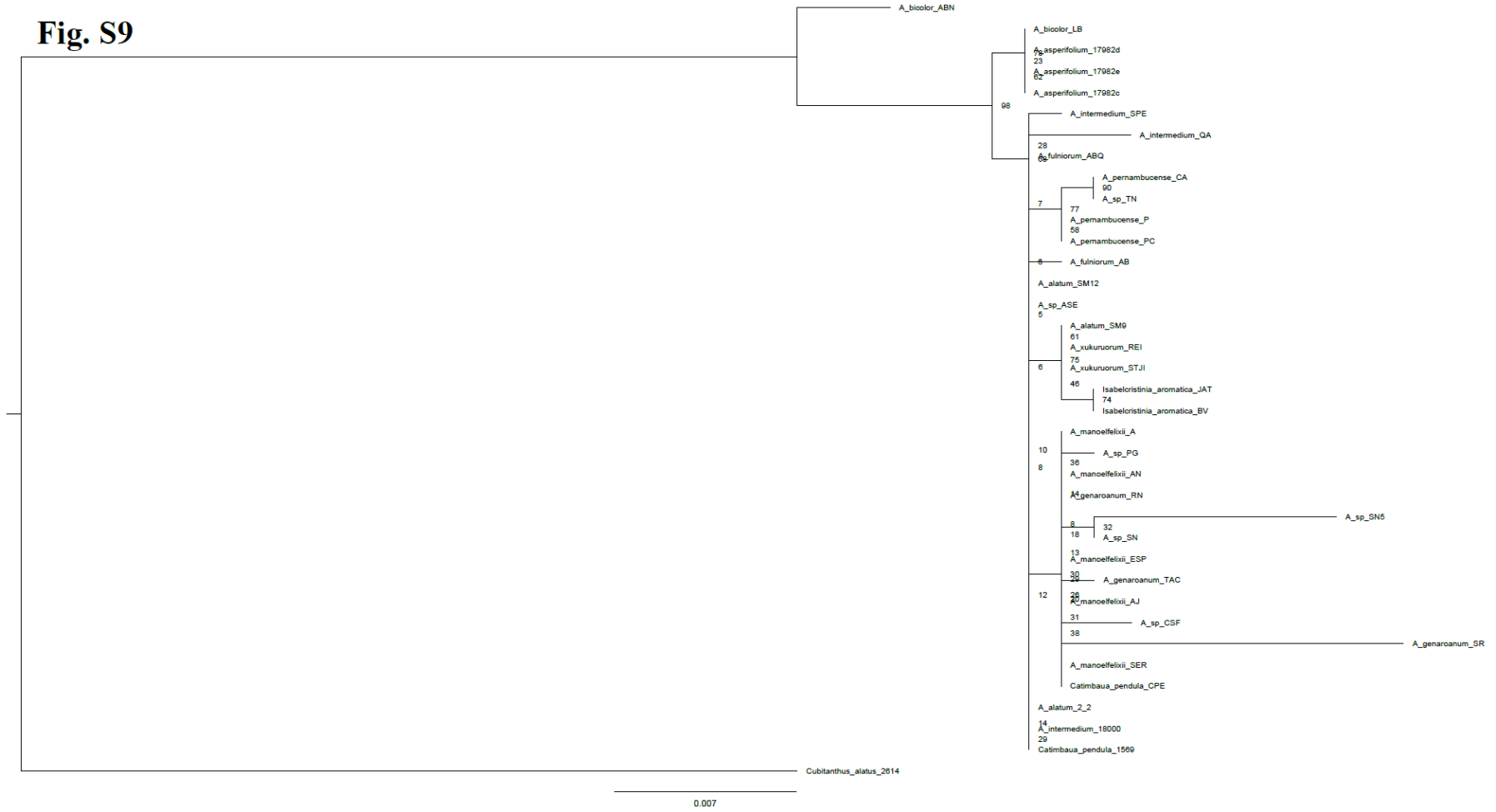


Figure S6. Phylogenetic relationships of the *Ameroglossum* clade based on maximum likelihood of the plastid *rps16-trnQ* dataset. With *Cubitanthus alatus* as outgroup.

Fig. S11

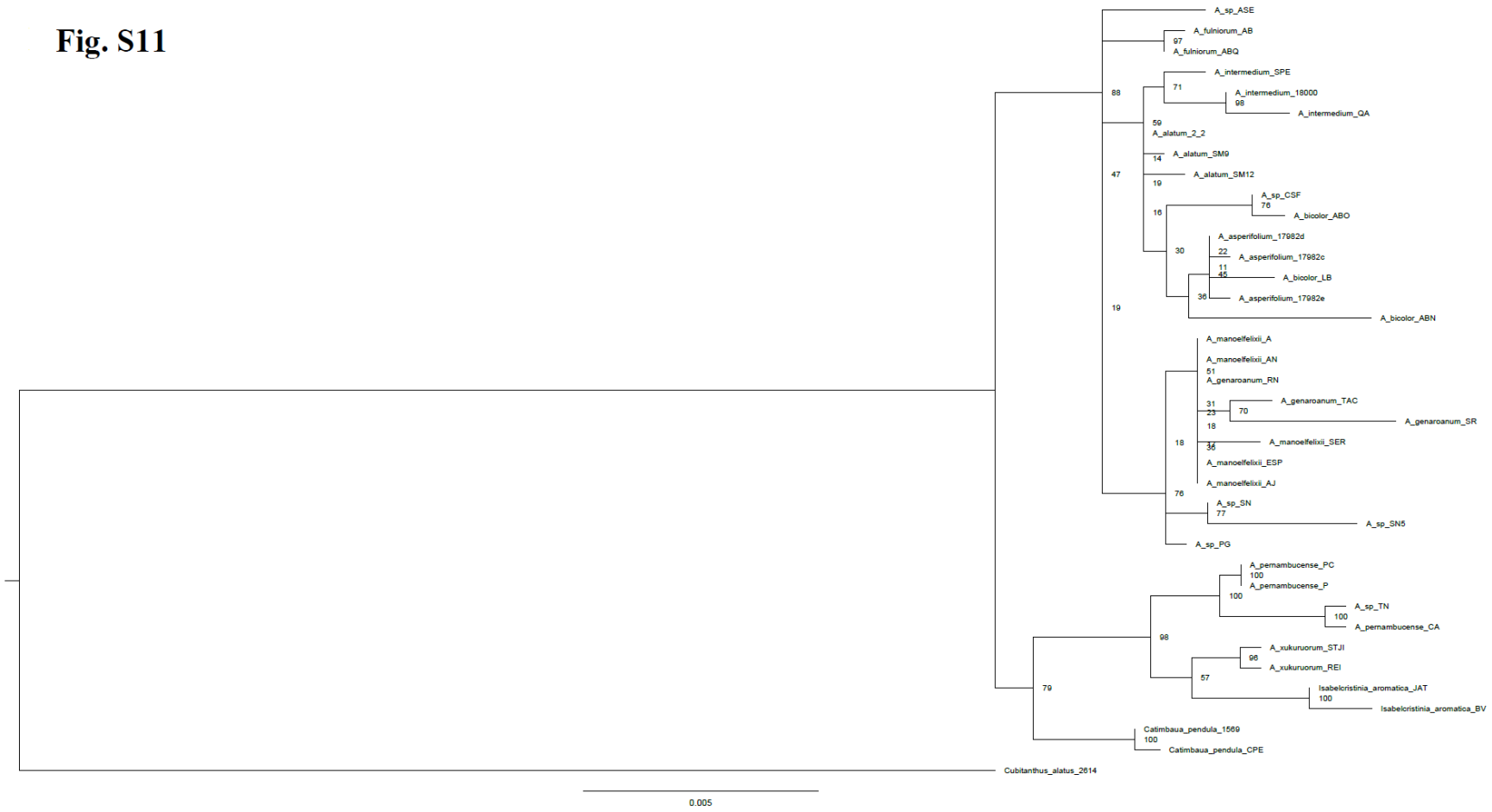


Figure S7. Phylogenetic relationships of the *Ameroglossum* clade (iii) based on maximum likelihood with the combination of nuclear (ITS) and plastid (*matK* and *rps16-trnQ*) markers, with *Cubitanthus alatus* as an outgroup.

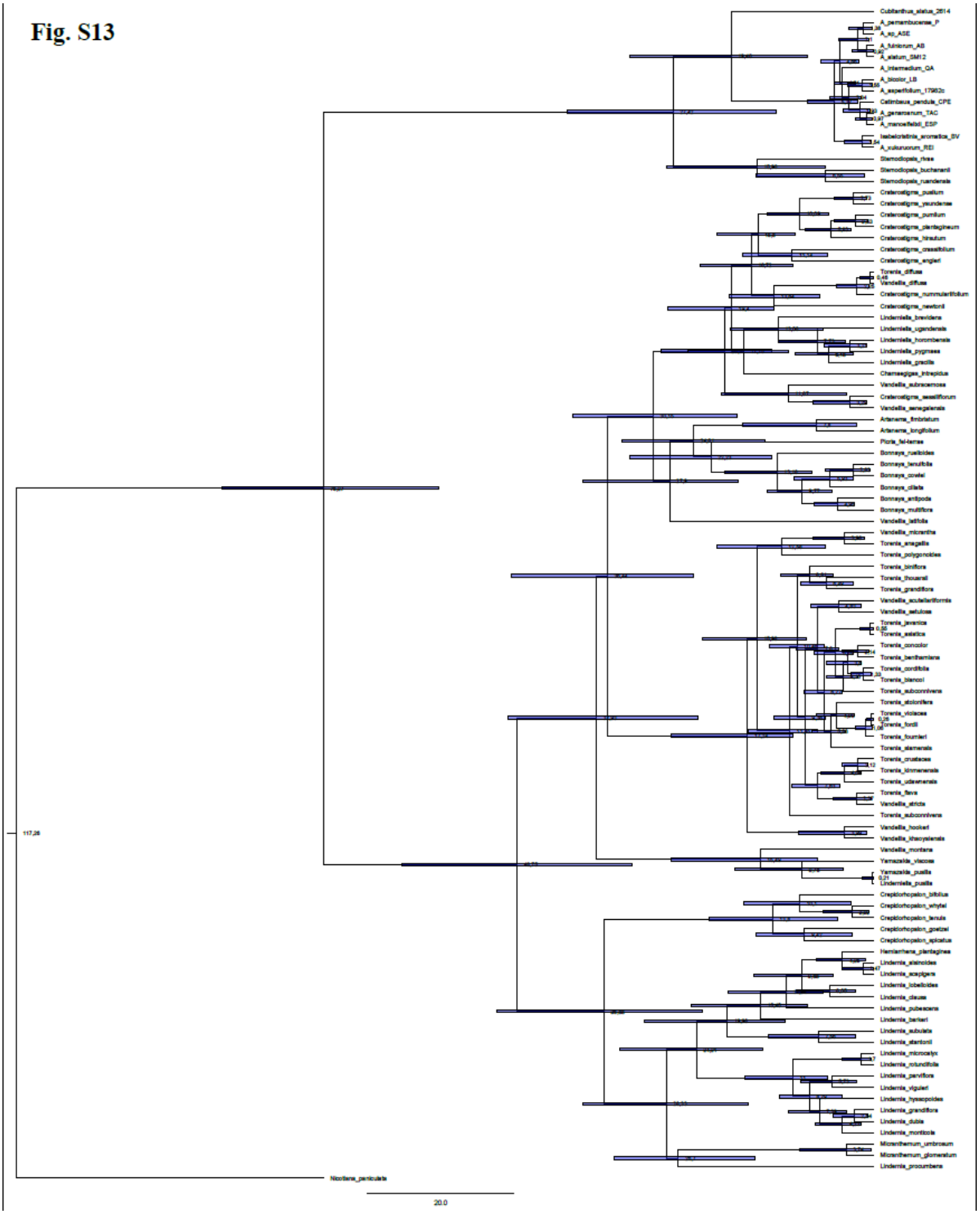


Figure S8. Phylogenetic relationships of Linderniaceae based on Bayesian Inference using the combined plastid dataset (*matK* and *rps16-trnQ*), with the date from Zhang *et al*. (2020). This tree was used as the basis for the biogeographical analysis in Figure S14.

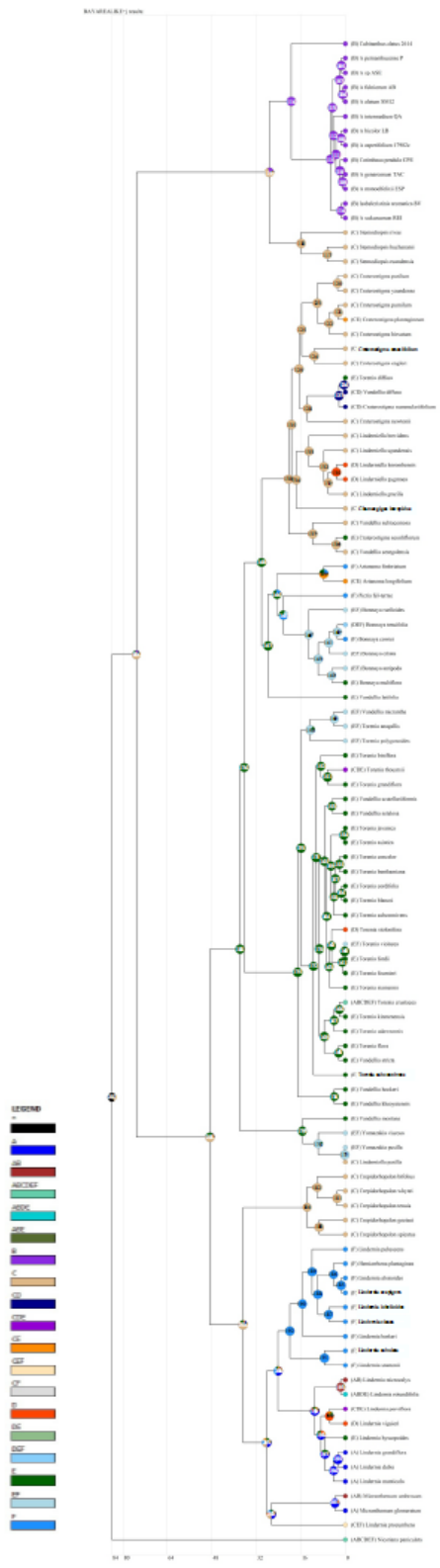


Figure S9. Phylogenetic relationships of Linderniaceae based on Bayarealike + J in RASP. This tree was used as the basis for the biogeographical analysis in Figure 4.

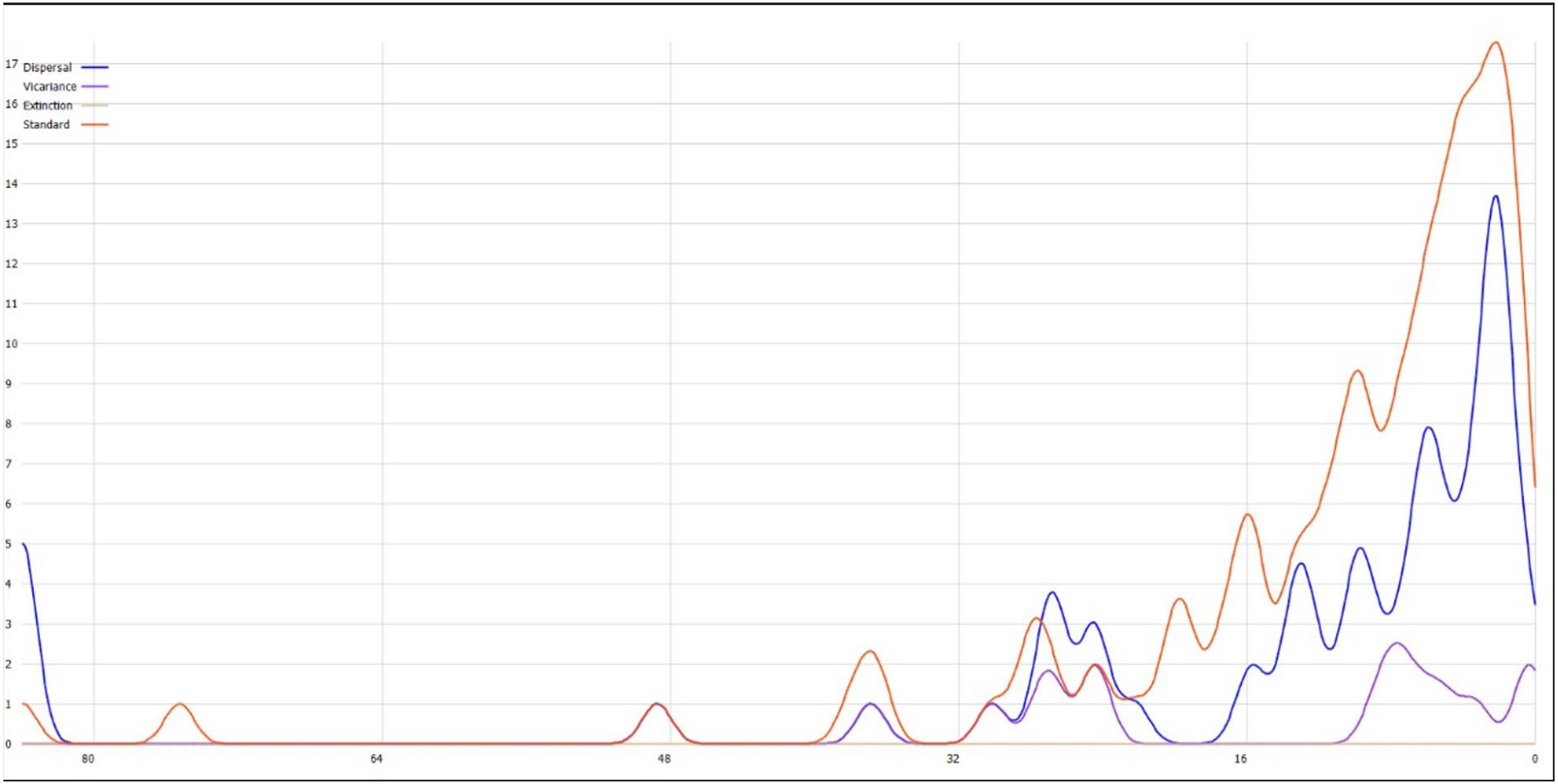
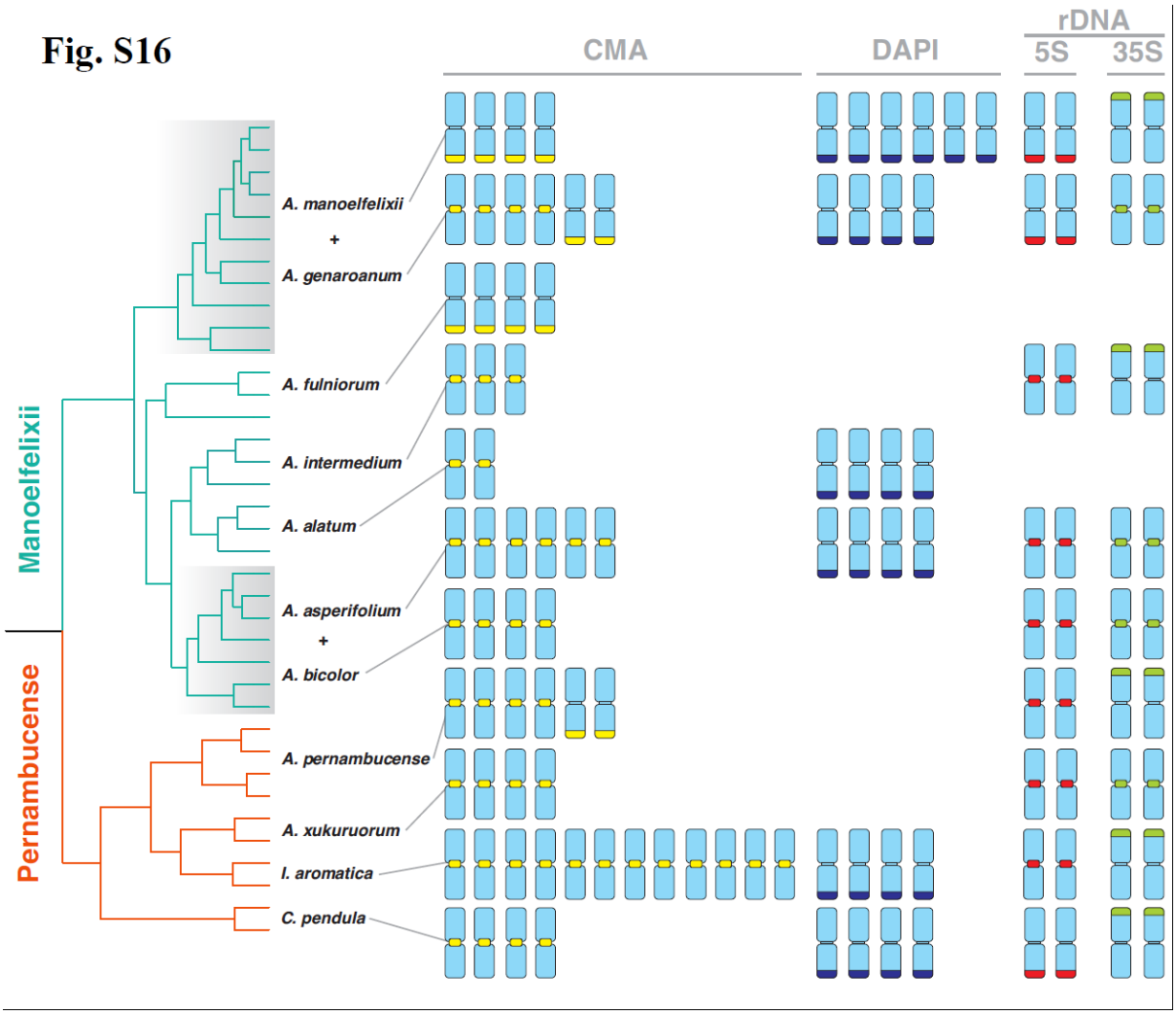


Figure S10. Graph generated in RASP from the dated tree used for biogeographic analysis based on Bayarealike + J, which shows that the long-distance dispersal event has the highest probability for the Linderniaceae dataset.



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SUPPLEMENTARY MATERIAL – TABLES

Table S1. Taxa, specimens and GenBank acession numbers for sequences used in the present study.

Taxa	*matK*	*rps16*	*ITS*
Acanthaceae			
Anisotes formosissimus (Klotzsch) Milne-Redh	JF270643.1	MK282280.1	KY632576.1
Ruellia simplex C.Wright	MF350155.1	MW697905	EF214458.1
Bignoniaceae			
Jacaranda mimosifolia D.Don	JX518220.1	JN686522.1	-
Tecoma stans (L.) Juss. ex Kunth	HQ384522.1	HQ385164.1	AY178636.1
Byblidaceae			
Byblis gigantea Lindl.	AB546635.1	JN686511.1	GU810491.1
Byblis rorida Lowrie & Conran	AB546634.1	-	GU810490.1
Calceolariaceae			
Jovellana violacea (Cav.) G. Don	HQ384542	HQ385178	HQ586831
Carlemanniaceae			
Silvianthus bracteatus Hook. f.	NC_047484	NC_047484	KR532566
Gesneriaceae			
Gesneria pedunculosa (DC.) Fritsch	KJ012607.1	JN686508.1	GU301122.1
Mitraria coccinea Cav.	JX196014.1	KY067886.1	KY067703.1
Streptocarpus holstii Engl.	HQ384539.1	HQ385175.1	AF316917.1
Lamiaceae			
Callicarpa dichotoma (Lour.) K.Koch	KP088986.1	AB751907.1	MT216836.1
Callicarpa nudiflora Hook. & Arn.	MK783316.1	MK783316.1	MG757679.1
Ocimum americanum L.	MK290584.1	AJ505350	GU810517
Vitex agnus-castus L.	HQ384496.1	HQ385143	EU785943
Lentibulariaceae			
Genlisea aurea A.St.-Hil.	MF593121	MF593121	-
Utricularia gibba L.	KC997777	MK611943.1	MT248957.1
Linderniaceae			
Ameroglossum alatum (2_2)	OQ305565	OQ223254	OQ240964
A. alatum (SM9)	OQ305566	OQ223255	OQ240965
A. alatum (SM12)	OQ305567	OQ223256	OQ240966
A. asperifolium (17982c)	OQ305568	OQ223257	OQ240967
A. asperifolium (17982d)	OQ305569	OQ223258	OQ240968
A. asperifolium (17982e)	OQ305570	OQ223259	OQ240969
A. bicolor (LB)	OQ305573	OQ223261	OQ240972
A. bicolor (ABN)	OQ305571	OQ223260	OQ240970
A. bicolor (CSF)	OQ305591	OQ223279	OQ240990
A. bicolor (ABO)	OQ305572	-	OQ240971
A. fulniorum (AB)	OQ305574	OQ223262	OQ240973
A. fulniorum (ABQ)	OQ305575	OQ223263	OQ240974
A. genaroanum (RN)	OQ305576	OQ223264	OQ240975
A. genaroanum (SR)	OQ305577	OQ223265	OQ240976
A. genaroanum (TAC)	OQ305578	OQ223266	OQ240977
A. intermedium (18000)	OQ305579	OQ223267	OQ240978
A. intermedium (QA)	OQ305580	OQ223268	OQ240979

Taxa	***matK***	***rps16***	***ITS***
A. intermedium (SPE)	OQ305581	OQ223269	OQ240980
A. manoelfelixii (A)	OQ305582	OQ223270	OQ240981
A. manoelfelixii (AJ)	OQ305583	OQ223271	OQ240982
A. manoelfelixii (AN)	OQ305584	OQ223272	OQ240983
A. manoelfelixii (ESP)	OQ305585	OQ223273	OQ240984
A. manoelfelixii (SER)	OQ305586	OQ223274	OQ240985
A. aff. manoelfelixii (PG)	OQ305592	OQ223280	OQ240991
A. aff. manoelfelixii (SN)	OQ305593	OQ223281	OQ240992
A. aff. manoelfelixii (SN5)	OQ305594	OQ223282	OQ240993
A. pernambucense (CA)	OQ305587	OQ223275	OQ240986
A. pernambucense (TN)	OQ305595	OQ223283	OQ240994
A. pernambucense (P)	OQ305588	OQ223276	OQ240987
A. pernambucense (PC)	OQ305589	OQ223277	OQ240988
A. xukuruorum (REI)	OQ305596	OQ223284	OQ240995
A. xukuruorum (STJI)	OQ305597	OQ223285	OQ240996
Ameroglossum sp. (ASE)	OQ305590	OQ223278	OQ240989
Catimbaua pendula (1569)	-	OQ223286	OQ240997
Catimbaua pendula (CPE	OQ305598	OQ223287	OQ240998
Cubitanthus alatus	JX196133.1	-	-
Cubitanthus alatus (2614)	OQ305599	OQ223288	OQ240999
Isabelcristinia aromatica (BV)	OQ305600	OQ223289	OQ241000
Isabelcristinia aromatica (JAT)	OQ305601	OQ223290	OQ241001
Lindernia anagallis (Burm.f.) Pennell	KF257401.1	KF257674.1	-
Lindernia dubia (L.) Pennell	KJ772896.1	KF257688.1	MG220205.1
Linderniella brevidens (Skan) Eb.Fisch., Schäferh. & Kai Müll.	KF257424.1	KF257697.1	
Stemodiopsis buchananii Skan	-	AJ609137.1	-
Stemodiopsis ruandensis Eb.Fisch.	FN773559.1	FN794110.1	-
Torenia benthamiana Hance	KF257452.1	KF257725.1	-
Torenia blancoi Merr.	KF257404.1	KF257677.1	-
Torenia concolor Lindl.	-	KF257727.1	KF257454.1
Torenia crustacea (L.) Cham. & Schltdl.	KF257414.1	KF257687.1	GU359049.1
Torenia fournieri Linden ex E. Fourn.	KF257458.1	KF257731.1	-
Vandellia hookeri C.B.Clarke ex Hook.f.	KF257417.1	KF257690.1	-
Vandellia montana (Blume) Benth.	KF257434.1	KF257707.1	-
Martyniaceae			
Craniolaria integrifolia Cham.	-	JN686536.1	JN686496.1
Ibicella lutea (Lindl.) Van Eselt.	HQ384525.1	HQ385167.1	MF963882.1
Proboscidea louisianica (Mill.) Thell.	AF531809.1	AJ609146.1	AY178642.1
Oleaceae			
Olea europaea L.	MT182984	MT182984	AJ585193
Orobanchaceae			
Phtheirospermum muliense C.Y. Wu & D.D. Tao	MG546880	MG546962	JQ910091
Phtheirospermum tenuisectum Bureau & Franch.	AY949770.1	-	JF746383
Pterygiella cylindrica Tsoong	JF955838.1	MG546985.1	JF978161.1
Pterygiella duclouxii Franch.	JF955844.1	MG546987.1	JN416365.1

Taxa	*matK*	*rps16*	*ITS*
Pterygiella muliensis (C.Y.Wu & D.D.Tao) Pinto-Carr., E.Rico & M.M.Mart.Ort.	MG546880.1	MG546963.1	MG523332.1
Paulowniaceae			
Paulownia catalpifolia T. Gong ex D.Y. Hong	MK392228.1	MK392302.1	-
Paulownia kawakamii T.Itô	MK392229.1	MK392303.1	-
Paulownia taiwaniana T.W. Hu & H.J. Chang	MK392227.1	MK392301.1	-
Pedaliaceae			
Sesamothamnus lugardii N.E. Br.	HQ384516.1	HQ385158.1	AY178660.1
Sesamum indicum L.	MT239315.1	HQ385159.1	KM210317
Uncarina grandidieri (Baill.) Stapf	HQ384517.1	FN794094.1	AY178659.1
Phrymaceae			
Dodartia orientalis L.	MK392230.1	JQ342982.1	JQ342980.1
Lancea hirsuta Bonati	MG551489	MG551489	-
Mazus pumilus (Burm.f.) Steenis	MH658928.1	KX807202.1	MH808609.1
Mazus reptans N.E. Br.	HQ384502.1	HQ385147.1	MH605198.1
Phryma leptostachya L.	MH660221.1	MH711667.1	AJ609150.1
Plantaginaceae			
Antirrhinum majus L.	KX783633.1	GQ997033.1	-
Digitalis purpurea L.	MK898788.1	AY492203.1	MK895648.1
Plocospermataceae			
Plocosperma buxifolium Benth.	AJ429315	FN794106	-
Schlegeliaceae			
Schlegelia fuscata A.H.Gentry	HQ384514	HQ385156	-
Schlegelia parviflora (Oerst.) Monach.	JQ589194.1	AJ609141.1	-
Thomandersia laurifolia (T.Anderson ex Benth.) Baill.	HQ384515	JN686550	JN686503
Scrophulariaceae			
Buddleja davidii Franch.	HQ384530.1	AJ609204.1	MG218897.1
Scrophularia canina L.	AM503830.2	KR827035.1	-
Scrophularia nodosa L.	JN896243.1	HQ130038.1	-
Verbascum nigrum L.	MK520793.1	HQ130034.1	-
Stilbaceae			
Anastrabe integerrima E.Mey. ex Benth.	KF147376.1	AJ609216.1	AJ616314.1
Halleria lucida L.	HQ384528.1	AJ609181.1	AF375149.1
Tetrachondraceae			
Polypremum procumbens L.	AJ429351	HQ385179	MF348875
Tetrachondra patagonica Skottsb.	AJ429352	AJ431064	-
Verbenaceae			
Lippia origanoides Kunth	MK248831	MK248831	-
Verbena officinalis L.	MH659079.1	AF225295.1	MH711753.1
Solanaceae			
Nicotiana paniculata L.	AB039988.1	BK010741.1	AJ492413.1

Table S2. Geographic distribution matrix of Linderniaceae applied for ancestral area reconstruction in BioGeoBEARS.

Genus	Specie	Geographic distribution					
		A	B	C	D	E	F
Ameroglossum	*A_alatum*_2_2		X				
	*A_alatum*_SM12		X				
	*A_alatum*_SM9		X				
	*A_asperifolium*_17982c		X				
	*A_asperifolium*_17982d		X				
	*A_asperifolium*_17982e		X				
	*A_bicolor*_ABN		X				
	*A_bicolor*_ABO		X				
	*A_bicolor*_LB		X				
	*A_bicolor*_CSF		X				
	*A_fulniorum*_AB		X				
	*A_fulniorum*_ABQ		X				
	*A_genaroanum*_RN		X				
	*A_genaroanum*_SR		X				
	*A_genaroanum*_TAC		X				
	*A_intermedium*_18000		X				
	*A_intermedium*_QA		X				
	*A_intermedium*_SPE		X				
	*A_manoelfelixii*_A		X				
	*A_manoelfelixii*_AJ		X				
	*A_manoelfelixii*_AN		X				
	*A_manoelfelixii*_ESP		X				
	*A_manoelfelixii*_SER		X				
	*A_aff_manoelfelixii*_PG		X				
	*A_aff_manoelfelixii*_SN		X				
	*A_aff_manoelfelixii*_SN5		X				
	*A_pernambucense*_CA		X				
	*A_pernambucense*_P		X				
	*A_pernambucense*_PC		X				
	*A_pernambucense*_TN		X				
	*A_sp*_ASE		X				
	*A_xukuruorum*_REI		X				
	*A_xukuruorum*_STJI		X				
Artanema	*A. fimbriatum*						X
	A. longiflorum			X		X	
Bonnaya	*B. antipoda*					X	X
	B. ciliata					X	X
	B. cowiei						X
	B. multiflora					X	
	B. ruellioides					X	X
	B. tenuifolia				X	X	x
Catimbaua	*Catimbaua_pendula*_1569		X				
	*Catimbaua_pendula*_CPE		X				
Craterostigma	*C. engleri*			X			
	C. hirsutum			X			
	C. newtonii			X			

	C. nummulariifolia			X	X		
	C. plantagineum			X		X	
	C. pumilum			X			
	C. pusillum			X			
	C. sessiliflorum					X	
	C. yaundense			X			
Crepidorhopalon	*C. bifolius*			X			
	C. goetzei			X			
	C. spicatus			X			
	C. tenuis			X			
	C. whytei			x			
Cubitanthus	*C. alatus*_CUB		X				
Hemiarrhena	*H. plantaginea*						X
Isabelcristinia	*Isabelcristinia_aromatica*_BV		X				X
	*Isabelcristinia_aromatica*_JAT		X				X
Lindernia	*L. alsinoides*						X
	L. barkeri						X
	L. crassifolia			X			
	L. dubia	X					
	L. grandiflora	X					
	L. hyssopioides					X	
	L. intrepida			X			
	L. microcalyx	X	X				
	L. monticola	X					
	L. parviflora			X	X	X	
	L. procumbens			X		X	X
	L. pubescens						X
	L. rotundifolia	X	X		X	X	
	L. stantonii						X
	L. viguieri				X		
Linderniella	*L. brevidens*			X			
	L. gracilis			X			
	L. horombensis				X		
	L. pusilla			X			
	L. pygmaea				X		
	L. ugandensis			X			
Micrantemum	*M. glomeratum*	X					
	M. umbrosum	X	X				
Picria	*P. fel-terrae*						X
Stemodiopsis	*S. buchananii*			X			
	S. rivae			X			
	S. ruandensis			X			
Torenia	*T. anagallis*					X	X
	T. asiatica					X	
	T. benthamiana					X	
	T. biniflora					X	
	T. blancoi					X	
	T. concolor					X	
	T. cordifolia					X	
	T. crustacea	X	X	X	X	X	X

	T. diffusa				X		
	T. flava				X		
	T. fordii				X		
	T. fournieri				X		
	T. grandiflora				X		
	T. javanica				X		
	T. kinmenensis				X		
	T. polygonoides				X	X	
	T. siamensis				X		
	T. stolonifera			X			
	T. subconnivens				X		
	T. thouarsii	X	X		X		
	T. udawnensis				X		
	T. violacea				X	X	
Vandellia	*V. clausa*					X	
	V. diffusa	X	X				
	V. hookeri				X		
	V. khaoyaiensis				X		
	V. latifolia				X		
	V. lobelioides					X	
	V. micrantha				X	X	
	V. montana				X		
	V. scapigera					X	
	V. scutellariiformis				X		
	V. senegalensis	X					
	V. setulosa				X		
	V. stricta				X		
	V. subracemosa	X					
	V. subulata					X	
Yamazakia	*Y. pusilla*				X	X	
	Y. viscosa				X	X	

* A = North America + Central America + South America; B = Brazilian Northeast; C = Africa + Middle East; D = Madagascar; E = Ásia; F = Oceania.

Table S3. Species of *Ameroglossum* with available morphological (mean flower tube length) and pollinator (main pollinator, mean pollinator bill length) data, retrieved from Wanderley et al. (2018).

Species	Provenance	Flower tube length	Main pollinator	Mean Pollinator Bill Length
A. bicolor	Agrestina, PE	3.90	*Eupetomena macroura*	2.37
A. intermedium	Quebrangulo, AL	3.41	*Eupetomena macroura*	2.37
A. manoelfelixii	Esperança, PB	3.88	*Eupetomena macroura*	2.37
	Serraria, PB	4.53	*Phaethornis pretrei*	3.50
	Alagoa Nova, PB	4.56	*Phaethornis pretrei*	3.50
A. aff manoelfelixii	Pedra do Guariba, Caruaru, PE	3.85	*Amazilia fimbriata*	1.97
A. pernambucense	Brejo da Madre de Deus, PE	2.89	*Amazilia fimbriata*	1.97
A. xukuruorum	Pesqueira, PE	2.76	*Chlorostilbon lucidus*	1.90

4 CONSIDERAÇÕES FINAIS

- As espécies aqui analisadas diferiram cariologicamente no número de cromossomos, padrão de bandas, aparente simetria do cariótipo e distribuição de sítios de rDNA.
- As espécies dos gêneros rupícolas, *Ameroglossum*, *Catimbaua* e *Isabelcristinia* possuem 2*n* = 60 cromossomos, com exceção de *A. genaroanum* com 2*n* = 64 e *Cubitanthus alatus* com 2*n* = 50. Já as espécies não rupícolas diferiram quanto ao nível de ploidia (6x versus 2x, respectivamente).
- Todas as espécies analisadas apresentam bandas CMA/5S sobrepostas, o que parece ser uma característica cariológica marcante desta família.
- O gênero *Ameroglossum* está filogeneticamente posicionado em Linderniaceae, formando um clado bem suportado com *Cubitanthus* e *Stemodiopsis*. Porém, o posicionamento dos gêneros *Catimbaua* e *Isabelcristinia* é incerto, uma vez que emergem dentro de *Ameroglossum*.
- As relações internas em *Ameroglossum* revelam dois clados, o clado A se destacou por ter maior suporte e maior diversidade morfológica entre suas espécies, enquanto o clado B ainda carece de melhor resolução interna.
- A estreita relação de *Ameroglossum* + *Cubitanthus* (Sul americanos) com *Stemodiopsis* (Africano) suporta a hipótese de que estes gêneros chegaram à América do Sul devido um evento de dispersão de longa distância oriundo da África, que ocorreu entre 27 a 19 Ma, com um aumento posterior na taxa de diversificação de *Ameroglossum*, potencialmente ligada à sua preferência por afloramentos rochosos.

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APÊNDICE A - Karyotype differentiation in *Ameroglossum* (Linderniaceae) and closely related genera endemic to Brazilian inselbergs

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Original Article

Karyotype differentiation in *Ameroglossum* (Linderniaceae) and closely related genera endemic to Brazilian inselbergs

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ABSTRACT

The genus *Ameroglossum* is composed of shrub plants endemic to inselbergs in northeastern Brazil, currently circumscribed in the Linderniaceae. Chromosomal counts for this family are few, but quite variable, ranging from 2*n* = 14 to 60. We investigated the chromosomal numbers of 14 species of Linderniaceae with emphasis on *Ameroglossum* and analyzed the distribution of heterochromatin and 5S and 35S rDNA sites for most species. We found 2*n* = 60 for the species of *Ameroglossum*, *Catimbaua* and *Isabelcristinia*, except *A. genaroanum* with 2*n* = 64, 2*n* = 50 for *Cubitanthus alatus*, and 2*n* = 28 for *Torenia thouarsii* and *Vandellia diffusa*. All of them had small similar chromosomes and 5S and 35S rDNA sites overlapping with CMA^+ bands. The species with 2*n* = 50-64 showed a single pair of 5S and 35S rDNA sites, *V. diffusa* had two pairs of 5S and one pair of 35S rDNA sites, and *T. thouarsii* revealed two pairs of each. Furthermore, other CMA^+ and $DAPI^+$ bands have been seen in almost all species. The karyotypical similarities among the species of *Ameroglossum*, *Catimbaua*, *Cubitanthus* and *Isabelcristinia* suggest a close relationship between representatives of Linderniaceae typical of inselbergs.

ADDITIONAL KEYWORDS: chromosome number - CMA/DAPI – heterochromatin - rDNA sites - *Torenia*

INTRODUCTION

Inselbergs or rocky outcrops are naturally occurring granitic or gneissic formations, similar to oceanic islands in flat or gently undulating land environments. The degree of geographic isolation of the inselbergs is quite varied, and may occur isolated or agglomerated, rising abruptly from the rest of the landscape, separated by a few or many kilometers away (Porembski & Barthlott, 2012). Inselbergs are found in tropical and temperate regions, and generally have low relative humidity and little soil accumulation. As a result, they are characterized by a vegetation cover that differs from the surrounding areas and serve as centers of diversity for geographically restricted and mostly threatened plant and animal species (Porembski *et al*., 2016). Among the species that occur in this type of environment, some are endemic, especially those from *Alcantarea* (E.Morren ex Mez) Harms, *Vellozia* Vand., *Euphorbia* L., *Pachypodium* Lindl. *Ameroglossum* Eb.Fisch., S.Vogel & A.V.Lopes, *Constatia* Barb.Rodr. and *Pseudolaelia* Porto & Brade (Porembski *et al*., 2007).

Ameroglossum is a small genus of shrubs growing in granitic rock, endemic to the northeastern Brazil, in enclaves of Atlantic rain forest "Brejo de altitude" *sensu* Andrade-Lima (Fischer, Vogel & Lopes, 1999) or totally immersed in the Caatinga (Almeida *et al*., 2016). The genus was initially inserted in the family Scrophulariaceae, as it shares some morphological similarities with some species of this family, such as type of inflorescence and shrub habit, although it differs from Scrophulariaceae in that it displays conspicuous staminoid and lower lip of the corolla, apparently minimally divided and trilobulated (Fischer *et al*., 1999). When Scrophulariaceae was recircunscribed, *Ameroglossum* remained unplaced to any family in Lamiales (Olmstead *et al*., 2001; Oxelman *et al*., 2005). Later, it was included in Linderniaceae together with the monotypic genus *Cubitanthus* Barringer, originally placed in Gesneriaceae based on unclear morphological characters (Christenhusz, Fay & Chase, 2017). Almeida *et al*. (2019) reinforced its placement within Linderniaceae based on the stamen form and texture

seeds, and included into the family two other genera recently described: *Catimbaua* L.P.Felix Christenh. & E.M.Almeida, and *Isabelcristinia* L.P.Felix, Christenh. & E.M.Almeida. The latter authors observed that *Ameroglossum*, *Catimbaua* and *Isabelcristinia* shared the same chromosome number, 2*n* = 60, quite distinct from all other Linderniaceae (Rice *et al*., 2015).

The chromosome number, when complemented with morphological and molecular data, has proven useful to understand the systematics and evolution of several groups of plants. In some genera, the chromosome number indicates the occurrence of quite different lineages, with low viability of interspecific hybrids and clear taxonomical delimitation (Sader *et al*., 2019; Burchardt *et al*., 2020). Therefore, a cytogenetic analysis of Linderniaceae representatives may help to understand the relationship between their genera and the placement of inselberg species within the family.

The family is cytologically poorly investigated, with chromosome data known for only 33 species (~13%) (Rice *et al*., 2015; Almeida *et al*., 2016, 2019). The chromosomal numbers of 33 belonging to the genera *Artanema* D. Don, *Bonnaya* Link & Otto, *Craterostigma* Hochst., *Lindernia* All., *Linderniella* Eb.Fisch., Schäferh. & Kai Müll., *Torenia* L., *Vandellia* L., and *Yamazakia* W.R.Barker, Y.S.Liang & Wannan, in addition to the *Ameroglossum*, *Catimbaua* and *Isabelcristinia*. Most karyotype analyses include, besides the chromosome number, other chromosome marks, as chromosome size and morphology, distribution of heterochromatin, rDNA sites, among others, which allow a better differentiation of individual karyotypes. The heterochromatin consists of chromosome regions, or bands, that vary in number, size, or distribution along the chromosomes. It is poor in genes and, therefore, evolutionarily more unstable (Liu *et al*., 2020). The band pattern contributed to understand the evolutionary relationships between species of different groups, such as *Anthurium* Schott. (Nascimento *et al*., 2019) and Cactaceae (Castro *et al*., 2020). The currently most used method for visualizing heterochromatin is the direct chromosome staining with the fluorochromes CMA

(chromomycin A3) and DAPI (4',6'-diamino-2-phenyl-indole), based on their preferential binding to GC- and AT-rich sequences, respectively (Sumner, 1990).

In addition to the CMA and DAPI bands, the location of the 5S and 35S ribosomal genes (rDNA) by fluorescent *in situ* hybridization (FISH) has also been widely used in molecular karyotyping of plant species. The only study of rDNA sites in Linderniaceae was done in *Torenia fournieri* Linden ex Fourn. and in *Torenia flava* Buch.-Ham. ex Benth. (sin. *Torenia baillonii* Godefroy ex André), where one and three chromosome pairs with 5S rDNA sites, respectively, were differentiated (Kikuchi *et al*., 2006). The detection of 5S and 35S rDNA sites simultaneously with the CMA/DAPI bands, may allow the characterization of several chromosomes or even each one of them, as for example in *Cuscuta monogyna* Vahl. (Ibiapino *et al*., 2020).

For the Linderniaceae group that occurs in inselbergs in northeastern Brazil, the chromosome number $2n = 60$ was reported for *Ameroglossum pernambucense* Eb.Fisch., S.Vogel & A.V.Lopes, *Ameroglossum manoelfelixii* L.P.Felix & E.M.Almeida¸ *Catimbaua pendula* L.P.Felix & E.M.Almeida and *Isabelcristinia aromatica* L.P.Felix & E.M.Almeida (Almeida *et al*., 2016, 2019). The CMA/DAPI staining of chromosomes of *A. pernambucense* and *A. manoelfelixii* revealed karyotypes with uniform DAPI staining and a single pair of heteromorphic CMA^+ bands in both species. However, in *A. pernambucense* the CMA^+ bands were much stronger than in *A. manoelfelixii* and occupied the entire short arm, providing an incipient karyotype differentiation between these two species (Almeida *et al*., 2016). The analysis of a larger sampling of *Ameroglossum* species and related genera may contribute to characterize these taxa karyotypically and shed some light on their relationships with other Linderniaceae genera.

This paper presents the chromosome numbers of 14 representatives of Linderniaceae, with emphasis on the genus *Ameroglossum*. A detailed analysis of CMA and DAPI bands and

number of 5S and 35S rDNA sites of 12 of these species was done to evaluate the interspecific variability of this group. In addition, we analyzed the karyotypical similarity between *Ameroglossum* and the other monospecific genera endemic to the northeastern Brazilian inselbergs (*Catimbaua*, *Isabelcristinia,* and *Cubitanthus*). For comparison purposes, we analyzed the karyotype of two other Linderniaceae species typical of humid environments, belonging to the genera *Torenia* and *Vandellia*.

MATERIAL AND METHODS

BOTANICAL COLLECTION AND DOCUMENTATION

Samples of each species of *Ameroglossum* and related genera were collected from different locations totaling 14 *taxa*: nine species of *Ameroglossum*, one species from *Isabelcristinia*, *Catimbaua*, *Cubitanthus*, *Torenia* and *Vandellia*. The specimens collected for cytological analysis were kept in cultivation at the Experimental Garden of the Plant Cytogenetics Laboratory of the Department of Biological Sciences, Center for Agricultural Sciences, Federal University of Paraíba (CCA-UFPB). Exsicates of all analyzed material were deposited in the Herbarium EAN.

CYTOLOGICAL ANALYSIS AND CHROMOSOME PREPARATION

Root tips were pre-treated with 8-hydroxyquinoline for 24h at 10 °C and fixed in ethanol-acetic acid (3:1; v/v) for 2h at room temperature. For mitotic analysis, the air-drying protocol was adopted according to Carvalho & Saraiva (1993) with some modifications. The roots were washed and incubated at 37 °C in a 2% cellulase enzyme solution (Onokuza) and 20% pectinase (Sigma) for 60 minutes. Subsequently, they were transferred to an inclined slide moistened with a cold, fresh fixative, and washed with this fixative during their fragmentation with the help of needles. The slides were air-dried and dipped in a 45% acetic acid solution for

30 minutes. The best preparations were stained with CMA (0.1 mg mL^{-1}) for 1h, mounted in glycerol: McIlvaine pH 7.0 buffer (1:1) containing 2.5 mM $MgCl_2$ plus DAPI (1µg mL^{-1}) (Vaio *et al*., 2018) and captured in a Leica DMLB epifluorescence microscope with a Cohu CCD camera equipped with Leica QFISH software.

FLUORESCENT *IN SITU* HYBRIDIZATION

CMA/DAPI stained chromosomes were destained in ethanol:acetic acid (3:1, v/v) for 30 min and absolute ethanol for 2 h, both at room temperature, and used for FISH. *In situ* hybridization was performed according to Pedrosa *et al*. (2002). For localization of the rDNA sites, a 500bp 5S DNA clone (D2) from *Lotus japonicus* (Regel) K.Larsen (Pedrosa *et al*., 2002) labelled with Cy3-dUTP (Amersham) and a 35S DNA clone 6.5kb (R2) of *Arabidopsis thaliana* (L.) Heynh. (Wanzenbock *et al*., 1997) labelled with digoxigenin 11-dUTP (Roche) were used as probes. The probes were labelled by nick translation and the 35S rDNA was detected with FITC-conjugated sheep anti-digoxigenin antibody (Roche) and amplified with FITC-conjugated rabbit anti-sheep antibody (Dako). The slides were mounted in glycerol-McIlvaine pH 7.0 buffer (1:1) containing 2.5 mM $MgCl_2$ and 1µg / mL of DAPI and the images were captured as previously described. All images were latter edited in Adobe Photoshop CS6 v.13.0 for brightness, contrast and sharpening.

RESULTS

GENERAL KARYOLOGICAL FEATURES

Of the nine species of *Ameroglossum* investigated, eight presented $2n = 60$ and only *Ameroglossum genaroanum* E.M.Almeida, J.M.P.Cordeiro & L.P.Felix presented $2n = 64$ (Table 1). *Catimbaua pendula* and *I. aromatica* also had $2n = 60$, while *Cubitanthus alatus*

(Cham. & Schltdl.) Barringer had 2*n* = 50, and *Vandellia diffusa* L. and *Torenia thouarsii* (Cham. & Schltdl.) Kuntze had 2*n* = 28. All species had small chromosome size and similar morphology, predominantly metacentric to submetacentric. Figure 1 shows the karyograms of *A. pernambucense*, *A. genaroanum*, *C. alatus* and *I. aromatica* with chromosome ordered from the largest to the smallest size. The karyotypes of *Ameroglossum*, *Isabelcristinia* and *Catimbaua* presented a small but gradual size variation between chromosomes, while in *Cubitanthus*, *Torenia* and *Vandellia* the difference between the smallest and the largest chromosome was much smaller. In *C. alatus*, excepting the largest pair, the difference among the other 24 pairs was very small. The two species with low chromosome numbers, *T. thouarsii* and *V. diffusa*, also had chromosomes very similar to each other (Fig. 2A, B). The chromosome pair with the highest variation among all species was that with the CMA^+ band colocalized with 35S rDNA sites. Figure 2C show that they varied substantially between species regarding their chromosome size, satellite size (more visible in DAPI images), and position of CMA^+ band (proximal or terminal).

Among species with 2*n* = 50-64, there was a small variation in the number of CMA/DAPI bands and rDNA sites, with only one 5S and one 35S rDNA sites located on separate chromosomes (Table 2). Only in two species, *Ameroglossum alatum* E.M.Almeida, A.M.Wanderley & L.P.Felix and *Ameroglossum fulniorum* E.M.Almeida, A.M.Wanderley & L.P.Felix, it was not possible to investigate the number of rDNA sites. The 5S and 35S rDNA sites were always co-located with CMA^+ bands (referred here as CMA/5S and CMA/35S bands), although the bands corresponding to the 5S rDNA sites were generally only slightly brighter with CMA than with DAPI. The CMA/35S bands were always located in one of the largest chromosomal pairs, forming secondary constrictions, and adjacent to one or two $DAPI^+$ bands of variable size, whereas the CMA/5S occupied a more variable relative position (Fig. 1). Aside from these bands, other CMA^+, $DAPI^+$ or chromatin blocks brilliant with both

fluorochromes have been seen in almost all species. After FISH, the weak $DAPI^+$ bands generally became brighter.

CMA/DAPI BANDS AND rDNA SITES IN SPECIES OF *AMEROGLOSSUM*

Besides the general characteristics, there were some karyotype particularities of each species, mainly related to the heterochromatic bands and rDNA sites. The two populations of *A. genaroanum* analyzed showed proximal CMA/35S bands, flanked by two $DAPI^+$ bands, and terminal CMA/5S bands located on much smaller chromosomes (Fig. 3A). In most metaphases, the CMA/35S band formed a slightly distended secondary constriction and a relatively large satellite. *Ameroglossum pernambucense* exhibited proximal CMA/5S and terminal CMA/35S bands (Fig. 3B) and one other chromosomal pair with proximal CMA^+ bands (Fig. 1A). *Ameroglossum xukuruorum* E.M.Almeida, Christenh. & L.P.Felix presented the CMA/35S band forming a well-distended secondary constriction and a small satellite strongly stained with CMA and entirely labelled with the 35S rDNA probe (Fig. 3C). The 5S rDNA sites stood out for being proximal and colocalized with a shiny CMA band, located on a medium-sized chromosome (Fig. 3C). In *Ameroglossum bicolor* E.M.Almeida, A.M.Wanderley & L.P.Felix the CMA/35S band was proximal, in the largest chromosomal pair, while the CMA/5S band was apparently proximal and located in one of the smallest chromosomal pairs of the karyotype. The chromosome with the CMA/35S band presented a poorly differentiated $DAPI^+$ band occupying half of the chromosome length (Fig. 3D, D´). In two samples of *Ameroglossum intermedium* E.M.Almeida, A.M.Wanderley & L.P.Felix, only three CMA^+ bands were observed. In the individual analyzed with FISH (LPF-14744), the CMA/35S band was terminal while the CMA/5S band was proximal and heteromorphic (Fig. 3E, E´), but only the larger 5S rDNA was seen as CMA^+, possibly because the 5S rDNA sites of *Ameroglossum* were, in general, poorly differentiated with CMA, making small like this hardly detectable. In

Ameroglossum asperifolium E.M.Almeida, J.M.P.Cordeiro & L.P.Felix, six chromosomes with proximal CMA^+ bands were observed. The chromosome pair with CMA/35S band had a very distended secondary constriction, separating the chromosomes in two halves, a $DAPI^+$ terminal band at one chromosome end and a neutral chromatin with DAPI at the other. After FISH, both chromosome termini were brilliant with DAPI (Fig. 3F, F´). The 5S rDNA site was terminal in this species.

In *A. alatum*, the secondary constriction was proximal, separating the chromosome into two halves, each with a $DAPI^+$ terminal band (inset in Fig. 3G), identical to *A. genaroanum.* *Ameroglossum manoelfelixii* presented a terminal CMA/35S band, with an adjacent $DAPI^+$ band (compare insets of the largest pair in Fig. 4A, A´), and at least six other heterochromatic blocks positively stained with both DAPI and CMA, including a CMA/5S band in a very small chromosome pair (Fig. 4A, A´). For *A. fulniorum*, a pattern of CMA^+ bands similar to *A. manoelfelixii* was observed (Fig. 4B), but its rDNA sites were not analyzed.

CMA/DAPI BANDS AND rDNA SITES IN OTHER LINDERNIACEAE

The representatives of *Isabelcristinia*, *Catimbaua*, and *Cubithanthus* presented chromosomes similar to *Ameroglossum*, with only a pair of 5S and 35S rDNA sites. In *I. aromatica* ($2n = 60$), four weak proximal $DAPI^+$ bands and eight to twelve CMA^+ bands were observed (Supporting Information, Fig. S1), including two proximal CMA/5S bands and two terminal CMA/35S bands (insets in Fig. 4C, C´). Some $DAPI^+$ bands were also slightly positive with CMA. *Catimbaua pendula* ($2n = 60$) presented four CMA^+ bands and four large $DAPI^+$ bands, two of them were adjacent to the CMA/35S bands (insets in Fig. 4D, D'). In this species, the largest and brightest CMA^+ bands were the CMA/5S (Fig. 4D´). *Cubitanthus alatus* ($2n = 50$) was the only species with $DAPI^+$ bands in the proximal region of all chromosomes, except those with CMA^+ bands (Fig. 4E). The CMA/35S bands were in the proximal region of the

largest chromosomal pair, showing a small satellite stained with DAPI (Fig. 2C), while the CMA/5S bands were located proximally in one of the smallest chromosome pairs (insets in Fig. 4F).

Vandellia diffusa and *T. thouarsii* presented 2*n* = 28 presented two pairs of very small CMA/5S and one or two pairs, respectively, of CMA/35S bands (Fig. 2A-C). *Torenia thouarsii* displayed $DAPI^+$ bands on the short arm of most chromosomes, which became more evident after FISH (Fig. 4G, G´), whereas in *V. diffusa* the pericentromeric proximal regions of eight to ten chromosomes were more intensely stained with DAPI than with CMA (Fig. 4H, H´).

DISCUSSION

NUMERICAL CHROMOSOMAL VARIATION IN LINDERNIACEAE

The present analysis confirmed the chromosome number 2*n* = 60 for *C. pendula, I. aromatica, A. manoelfelixii* and *A. pernambucense* (Almeida *et al*., 2016, 2019) and report for the first time the chromosome numbers of *C. alatus, V. diffusa, T. thouarsii, A. alatum, A. asperifolium, A. bicolor, A. fulniorum, A. genaroanum, A. intermedium* and *A. xukuruorum*. In the two most studied genera, *Torenia* and *Vandellia*, dominate the numbers 2*n* = 18 and 2*n* = 16, respectively, while *Artanema* and *Craterostigma* had, respectively, one (2*n* = 40) and two species (2*n* = 42 and 2*n* = 112) counted. However, species with *n* = 10 or multiples of 10 (2*n* = 20, 30, 40, 50, 60) occur in all genera, except *Craterostigma*, suggesting that the basic number of the family could be *x* = 10 (Supporting Information, Table. S1). In this case, the species of *Ameroglossum*, *Catimbaua* and *Isabelcristinia*, with 2*n* = 60, would be hexaploids, while those of *Lindernia, Torenia, Artanema, Bonnaya* and *Vandellia,* would be diploids or tetraploids predominantly with secondary basic numbers derived from *x* = 10. *Vandellia diffusa* and *T. thouarsii* with 2*n* = 28 may be tetraploids, since *n* = 7 have been reported for other species of this group, including two species of *Torenia* (Table S1). *Cubitanthus alatus* with 2*n* = 50 is

quite similar in several chromosomal features to the $2n = 60$ group, suggesting that it is a hexaploid derived from $2n = 60$ by dysploidy. Dysploid reductions are common in several other polyploid groups and seem to have played an important role in the evolution and maintenance of polyploid lineages (Levin, 2020).

Aside from the karyotype similarity, *Cubitanthus* and the other hexaploid Linderniaceae have some particularity that distinguish them from the rest of the family, as a distribution in small populations widely separated from each other and restricted to inselbergs of northeastern Brazil (Almeida *et al*., 2019). They are perennial subshrubs and adapted to extreme environments, with limited availability of water, as many other inselberg plants (Porembski, 2007). In contrast, the other genera of Linderniaceae that occur in Brazil are represented by herbaceous species that grow associated with open, submerged or coastal aquatic environments (Souza & Giulietti, 2009). All these data suggest the separation of the saxicolous genera into a distinct clade within Linderniaceae. The distribution of these genera in inselbergs may be related to the higher tolerance of polyploids to extreme habitats (Ramsey & Ramsey, 2014). Polyploids with high chromosome number have the potential advantage of the heterosis effect, higher phenotypic variation and gene redundancy to invade new environments (Pandit, White & Pocock, 2014). However, analyses of several plant taxa characteristics of inselberg did not reveal a higher abundance of polyploids in relation to the same taxa from terrestrial environments (Pitrez *et al*., 2014). Therefore, the potential advantage of superior polyploids is not enough to guarantee a successful invasion of such an extreme habitat as the tropical inselbergs.

VARIATION OF rDNA SITES AND HETEROCHROMATIC BANDS

The number of rDNA sites was reduced to a single pair of each sequence inside the hexaploid group, whereas in both putative tetraploids there were two pairs of 5S rDNA and one

pair of 35S rDNA sites. The small number of sites in hexaploids is probably due to a general trend among angiosperms for a reduction to only one pair of 5S and 35S rDNA sites per karyotype (Roa and Guerra, 2012). In this case, the reduction was stronger among hexaploids than in tetraploids.

The number of rDNA sites has been reduced to a single pair in the hexaploid group, whereas in both putative tetraploids there were two pairs of 5S rDNA and one pair of 35S rDNA sites. This apparent contradiction seems to be part of the diploidization process, which often includes reduction of the monoploid genome size, loss of rDNA repeats (Leitch and Bennett, 2004), and also reduction in the number of rDNA sites and CMA^+ bands. Similar results have been observed in several other genera, as *Passiflora* (Melo and Guerra, 2003) and *Cuscuta* (Ibiapino *et al*., 2019).

The staining intensity of CMA and DAPI bands depends on the GC/AT content of their repetitive sequences and the condensation level of the chromatin, which in metaphase is usually uniform. The 35S rDNA repeat has often a high GC content, resulting in $CMA^+/DAPI^-$ bands, whereas the 5S rDNA has a lower and more variable GC content (see, *e.g*., Grabiele *et al*., 2012; Waminal *et al*., 2014) and is more commonly undifferentiated by CMA/DAPI staining. Thus, CMA/5S bands are less common in angiosperms, but they are generally conserved throughout the genus (Bareka *et al*., 2012; Marinho *et al*., 2018). In the Linderniaceae investigated here, the 5S rDNA sites were positively stained with CMA, but in a highly variable intensity suggesting that the more variable component of the 5S rDNA, the non-transcribed spacers (NTS), probably differ in its GC content among species, potentially explaining the differential staining patterns. In addition, the presence of CMA^+ bands non-colocalized with rDNA sites reveals the existence of other GC-rich DNA sequences, as observed in other genera (Barros e Silva *et al*., 2010; Van-Lume *et al*., 2017).

CYTOTAXONOMIC CONSIDERATIONS

Although a molecular phylogenetic analysis is necessary to reconstruct the evolutionary history of these groups, the chromosomal number, apparent karyotype symmetry, and heterochromatin pattern of *Ameroglossum*, *Isabelcristinia*, *Catimbaua* and *Cubitanthus* species suggest a close relationship between all representatives of Linderniaceae typical of inselbergs with known chromosome number. Differences in the location of the CMA/5S and CMA/35S bands among these species may be related to structural changes fixed by chance during their geographic and ecological isolation, as observed for example in the Caesalpinia group (Van-Lume *et al*., 2017). In general, karyotype differences were more evident in species ecologically more differentiated, as *A. genaroanum*, which has a more northern distribution and occurs in lower altitudes (Almeida *et al.*, 2021) and was the only species with $2n = 64$. Morphometric and microsatellite analysis of *A. pernambucense* and *A. manoelfelixii* showed that genetic and phenotypic variations are primarily associated with climatic differences and geographical distance (Wanderley *et al*., 2018). On the other hand, morphologically close species, such as *A. pernambucense* and *A. xukuruorum*, which occur in the higher regions of the Borborema Plateau (Almeida *et al.* 2021), differed in the location of CMA/5S bands (proximal in *A. xukuruorum* and terminals in *A. pernambucense*). Therefore, in spite of the stable chromosome number and reduced chromosome size, the hexaploid species revealed several small differences in the number, position and size of CMA/DAPI bands and 5S and 35S rDNA sites, which may represent the chromosomal changes fixed by chance during their geographic and ecological isolation.

Among the genera studied, *Isabelcristinia* and *Ameroglossum* presented more similar karyotypes suggesting that they are phylogenetically closer. Moreover, *I. aromatica* and *A. pernambucense* occupy geologically similar habitats (granitic outcrops) and geographically close. *Catimbaua pendula* occurs in sandstone outcrop, differed from the other polyploid genera

mainly by its large DAPI$^+$ blocks, whereas *Cubitanthus alatus*, the cytologically most divergent species, presented reduced chromosome number, more similar chromosome sizes, and presence of proximal DAPI$^+$ bands in almost all chromosomes. *Torenia* and *Vandellia*, on the other hand, typical of a humid environment, were karyotypically more similar to each other than to the hexaploids, mainly by their lower chromosome number ($2n = 28$) and higher number of rDNA sites.

CONCLUSIONS

The Linderniaceae species differed karyologically in chromosome number, banding pattern, apparent karyotype symmetry, and distribution of rDNA sites. Inselberg genera differed from non-inselberg ones by ploidy level ($6x$ versus $2x$, respectively), whereas absolute chromosome number distinguish *Cubitanhus alatus*, $2n = 50$, and *A. genaroanum*, $2n = 64$, from the karyotype of the remaining inselberg species, $2n = 60$. Small variations in the karyotype symmetry and in the number and position of CMA/DAPI bands and rDNA sites distinguish less clearly most other species. Differently, the non-inselberg Linderniaceae genera, *Torenia* and *Vandellia*, with $2n = 28$ and aquatic habitat, were more similar to each other than with the rupicolous genera of Linderniaceae, suggesting the existence of at least two main groups of species within the family. On the other hand, the presence of CMA/5S bands in all analyzed species seems to be a striking karyological feature of this family. Karyotype analyses of a larger sample of non-inselberg Linderniaceae is necessary to understand their relationship with the inselberg species.

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LEGENDS

Figure 1. Karyograms of *Ameroglossum pernambucense* (A), *A. genaroanum* (B), *Cubitanthus alatus* (C), and *Isabelcristinia aromatica* (D). The cells were simultaneously stained with DAPI (blue) and CMA (yellow) but the DAPI brightness was intensified, in order to show even the smallest and palest chromosomes. Therefore, only the brightest CMA^+ bands, which were co-localized with 5S or 35S rDNA sites, are visible and indicated at the top of the chromosomes. Bar in D corresponds to 2.5 µm.

Figure 2. Karyograms of *Vandellia diffusa* (A) and *Torenia thouarsii* (B) stained with CMA/DAPI and DAPI (blue) and a comparison of chromosomes bearing the CMA/35S band, with respective $DAPI^-$ band, in all 14 species (C). DAPI brightness (blue) was intensified, as in Figure 1, and only the brightest CMA^+ bands (yellow), co-localized with 5S or 35S rDNA sites (see Figures 3 and 4), are visible and indicated at the top of the chromosomes. Abbreviations in C: Abi, *A. bicolor*; Age, *A. genaroanum*; Aas, *A. asperifolium*; Axu, *A. xukuruorum*; Aal, *A. alatum*; Amf, *A. manoelfelixii*; Ape, *A. pernambucense*; Afu, *A. fulniorum*; Cal, *C. alatus*; Iar, *I. aromatica*; Cpe, *C. pendula*; Vdi, *V. diffusa*; Tth, *T. thouarsii*. Bar in C corresponds to 2.5 µm.

Figure 3. Metaphases and prometaphases of *Ameroglossum* showing CMA/DAPI images and rDNA sites. A - *A. genaroanum*; B - *A. pernambucense*; C - *A. xukuruorum*; D - *A. bicolor*; E - *A. intermedium*; F - *A. asperifolium*; G - *A. alatum*. Insets show magnified chromosomes with CMA^+ bands (yellow), 5S (red) and 35S (green) rDNA sites. For *A. bicolor*, the picture in D shows the chromosome size and morphology and CMA/DAPI bands whereas D' shows 5S and 35S rDNA merged images in another cell. Distended secondary constrictions were highlighted

in C-C', F-F', and G. Observe DAPI$^+$ bands adjacent to the CMA/35S bands in most insets. Asterisks in A' and F' indicate background. Bar in D' and G corresponds to 2.5 μm.

Figure 4. Metaphases and prometaphases showing CMA/DAPI bands and rDNA sites in *Ameroglossum manoelfelixii* (A), *A. fulniorum* (B), *Isabelcristinia aromatica* (C), *Catimbaua pendula* (D), *Cubitanthus alatus* (E, F), *Torenia thouarsii* (G), and *Vandellia difusa* (H). Insets highlight chromosomes with CMA bands (yellow), 5S (red) and 35S (green) rDNA sites and DAPI bands after FISH in G'. Arrows in D, E and G' points chromosome with DAPI$^+$ bands. In H arrows point to CMA/35S bands. Asterisks in A´ and D' point to background. Bar in H corresponds to 2.5 μm.

Figure S1. Metaphase of *Isabelcristinia aromatica* showing some DAPI$^+$ bands (A), CMA$^+$ bands (A'), and merged CMA/DAPI images (A''). There are four large CMA$^+$ bands adjacent to DAPI$^+$ bands (arrowheads). Arrows indicate two of the few DAPI$^+$ bands not associated to CMA$^+$ bands. The remaining bands are CMA$^+$ only or CMA$^+$ bands collocated or tightly associated to DAPI$^+$ bands (asterisks). Bar in A" corresponds to 2.5 μm.

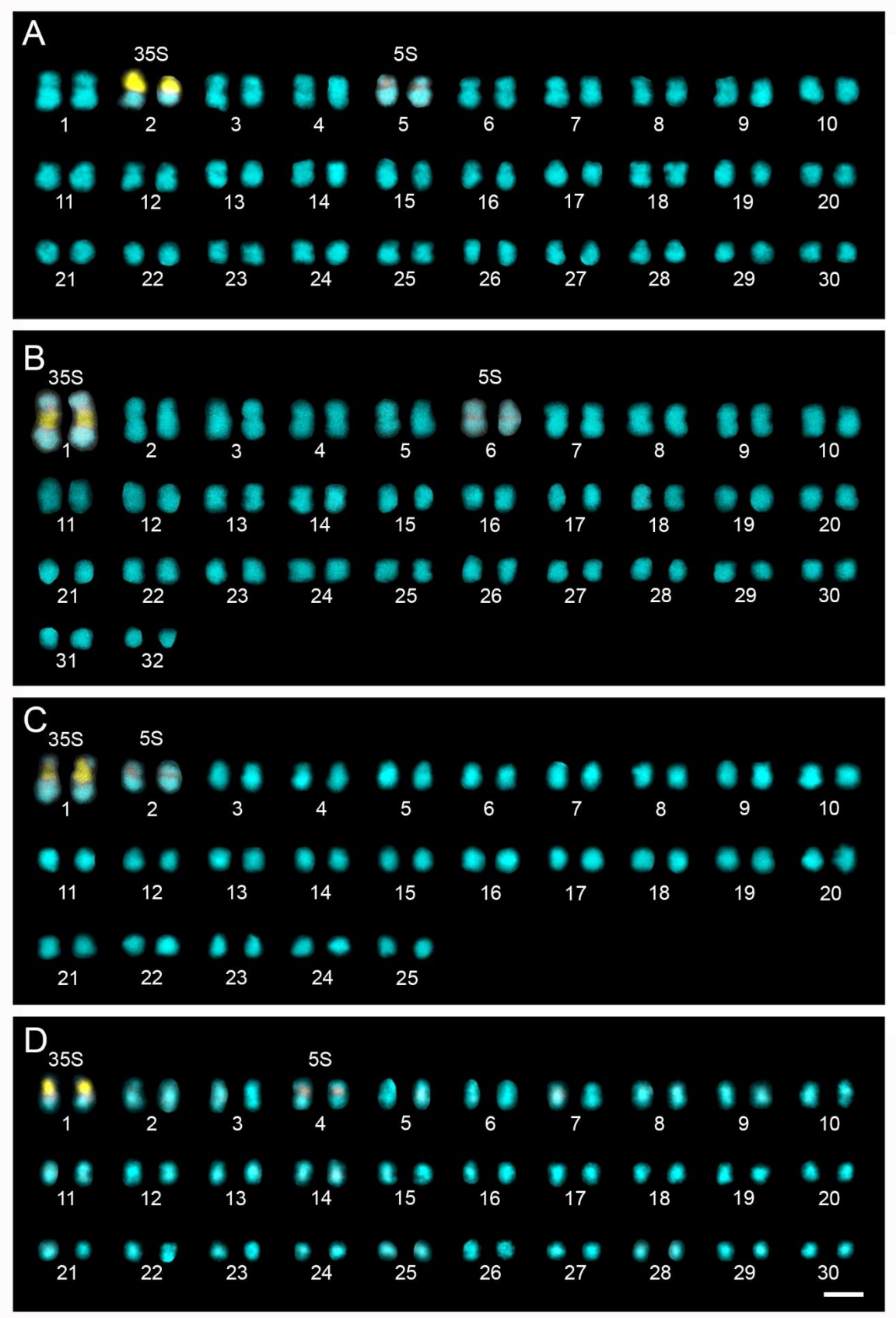

Figure 1. Karyograms of *Ameroglossum pernambucense* (A), *A. genaroanum* (B), *Cubitanthus alatus* (C), and *Isabelcristinia aromatica* (D). The cells were simultaneously stained with DAPI (blue) and CMA (yellow) but the DAPI brightness was intensified, in order to show even the

smallest and palest chromosomes. Therefore, only the brightest CMA^+ bands, which were co-localized with 5S or 35S rDNA sites, are visible and indicated at the top of the chromosomes. Bar in D corresponds to 2.5 µm.

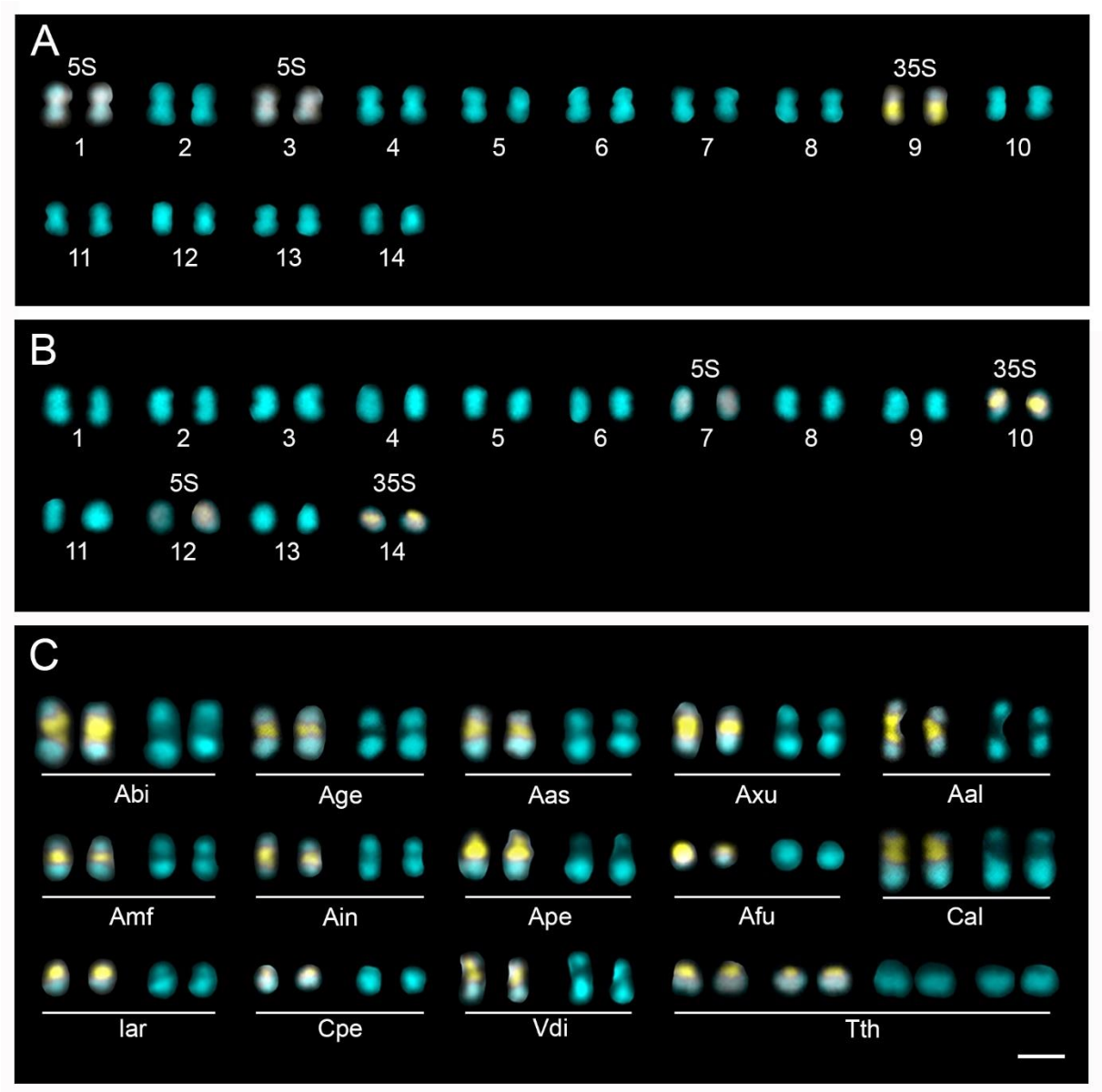

Figure 2. Karyograms of *Vandellia diffusa* (A) and *Torenia thouarsii* (B) stained with CMA/DAPI and DAPI (blue) and a comparison of chromosomes bearing the CMA/35S band, with respective $DAPI^-$ band, in all 14 species (C). DAPI brightness (blue) was intensified, as in Figure 1, and only the brightest CMA^+ bands (yellow), co-localized with 5S or 35S rDNA sites (see Figures 3 and 4), are visible and indicated at the top of the chromosomes. Abbreviations in C: Abi, *A. bicolor*; Age, *A. genaroanum*; Aas, *A. asperifolium*; Axu, *A. xukuruorum*; Aal, *A. alatum*; Amf, *A. manoelfelixii*; Ape, *A. pernambucense*; Afu, *A. fulniorum*; Cal, *C. alatus*; Iar, *I. aromatica*; Cpe, *C. pendula*; Vdi, *V. diffusa*; Tth, *T. thouarsii*. Bar in C corresponds to 2.5 µm.

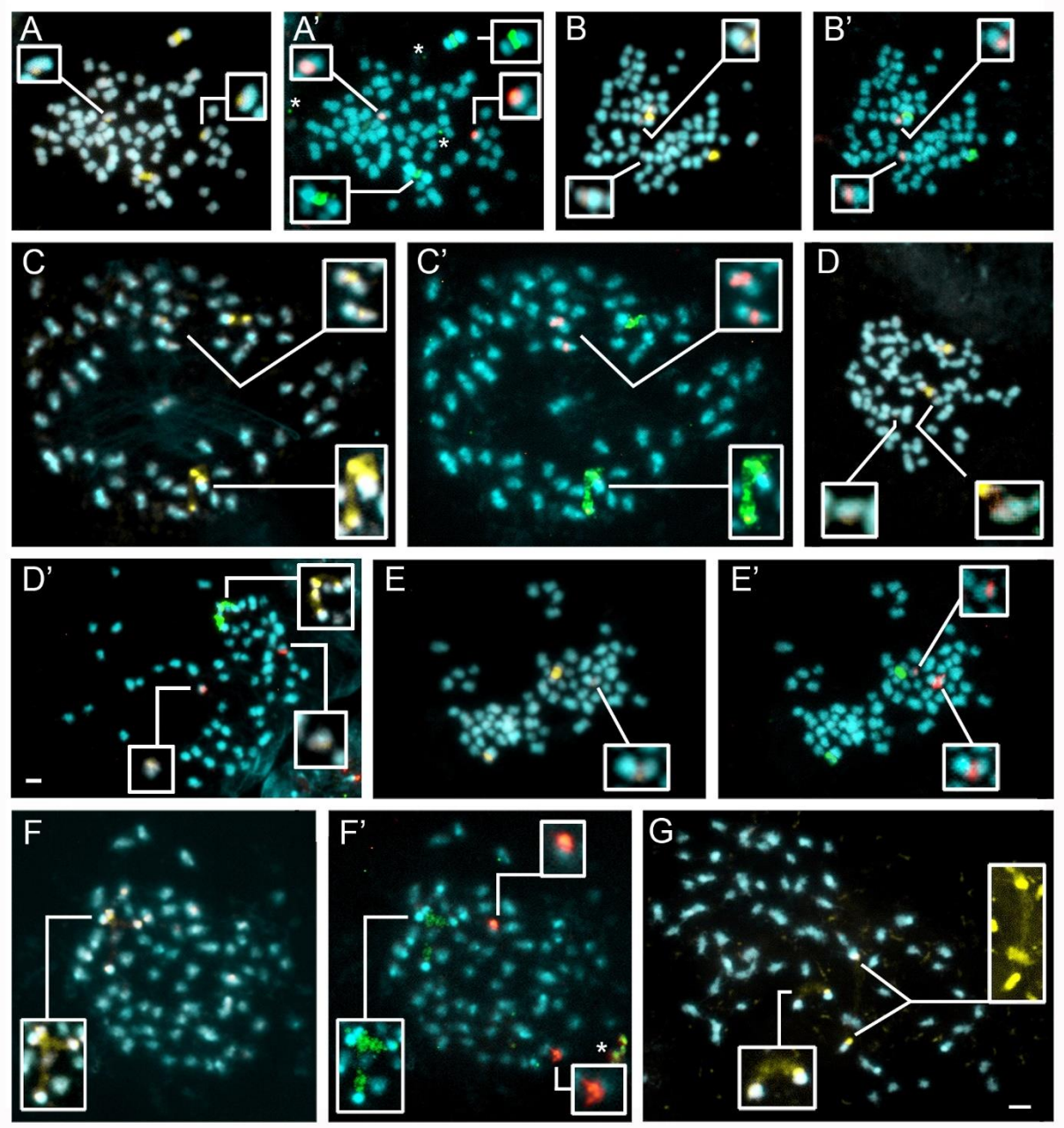

Figure 3. Metaphases and prometaphases of *Ameroglossum* showing CMA/DAPI images and rDNA sites. A - *A. genaroanum*; B - *A. pernambucense*; C - *A. xukuruorum*; D - *A. bicolor*; E - *A. intermedium*; F - *A. asperifolium*; G - *A. alatum*. Insets show magnified chromosomes with CMA^+ bands (yellow), 5S (red) and 35S (green) rDNA sites. For *A. bicolor*, the picture in D shows the chromosome size and morphology and CMA/DAPI bands whereas D' shows 5S and 35S rDNA merged images in another cell. Distended secondary constrictions were highlighted in C-C', F-F', and G. Observe $DAPI^+$ bands adjacent to the CMA/35S bands in most insets. Asterisks in A' and F' indicate background. Bar in D' and G corresponds to 2.5 μm.

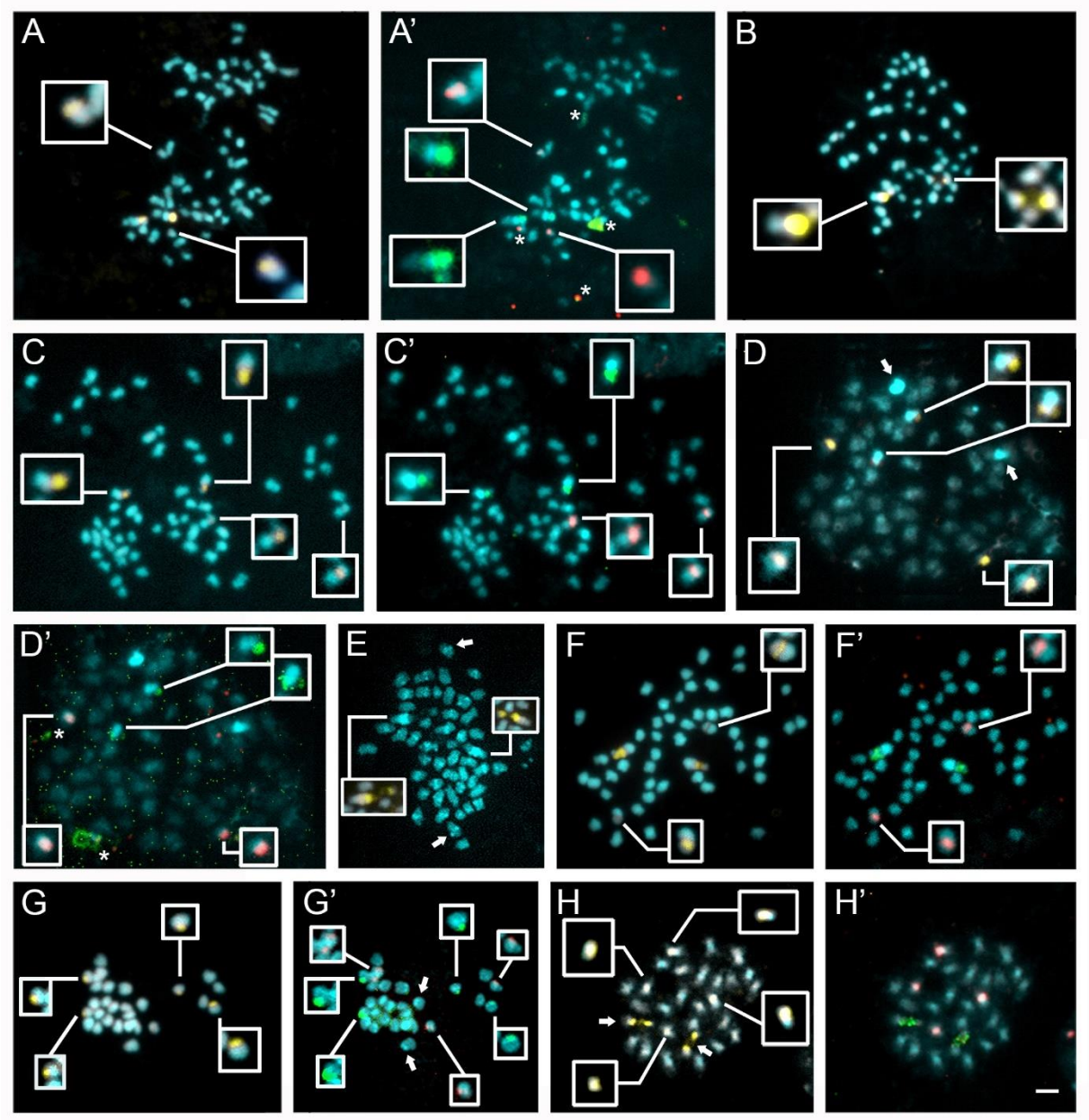

Figure 4. Metaphases and prometaphases showing CMA/DAPI bands and rDNA sites in *Ameroglossum manoelfelixii* (A), *A. fulniorum* (B), *Isabelcristinia aromatica* (C), *Catimbaua pendula* (D), *Cubitanthus alatus* (E, F), *Torenia thouarsii* (G), and *Vandellia difusa* (H). Insets highlight chromosomes with CMA bands (yellow), 5S (red) and 35S (green) rDNA sites and DAPI bands after FISH in G'. Arrows in D, E and G' points chromosome with $DAPI^{+}$ bands. In H arrows point to CMA/35S bands. Asterisks in A´ and D' point to background. Bar in H corresponds to 2.5 µm.

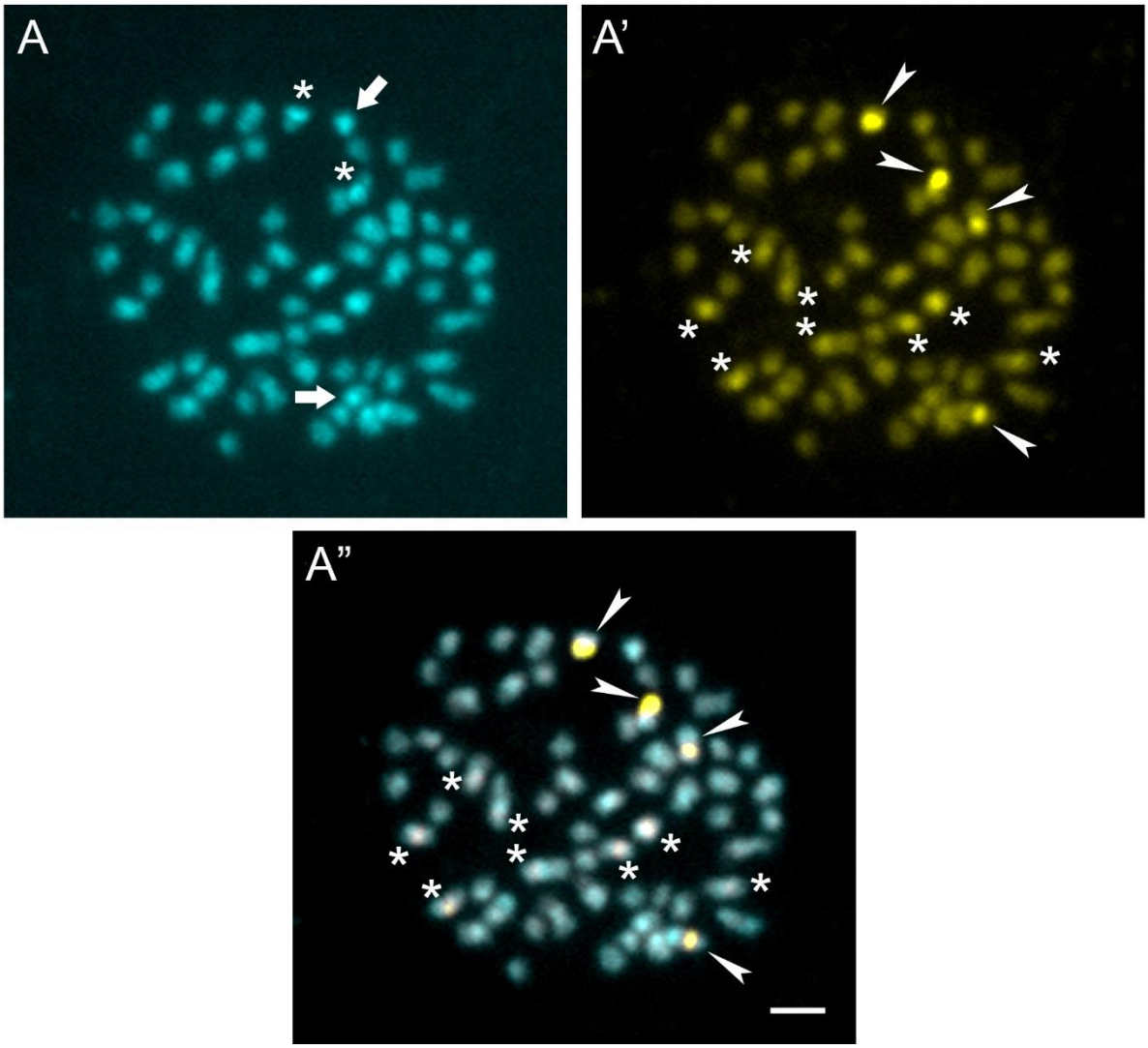

Figure S1. Metaphase of *Isabelcristinia aromatica* showing some $DAPI^+$ bands (A), CMA^+ bands (A'), and merged CMA/DAPI images (A''). There are four large CMA^+ bands adjacent to $DAPI^+$ bands (arrowheads). Arrows indicate two of the few $DAPI^+$ bands not associated to CMA^+ bands. The remaining bands are CMA^+ only or CMA^+ bands collocated or tightly associated to $DAPI^+$ bands (asterisks). Bar in A" corresponds to 2.5 µm.

Table 1. Provenance, voucher numbers, and chromosome numbers (2*n*) of the Linderniaceae species analyzed.

TAXON	PROVENANCE[a]	VOUCHER[b]	2*n*
Ameroglossum Eb.Fisch., S.Vogel & A.V.Lopes			
A. alatum E.M.Almeida, A.M.Wanderley & L.P.Felix	Maravilha, AL	EMA 450	c. 60
A. asperifolium E.M.Almeida, J.M.P.Cordeiro & L.P.Felix	Ibateguara, AL	LPF 15160	60
A. bicolor E.M.Almeida, A.M.Wanderley & L.P.Felix	Agrestina, PE	LPF 17160	60
A. fulniorum E.M.Almeida, A.M.Wanderley & L.P.Felix	Águas Belas, PE	EMA 439	60
A. genaroanum E.M.Almeida,	Tacima, PB	EMA 2537	64[c]
J.M.P.Cordeiro & L.P.Felix	Serrinha, RN	EMA 397	64[c]
A. intermedium E.M.Almeida,	Quebrangulo, AL	LPF 14744	60
A.M.Wanderley & L.P.Felix	Quebrangulo, AL	EMA 461	60
A. manoelfelixii E.M.Almeida, A.M.Wanderley & L.P.Felix	Esperança, PB	EMA 785	60
A. pernambucense Eb.Fisch., S.Vogel & A.V.Lopes	Barra de Santana, PB	EMA 816	60
A. xukuruorum E.M.Almeida, Christenh. & L.P.Felix	Pesqueira, PE	LPF 15647	60
Catimbaua L.P.Felix & E.M.Almeida			
C. pendula L.P.Felix & E.M.Almeida	Buíque, PE	EMA 1569	60
Cubitanthus Barringer			
C. alatus (Cham. & Schltdl.) Barringer	Ubaitaba, BA	EMA 2614	50[c]
Isabelcristinia L.P.Felix & E.M.Almeida			
I. aromatica L.P.Felix & E.M.Almeida	Belo Jardim, PE	LPF 15963	60
	Belo Jardim, PE	LPF 17812	60
Torenia L.			
T. thouarsii (Cham. & Schltdl.) Kuntze	Água Preta, PE	EMA 2858	28[c]
Vandellia L.			
V. diffusa L.	Ubaitaba, BA	EMA 2618	28[c]

a AL, Alagoas; PE, Pernambuco; PB, Paraíba; RN, Rio Grande do Norte; BA, Bahia
b EMA, Erton M. Almeida; LPF, Leonardo P. Felix; JMPC, Joel M. P. Cordeiro
c First counting

Table 2. Main karyotype features of the Linderniaceae species analyzed: chromosome number (2*n*), number and position of CMA and DAPI bands and 5S and 35S rDNA sites. Position of bands and sites are indicated as P (proximal) or T (terminal).

TAXON	2*n*	CMA	DAPI	5S	35S
Ameroglossum					
A. alatum	c. 60	2P	4T	-	-
A. asperifolium	60	6P	4T	2P	2P
A. bicolor	60	4P	-	2P	2P
A. fulniorum	60	4T	-	-	-
A. genaroanum	64[c]	4P + 2T	4T	2T	2P
A. intermedium	60	3P	-	2P	2T
A. manoelfelixii	60	4T	6T	2T	2T
A. pernambucense	60	4P + 2T	-	2P	2T
A. xukuruorum	60	4P	-	2P	2P
Catimbaua					
C. pendula	60	4T	4T	2T	2T
Cubitanthus					
C. alatus	50	4P	~30P	2P	2P
Isabelcristinia					
I. aromatica	60	12P	4P	2P	2T
Torenia					
T. thouarsii	28	4P	~8T	4P	4T
Vandellia					
V. diffusa	28	6P	~8P	4P	2P

Table S1. Chromosome number variation in the family Linderniaceae, arranged according to Biffin *et al*. (2018) and Almeida *et al.* (2021)

Genus / Species	*n*	2*n*	References*
Ameroglossum			
A. alatum		60	01
A. asperifolium		60	01
A. bicolor		60	01
A. fulniorum		60	01
A. genaroanum		64	01
A. intermedium		60	01
A. manoelfelixii		60	02
A. pernambucense		60	02, 03
A. xukuruorum		60	01
Artanema D.Don			
A. longifolium (L.) Vatke		40	04
Bonnaya Link & Otto			
B. ciliata Spreng.	7, 9, 12	18	05, 06, 07
B. oppositifolia Spreng.		18	08
B. ruelloides (Colsm.) Spreng.		18	06
B. tenuifolia Spreng.	8, 9	16, 18	06, 08
Catimbaua			
C. pendula		60	03
Cubitanthus			
C. alatus		50	01
Craterostigma Hochst.			
C. nummulariifolium (D. Don) Eb.Fisch., Schäferh. & Kai Müll	12		09
C. plantagineum Hochst.		112	10
Isabelcristinia			
I. aromatica		60	03
Lindernia All.			
L. antipoda (L.) Alston	9, 11, 18	18, 22, 36	05, 06
L. dubia (L.) Pennell	10	20	11
L. hyssopoides (L.) Haines	10	18	04, 06
L. parviflora Haines	10, 13	20, 26	06, 12
L. procumbens (Krock.) Philcox		24, 30	06, 13
Linderniella Eb.Fisch., Schäferh. & Kai Müll.			
L. brevidens (Skan) Eb.Fisch., Schäferh. & Kai Müll.		28	10

Torenia			
T. anagallis (Burm.f.) Wannan, W.R.Barker & Y.S.Liang	9, 11	22	05, 06
T. asiatica L.	10	20	06
T. benthamiana Hance		36	14
T. bicolor Dalzell		16	04
T. concolor Lindl.		34	14
T. cordifolia Roxb.	16		09
T. crustacea (L.) Cham. & Schltdl.	7, 16, 21	14, 28, 42	07, 08, 15, 06
T. flava Buch.-Ham. ex Benth.		16	16
T. fournieri Linden ex E. Fourn.	9	18	16
T. thouarsii		28	01
T. violacea (Azaola) Pennell		18	17
Vandellia			
V. diffusa		28	01
V. micrantha (D.Don) Eb.Fisch., Schäferh. & Kai Müll.	9	18	06
V. subracemosa (De Wild.) Eb.Fisch., Schäferh. & Kai Müll.	28		10
V. vogelii (Skan) Eb.Fisch., Schäferh. & Kai Müll.		14	18
Yamazakia W.R.Barker, Y.S.Liang & Wannan			
Y. pusilla (Willd.) W.R.Barker, Y.S.Liang & Wannan	20	20, 40	06
Y. viscosa (Hornem.) W.R.Barker, Y.S.Liang & Wannan	10, 19	38	06, 19

* **01**: This work; **02**: Almeida *et al*., (2016); **03**: Almeida *et al*., (2019); **04**: Chandran & Bhavanandan (1981); **05**: Chandran & Bhavanandan (1983); **06**: Bhattacharyya (1968); **07**: Bala & Gupta (2012); **08**: Subramanian & Pondmudi (1987); **09**: Mehra & Vasudevan (1972); **10**: VanBuren *et al*., (2018); **11**: Delgado, Gallego & Rico (2015); **12**: Bir & Sidhu (1980); **13**: Probatova *et al*., (2008); **14**: Hsieh & Yang (2002); **15**: Vij & Kashyap (1976); **16**: Kikuchi *et al*., (2006); **17**: Darlington & Wylie (1956); **18**: Mangenot & Mangenot (1962); **19**: Sarkar, Datta & Chatterjee (1980).

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ANEXO A – Normas de submissão da revista Botanical Journal of the Linneas Society - https://academic.oup.com/botlinnean/pages/General_Instructions

Author Guidelines

Introduction

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Manuscripts that are not instantly rejected are sent out for peer review, usually to two independent reviewers. Based on the feedback from these reviewers and the Editors' judgment a decision is given on the manuscript. The average time from submission to first decision is c. nine weeks.

ANEXO B – Normas de submissão da revista Molecular Phylogenetics and Evolution – https://www.elsevier.com/journals/molecular-phylogenetics-and-evolution/1055-7903/guide-for-authors

MOLECULAR PHYLOGENETICS AND EVOLUTION

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DESCRIPTION

Molecular Phylogenetics and Evolution is dedicated to bringing Darwin's dream within grasp - to "have fairly true genealogical trees of each great kingdom of Nature." The journal provides a forum for molecular studies that advance our understanding of **phylogeny** and **evolution**, further the development of phylogenetically more accurate **taxonomic classifications**, and ultimately bring a **unified classification** for all the ramifying lines of life.

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