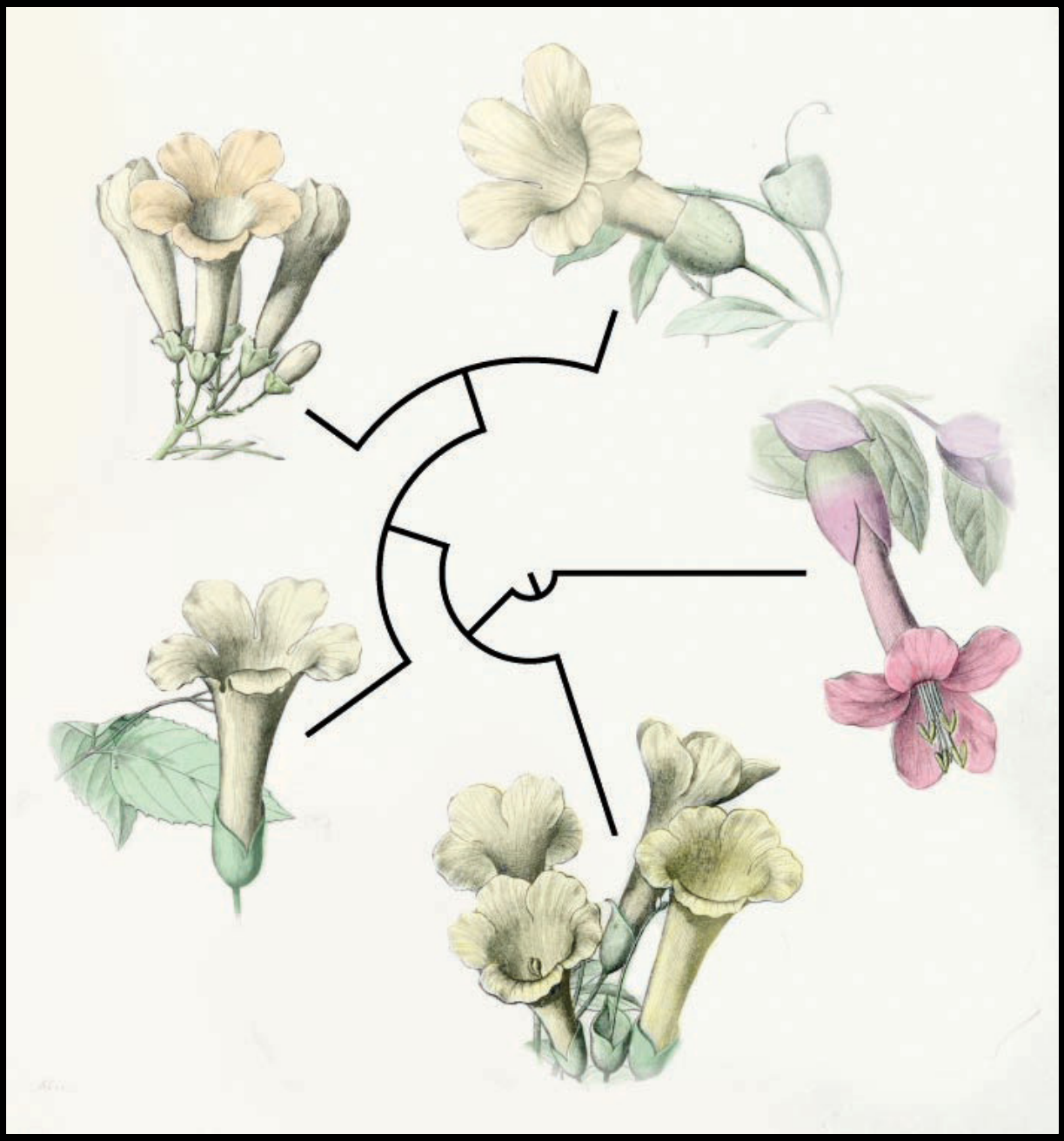
Luiz Henrique Martins Fonseca

Filogenia molecular do gênero *Dolichandra s.l.* (Bignonieae, Bignoniaceae)



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Filogenia molecular do gênero *Dolichandra s.l.* (Bignonieae, Bignoniaceae)

Molecular phylogeny of the genus *Dolichandra s.l.* (Bignonieae, Bignoniaceae)

Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Mestre em Ciências, na Área de Botânica.

Orientadora: Lúcia G. Lohmann

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“ E não há melhor resposta

que o espetáculo da vida:

vê-la desfiar seu fio,

que também se chama vida,

ver a fábrica que ela mesma,

teimosamente, se fabrica...”

Morte e Vida Severina
(João Cabral de Melo Neto)

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RESUMO

Dolichandra s.l. é um gênero de lianas com distribuição neotropical pertencente a família Bignoniaceae, tribo Bignonieae. A atual circunscrição do gênero foi estabelecida com base em uma filogenia molecular da tribo, a qual foi interpretada a luz de caracteres morfológicos. Atualmente são reconhecidas 10 espécies, duas delas propostas nesta dissertação. Espécies de *Dolichandra s.l.* apresentam grande variação em sua distribuição geográfica e habitats ocupados, bem como em caracteres morfológicos relacionados aos sistemas de polinização e dispersão. O presente estudo teve como objetivos: (1) reconstruir a filogenia de *Dolichandra s.l.* utilizando os marcadores moleculares plastidiais *ndh*F e *rps*32-*trn*L e o marcador nuclear *Pep*C, e (2) utilizar a filogenia como base para inferir os processos que atuaram na especiação do gênero. Amostras de quatro grupos externos e 20 indivíduos de *Dolichandra s.l.* foram obtidas, totalizando nove espécies. Filogenias bem resolvidas para os três marcadores amostrados revelaram que tanto o gênero, como as espécies com mais de um indvíduo amostrados são monofiléticas. *D. unguiculata* é a primeira linhagem à divergir dentro da família, sendo grupo-irmão das demais espécies do gênero em todas as topologias. *D. acuminata* e *D. quadrivalvis* formam um clado bem sustentado em todas as topologias. Esse clado emergiu para todos os marcadores e critérios de reconstrução como irmão do clado formado por *D. dentata, D. hispida, D. uncata*, and *D. unguis-cati.* Nesse último clado, *D. uncata* sempre aparece como irmã das espécies restantes em todas as topologias. Por outro lado, o posicionamento de *D. chodatii, D. cynanchoides, D. dentata* e *D. hispida* não está bem sustentado na árvore combinada, refletindo diferenças na topologia entre os marcadores plastidiais e o marcador nuclear. Todos os nós da filogenia apresentam algum grau de simpatria entre suas linhagens, indicando a importância da diferenciação de nichos para a diversificação do gênero e a prevalência de especiação simpátrica no grupo.

PALAVRAS CHAVE: Biologia comparada – Neotrópicos – Especiação – Lianas – Diversificação – *D. acuminata* – *D. hispida* – Chave taxonomica – *ndh*F – *Pep*C – *rpl*32-*trn*L.

ABSTRACT

Dolichandra s.l. is a genus of lianas found in the Neotropics. It belongs to the family Bignoniaceae, tribe Bignonieae. The actual circumscription of the genus was based on a molecular phylogeny of the tribe and morphological synanpomorphies. Currently the genus comprises ten species, two of than proposed in this dissertation. Species of *Dolichandra s.l.* have great variation in geographic distribution, habitats occupied, as well as, morphological characters related to pollination and dispersal events. The goals of this study are: (1) Reconstruct the phylogeny of *Dolichandra s.l.* using the plastid markers *ndh*F e *rps*32-*trn*L and the nuclear marker *Pep*C, and (2) use the phylogeny as base to infer process that acted in genus diversification. A sample from four outgroups and 20 individuals were obtained, accounting nine species of *Dolichandra s.l.* Well resolved phylogenies for the three markers revealed that the genus and the multiple sampled species are monophyletic. *D. unguiculata* is the first species to diverge, being sister of a clade with the rest of the genus in all topologies. *D. acuminata* as sister of *D. quadrivalvis* is a well supported clade in all topologies. This clade emerged for all markers and criteria as sister of clade with *D. dentata, D. hispida, D. uncata*, and *D. unguis-cati.* In the latter clade, *D. uncata* emerged as sister of the other species in all topologies. On the other hand, the phylognetic position of *D. chodatii, D. cynanchoides, D. dentata* and *D. hispida* is not well supported in the combined analysis, revealing differences in topology between plastid and nuclear markers. All the nodes of the phylogeny have some degree of sympatry between lineages, indicating the importance of niche differences for the genus diversification and the prevalence of sympatric speciation.

KEY WORDS: Comparative biology – Neotropics – Speciation – Lianas – Diversification – *D. acuminata* – *D. hispida* – Taxonomic key – *ndh*F – *Pep*C – *rpl*32-*trn*L.

SUMÁRIO

INTRODUÇÃO GERAL

GRUPO DE ESTUDO

Dolichandra s.l. é um gênero com distribuição neotropical que pertence à família Bignoniaceae. As Bignoniaceae apresentam distribuição pantropical e incluem aproximadamente 112 gêneros e cerca de 800 espécies (Lohmann & Ulloa, 2006 onwards). No Brasil ocorrem 32 gêneros e 391 espécies (Lohmann, 2010). A família constitui um grupo monofilético sustentado por caracteres morfológicos e dados moleculares dos genes *ndh*F, *rbc*L e *trn*L–F (Spangler & Olmstead, 1999; Olmstead *et al*., 2009). É composta por arbustos, árvores e lianas (Fig. 1**A–C**) e caracterizada, em sua maioria, pela presença de estames didínamos, flores grandes com corolas bilabiadas e sementes aladas, bem como pelas folhas opostas compostas (Fig. 1**D–G**) (Gentry, 1973a). O centro de diversidade da família é neotropical, região que abriga um grande número de representantes da tribo Bignonieae, endêmica da região e composta por 392 espécies e 21 gêneros (Lohmann & Taylor, no prelo).

Bignonieae é um grupo monofilético, bem sustentado por caracteres morfológicos e moleculares. Seus representantes se distribuem por áreas de cerrado e florestas úmidas e são predominantemente lianas, constituindo o maior clado de lianas neotropicais (Lohmann, 2006). Representantes de Bignonieae distinguem-se de outras tribos de Bignoniaceae pela gavinha na posição do folíolo terminal, anatomia caulinar com o floema formando 4–32 cunhas e fruto com deiscência septicida (Fig. 1**H–J**) (Lohmann, 2006). *Dolichandra s.l.* é o único gênero da tribo que apresenta frutos com deiscência tanto septicida como loculicida (Fig. 2**B**) e anatomia caulinar composta for cunhas de floema múltiplo-dissectadas.

A atual circunscrição de *Dolichandra s.l.* é resultado de uma reclassificação genérica da tribo Bignonieae, onde foram reconhecidos 21 gêneros (Lohmann & Taylor, no prelo). Essa reclassificação foi baseada em um estudo filogenético onde um terço das espécies da tribo foram amostradas (Lohmann, 2006). A nova circunscrição de *Dolichandra s.l.* inclui representantes dos gêneros *Dolichandra* Cham., *Macfadyena* A. DC.*, Melloa* Bureau e *Parabignonia* Bureau ex K. Schum. A similaridade morfológica entre esses gêneros já era reconhecida devido à um grande número de

caracteres vegetativos, reprodutivos e palinológicos (Gentry & Tomb, 1979; Gentry, 1979). Essa hipótese morfológica de parentesco entre os gêneros foi então corroborada por estudos filogenéticos moleculares (Lohmann, 2006), levando o clado inteiro a ser reconhecido como um único gênero, tratado como *Dolichandra s.l.* (Lohmann, 2003; Lohmann & Taylor, no prelo). Na filogenia de Bignonieae (Lohmann, 2006), os caracteres: presença de crescimento dimórfico, frutos com quatro linhas de deiscência, floema múltiplo-dissectado (dos Santos, 1995), gavinha trífida uncada (Fig. 2**A-D**) e pólen tricopado com exina psilada (Gentry & Tomb, 1979), emergiram como sinapomorfias de *Dolichandra s.l.*. Além disso, o cálice conspícuo também representa uma característica diagnóstica marcante (mas não sinapomórfica) do gênero (Lohmann, 2003; Lohmann & Taylor, no prelo). Apesar de *Dolichandra s.l.* emergir como um clado com alta sustentação, seu posicionamento dentro da tribo ainda é incerto (Anexo 1) (Lohmann, 2006).

Ao todo, dez espécies de lianas são reconhecidas em *Dolichandra s.l.* (Capítulo 2; Lohmann, 2003; Lohmann & Taylor, no prelo), quatro das quais apresentam caracteres conspícuos e únicos. Enquanto *D. chodatii* (Hassl.) L.G. Lohmann se caracteriza pelo cálice tri-lobado; *D. cynanchoides* Cham. se caracteriza por apresentar uma corola vermelha-magenta e por os estames e estigma excertos; *D. hispida* (Seem.) L.H. Fonseca & L.G. Lohmann caracteriza-se pelo indumento hispido e *D. unguis-cati* (L.) L.G. Lohmann é reconhecida pelo cálice truncado e profilos ovado-estriados (Capítulo 2; Gentry, 1973a, 1973b; Lohmann, 2003; Lohmann & Taylor, no prelo). Por outro lado, as demais espécies do gênero, só podem ser reconhecidas por uma combinação de caracteres, como a presença de folíolos acuminados, pecíolos e peciólolos curtos e ausência de gavinha lenhosa em *D. acuminata* L.H. Fonseca & L.G. Lohmann; folíolos com margem denteada, cálice espatáceo e sementes lenhosas em *D. dentata* (K. Schum.) L.G. Lohmann; folíolos ovado-lanceolados, cálice com apículo recurvado e gavinhas lenhosas em *D. quadrivalvis* (Jacq.) L.G. Lohmann; e folíolos lanceolados acuminados, cálice espatáceo e sementes lenhosas em *D. uncata* (Andrews) L.G. Lohmann. Por fim, *D. steyermarkii* (Sandwith) L.G. Lohmann e *D. unguiculata* (Vell.) L.G. Lohmann

são reconhecidas no gênero pela presença de um cálice penta-lobado, ramos descamantes e folíolos coriáceos, e se diferenciam pela morfologia do cálice e distribuição geográfica (vide Fig. 3**A–V**).

Entre os caracteres morfológicos encontrados nas espécies de *Dolichandra s.l.* chamam atenção os caracteres associados com a morfologia floral e morfologia da semente (Gentry, 1973a, 1973b; Lohmann, 2003; Lohmann & Taylor, no prelo), estruturas reprodutivas envolvidas respectivamente nos eventos de fecundação e dispersão. Como tais eventos estão diretamente relacionados ao sucesso reprodutivo dos indivíduos, essas estruturas estão sujeitas a intensa atuação da seleção natural exercida tanto pela interação entre plantas e animais como pela interação entre plantas e o ambiente (p. ex., Darwin, 1862; Fægri & van der Pijl, 1966; van der Pijl, 1982). Na tribo Bignonieae, a interação entre a morfologia floral e animais, polinizadores ou antagonistas, é bem conhecida (Alcantara & Lohmann, 2010; Gentry, 1974a), com tipos florais (*sensu* Gentry 1974) frequentemente correspondendo a síndromes de polinização (Alcantara & Lohmann, 2010; *sensu* Fægri & van der Pijl, 1966). No caso das sementes, duas síndromes de dispersão são conhecidas para a tribo, anemocoria e hidrocoria (Gentry, 1974a).

Em *Dolichandra s.l.* especificamente, duas síndromes de polinização são conhecidas: melitofilia e ornitofilia (Gentry, 1974a). *D. chodatii* é uma exceção dentro no gênero pois apresenta uma morfologia floral mista, com caracteres relacionados tanto à polinização por abelhas, quanto por aves. Em termos da dispersão de sementes, o gênero possui ambos os modos descritos para a tribo. Enquanto *D. dentata* e *D. uncata* são dispersas pela água, ocorrendo obrigatoriamente em regiões pantanosas ou beira de rios, as demais espécies do gênero apresentam sementes aladas, sendo encontradas tanto em regiões pantanosas e beiras de rios como em regiões não alagáveis (Gentry, 1973a, 1973b; Lohmann, 2003; Lohmann & Taylor, no prelo).

A distribuição geográfica das espécies de *Dolichandra s.l.* compreende florestas úmidas e secas, do México ao norte da Argentina (Lohmann, 2003; Lohmann & Taylor, no prelo), com um maior número de ocorrências e espécies nas florestas tropicais sazonalmente secas (Lohmann, 2003; Lohmann & Taylor; no prelo; veja também Särkinen *et al.*, 2011). A maior riqueza de espécies está no

norte da Argentina, Bolívia, sul do Brasil e Paraguai, onde ocorrem 7 espécies (Fig. 4). A abrangência da distribuição das espécies varia. *Dolichandra quadrivalvis*, *D.uncata* e *D. unguis-cati*, apresentam distribuição ampla sendo encontradas do México ao norte da Argentina e sul do Brasil. *Dolichandra steyermarkii* e *D. unguiculata* (restrita à Mata Atlântica) apresentam distribuições um pouco mais restritas, com *D. steyermarkii* ocorrendo na Costa Rica, Colômbia, Equador e Venezuela, enquanto *D. unguiculata* é restrita à Mata Atlântica. Por outro lado, *D. chodatti, D. cynanchoides, D. dentata* e *D. hispida* se distribuem na porção sul da América do Sul, tornando essa a região, a área com maior riqueza de espécies para o gênero. *Dolichandra acuminata* é uma exceção, sendo endêmica da porção sul da mata atlântica (Capítulo 2; Gentry, 1974b; Lohmann, 2003; Lohmann & Taylor, no prelo).

ESPECIAÇÃO EM *DOLICHANDRA S.L.*

A diversidade de formas presente no gênero em caracteres florais e das sementes, estruturas associadas ao sucesso reprodutivo das espécies, somada à variação na distribuição geográfica e de habitats ocupados pelas diversas espécies do gênero levantam questões sobre os modos de especiação associados à diversificação no gênero (Capítulo 2). Historicamente uma grande importância foi dada ao isolamento geográfico como o responsável pela interrupção do fluxo gênico entre populações (Coyne & Orr, 2004). Neste caso, populações isoladas então estariam sujeitas à atuação da seleção natural e da deriva genética levando a formação de novas espécies, ou ocasionalmente a extinção de uma ou ambas linhagens (Grant, 1971; Mayr, 1942, 1963). A importância do isolamento geográfico é observada na clássica separação da especiação em modos geográficos, com ênfase sendo dada à especiação alopátrica, padrão originado da vicariância ou dispersão à longa distância (Coyne & Orr, 2004; Mayr, 1963; Templeton, 1981).

No entanto, estudos de especiação mais recentes têm focado em fatores ecológicos como geradores de barreiras que promovam o isolamento reprodutivo, com o isolamento meramente geográfico apresentando um papel menor (Coyne & Orr, 2004; Sobel *et al*., 2010). Neste contexto, especiação simpátrica e parapátrica ganham um maior destaque, com novas barreiras ao fluxo gênico

sendo consideradas, bem como a possibilidade de especiação na presença de algum fluxo gênico (Coyne & Orr, 2004; Rieseberg & Willis, 2007). Em plantas, as principais barreiras promotoras do isolamento reprodutivo são geográficas ou ecológicas, tais como diferenças nos tipos de polinizadores, modos de dispersão e fenologia. Variáveis ecológicas abióticas, como altitude, solo, precipitação e temperatura também representam potenciais barreiras ao fluxo gênico, promovendo o isolamento de habitat (Struwe *et al.,* 2011). Outro fator muito importante em plantas é a poliploidia, cujo papel na especiação tem sido amplamente documentado na literatura (Coyne & Orr, 2004; Hardy & Linder, 2005; Kay & Sargent, 2009; Rieseberg & Willis, 2007; Sobel *et al.,* 2010; Soltis & Soltis, 2009; Struwe *et al.,* 2011).

A alta quantidade de possíveis barreiras ao fluxo gênico existentes em plantas indica a necessidade de uma abordagem integrada, incluindo variáveis fenotípicas, ecológicas e geográficas para o teste de hipóteses de especiação em plantas. Filogenias detalhadas, incluindo amostragens completas de espécies representam os padrões de cladogênese que levaram às espécies viventes, possibilitando o estudo dos processos envolvidos na origem de taxa (Barraclough & Vogler, 2000; Losos & Glor, 2003; Perret *et al.,* 2007; Struwe *et al.,* 2011) e a integração de dados de diferentes naturezas (p.ex., ambiental, fenotípica e geográfica) no teste de hipóteses de especiação.

OBJETIVOS

O presente estudo visou reconstruir a filogenia de *Dolichandra s.l.* para (1) testar o monofiletismo do gênero e estabelecer a relação de parentesco entre as espécies que o compõe; e, (2) inferir potenciais barreiras ao fluxo gênico que estariam relacionadas aos processos de especiação no gênero utilizando dados ambientais, fenotípicos e geográficos.

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FIGURAS

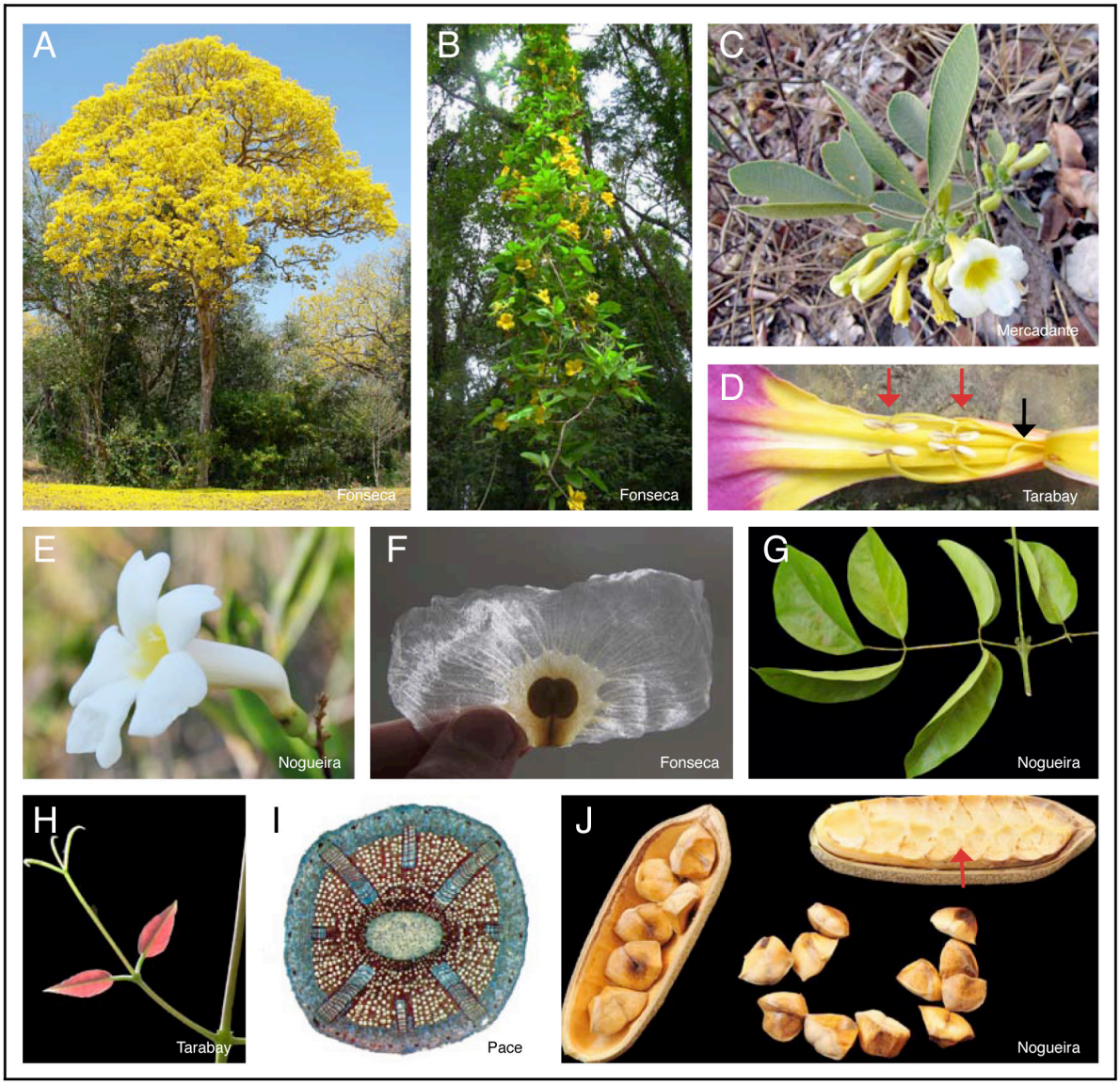

Fig. 1. Hábito e caracteres diagnósticos de Bignoniaceae e Bignonieae. **A,** *Handroanthus* sp., hábito arbóreo. **B,** *Dolichandra hispida*, hábito lianescente. **C,** *Anemopaegma glaucum*, hábito arbustivo. **D,** *Dolichandra unguiculata,* estames didínamos (setas vermelhas), e estaminódio (seta preta). **E,** *Amphilophium elongatum*, flor bilabiada, pentâmera e tubular. **F,** *Amphilophium* sp., semente alada. **G,** *Adenocalymma sp.*, folha oposta e composta. **H,** *Dolichandra unguiculata*, folha composta por dois folíolos e gavinha terminal. **I,** *Bignonia binata,* oito cunhas de floema. **J,** *Amphilophium* sp., posição do septo (seta vermelha), evidenciando a deiscência septicida.

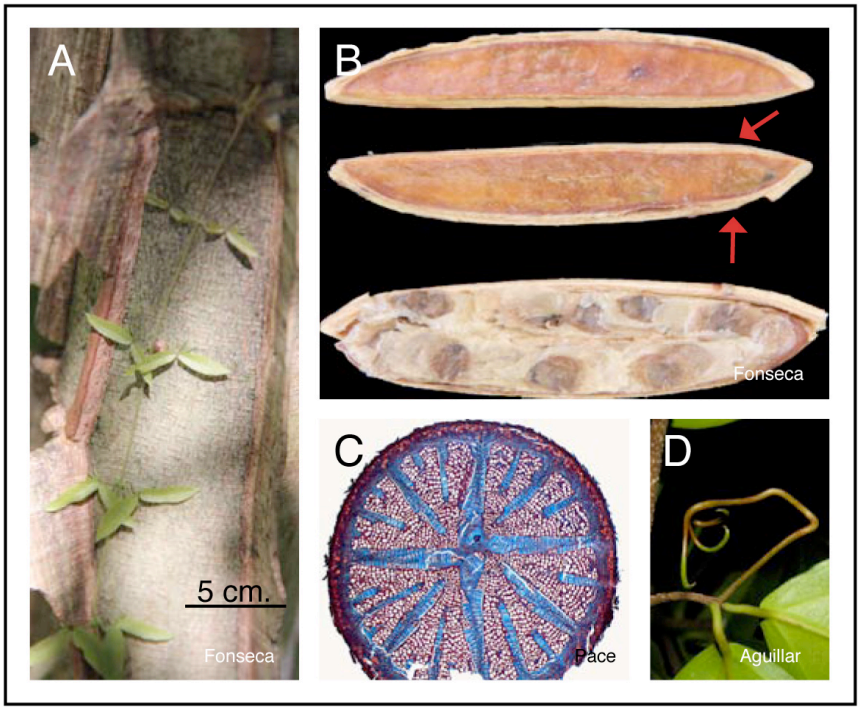

Fig. 2. Sinapomorfias de *Dolichandra s.l.* **A,** Crescimento dimórfico em *Dolichandra unguis-cati.* **B,** Deiscência septicida e loculicida do fruto de *Dolichandra quadrivalvis* (setas vermelhas). **C,** Secção do caule com floema multidissectado em *Dolichandra unguis-cati* . **D,** Gavinha trífida uncinada de *Dolichandra unguiculata*

Fig. 3. Características reprodutivas e vegetativas de *Dolichandra s.l.* **A–C,** *D. unguiculata*. **A**, Flores em vista lateral e inferior. **B**, Fruto maduro. **C,** Gavinha. **D**, Flor de *D. steyermarkii*. **E–F**, *D. chodatii*. **E**, Gavinha. **F**, Inflorescência. **G–I**, *D. cynanchoides*. **G,** Flor em vista lateral. **H,** A seta amarela indica uma flor ainda em desenvolvimento e a seta vermelha uma flor madura. **I**, Fruto maduro. **J–K**, *D. acuminata*. **J**, Flor. **K**, Flor em vista lateral; a seta indica a posição da bractéola. **L**, Fruto de *D. quadrivalvis*. **M–O**, *D. uncata*. **M**, Ramo. **N**, Flor em vista lateral. **O**, Fruto maduro revelando as sementes lenhosas. **P–R**, *D. hispida*. **P**, Ramo. **Q**, Flor em vista lateral. **R**, Ramo em detalhe mostrando a alta concentração de tricomas e os profilos lanceolados. **S–V**, *D. unguis-cati*. **S**, Ramo. **T**, Flor em vista lateral. **U**, Ramo. **V**, detalhe da gavinha.

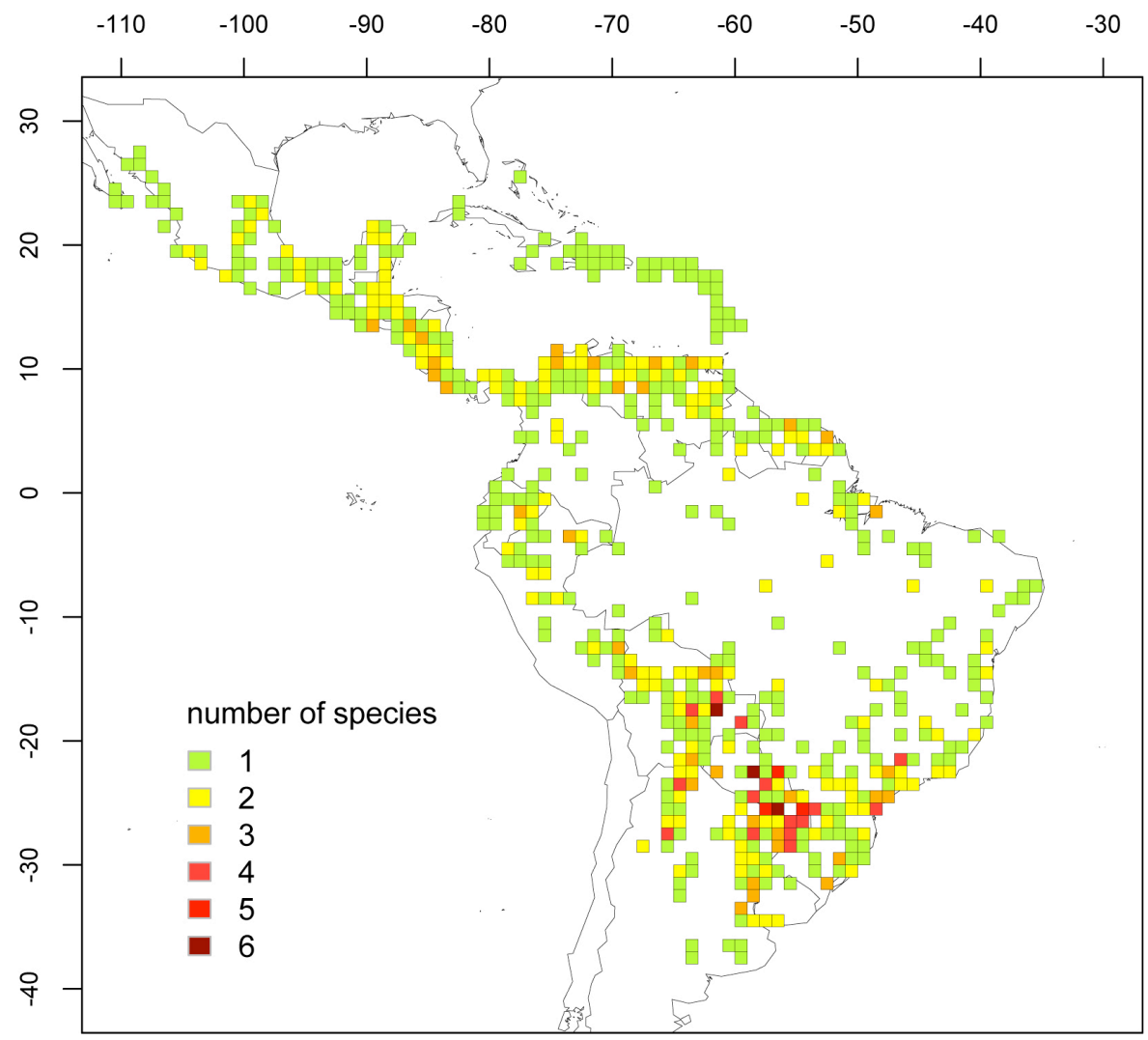

Fig. 4. Distribuição geográfica das espécies de *Dolichandra s.l.* na América Central e do Sul utilizando como representação células de 1º x 1º. O gradiente de cores indica o número de espécies diferentes observadas em cada quadrante.

Capítulo 1

Molecular Phylogeny and diversification of *Dolichandra s.l.* (Bignonieae, Bignoniaceae).

(a ser submetido à revista Botanical Journal of the Linnean Society)

Molecular Phylogeny and diversification of *Dolichandra s.l.* (Bignonieae, Bignoniaceae).

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ABSTRACT

Dolichandra s.l. is a genus of lianas found in dry and wet forests of the Neotropics. The genus includes ten species and is recognized by molecular and morphological synanpomorphies. In this study, we reconstructed the phylogeny of *Dolichandra s.l.* and used this framework as basis to further understand the history of diversification of the genus. For the phylogenetic component of this project, we used two cpDNA makers (the gene *ndh*F and the intergenic region *rpl*32-*trn*L) and one nuclear intron (*Pep*C) and analyzed the DNA sequences from the individual partitions separately and in combination using Maximum parsimony and Bayesian inference. Modes of speciation were inferred using detailed distribution data and Age-Range Correlation (ARC), as well as environmental data and Spatial Evolutionary and Ecological Vicariance Analysis (SEEVA), in combination with Bayesian ancestral character states reconstructions. *Dolichandra s.l.* and all species included herein emerged as monophyletic in all analysis. Relationships among species are resolved, although poorly supported in some instances. Sympatric speciation was shown to represent the predominant mode of speciation inferred for the genus, with ecological factors acting as potential barriers that may have influenced the patterns of diversification in the genus.

ADDITIONAL KEYWORDS: comparative biology – lianas – modes of speciation – Neotropical – sympatric speciation.

INTRODUCTION

Dolichandra s.l. Cham. is a small genus that belong to the plant family Bignoniaceae (tribe Bignonieae). The genus currently includes all species previously placed in *Dolichandra s.s., Macfadyena* A. DC., *Melloa* Bureau and *Parabignonia* Bureau ex K. Schum. These generic realignments were proposed based on a molecular phylogeny of Bignonieae that included one fourth of all species currently recognized in the tribe (Lohmann, 2006; Lohmann & Taylor, in press). In this phylogeny, *Dolichandra*, *Macfadyena*, *Melloa* and *Parabignonia* appear as monophyletic and strongly supported by molecular and morphological traits. In particular, the unique multiple dissected phloem wedges, trifid and uncinate tendrils, fruits with four lines of dehiscence, a dimorphic growth form and colpate pollen with a psilate exine (Gentry & Tomb, 1979; Gentry, 1979; Lohmann, 2003; Lohmann & Taylor, in press) represent morphological synapomorphies for this clade. The large calyx also represents an additional diagnostic character of representatives of the genus, although this feature does not represent a synapomorphy for the clade (Lohmann, 2003; Lohmann & Taylor, in press).

The most recent classification of *Dolichandra s.l.* recognized eight species (Lohmann, 2003; Lohmann & Taylor, in press). However, one new species was recently described (*D. acuminata* L.H. Fonseca & L.G. Lohmann; Chapter 2) while a species previously placed in synonymy is now treated as a separate taxon (*D. hispida* L.H. Fonseca & L.G. Lohmann; Capítulo 2), leading to a total of 10 species in the genus. The geographic distribution of *Dolichandra s.l* is Neotropical and centred in southern Brazil, northern Argentina and Paraguay, where seven species occur and two are endemic, *Dolichandra cynanchoides* Cham. and *Dolichandra dentata* (K. Schum.) L.G. Lohmann. Of the remaining five taxa, three are widely distributed [*D. quadrivalvis* (Jacq.) L.G. Lohmann, *D. uncata* and *D. unguis-cati* (L.) L.G. Lohmann], while the remaining two either extend to the forests of Bahia [*Dolichandra chodatii* (Hassl.) L.G. Lohmann] or to the Brazilian states of Mato Grosso and Minas Gerais [*D. hispida* (Seem.) L.G. Lohmann]. Two other species of *Dolichandra s.l.* [*Dolichandra acuminata* L.H. Fonseca & L.G. Lohmann and *D. unguiculata* (Vell.) L.G. Lohmann] are endemic to the Brazilian Atlantic Forests, while *Dolichandra steyermerkii* (Sandwith) L.G. Lohmann is

exclusively found in Western Amazonia. Some species of *Dolichandra s.l.* have clear ecological preferences, representing conspicuous components of Seasonally Dry Tropical Forests (Gentry, 1974a; Lohmann, 2003; Lohmann & Taylor, in press; see also Särkinen *et al.,* 2011), or of Neotropical moist forests (Gentry, 1974a; Lohmann, 2003; Lohmann & Taylor, in press).

Species of *Dolichandra s.l.* are variable in both vegetative and reproductive characters, with morphological structures involved in pollination and dispersal events being particularly variable (Gentry, 1973a, 1973b; Lohmann, 2003; Lohmann & Taylor, in press). Field observations in combination with inferences based on flower morphology indicate that *D. cynanchoides* is pollinated by birds, while all other species are bee pollinated (Alcantara & Lohmann, 2010; Gentry, 1974a). *Dolichandra chodatii* is an exception with a mixed morphology. While *D. dentata* and *D. uncata* are dispersed by water, occurring in wetlands or river banks, all other species have winged seeds and are dispersed by wind, being found in various habitats other than flooded areas (Gentry, 1973a, 1973b; Lohmann, 2003; Lohmann & Taylor, in press).

The diversity of forms in floral and seed traits among species of *Dolichandra s.l.*, coupled with the patterns of variation in distribution and habitats occupied by the various taxa, raise questions about the modes of speciation associated with the diversification in the genus. Traditionally, speciation studies have focused on the potential effectiveness of geographical barriers for the reproductive isolation of populations, emphasizing allopatric speciation (Grant, 1971; Mayr, 1942, 1963). More recently, speciation studies have considered ecological factors as generators of barriers to gene flow, placing greater emphasis on natural selection instead of purely geographic isolation (reviewed in Coyne & Orr, 2004; Sobel *et al.*, 2010). Among the most effective ecological barriers to gene flow in plants are abiotic variables such as phenology, pollination and dispersal systems, as well as abiotic factors such as altitude, soil, rainfall and temperature. In this context, great attention has also been placed in polyploidy, as another key gene flow barrier in plants (Coyne & Orr, 2004; Hardy & Linder, 2005; Kay & Sargent, 2009; Rieseberg & Willis, 2007; Sobel *et al.,* 2010; Soltis & Soltis, 2009; Struwe *et al.,* 2011). Even though polyploidy has been documented in *D. unguis-cati* (4N = 80), *D.*

cynanchoides, D. dentata and *D. uncata,* and the majority of individuals of *D. unguis-cati* are diploids (2N = 40) (Jullier, 1989; Piazzano, 1998), suggesting that polyploidy did not play an important role during the diversification history of the genus.

In this study, we reconstruct the phylogeny of *Dolichandra s.l.* and use this phylogenetic framework as basis to further understand gene flow barriers that may have been associated with the diversification of the genus. More specifically, we test the hypothesis that environmental, phenotypic and geographic factors have played important roles during the diversification history of *Dolichandra s.l.*

MATERIAL AND METHODS

TAXON SAMPLING

We sampled twenty-four accessions, including nine species of *Dolichandra s.l.* and four outgroups (Table 1). Only *D. steyermarkii* was not included in our analysis as we were unable to encounter this species in the field and all of our attempts to obtain sequences of this species from herbarium specimens failed. For all other taxa, we sampled multiple accessions per species, representing different morpho-types as well as different portions of the species' distribution ranges in order to further evaluate species limits (Table 1). *ndh*F and *Pep*C sequences of *D. cynanchoides, D. unguis-cati* and *Fridericia speciosa* Mart. derived from an earlier study (Lohmann, 2006), were also incorporated into our data matrix. Given the uncertain placement of *Dolichandra s.l.* in the tribe (Supplementary material 1), we sampled representatives of all major clades of Bignonieae as outgroups, including: *Adenocalymma marginatum* DC. (*Adenocalymma-Neojobertia* clade), *Fridericia speciosa* (*Fridericia* and Allies clade), *Mansoa onohualcoides* A.H. Gentry (Multiples of four clade) and *Stizophyllum riparium* (Kunth) Sandwith (Lohmann, 2006).

MOLECULAR SAMPLING

The nuclear *Pep*C (Matsuoka and Minami, 1989) and the cpDNA markers *ndh*F and *rpl*32–*trn*L (Sugiura, 1992) were selected after a careful pilot study that tested eight additional molecular markers,

including four regions of the PPR gene family (Yuan *et al.,* 2009), three COSII genes (Wu *et al.,* 2006; Tepe & Bohs, 2010), and the plastidial intergenic region *trn*Q–*rps*16 (Sugiura, 1992). These markers showed high number of copies, low phylogenetic information or problems with amplification in Bignonieae and were discarded from the present study (Anexo 2).

DNA was extracted from silica-dried leaflets or herbarium specimens using Invisorb® Spin Plant Mini Kit (Invitek, Berlin, Germany). Plant samples (60 ng) were pulverized with Tissuelyzer® (Qiagen, Duesseldorf, Germany) and the protocol of extraction was followed according to the Spin Plant Mini Kit manufacturer's instructions. PCR primers and conditions for the amplification and sequencing of *ndh*F and *Pep*C from silica-dried samples followed Lohmann (2006), while amplification and sequencing of *rpl*32–*trn*L followed Shaw *et al.* (2007) (Table 2). New primers were developed for herbarium materials and amplicons of approximately 500 bp were obtained (Table 2). For all markers, PCR conditions for herbarium specimens were as follows: 5 min at 94º C as a HotStart, followed by 39 cycles of 94º C for 1 min, 54º C for 30 sec and 72º C for 1 min, with a final extension of 10 min at 54º C. Some species of *Dolichandra s.l.* presented small homopolymeric regions (10–12 bp) in the *rpl*32–*trn*L intergenic spacer, leading to poor quality sequences. New primers were developed immediately after the homopolymeric regions (Table 2) in order to ensure that double-stranded sequences could be obtained. Amplifications were conducted in 20 µl PCR reactions including: 4 µl of 5x Mg-Free buffer, 2,5 mmol/L of MgCl2, 0.25 mmol/L of dNTP, 0.10 µmol/L of each primer, 1 µl of DNA template and 1.5 unit of GoTaq® DNA polymerase or GoTaq® HotStart DNA polymerase (Promega, Madison, Wisconsin, USA). For recalcitrant samples, DNA stocks were diluted from 1/10 to 1/100 and 0.4 µl of DMSO was added. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) or with exonuclease I and shrimp alkaline phosphatase (Exo/SAP). All samples were sequenced in both directions by Macrogen Inc. (Seul, Korea).

Forward and reserves sequences, and at least 85% of double-stranded sequences were obtained

for all taxa. Contigs were built using Geneious 4.7.5 (Drummond *et al.*, 2010). Sequences were aligned using Muscle (Edgar, 2004), implemented in Geneious 4.7.5, followed by visual inspection. Gaps of non-coding regions were coded using SeqState (Müller, 2005) following the simple indel coding method (Simmons & Ochoterrena, 2000) indicated by Simmons, Müller, & Norton (2007) in a performance test of indel-coding methods. Gaps were added to the matrix as binary characters. For *rpl*32–*trn*L, a reversion was identified and manually coded; the reverted portion of the sequence was excluded from the final analyses. All trees were rooted with *A. marginatum*.

PHYLOGENETIC ANALYSIS

Data partitions were analyzed separately and combined. Two different analyses were conducted for the individual data partitions, one including all sampled individuals and the other including a single individual per species. Phylogenetic analyses were carried out using maximum parsimony (MP; Farris, Kluge & Eckardt, 1970) and Bayesian inference (BI; Mau, Newton & Larget, 1999). Trees from both methods were compared to detect areas of greater resolution. MP analyses were performed in PAUP*4.0B.10 (Swofford, 2002). Heuristic searches used 10,000 random taxon addition replicates and TBR branch swapping. All characters were equally weighted, and gaps were treated as missing data. Support for clades was estimated by bootstrap (BP) (Felsenstein, 1985), with 1,000 replicates and exclusion of uninformative sites. One thousand replications for each bootstrap replicate were conducted in a heuristic search with the same parameters of the tree search.

Bayesian Inference (BI) was conducted in MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001). The best-fitted substitution model was determined for each data set in JModelTest 0.1.1 (Posada, 2008; see also Guindon & Gascuel, 2003) using a maximum likelihood tree as reference and the Akaike Information Criterion (Akaike, 1974), as suggested by Luo *et al.* (2010). The substitution model recovered for *ndh*F was TVM + G (Table 3). However, since this model is not implemented in MrBayes 3.1.2, the model was replaced by the default six parameters (GTR) with gamma distribution. The best-fit model of nucleotide substitution for *rpl*32–*trn*L was GTR, while GTR + I + G was the

best-fit model of nucleotide substitution for *Pep*C (Table 3). Four independent MCMC runs were conducted, each with four linked chains of 10^7 generations, sampling every 1,000 generations to minimize autocorrelation among samples. Post analyses were carried out in Tracer 1.5 (Rambaut & Drummond, 2007) to check if initial burn-in is sufficient. Posterior probabilities were used to estimate node support. The average standard deviation of split frequencies was < 0.01 for all data sets. Nodes with BP ≥ 80 and PP ≥ 0.95 were considered well supported. Both measures of support were considered, given that PP values are often inflated in relation to BP values (Erixon *et al.,* 2003).

DIVERGENCE TIME ESTIMATES

Divergence times were estimated for the reduced tree using the Penalized Likelihood method proposed by Sanderson (2002). To calculate the λ parameter we used the method proposed by Paradis (Second Edition: 2011). The analysis was implemented in Ape package (Paradis *et al.,* 2004) for R (R development core team, 2003). We acknowledge that more sophisticated and parameterized methods using Bayesian MCMC approach are available, however our objective here was to obtain approximate divergence times for a large-scale comparison. The input tree was calibrated using the mean divergence time value of 22.54 Mya available for *D. cynanchoides* (Lohmann *et al.,* in press) and a λ value of 10^4.

GEOLOCATION AND GEOGRAPHIC SPECIATION

The geographic distribution of each species was compiled from 2143 accessions (Lohmann, unpubl. data set) from ten herbaria (BOTU, ESA, FUEL, INPA, MBM, MO, NY, SP, SPF, UPCB) using a presence/absence coding scheme in a grid divided into quadrants of 1.5-degree intervals of latitude and longitude. These dimensions were selected to accommodate the significant differences in the geographic distribution of species found in *Dolichadnra s.l.*, the asymmetrically distributed records through species (e.g., with nearly half of the samples examined belonging for *D. unguis-cati* and only five specimens belonging to *D. acuminata*), and the total number of samples available. Distribution data for each species was modelled with Raster package 2.0 (Hijmans & Etten, unpubl. data) for R (R

Development core team, 2003).

To examine the potential role of geographic isolation in the diversification history of the genus, we used the Age–Range Correlation (ARC) approach proposed by Barraclough & Vogler (2000; previous implemented in Barraclough, 1998), which considers the degree of sympatry for each pair of independent sister clades of the phylogeny in relation to age node. When speciation is predominantly sympatric, then the range overlap is expected to be close to 100% in recent nodes and to decrease through time. Because sympatric speciation can occur anywhere within a species range, the degree of sympatry was calculated by dividing the range overlap between sister clades by the range size of the clade with the smaller ranges (Barraclough & Vogler, 2000). The geographic distribution of clades in *Dolichandra s.l.* was estimated by the sum of the distribution of species within that particular clade. The degree of sympatry varied between zero (indicating allopatry and no range overlap) and one, (indicating complete sympatry, when the narrow distributed clade is entirely encompassed by the range of broader one). Because the measure of sympatry is bounded between zero and one, the degree of sympatry was arcsine transformed (Sokal & Rohlf, 1995). A regression line was fitted to the plot between range overlap and node ages and the intercept of the y-axis used to indicate the predominant mode of speciation, while the slope of the regression line was used to estimate the degree of range movement subsequent to speciation. A randomization approach was used to explore the potential loss of geographic signature of speciation due to drastic movements of species range over time, with environmental changes effacing the biogeography of speciation. This method consists of a random shuffle of distributions among tips 1000 times, each time recalculating the intercept. The *P* value represents the proportion of tests with an intercept more extreme than the one observed and the rejection of null hypothesis means that there is phylogenetic signal in the data (Perret, 2007).

ENVIRONMENTAL DATA

We used raster files from WorldClim v.1.4 (http://www.worldclim.org; Hijmans *et al.,* 2005) to estimate abiotic ecological variables for the various taxa. In particular, we examined: annual mean

temperature (in ºC x 10; 30 arc second); maximum temperature of warmest month (in ºC x 10; 30 arc second); minimum temperature of the coldest month (in ºC x 10; 30 arc second); annual range temperature (in ºC; 30 arc second); annual precipitation (mm precipitation; 30 arc second); precipitation of the wettest month (mm precipitation; 30 arc second) and precipitation of the driest month (mm precipitation; 2.5 arc-minutes). Resolutions for these layers are the highest available in WorldClim, being consistent with our goal to elucidate broad patterns associated with cladogenesis. Variables were selected according to individual species habitats and the potential variables conditioning their distributions.

We used geo-referenced locations for each separate species and the Raster package 2.0 (Hijmans & Etten, unpubl.) for R (R Development core team, 2003) to extract data from environmental variables. The environmental data for each collection were archived in a matrix with all species and variables and analysed using Spatial Evolutionary and Ecological Vicariance Analysis (SEEVA), implemented in SEEVA 1.01 (available in http://seeva.heiberg.se/; Struwe *et al.,* 2011). The method assumes the premise that cladogenesis can be associated with ecological splits and proposes the divergence index (*D*) metric scaled from 0.0 (no divergence between sister clades) to 1.0 (maximum possible difference between clades) to evaluate the role of each environmental variable for each lineage split (Struwe *et al.,* 2011). A null hypothesis assumes no divergence of feature states between the two sister clades and a *P*-value is computed for each node and variable using a Bonferroni correction (Rice, 1989). The significance of the results is evaluated by permuting specimen values through species (Struwe, 2011).

FLORAL AND SEED EVOLUTION

Putative pollination syndromes were inferred from a set of floral characters (Table 4), including corolla colour (non-red – 0, red – 1); corolla shape (infundibuliform – 0, tubular – 1); corolla texture (membranous – 0, coriaceous – 1); position of sexual whorls (included – 0; exerted – 1); presence of landing platform (absent – 0, present – 1); presence of scent (absent – 0, present – 1); presence of

tactile cues (absent – 0, present – 1); and amount of reward, inferred from the volume of nectaries (< 10 mm^2 – 0, ≥ 10 mm^2 – 1) (Alcantara & Lohmann, 2010; Fægri & van der Pijl, 1966; Gentry, 1974a; van der Pijl, 1982). This data was originally compiled from the literature (Alcantara & Lohmann, 2010; Gentry, 1974b; Lohmann, 2003, Rivera, 2000) and complemented with additional obervations from the present study. Ornitophylly and melitophylly were inferred for the genus (Gentry, 1974b) based on comparisons among similar morphologies in the tribe. Similarly, dispersal syndromes were inferred (Table 5) based on the texture of the wings (membranous – 0, woody – 1) and the thickness of the seed body (thin – 0, thick – 1) (Gentry, 1974b; van der Pilj, 1982).

The evolutionary history of characters was inferred by estimating the marginal posterior probability of ancestral states under a Bayesian approach (Pagel, Meade & Barker, 2004). Ancestral states were estimated using BayesTraits 1.0 (Pagel, 2004) with a set of 1000-post burn-in sample of trees selected from the first run. All four runs converged, indicating that any run could have been chosen for the tree selection. Outgroups of all 1000 trees were pruned due to the uncertainty of the placement of Bignonieae (Anexo 1). Transitions rates were set to be equal with an exponential prior for rate coefficient. Ancestral states for nodes with posterior probabilities < 1.0 were estimated using the MRCA approach (Pagel, 2004). To ensure adequate mixing, the rate deviation of the normal distribution was adjusted manually to obtain acceptance rates between 20%–40% for all characters. The MCMC was run for 10^7 generations, with the first 2.5 x 10^5 generations discarded as burn-in. Values were saved for every 1000th generation to avoid any sort of auto-correlation within values. A total of 7,500 generations were saved and used to compute mean values of frequency for each character state.

RESULTS

PHYLOGENETIC ANALYSIS OF *NDH*F

Complete sequences were obtained for 18 ingroup accessions and three outgroup accessions of *ndh*F. In addition, sequences of *D. cynanchoides1, D. unguis-cati1* and *F. speciosa* from a previous study (Lohmann, 2006) were also added to our data matrix. Raw sequences of *ndh*F ranged from 2026 to

2125 bp in length. The aligned *ndh*F data set included 1995 characters of which 1761 were constant, 233 were variable, and 127 were parsimony-informative. An insertion in *S. riparium* of three codons was deleted from the final matrix and no other indels were observed. The heuristic search recovered a single tree with 171 steps, consistency index (CI) = 0.8363 and retention index (RI) = 0.9213 (Table 3). The MP tree is identical to the Bayesian majority rule consensus tree (Fig. 5).

In all analysis, *Dolichandra s.l.* emerges as monophyletic (BS = 100, PP = 1.0) and placed inside within a polytomy with *F. speciosa*, *M. onohualcoides* and *S. riparium*. All species with multiple accessions were strongly supported as monophyletic (BS ≥ 80 and PP ≥ 0.95). *Dolichandra unguiculata* is the first species to diverge (BS = 100, BI = 1.0), being sister of a clade that includes all other species in the genus (BS = 93, PP = 1.0). The next species to diverge is *D. chodatii* (BS = 100, PP = 1.0) and subsequently *D. cynanchoides* (BS = 100, PP = 1.0). *Dolichandra cynanchoides* is sister to the clade that includes the remaining species of the genus (BS = 63, PP = 0.7). This clade includes two main lineages. The first (BS = 100, PP = 1.0) is composed of *D. acuminata*, which is sister to *D. quadrivalvis* (BS = 88, PP = 1.0). The second (BS = 100, PP = 1.0) includes *D. uncata* (BS = 100, PP = 1.0), which appears as sister of a clade composed of *D. dentata*, *D. hispida* and *D. unguis-cati* (BS = 68, PP = 0.68). Within this clade, *D. unguis-cati* (BS = 99, PP = 1.0) emerges as sister of *D. dentata* and *D. hispida* (BS = 70, PP = 0.81) (Fig. 5).

PHYLOGENETIC ANALYSIS OF *RPL*32-*TRN*L

Complete sequences of *rpl*32–*trn*L were obtained for all 20 ingroup accessions and four outgroups. Raw sequences for *rpl*32–*trn*L ranged from 926 to 1120 bp. The aligned *rpl*32–*trn*L data set included 921 characters of which 726 were constant, 195 were variable, and 104 were parsimony-informative. Nine hundred and two characters were DNA base pairs while 19 characters represent coded indels. A heuristic search recovered 10 most parsimonious trees in a single island with 137 steps, CI = 0.8467 and RI = 0.9250 (Table 3). The strict-consensus trees that resulted from the MP analysis were less resolved than the majority rule tree of BI. However, all clades recovered in the tree that resulted from

the MP analysis, were strongly supported in the topology that resulted from the BI analysis. Clades that were only recovered in the tree resulting from the BI analysis were poorly supported (Fig. 5).

Dolichandra s.l. appeared as monophyletic in all analyses and placed within a polytomy with *F. speciosa* and *M. onohualcoides* (BS = 84, PP = 1.0). All species with multiple accessions were monophyletic with high support values. *Dolichandra unguiculata* is the first species to emerge (BS = 100, PP = 1.0), being followed by *D. chodatii* (BS = 100, PP = 1.0) and *D. cynanchoides* (BS = 86, PP = 1.0), which is sister to the rest of the taxa included in the genus (BS = 65, PP = 0.82). The remaining species are placed in two lineages: A clade composed of *D. acuminata* and *D. quadrivalvis* (BS = 84, PP = 1.0), and a clade composed of *D. uncata* (BS = 100, PP = 1.0), *D. dentata*, *D. hispida* (BS = 100, PP = 1.0) and *D. unguis-cati* (BS = 94, PP = 1.0) (Fig. 5).

PHYLOGENETIC ANALYSIS OF *PEP*C

Our final *PepC* matrix is composed of 17 sequences, of which 14 represent newly generated sequences for the present study. Of these 14 sequences, 11 are members of *Dolichandra s.l.* and three are outgroups. In addition, sequences of *D. cynanchoides1, D. unguis-cati1* and *Fridericia speciosa* generated in a previous study (Lohmann, 2006) were added to our dataset. Unfortunately, we were unable to obtain *Pep*C sequences for *D. chodatii2, D. cynanchoides2, D. hispida2, D. quadrivalvis2, D. uncata2, D. unguis-cati3* and *D. unguis-cati6*. Raw sequences ranged from 688 to 810 bp in length. The aligned *Pep*C data set included 751 characters of which 518 are constant, 223 are variable, and 91 are parsimony-informative. Seven hundred and thirty seven characters are DNA bases and 13 are coded indels. A heuristic search recovered six most parsimonious trees with 180 steps, CI = 0.6000 and RI = 0.7154 (Table 3). The strict-consensus tree that resulted from the parsimony analysis and the majority rule topology that resulted from the Bayesian analyses are identical (Fig. 6).

In all analysis, the genus is monophyletic (BS = 100, PP = 1.0), placed in a polytomy with *F. speciosa* and *M. onohualcoides*. All species with multiple accessions are monophyletic. *Dolichandra unguiculata* represents the earliest diverging lineage in the genus (BS = 100, PP = 1.0). This basal

split is followed by the divergence of a small clade composed of *D. chodatti* and *D. cynanchoides* (BS = 84, PP = 0.98) and another clade that includes the remaining species in the genus (BS = 69, PP = 0.89). This larger clade includes a smaller subclade composed of *D. acuminata* and *D. quadrivalvis* (BS = 89, PP = 1.0) and another clade that is composed of *D. uncata*, *D. hispida, D. dentata* and *D. unguis-cati* (BS = 90, PP = 0.99). Relationships within this clade are only moderately or poorly supported (Fig. 6).

COMBINED ANALYSIS

A visual comparison among topologies reveals a congruent pattern for all markers, with a few exceptions. For instance, small differences are observed in the placement of *D. cynanchoides* and *D. chodatii.* For the plastid markers, *D. chodatii* appears as sister to a clade composed of *D. cynanchoides* and all other species of *Dolichandra s.l.*, except for *D. unguiculata*, which is the earliest diverging lineage in this topology. This topology is poorly supported for both markers (Fig. 5). In the *Pep*C topology, however, *D. chodatii* and *D. cynanchoides* form a moderately supported clade (BS = 85, PP = 0.98) (Fig. 6). Differences in the placement of *D. dentata* and *D. hispida* are also observed. While both taxa form a clade in both cpDNA topologies, this relationship is not recovered in the *Pep*C topology (Fig. 6). This incongruence is, however, only poorly supported, with all strongly supported clades being recovered in all topologies.

Since the topologies of the individual data sets were not visually different from each other, all markers were combined in a single matrix. The combined data set included 24 individuals, 3634 characters, of which 654 were variable, and 323 were parsimony-informative. The MP heuristic search recovered 18 most parsimonious trees with 496 steps, CI = 0.7419 and RI = 0.8555 (Table 3). The strict-consensus tree (MP) and the majority-rule consensus (BI) are identical (Fig. 7).

The phylogeny derived from the analysis of the combined data set also recovers a monophyletic *Dolichandra s.l.* within a polytomy with *F. speciosa* and *M. onohualcoides*. All species with multiple accessions are monophyletic. *Dolichandra unguiculata* represents the earliest diverging

lineage within *Dolichandra s.l.* (BS = 100, PP = 1.0), and is sister to a clade with the remaining species of the genus (BS = 100, PP = 1.0). This clade includes two main lineages: A poorly supported clade (BS = 74, PP = 0.61) composed of *D. chodatii* (BS = 100, PP = 1.0) and *D. cynanchoides* (BS = 100, PP = 1.0), plus a strongly supported clade (BS = 96, PP = 0.99) composed of *D. acuminata*, *D. quadrivalvis*, *D. uncata*, *D. hispida*, *D. dentata* and *D. unguis-cati*. Within this clade, *D. acuminata* and *D. quadrivalvis* form a strongly supported clade (BS = 100, PP = 1.0) that is sister to the remaining taxa. In turn, *D. uncata* (BS =100, PP = 1.0) is sister to a clade composed of *D. hispida*, *D. dentata* and *D. unguis-cati* (BS = 97, PP = 1) (Fig. 7).

The simplified combined data set included a single accession per species, 613 variable sites and 171 parsimony-informative characters. The parsimony analysis of this data set led to three most parsimonious trees with 289 steps, CI = 0.6678 and RI = 0.7363 (Table 3). The strict-consensus tree (MP) and majority-rule phylogram (BI) are identical (Fig 9). *Dolichandra s.l.* is monophyletic in all analysis (BS = 100, PP = 1.0) and *D. unguiculata* is the first species to diverge, appearing as sister to the remaining species (BS = 100, PP = 1.0). The following clade includes a moderately supported clade (BS = 75, PP = 0.74) composed of *D. chodatii* and *D. cynanchoides*; and a strongly supported clade (BS = 100, PP = 1.0) composed of *D. acuminata* and *D. quadrivalvis*, which is sister to a clade composed of *D. uncata-D. hispida-D. dentata-D. unguis-cati* (BS = 100, PP = 1.0). Within this clade, *D. uncata* is the first species to diverge and strongly supported (BS = 97, PP = 1.0) as sister to the rest; In turn, *D. hispida* is moderately supported as sister (BS = 74, PP = 0.98) to a clade composed of *D. dentata* and *D. unguis-cati* (Fig. 8).

GEOGRAPHIC MODE OF SPECIATION

The observed intercept of the regression line between the degree of sympatry and node ages is 0.69 with a negative slope value of -0.0076 (Fig. 9). The comparison of the intercept value with the null distribution obtained with a simulation approach reveals a significant result with a p-value of 0.0498. The null distribution interval of intercept values ranges from 0.36 to 1.0. The general mode of

geographic speciation inferred for the genus is sympatric, with a decrease of sympatry levels trough time, as indicated by the negative slope value of the regression. All nodes of the phylogeny of *Dolichandra s.l.* present some degree of sympatry (Table 6), including the most recent phylogenetic splits such as nodes E, F, G and H, with ages ranging from 5 to 10 million years (Fig. 8).

ECOLOGICAL DIVERGENCE WITHIN CLADES

Four precipitation and three temperature variables were analysed using SEEVA. Eight nodes were selected for this analysis, leading to a total of 56 comparisons. All results present a degree of diverge between nodes, with 44 significant differences after a Bonferroni correction ($p \leq 0.0085$, indicated by * in Table 6). Despite the high number of significant variables, few diverge index values were greater than 0.5, indicating moderate to low ecological divergence between lineages. Temperature variables present slightly larger amounts of significant differences (ranging from 0.03 to 0.57), with the minimum temperature of the coldest month being significant for every node and the maximum temperature of the hottest month presenting the lowest percentage of significant nodes. Precipitation values in the driest month are significant for all nodes, except for node C. In addition, precipitation of the coldest month is the less significant variable within nodes (Table 6).

Nodes A and B present ecological divergence for all variables, with *D* values ranging from 0.25 to 0.5 and 0.05 to 0.43, respectively. Node C presents four significant *D* values: (1) mean annual temperature, (2) minimum temperature of the coldest month, (3) temperature annual range and (4) precipitation of the wettest month. Significant *D* values for node C range from 0.04 to 0.19. All values are significant for node D, except for precipitation of the wettest month, which presented values that ranged from 0.03 to 0.12. Node E presented significant ecological differences in two variables, annual precipitation and precipitation of the driest month, with *D* values ranging from 0.55 to 0.63, respectively. Nodes F and H presented significant *D* values for all variables tested, ranging from 0.11 to 0.29 and from 0.2 and 0.57, respectively. Node G presented four significant *D* values ranging from 0.2 to 0.57 (Table 6). The nodes with the lowest amounts of significant *D* values were all composed of

species pairs (e.g., nodes C and E) or included three species (i.e., node G). These are the nodes with highest mean values of *D* and also the highest individual values for some of the variables examined (Table 6).

EVOLUTION OF FLORAL AND SEED CHARACTERS

The results of ancestral character state reconstructions are summarized in Fig. 10. Pie charts depict mean values for each state reconstructed based on the Bayesian MCMC method. Density distribution state values for each character are also shown. Differences among lineages are summarized in Table 6. These reconstructions indicate that a single evolution of hummingbird pollination occurred from a bee pollinated ancestral, as well as a reversal from a hummingbird pollinated ancestor to a bee pollinated species in *D. chodatii* (Table 6). A pollinator shift is depicted by the transition from node B to node C. While node B presented flowers with included stamens and stigma (0.99), membranous corolla (0.57) color other than red (0.99), landing plataforms (0.99), scent (0.57), small floral nectaries (0.57) and tactile cues (0.99), node C presented flowers with exerted stamens and stigma (0.53), coriaceous (0.99) red corollas (0.53), big floral nectaries (0.99), lack of a landing plataform (0.53), and presence of tactile cues (0.99). A reversal from a hummingbird pollinated ancestor to a bee pollinated species in node C is inferred based on the included stamens and stigma, corolla with color other than red, landing plataform and tactile cues in *D. chodatii* (Fig. 10).

The presence of seed wings and thickness of the seed were strongly correlated, with the same frequency values of states between those traits (Fig. 10). Two evolutions of water dispersed seeds from a wind dispersed ancestral are estimated with two reversals for the wind dispersed condition (Table 6). The first transition of dispersal mode is observed between nodes D and F, from wind dispersal in node D (0.99) to water dispersal in node F (0.53). A reversion to wind-dispersal seed is estimated from node F to G (0.99), while a new evolution of water-dispersed seeds is estimated from node G to H (0.59). Finally, a reversion to wind dispersal from water-dispersed seeds is observed in node H (Fig. 10).

DISCUSSION

In this study, we used three molecular markers to investigate phylogenetic relationships within *Dolichandra s.l.* Four outgroups representing major lineages in Bignonieae were sampled and the genus emerged as monophyletic in all gene trees and in the combined molecular phylogeny. All species for which multiple accessions were sampled also appeared as monophyletic (Figs. 6–8). A speciation study revealed that geography alone does not explain all clodogenetic splits in *Dolichandra s.l.* (fig. 8), with niche isolation emerging as a possible speciation driver in the genus, in combination with abiotic and biotic factors (Fig. 9–10; Table 6).

PHYLOGENETIC POSITION AND MONOPHYLY OF *DOLICHANDRA S.L.*

The genus *Dolichandra s.l.* was strongly supported as monophyletic, corroborating earlier findings (Lohmann, 2006). In the combined BI phylogeny, *Dolichandra s.l.* emerged in a polytomy with *M. onohualcoides* (multiples-of-four clade) and *F. speciosa* (*Fridericia* and allies clade). This relationship was not recovered in the MP tree (Fig. 7), nor in a previous phylogenetic study of Bignonieae, in which *Dolichandra s.l* emerged as sister of multiples-of-four clade (Lohmann, 2006). Although the sister relationship between *Dolichandra* and the multiples-of-four clade only received poor support (Lohmann, 2006), this relationship was further corroborated by wood anatomical traits; in particular, the presence of multi-dissected phloem edges (Lohmann, 2006; Pace, Lohmann & Angyalossy, 2009). This unique pattern of phloem development represents the final step in a developmental series that goes through intermediate steps that are exclusive of the multiples-of-four clade, further supporting the sister relationship between *Dolichandra* and the multiples of four clade (Pace *et al.*, 2009).

PHYLOGENETIC RELATIONSHIPS WITHIN *DOLICHANDRA S.L.*

Dolichandra unguiculata was strongly supported as sister to the rest of the genus in all analyses. In all topologies that resulted from the analysis of the plastid markers, *D. chodatii* and *D. cynanchoides* were the following lineages to diverge. However, in the topology that resulted from the analysis of the

nuclear marker (i.e., *Pep*C), *D. chodatii* and *D. cynanchoides* emerged as a well-supported clade, sister to the rest of the tribe. This clade was held in the combined analyses, although with moderate support (BS = 74, PP = 0.67). Morphological features such as corolla texture, presence of large nectaries, or inflated calyx provide further support for this relationship.

The clade formed by *D. quadrivalvis* and *D. acuminata* was highly supported in all topologies (Figs. 6–9). Both species are very similar vegetatively and reproductively, differing from each other in their leaflet dimensions, leaflet shape and calyx morphology. This clade is sister to a clade that includes all former representatives of *Macfadyena* (*sensu* Gentry, 1973a), including *D. uncata*, *D. dentata*, *D. hispida* and *D. unguis-cati*. This whole lineage is here called yellow-flowered clade (Fig. 7). The clade that includes all species previously included in *Macfadyena* is strongly supported as monophyletic by a series of molecular synapomorphies and by morphological features such as the linear fruits, winged seeds that are poorly demarcated from the seed body, filiform bracts (versus foliaceous in *D.quadrivalvis* and *D. acuminata*) and tendril branches that do not become woody and thick in age. Relationships within this clade are not well defined, varying across topologies (Figs. 6–7). The only relationship within this clade that was recovered in all topologies is the sister relationship between *D. uncata* and the remaining taxa in this clade. Although this relationship is strongly supported by molecular characters, it is less supported by morphological traits, with no obvious morphological synapomorphies. The placement of *D. dentata, D. hispida* and *D. unguis-cati* varied across trees. In the combined analysis, *D. hispida* is sister of a clade composed of *D. dentata* and *D. unguis-cati* (Figs. 8–9); these relationships were also recovered in the *PepC* topology.

Multiple accessions were sampled for *D. chodatii, D. cynanchoides, D. hispida, D. quadrivalvis, D. uncata, D. unguiculata* and *D. unguis-cati*, all of which emerged as monophyletic in all topologies (Figs. 6–8). Reciprocal monophyly is not a necessary property of species, yet it is generally interpreted as an evidence of such. Species are evolutionary lineages that acquire properties through time, reaching a status of reciprocal monophyly in advanced stages of the speciation process, when alleles are fixed between lineages and no gene flow is observed to shuffle genes histories (de

Queiroz, 2007; Funk & Omland, 2003). Therefore reciprocal-monophyly criterion provides a reasonable test for species hypotheses proposed exclusively based on morphological similarity. Morphologically and geographically distant individuals of *Dolichandra s.l.* were sampled in the present study in order to test species limits within the genus. Our results corroborate previous species hypothesis, with all previously recognized species forming strongly supported clades.

SPECIATION IN *DOLICHANDRA S.L.*

Simulation studies detected a significant phylogenetic signal in the distribution data and consequently the retention of geographical signatures of speciation in present-day distributions, suggesting that sympatric speciation is the most likely mode of speciation in the genus. More specifically, all nodes of the species level phylogeny of *Dolichandra s.l.* presented some level of sympatry between lineages, with only nodes C and H presenting values that were lower than 0.5 (Fig. 9). Those results indicate that sympatric speciation cannot be excluded for all nodes of the phylogeny and that diversification studies of *Dolichandra s.l.* need to take into account the possibility of sympatric speciation.

Significant divergence values (*D*) were detected for all nodes in the phylogeny, indicating non-random associations between ecological (i.e., environmental variables) and phylogenetic splits, highlighting the complex interactions between environmental variables and phylogenetic history. These results, in association with the fact that all nodes are partially or totally sympatric, indicate that at least some nodes may have resulted from speciation due to habitat isolation. In particular, nodes E and H represent the most recent nodes of the phylogeny and the ones to present the highest significant *D* values, corroborating the hypothesis of habitat isolation in those lineages. Habitat isolation in other nodes of the phylogeny should be analysed with caution, since habitat divergences between lineages may have simply resulted from habitat changes that may have occurred after speciation. It is unlikely that small habitat differences can reduce reproductive encounters by adaptation or preferences for ecologically different parts of this area (e.g., Coyne & Orr, 2004).

Ancestral character state reconstructions indicated that pollinator shifts are present in nodes B

and C of the phylogeny of *Dolichandra s.l.* In node B, the evolution of a hummingbird-pollinated species from a bee pollinated ancestral was detected. This pollination switch is common in the angiosperms (Tripp & Manos, 2008; Whitall & Hodges, 2007) and also known from other lineages of Bignonieae (Alcantara & Lohmann, 2010). In node C, on the other hand, a reversal from a hummingbird ancestral to a bee-pollinated morphology was inferred, an uncommon direction of pollination shift in angiosperms and Bignonieae (Alcantara & Lohmann, 2010). This reversal was not detected in all characters associated with pollination though, with coriaceous corollas, lack of scent and presence of giant floral nectaries (with at least 30 mm^2), being present in both taxa derived from that divergence event (*D. chodatii* and *D. cynanchoides*). All of these traits are associated with both melitophyly and ornitphyly (Gentry, 1974a). Pollinator specialization shifts are often thought to be associated with speciation in sympatry, given that gene flow is reduced when different populations use different functional groups of pollinators (Coyne & Orr, 2004; Kay & Sargent, 2009).

Apart from differences in pollination systems, two evolutions of hydrocoric seeds from anemocoric seeds were observed in *Dolichandra s.l.*, as well as two reversals to anemocory from hydrocoric ancestors. Transitions from anemocoric to hydrocoric dispersal systems were observed in nodes D and G, while the reversals were encountered in nodes F and H. This result suggests that dispersal specialization may have played a role in at least four divergence events in *Dolichandra s.l.* While pollination syndromes have been extensively studied in speciation studies (see Coyne & Orr, 2004; Kay & Sargent, 2009), much less attention has been given to specialized dispersal syndromes. Little is still known about the gene flow barriers that can be created due to changes among dispersion syndromes; however, it is possible that these barriers may be caused by selection against hybrids and their mal-adapted "wings" (Coyne & Orr, 2004).

CONCLUSIONS AND FUTURE DIRECTIONS

This study presents a phylogenetic hypothesis of *Dolichandra s.l.* based on molecular characters that is also corroborated by a series of morphological synapomorphies. While several relationships among

species of *Dolichandra s.l.* are strongly supported, the exact phylogenetic position of *D. chodatii, D. cynanchoides, D. dentata* and *D. hispida* remain to be explored with an increased taxon and character sampling. An increase in molecular sampling would also be necessary to increase the support for the current placement of *Dolichandra s.l.* within Bignonieae. Despite that, our results are sufficiently robust for a taxonomic revision of the genus (Fonseca *et al.,* in prep.), as well as provide the basis for evolutionary studies in the genus.

Our results suggest that *Dolichandra s.l.* has had a complex speciation history, with multiple factors being important for the establishment of gene flow barriers. From the eight divergence events within *Dolichandra s.l.*, at least three present habitat differences, two present differences in pollination syndromes and four present differences in dispersal syndromes. This complex speciation history is confirmed by the observed high degree of sympatry across nodes of the phylogeny, suggesting a limited role of geographic isolation for the diversification history of the genus. With the evidence collected, we believe that ecological isolation changes in habitat, pollinator and dispersal also seem to also have played important roles for the speciation history of *Dolichandra s.l.*

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FIGURES

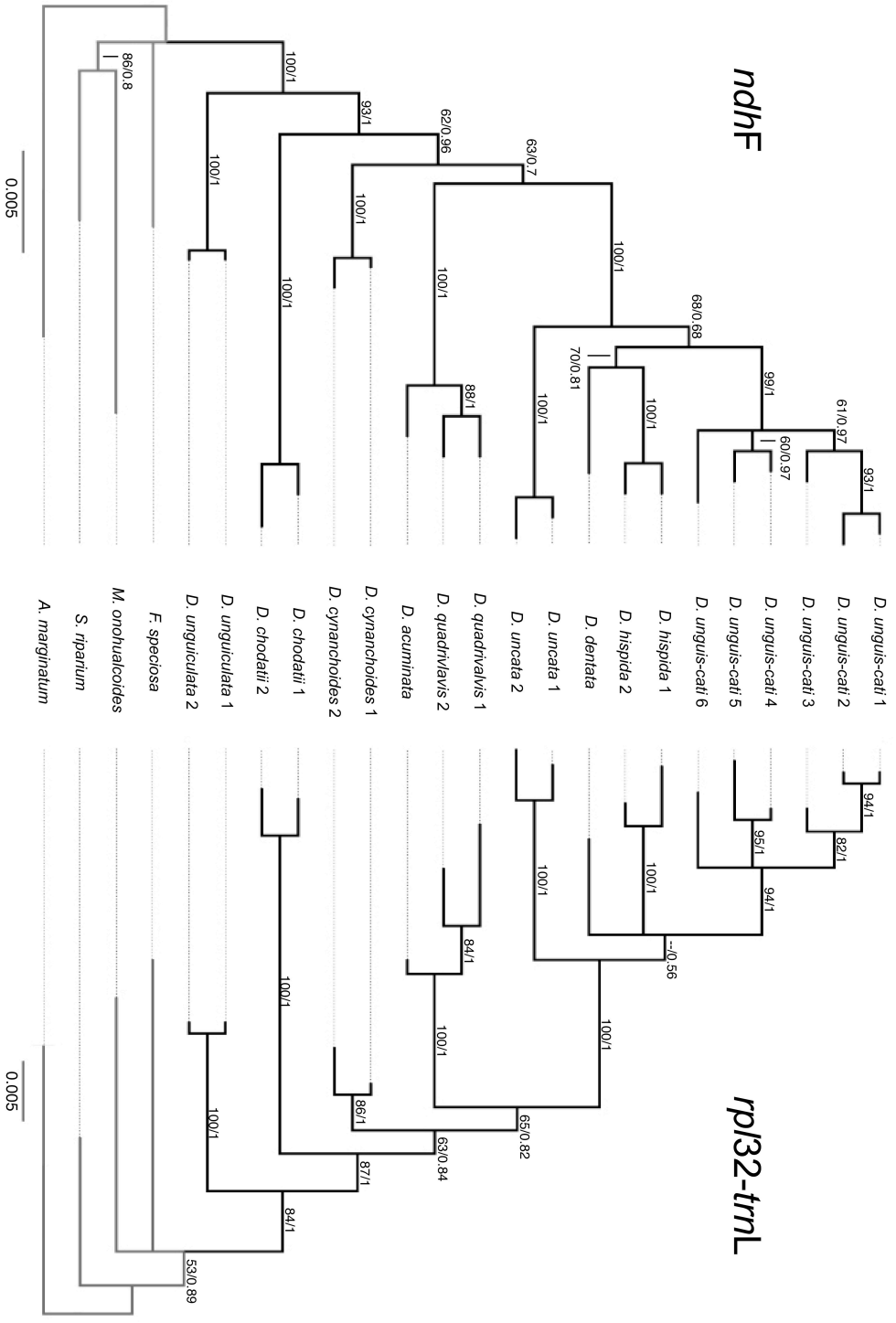

Fig. 5. Comparison of the 50% majority-rule post burn-in consensus trees derived from the Bayesian analyses of the *ndh*F and *rpl*32-*trn*L data partitions. Bootstrap values greater than 50% and Posterior Probabilities greater than 50% are presented above branches

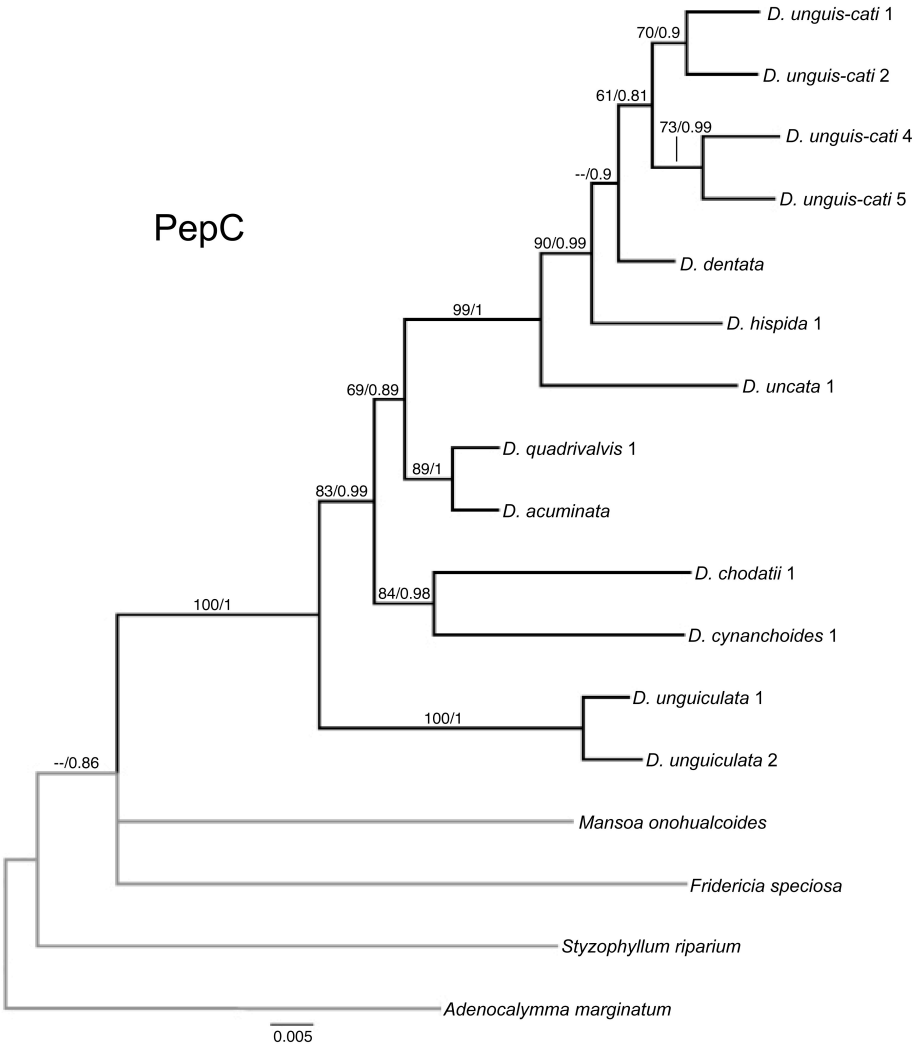

Fig. 6. 50% majority-rule post burn-in consensus tree derived from the Bayesian analysis of the *Pep*C data set. Bootstrap values and Posterior Probabilities greater than 50% are provided above branches.

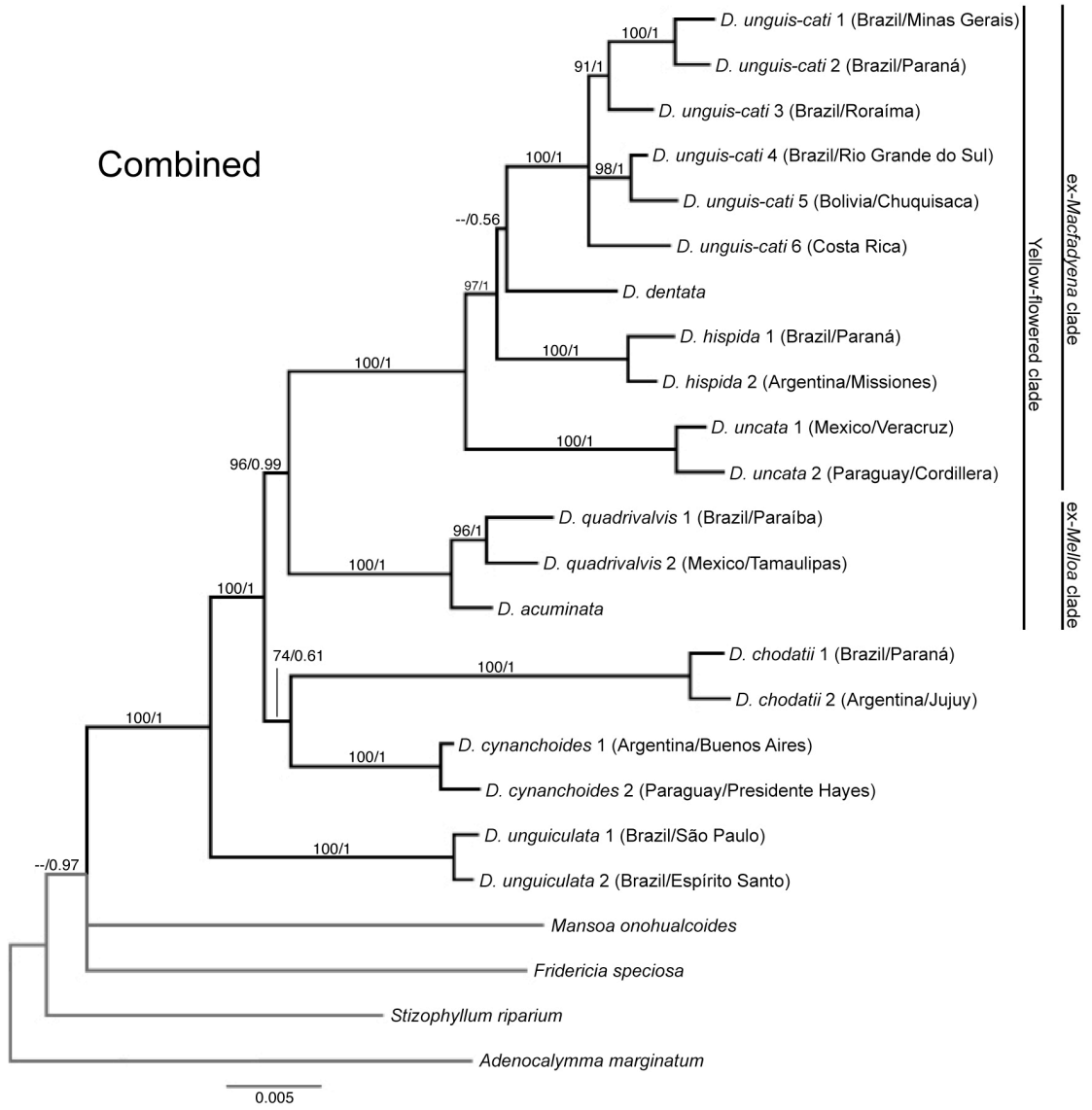

Fig. 7. 50% majority-rule post burn-in consensus tree derived from the Bayesian analysis of the combined data set. Bootstrap values and Posterior Probabilities greater than 50% are provided above branches.

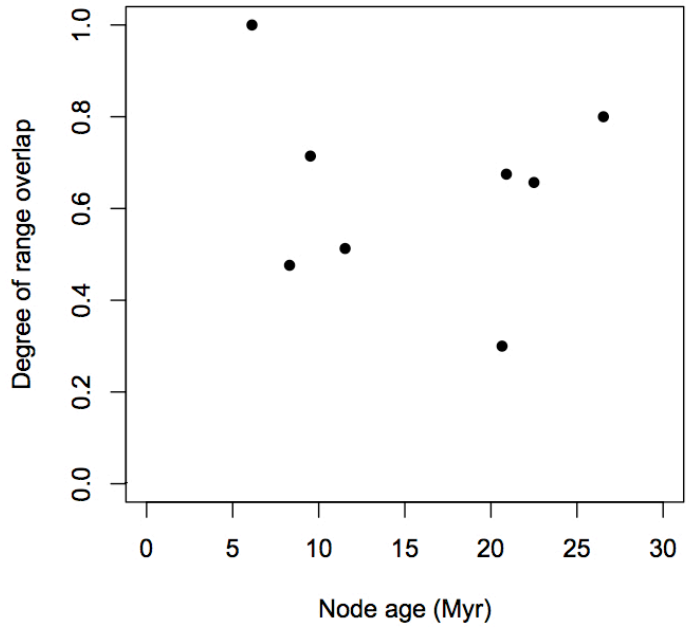

Fig. 8. Plot of the degree of sympatry (y-axis) against node age (x-axis) in *Dolichandra s.l.*

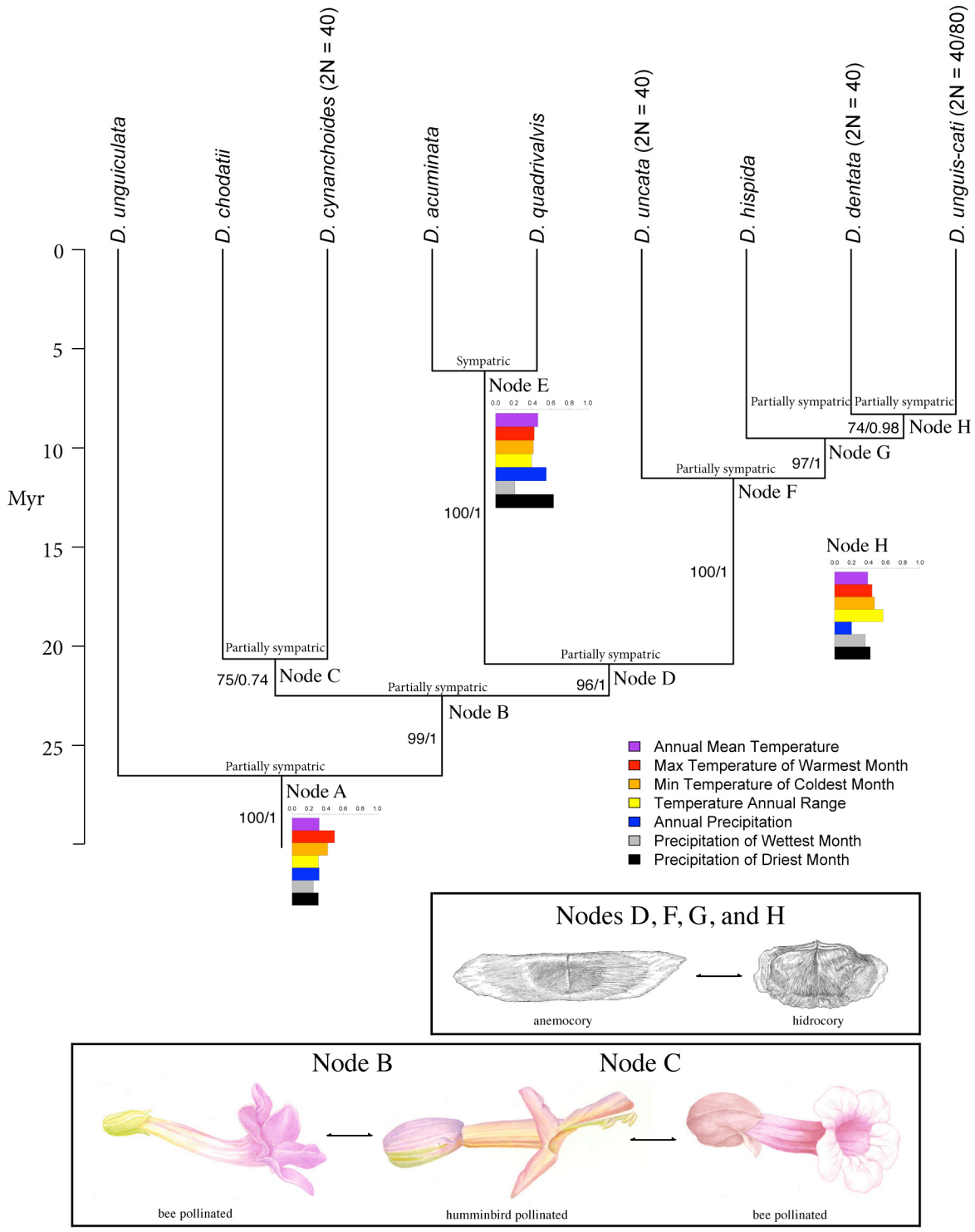

Fig. 9. Simplified phylogeny of *Dolichandra s.l.* including a single sample per species. Branches are proportional to time. Bootstrap values and Posterior Probabilities greater than 50% are provided above branches. *D* values for each environmental variable are presented in nodes A, E, and H. Changes in floral and seed characters are presented in boxes.

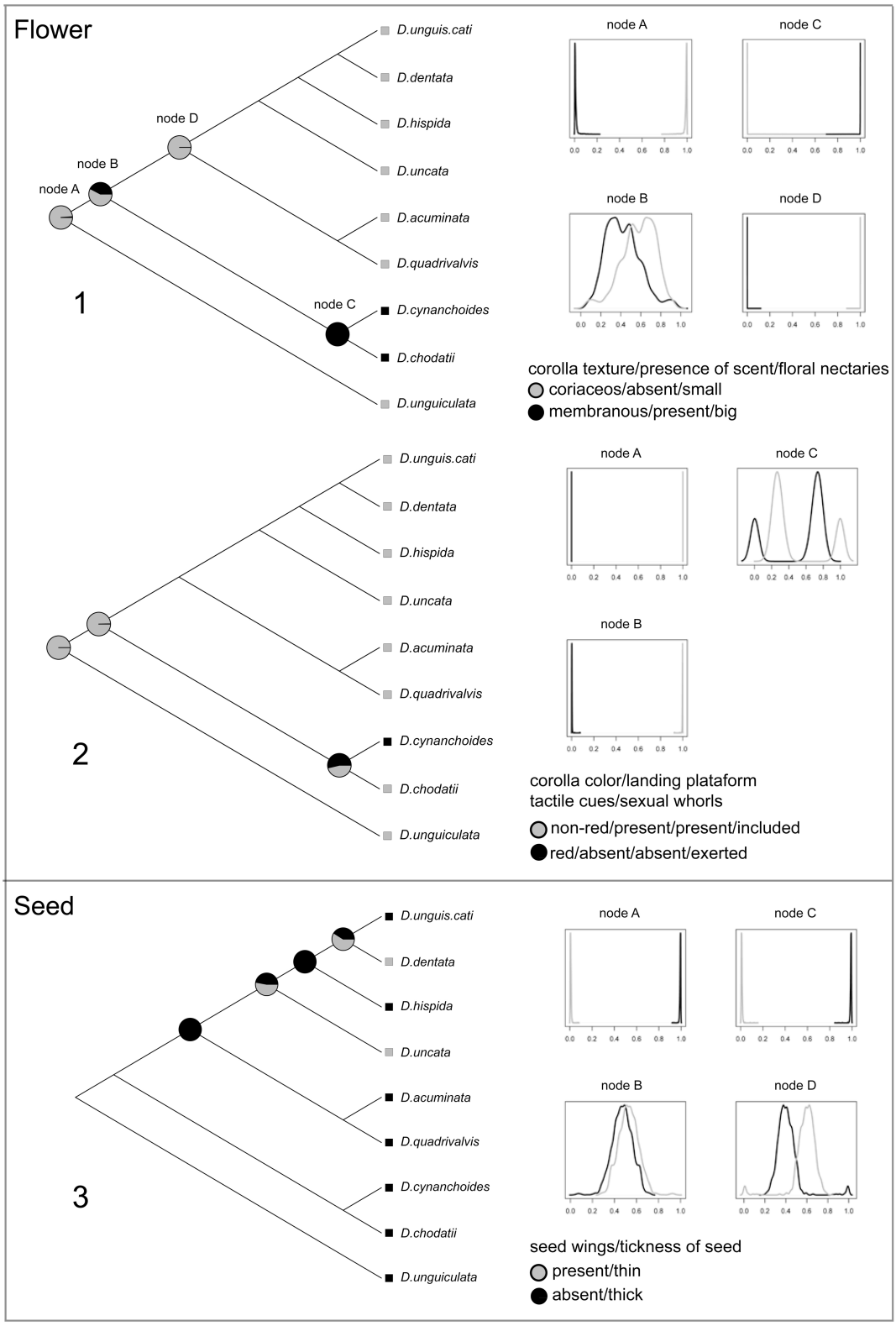

Fig. 10. Cartoon of the phylogeny of *Dolichandra s.l.* showing Bayesian ancestral state reconstructions of floral and seed characters. Plots with density curves represent the posterior probability distribution of each state at the nodes indicated. Plots 1 and 2: Ancestral state reconstructions of floral characters. Plot 3: Ancestral state reconstructions of seed characters.

TABLES

Table 1. Taxa, voucher, and collection sites for sampled *Dolichandra* and outgroups. Samples presented in bold correspond to DNA sequences obtained from Lohmann (2006).

Genus	Species	Collector	Number	Country	Major area	Minor area
Dolichandra	*acuminata*	Fonseca	161	Brasil	São Paulo	Iporanga
Dolichandra	*chodatii* 1	Fonseca	136	Brasil	Paraná	Londrina
Dolichandra	*chodatii* 2	Ferraro	7464	Argentina	Jujuy	Parque Nacional Calilegua
Dolichandra	***cynanchoides* 1**	**Galetto**	**1019**	**Argentina**	**Buenos Aires**	**Buenos Aires**
Dolichandra	*cynanchoides* 2	Silva	60	Paraguai	Presidente Hayes	Pozo Arias
Dolichandra	*dentata*	Fonseca	124	Brasil	Santa Catarina	Itapiranga
Dolichandra	*quadrivalvis* 1	Lohmann	686	Brasil	Paraíba	Curral de Cima
Dolichandra	*quadrivalvis* 2	Martinez	582	Mexico	Tamaulipas	Gomez Farias
Dolichandra	*hispida* 1	Fonseca	27	Brasil	Paraná	Morretes
Dolichandra	*hispida* 2	Fonseca	111	Brasil	Paraná	Foz do Iguaçu
Dolichandra	*uncata* 1	Trigos	2773	México	Veracruz	Catemaco
Dolichandra	*uncata* 2	Zardini	21585	Paraguai	Cordillera	Estero de Ypoá
Dolichandra	*unguiculata* 1	Fonseca	166	Brasil	São Paulo	Bertioga
Dolichandra	*unguiculata* 2	Fonseca	159	Brasil	São Paulo	Apiaí
Dolichandra	***unguis-cati* 1**	**Lombardi**	**2432**	**Brasil**	**Minas Gerais**	**Parque Estadual do Rio Doce**
Dolichandra	*unguis-cati* 2	Fonseca	104	Brasil	Paraná	Londrina
Dolichandra	*unguis-cati* 3	Fonseca	140	Brasil	Roraima	Caracaraí
Dolichandra	*unguis-cati* 4	Fonseca	131	Brasil	Rio Grande do Sul	São Borja
Dolichandra	*unguis-cati* 5	Rodriguez	4798	Costa Rica	Puntarenas	Golfito
Dolichandra	*unguis-cati* 6	Gutierrez	990	990 Bolívia	Chiquisaca	Chiquisaca Tomina Tomina
Adenocalymma	*marginatum*	Fonseca	117	Brasil	Paraná	Londrina
Fridericia	***speciosa***	**Lombardi**	**2521**	**Brasil**	**Minas Gerais**	**Rio Doce**
Mansoa	*onohualcoides*	Zuntini	276	Brasil	Espírito Santo	Linhares
Styzophyllum	*riparium*	Fonseca	103	Brasil	Paraná	Londrina

Table 2. Primers used for the amplification and sequencing of *ndh*F,*trn*L-*rpl*32 and *Pep*C loci in *Dolichandra* and outgroups.

Region	Genome	Primer	Ref.
*ndh*F	Plastid	5F: 5'-ATG GAA CAG ACA TAT CAA TAT GSG TGG-3'	Olmstead & Reeves (1995)
*ndh*F	Plastid	1318R: 5'-CGA AAC ATA TAA AAT GC(G/A) GTT AAT CC-3'	Olmstead & Sweere (1994)
*ndh*F	Plastid	972F: 5'-ATC ATA TAA CCC AAT TGA GAC-3'	Olmstead & Sweere (1994)
*ndh*F	Plastid	3R: 5'-CCC (T/C)A(C/G) ATA TTT GAT ACC TTC (T/G)CC G-3'	Olmstead & Reeves (1995)
*ndh*F	Plastid	370F: 5'-TTC CAT GTT GGG ATT AGT TAC TAG C-3'	Zuntini (ined.)
*ndh*F	Plastid	478R: 5'-AGGTCGTGTGAACCAAAACC-3'	Zuntini (ined.)
*ndh*F	Plastid	808F: 5'-AGC TCG CCT TCT TCC TCT TT-3'	Zuntini (ined.)
*ndh*F	Plastid	849R: 5'-GGC CTA TCA AAG AGA TAA AAT TCA-3'	Zuntini (ined.)
*ndh*F	Plastid	1290F: 5'-CAG CAG GAT TAA CCG CAT TT-3'	Zuntini (ined.)
*ndh*F	Plastid	1835R: 5'-CGC TAA AAA TAT TCC GAA ATA AGC-3'	Zuntini (ined.)
*rpl*32-*trn*L	Plastid	trnL: 5'-CTG CTT CCT AAG AGC AGC GT-3'	Shaw *et al*, 2007
*rpl*32-*trn*L	Plastid	rpl32: 5'-CAGTTCCAAAAAAACGTACTTC-3'	Shaw *et al,* 2007
*rpl*32-*trn*L	Plastid	365F: 5'- TGC CTG GAT TGA TGG YGA GAG A -3'	Fonseca (ined.)
*rpl*32-*trn*L	Plastid	478R: 5'- TAG AAG GGC GGA TAG AAA ATC T -3'	Fonseca (ined.)
*rpl*32-*trn*L	Plastid	682F: 5´- CGG ACG ATC GAG TTT TAC AAG AGT - 3´	Fonseca & Zuntini (ined.)
*rpl*32-*trn*L	Plastid	619R: 5´- TTC TTT TAA TGA ACT GTT TTT GA - 3´	Fonseca & Zuntini (ined.)
*rpl*32-*trn*L	Plastid	241F: 5´ - ATC ATT TCC AAG CCG AGG A - 3´	Fonseca & Zuntini (ined.)
*rpl*32-*trn*L	Plastid	146R: 5´ - TMA CAT CTG GYT CCC AYC CT - 3´	Fonseca & Zuntini (ined.)
*Pep*C	Nuclear	4F: 5'- ACT CCA CAG GAT GAG ATG AG -3'	Gaskin (unpublished)
*Pep*C	Nuclear	5R: 5'- GCA GCC ATC ATT CTA GCC AA -3'	Gaskin (unpublished)

Table 3. Statistics of individual data partitions and combined data sets.

Locus	Raw seq. length (bp)	Aligned length	Nº of MPT	Length of MPT	Variable sites	PIC	CI	RI	Substitution model
*ndh*F	2026-2125	1995	1	171	232	127	0.8363	0.9213	TVM+G
*rpl*32-*trn*L	926-1120	921	10	137	195	104	0.8467	0.9250	GTR
*Pep*C	688-810	737	6	180	223	91	0.6000	0.7154	GTR+I+G
Combined	---	3634	18	496	654	323	0.7419	0.8555	---
Simplified	---	3634	3	289	613	171	0.6678	0.7363	---

CI = Consistency index; MPT = Most parsimonious tree; RI = Retention index; CI, MPT and RI were computed with uninformative sites excluded.

Table 4. Expected morphological traits under the various pollination syndromes (Based on: Faegri and Van der Pijl, 1979; Gentry; 1974; Glover, 2008).

	Melittophily (Small bees)	Melittophily (Medium/large bees)	Ornitophily
Corolla colour	Blue/yellow/ultraviolet	Blue/yellow/ultraviolet	Red/orange
Corolla texture	Menbranous	Menbranous/Coriaceos	Coriaceos
Corolla tubes	Flattened	Flattened/Rounded	Rounded
Reward	Less nectar	Much nectar	Much nectar
Nectar guides	Present	Present	Present/Absent
Tactile cues	Present	Present	Absent
Landing plataform	Present	Present	Absent
Scent	Minimal or some	Minimal or some	Minimal or absent
Sexual whorls	Included	Included	Exerted

Table 5. Expected morphological traits under the various dispersal syndromes (Based on: Gentry, 1974).

	Anemocory	Hidrocory
Seed wings	Membranous	Woody
Seed thickness	Thin	Thick

Table 6. Results from the analyses of speciation. Degree of sympatry, Index of Divergence (*D*), and changes of syndromes are provided for each node of the simplified phylogeny presented in Figure 9.

		Node A	Node B	Node C	Node D	Node E	Node F	Node G	Node H
Degree of sympatry		0.62	0.80	0.37	0.73	1	0.67	0.75	0.47
Node ages (Myr)		26.54	22.5	20.64	20.9	6.12	11.53	9.51	8.30
Mean annual temperature	Index of divergence (D)	0,32*	0,31*	0,07*	0,08*	0,46	0,19*	0,21*	0,39*
Max temperature of warmest month		0,49*	0,05*	0,04	0,03*	0,42	0,11*	0,08	0,44*
Min temperature of coldest month		0,42*	0,39*	0,04*	0,12*	0,41	0,19*	0,26*	0,47*
Temperature annual range		0,31*	0,43*	0,16*	0,11*	0,39	0,13*	0,36*	**0,57***
Annual precipitation		0,32*	0,24*	0,08	0,07*	**0,55***	0,29*	0,1	0,20*
Precipitation of the wettest month		0.25*	0,39*	0,19*	0,02	0,21	0,19*	0,08	0,36*
Precipitation of the driest month		0.31*	0,08*	0,11	0,09*	**0,63***	0,15*	0,15*	0,42*
Mean values of *D* for each node		0.35	0.27	0.1	0.075	0.44	0.18	0.18	0.41
Difference in pollination syndrome		no	yes	yes	no	no	no	no	no
Difference in dispersal syndrome		no	no	no	yes	no	yes	yes	yes

Capítulo 2

Taxonomic novelties in *Dolichandra s.l.* (Bignonieae, Bignoniaceae).

(a ser submetido à revista Phytotaxa)

Taxonomic novelties in *Dolichandra s.l.* (Bignonieae, Bignoniaceae).

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ABSTRACT

Dolichandra s.l. is a genus of lianas found in dry and wet forests of the Neotropics. The genus includes ten species and is recognized by molecular and morphological synanpomorphies. Here, we describe a new species, propose a new combination and present a taxonomic key for all species included in the genus. *D. acuminata* is a new species that resembles *D. quadrivalvis*. Both species are differentiated from other species of *Dolichandra s.l.* by the foliaceous bracts of the inflorescence, the subspathaceous calyx with a recurved apicule, and the oblong-elliptic woody fruits. However, they differ from each other by the caducous tendrils and elliptic leaflets with long acuminate apex of *D. auminata*, opposed to the permanet and woody tendrils, and obvate and acute leaflets of *D. quadrivalvis*. Other differences between those taxa are in the petiole and petiolule length, indument type, and geographic distribution. *Macfadyena hispida* is here considered taxonomically different from *D. uncata* based on the presence of the hispid indument, long fruits, and winged seeds. This species is thus removed of synonymy and the new combination *Dolichandra hispida* is proposed.

KEY WORDS: Atlantic Forest – Lianas – Taxonomic key – *Dolichandra acuminata* – *Dolichandra hispida*

INTRODUCTION

Dolichandra Chamisso (1832: 657) is a genus of lianas that belongs to the tribe Bignonieae, in the plant family Bignoniaceae (Lohmann 2006, Lohmann & Taylor in press). The family comprises 80 genera and more than 800 species, being an important component of Neotropical forests (Lohmann & Ulloa 2006, onwards). Brazil is the center of diversity for the family and for the tribe Bignonieae (Gentry 1992, Lohmann & Ulloa 2006 onwards).

The current circumscription of *Dolichandra s.l.* (Lohmann 2003; Lohmann & Taylor in press) is based on molecular phylogenetic data and morphological synapomorphies (Lohmann 2006). In this circumscription, the genus is composed of ten species (Lohmann 2003; Lohmann & Taylor in press) previous described in *Dolichandra s.s.*, *Macfadyena* DC. (1845: 179), *Melloa* Bureau (1868: 379), and *Parabignonia* Bureau ex K.Schum in Engler (1894: 229). *Dolichandra s.l.* is characterized by unique multiple dissected phloem wedges, trifid and uncinate tendrils, fruits with four lines of dehiscence, a dimorphic growth form, and colpate pollen with a psilate exine (Lohmann 2003; Lohmann & Taylor in press). An additional diagnostic character is the large and membranaceous calyce (Lohmann 2003; Lohmann & Taylor in press).

The genus is distributed in wet and dry Neotropical forests, from México to northern Argentina (Lohmann 2003; Lohmann & Taylor in press), being a conspicuous component of seasonally dry forests (Särkinen *et al.* 2011). The geographic distribution of *Dolichandra s.l.* is centered in southern Brazil, northern Argentina and Paraguay, where up to seven species are found. The geographic distribution of species is highly variable, with species found throughout the Neotropics, like the ubiquitous *D. unguis-cati* (Linnaeus 1753: 623) L.G. Lohmann (in press), and species with restricted distribution, like the endemic *D. dentata* (Schumann 1894: 227) L.G. Lohmann (in press) (Lohmann & Taylor in press).

During the course of a phylogenic study of *Dolichandra s.l.,* a new species was identified. In addition, a previous described taxon, *Spathodea hispida* (DC. in Candolle 1845: 205), was here considered distinct from *D. uncata* (Andrews 1808: 530) L.G. Lohmann (in press) and recognized as a

separate taxon. Here we present a description of the newly described species and the new combination proposed for *Macfadyena hispida*. We also provide a taxonomic key for the identification of species within the genus.

MATERIAL AND METHODS

This study was based on herbarium collections from eight herbaria (ESA, FUEL, INPA, MBM, MO, SP, SPF, and UPCB). Collections of the whole geographic distribution of *D. quadrivalvis* (Jacquin 1809: 37) L.G. Lohmann (in press) and *D. uncata* where used for comparisons. Morphological studies were carried out under a stereomicroscope using dried and fresh specimens.

Dolichandra acuminata **L.H. Fonseca & L.G. Lohmann, *sp. nov.*** (Figs. 11–13).

Dolichandra acuminata is morphologically similar to D. quadrivalvis, however it can be distinguished by the caducous tendrils, and the elliptic leaflets, with long acuminate apex. Short petioles ((5–)6–16 mm long), and petiolules (2–7 mm long), and puberulent indument are always present.

Type:—BRAZIL. São Paulo: Iporanga, estrada entre Apiaí e Iporanga, à 5 km de Iporanga, beira do Rio Bethary, floresta ombrófila densa, 24º35′18′′S, 48º37′39′′O, 100 m elev., 23 October 2010, *L.H.M. Fonseca & D. Tarabay 161* (holotype SPF, isotype MO).

Liana, 3–15 m high. Brachlets terete, striate, glabrous to puberulous; interpetiolar region with ridges and glandular fields with peltate glands on young branchlets; older stems and branchlets glabrous and lenticellate; prophylls 2.5–4 mm long, subulate, apiculate and puberulous with simple trichomes. Leaves bifolialate; petioles semi-terete, puberulous, (5–)6–16 mm long, with simple trichomes; petiolules terete, 2–6 mm long, puberulous, with simple trichomes; tendrils trifid and uncinate (sometimes woody); leaflets elliptic, apex long acuminate with a drip tip, base cuneate (sometimes acute) symmetric or slight asymmetric, 2.7–11.8 × 0.6–4.5 cm, margin entire, chartaceous, the abaxial surface glabrescent with peltate glands concentrated around the main vein, the adaxial surface glabrous, primary venation straight, unbranched, secondary venation brochidodromous, sometimes

with smooth increase of angles, tertiary venation percurrent. Inflorescence a terminal or axillary raceme with up to six orders; axis of the inflorescence puberulent, with simple trichomes; bracts of the inflorescence caducous, foliaceous, 1.7–2.4 cm long; bracteoles caducous, elliptic–foliaceous, 0.7–1.2 cm long. Flowers with calyx membranaceous, green, 2.0–3.2 × 0.8–1.6 cm, with a long recurved apicule, puberulent throughout (with more trichomes at the base), trichomes simple; corolla yellow, narrow infundibuliform, flattened (sometimes slightly circular), 6.5–9 × 1.5–2.4 cm wide at opening of the tube, tube 4.6–5.8 cm long, with nectar guides dark yellow, glabrous outside and hispid inside at the level of the stamens insertion, trichomes simple and glandular; lobes obcordate, 1.7–2.7 × 1.5–3.5 cm, with pubescent margins, glabrous; stamens attached at the same height from the base of the corolla, 6–9 mm from the base, shorter filaments 1.5–1.8 cm long, long filaments 2.2–2.4 cm long, the filaments glabrous, the staminode 0.6–1.0 cm long, placed at 7–9 mm from the base of corolla, glabrous, anthers 4–5 mm, pale-yellow or white, glabrous; pistil 3.5–4.5 cm, ovary 2.5–4.8 × 2.3–3.2 mm, elliptical–ovoid, ribbed, lepidote, with peltate trichomes, four series of ovules per placenta, style 3.3–4 cm long, ovate, glabrous. Immature fruit elliptic–oblong, 7.4 × 4.2 cm, acute in base and apex, rough, glabrous. Mature fruits and seeds are unknown.

Distribution and habitat:—Present in the Atlantic Rainforest on the coastal plain of the states of Paraná and São Paulo (Fig. 3), from sea level to 100 m elev., representing the most restricted species of the genus. All specimens available were collected in riverbanks, although young individuals were also observed hundreds of meters from the riverbanks, indicating that this species also occurs in environments other than riverbanks. Seeds could be dispersed both by water and wind, like many other species of *Dolichandra s.l,* including the sister species *D. quadrivalvis*.

Phenology:—Flowering samples were collected from September to December. One immature fruit was collected in March.

Etymology:—The epithet *acuminata* refers to the long acuminate leaflet apex of the species.

Observations:—The morphologically similar *D. acuminata* and *D. quadrivalvis* formed a robust clade in a molecular phylogenetic study of the genus (Capítulo 1; Fonseca & Lohmann, in

prep.). Potential morphological synapomorphies for this clade are the presence of foliaceous bracts and bracteoles, a subspathaceous calyx, with a recurved apicule and elliptic–oblong woody fruits. Diagnostic characters are the large tendrils and flowers with deep yellow nectar guides.

Remarkable geographic differences are observed between *D. acuminata* and *D. quadrivalvis*. The latter is a widely distributed species, ranging from Mexico to southern Brazil, Paraguay, Uruguay and Argentina, occurring between sea level to 1300 (1800) m, mainly in deciduous and dry forests of Bolivia, Brazil, Costa Rica and Paraguay, subtropical forests of Argentina and the "caatingas" of eastern Brazil. In contrast, *D. acuminata* is restricted to the southern portions of the Atlantic forest, being found between sea level to 100 m elevation in the costal plains of São Paulo and Paraná.

Additional material examined (paratypes):—BRAZIL. **Paraná**: Morretes, Estrada do Barreiro, Porto de Cima, 10 December 1949, *G. Hatschbach 1656* (MBM). **São Paulo**: Cananéia, Distrito de Itapitangui, Parque Estadual de Jacupiranga, Núcleo do Cedro, Trilha do Rio das Minas, Sítio Paraíso das Minas, Floresta ombrófila densa baixo montana muito degradada, sea level, 24º59′31″S, 48º07′38″W, 26 March 2005, *Destefani et al. 145* (ESA); Cananéia, Estrada Pariquera-açu–Cananéia (via ponte) km 3, a 19 km de Pariquera-açu, Beira de rio, 24º51′51.8″ S, 47º52′50″W, 06 September 1994, *Wongtschowsky et al. 12* (ESA, MO); Juquiá, Capueiras à beira do Rio Juquiá, 26 November 1954, *M. Kuhlmann 3103* (SP).

Dolichandra hispida **(DC.) L.H. Fonseca & L.G. Lohmann,** ***comb. nov***. Basionym: *Spathodea hispida* DC. in Candolle (1845: 205). *Macfadyena hispida* (DC.) Seemann (1863: 227). Type:—BRAZIL. Mato Grosso: Cuiabá "Cujaba", 1832, *A. Silva Manso 105A* (holotype G-

Macfadyena pubescens Moore (1895: 418). Type:— PARAGUAY. "inter Villa Maria et Corumbá," December 1891–92, *Moore 1021* (holotype BM!, isotype NY!).

Spathodea mollis Sonder (1849: 561). *Macfadyena mollis* (Sond.) Seemann (1863: 227). Type:— BRAZIL. Minas Gerais: Caldas, *Regnell I-292* (holotype S!, isotype MO!).

D. hispida can be differentiated from other species of *Dolichandra* by the unique hispid indument found in vegetative and reproductive portions of this species, as well as the incurved apiculated calyx and the lack of nectar guides in the corolla. *D. hispida*, has been treated as synonyms of *D. uncata*

since Gentry (1973).

Here, we recognize *D. hispida* as a different species from *D. uncata* based on the hispid indument, in comparison to the glabrous to puberulous indument of *D. uncata*. In addition, the hyaline seed wings (vs woody seed wings of *D. uncata*) and a striking difference in fruit length (77–125.8 cm in *D. hispida* vs 9.2–38.5 cm in *D. uncata*) further corroborates the distinction of these taxa. With *D. unguis-cati*, *D. hispida* presents the longest fruit of Bignoniaceae (Lohmann & Taylor in press) and possibly one of longest capsule within Angiosperms. *D. hispida* and *D. uncata* also differ geographically, with *D. hispida* occuring in northern Argentina, southern and southwestern Brazil, Paraguay and Bolivia and *D. uncata* occuring from Mexico to northern Argentina and southern Brazil. *D. uncata* is adapted to riverbanks, swamps and mangroves, presenting modified seeds for water dispersal. On the other hand, *D. hispida* is more common in non-flooded areas, presenting seeds that are adapted to wind dispersal. *M. pubescens* and *S. mollis* were also sinonimized in *D. uncata* by Gentry (1973) and are now treated as synonimous of *D. hispida*.

Additional material examined:—BRAZIL, **Mato Grosso**: Alta Floresta, Fazenda Mogno, Ponte do 27, margem direita, mata de capoeira, solo arenoso, 18 September 1991, *Macedo et al. 3009* (UFMT, INPA). **Paraná**: Antonina, Rio Mergulhão, 31 October 1973, *G. Hatschbach 29172* (MBM); Foz do Iguaçu, Parque Nacional das Cataratas do Iguaçu, 14 October 1962, *G. Hatschbach 9378* (MBM); Guaraqueçaba, Tagaçaba de Cima, Rio Tagaçaba, orla da Floresta Atlântica, 20 November 2003, *G. Hatschbach et al. 76720* (MBM); Irati, Riozinho, 01 October 1982, *Hatschbach 45518* (MBM); Laranjeiras do Sul, Salto Santiago, 07 March 1991, *Silva et al. 955* (UPCB, SP); Londrina, Fazenda Santa Ana, 31 October 1985, *Dias s.n.* (FUEL, MO); Morretes, início da Estrada do Itupava, beira do Rio Nhumdiaquara, próximo à ponte de Morretes, 29 m elev., 25°26′1.31″S, 48°52′26.31″W, *L.H.M. Fonseca et al. 27* (SPF, MBM); Pinhão, Vale do Rio Iguaçu, Córrego Estreito, 22 February 1996, *G. Hatschbach et al. 64429* (MBM); Rio Bonito do Iguaçu, Fazenda Giacomet-Marodin, Pinhal Ralo, 23 June 1995, *Poliquesi & Cordeiro 328* (MBM, SPF); Tibagi, 696 m elev., 12 October 1959, *G. Hatschbach 6373* (MBM). **Rio Grande do Sul**: Morrinhos do Sul, Morro do Forno, trepadeira em

borda de Mata Atlântica de encosta, 19 October 1997, *Jarenkow & Sobral 3204* (PEL, MBM). **Santa Catarina**: Apiúna, floresta ombrófila densa, 549 m elev., 27º10′27″S, 49º18′08″W, 11 October 2009, *Korte Kniess 561* (FURB, SPF). **São Paulo**: Iporanga, estrada entre Apiaí e Iporanga, floresta ombrófila densa, próximo ao Rio Bethary, 240 m elev., 24º32′55″S, 48º41′09″W, 23 October 2010, *L.H.M. Fonseca & D. Tarabay* 157 (SPF, SP, MBM, MO).

DISCUSSION

The morphological proporties of *D. hispida* allows one to readly recognize this species. Other species of the genus also present conspicuous and unique features that are easily recognized. This is the case of *D. chodatii* (Hassler 1907: 720) L.G. Lohmann (in press) and its dark purple corolla and tri-lobed calyx, or *D. cynanchoides* and its red corolla and exerted stamens (Lohmann & Taylor in press). Nevertheless, some species of *Dolichandra s.l.* are only recognized by combinations of features such as the leaflets with toothed margins, subspathaceously split calyx, and woody seeds of *D. dentata*. *Dolichandra acuminata* is another example, since the diagnostic features used to separate it from the sister-species *D. quadrivalvis* (Capítulo 1, Fonseca & Lohmann in prep.) are also present in other species of the genus (Lohmann & Taylor in press).

This intricate morphological variation in the genus complicates the identification of collections of its taxa often leading to misidentified specimens in Herbarium collections. We present a taxonomic key with vegetative, and reproductive traits of all taxa currently recognized in *Dolichandra s.l.* in order to facilitate the identification of taxa.

KEY TO *DOLICHANDRA S.L.*

Phloem wedges multiple dissected in cross section; trifid and uncinate tendrils; fruits with four lines of dehiscence ..... *Dolichandra*

1 Brachlets with a flaky bark; leaflets chartaceous; calyx 5-lobed; corolla purple 2

2 Bracts linear–lanceolate to subulate, less than 1 mm wide; calyx lobes rounded and shortly mucronate, magenta, puberulent; corolla puberulent outside with peltate trichomes at

the lobes; Colombia, Costa Rica, and Equador ..... *D. steyermarkii*
2' Bracts elliptic or lanceolate, 2–3 mm wide; calyx lobes ovate–lanceolate, attenuate then mucronate, green, glabrous (except at margin); corolla glabrous outside (although sparsely pubescent at apex); Brazilian Atlantic Forest ..... *D. unguiculata*

1' Brachlets without a flaky bark; leaflets chartaceous or membranaceous; calyx 2–3-lobed; corolla yellow or red 3

3 Leaflet margins toothed; seed wings woody with a narrow hyaline margin; prophylls subulate and smooth; riverbanks of Uruguay River basin ..... *D. dentata*
3' Leaflet margins entire, rarely toothed; seed wings hyaline, rarely woody but then, never presenting a hyaline margin; prophylls ovate, subulate or lanceolate, striate or smooth; 4

4 Anthers and stigma exserted; corolla bilabiate with the upper 2 lobes forward projected and the lower 3 lobes reflexed, red; fruit elliptic and coriaceous ..... *D. cynanchoides*
4' Anthers and stigma included; corolla bilabiate, with the upper 2 lobes reflexed and the lower 3 lobes forward, yellow or purple; fruits linear and woody, rarely e lliptic, but then woody 5

5 Leaflet chartaceous; calyx 3-lobed, comprising approximately 1/3 of the corolla; corolla purple ..... *D. chodatii*
5' Leaflet membranaceous; calyx 2-lobed or truncated comprising approximately 1/4 or 1/5 of the corolla; corolla yellow..... 6

6 Inflorescence bracts foliaceous; calyx with a recurved apicule; fruit oblong-elliptic capsules..... 7

7 Tendrils permanent, woody and thick; leaflet apex short acuminate or acute; vegetative and reproductive portions glabrous, rarely puberulent; petioles 1.2–5 cm long and petioloules 0.5–3.5 cm long; widespread from Mexico to North Argentina and South Brazil ..... *D. quadrivalvis*
7' Trendrils caducous; leaflet apex long acuminate; vegetative and reproductive portions puberulent; petioles (5–)6–16 mm long and petiolules 2–7 mm long; restricted to South São Paulo and Paraná, in coastal lowlands ..... *D. acuminata*

6’ Inflorescence bracts filiform; calyx without apicule, or apicule incurved; fruit a narrow, linear capsule..... 8

8 Calyx cupular, truncate to sinuous; leaflets acute or short acuminate; prophylls ovate and striate ..... *D. unguis-cati*
8’ Calyx usually subspathaceously split, often with an incurved apicule; leaflets long-acuminate; prophylls subulate-lanceolate or subulate and smooth 9

9 Indument hispidous; ovary vinaceous; fruits long (77–124 cm long); seeds with hyaline wings; deciduous forests of northern Argentina, southern and southwestern Brazil, Paraguay and Bolivia ..... *D. hispida*
9’ Species glabrous to puberulous, never hispid; ovary green; fruits short (9.2–38.5 cm long); seeds woody, opaque, without hyaline wings; mangroves and swamps from Mexico to Argentina and Trinidad ..... *D. uncata*

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FIGURAS

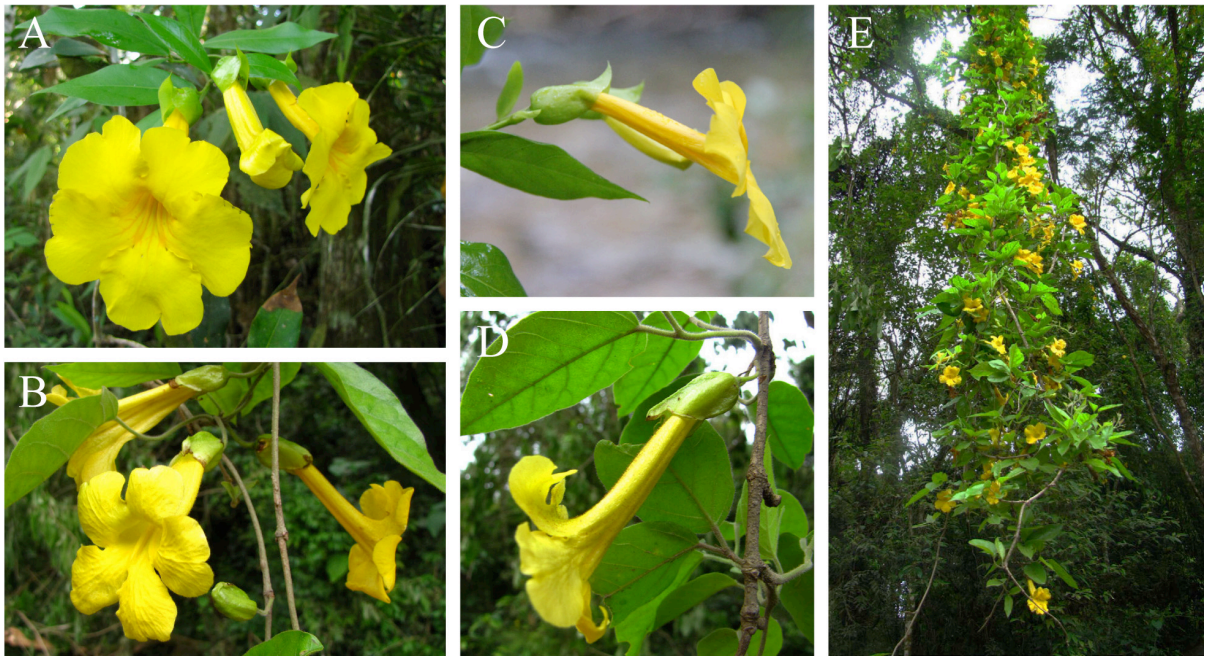

Fig. 11. A–B: *Dolichandra acuminata*; C–E: *Dolichandra hispida*. A. flowers, flower bud and leaves; B. lateral view of flower showing the recurved apiculate calyx and foliaceous bract and bracteoles; C. flowers and flower bud; D. lateral view of flower showing the incurved apiculate calyx; E. habit.

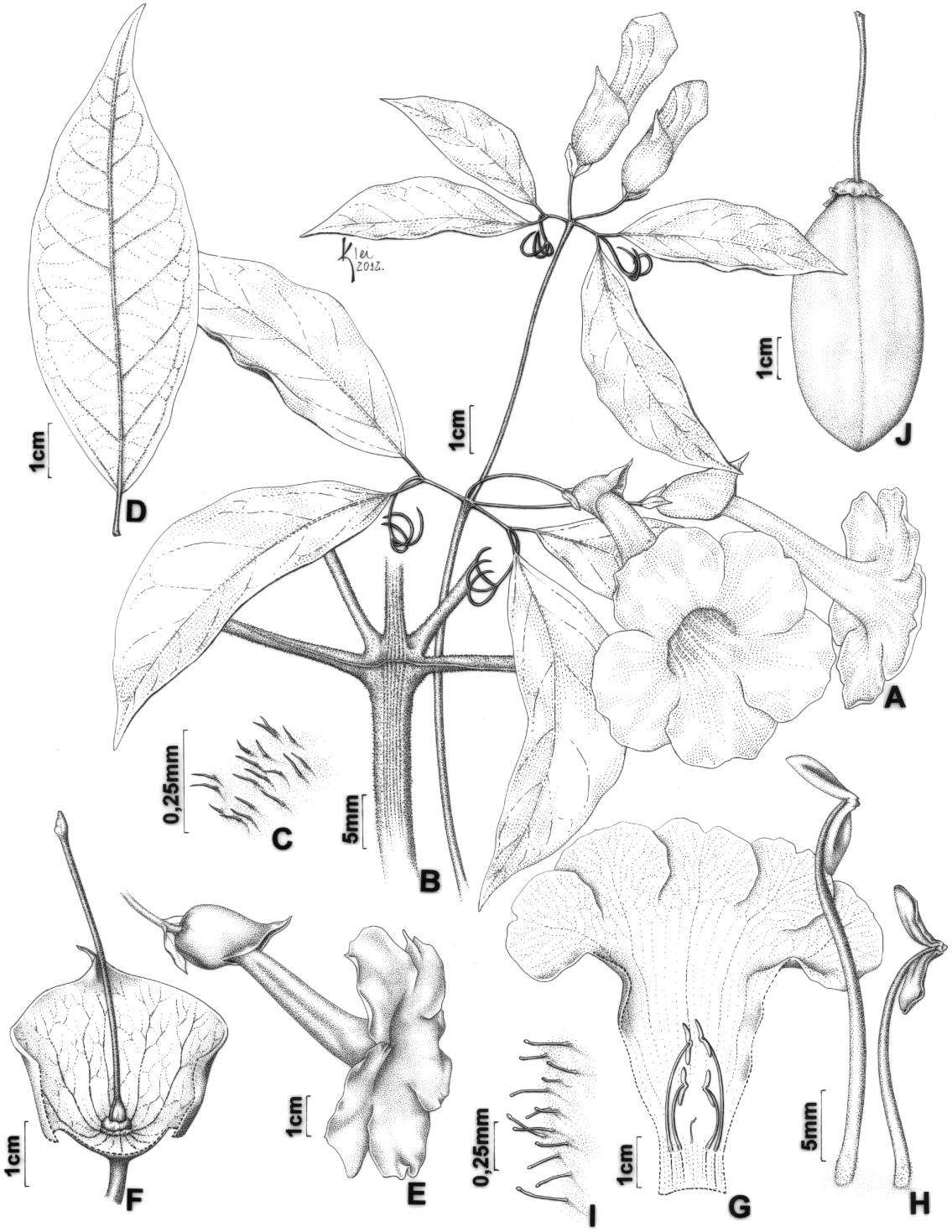

Fig. 12. *Dolichandra acuminata* (from the holotype by Klei Souza). A. flowering branchlet and leaves; B. node branch; C. detail of indument; D. acuminate leaf; E. lateral view of flower; F. open calyx showing the ring nectary and pistil; G. open corolla and stamens; H. didinamous stamens; I. detail of trichomes in stamens base.

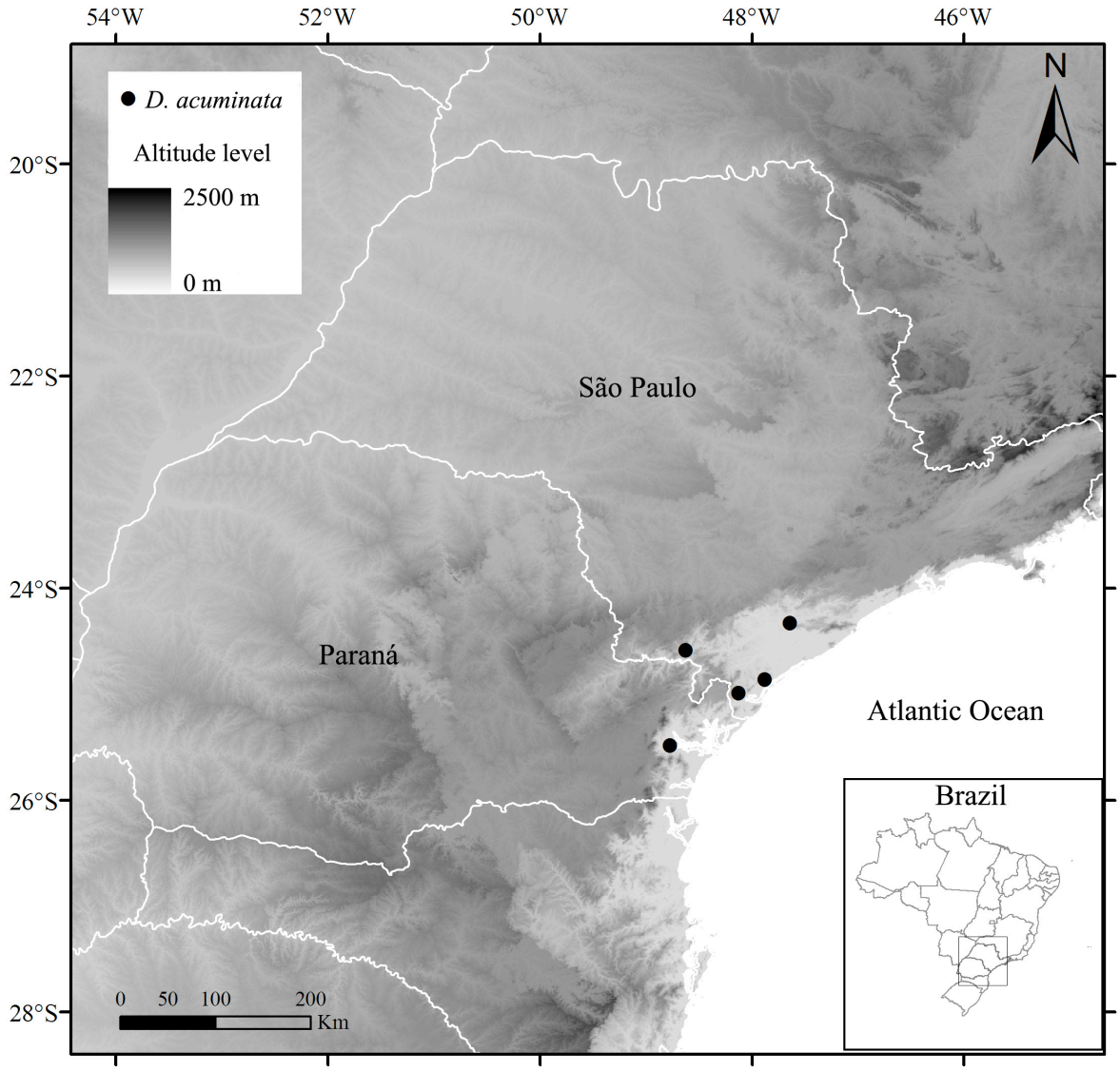

Fig. 13. Map of the states of Paraná and São Paulo, Brazil, showing the distribution of *Dolichandra acuminata*.

Anexos

Anexo 1: Posicionamento do gênero *Dolichandra s.l.* dentro em Bignonieae.

INTRODUÇÃO E OBJETIVOS

No único estudo filogenético compreensivo da tribo Bignonieae (Lohmann, 2006), o gênero *Dolichandra s.l.* emergiu como grupo irmão do clado denominado Múltiplos-de-Quatro. No entanto, essa relação de parentesco apenas apresenta baixa sustentação por índices de bootstrap para parcimônia, bootstrap para máxima verossimilhança e probabilidades posteriores (Lohmann, 2006). Apesar desta baixa sustentação com base em caracteres moleculares, *Dolichandra s.l.* apresenta floema multidissectado, com diversas cunhas de floema (dos Santos, 1995; Lohmann, 2003; Pace et al. 2009), condição que corrobora o posicionamento de *Dolichandra s.l.* dentro do clado Múltiplos-de-Quatro.. Esse tipo de anatomia caulinar foi inferido como condição final em uma série ontogenética que recapitularia estágios presentes em todos os gêneros de Bignonieae e o estágio com cunhas em número múltiplo de quatro, presente somente no clado Múltiplos de Quatro (Pace et al., 2009). No entanto, dada a incerteza no posicionamento de *Dolichandra s.l.*, buscamos utilizar uma maior amostragem do gênero visando testar a hipótese de que o gênero seria um dos representantes do clado Múltiplos-de-Quatro.

MATERIAL E MÉTODOS

Para o posicionamento do gênero dentro de Bignonieae, utilizamos as 103 espécies da tribo com sequências para os marcadores *ndh*F e *Pep*C disponíveis, totalizando três sequências do gênero: *Dolichandra cynanchoides*, *Dolichandra quadrivalvis* e *Dolichandra unguis-cati* (Lohmann, 2006). À essas sequências foram somadas sequências de ambos marcadores para seis espécies de *Dolichandra s.l.*: *Dolichandra acuminata, Dolichandra chodatii, Dolichandra dentata, Dolichandra hispida, Dolichndra uncata* e *Dolichandra unguiculata*. Protocolos de extração, amplificação e sequenciamento foram delineados em Lohmann (2006) ou estão detalhados no corpo da dissertação. A metodologia utilizada para o alinhamento das sequências, codificação de gaps e busca dos modelos

evolutivos também segue a metodologia descrita no corpo da dissertação. codificação de gaps e busca dos modelos evolutivos também segue a metodologia descrita no corpo da dissertação.

Para a reconstrução filogenética utilizamos a metodologia bayesiana, incluindo quatro corridas independentes, cada uma com quatro cadeias por 5 x 10^6 de gerações, com amostras a cada 1000 gerações. Dessas árvores retidas, as 25% iniciais foram descartadas como burn-in. Análises posteriores do conjunto de árvores retidas e estatísticas associadas à análise bayesiana também seguiram a metodologia descrita no corpo da dissertação.

RESULTADO E DISCUSSÃO

A topologia obtida para a tribo é identica à aquela descrita por Lohmann (2006), exceto no que se refere ao posicionamento do gênero *Dolichandra s.l.*, o qual emergiu em meio à uma politomia. Essa politomia é formada por *Dolichandra s.l.* mais os clados: (1) Múltiplos-de-Quatro, (2) o clado formado por *Fridericia* e gêneros próximos, (3) *Pachyptera*, (4) *Manaosella* e (5) *Pleonotoma*. Há contudo que se levar em consideração que o único clado que apresenta sustentação robusta (> 0.95) e contém *Dolichandra s.l.* é aquele que compreende todas as Bignonieae, exceto o clado SMANG (Fig. 1). Dessa forma o posicionamento do gênero permanece incerto e a hipótese de que esse seja grupo-irmão do clado Múltiplos-de-Quatro não pôde ser descartada nem corroborada com os dados disponíveis. Concluímos assim que uma amostragem mais completa de taxa e marcadores moleculares será necessária para posicionar o gênero em meio à tribo Bignonieae com maior sustentação.

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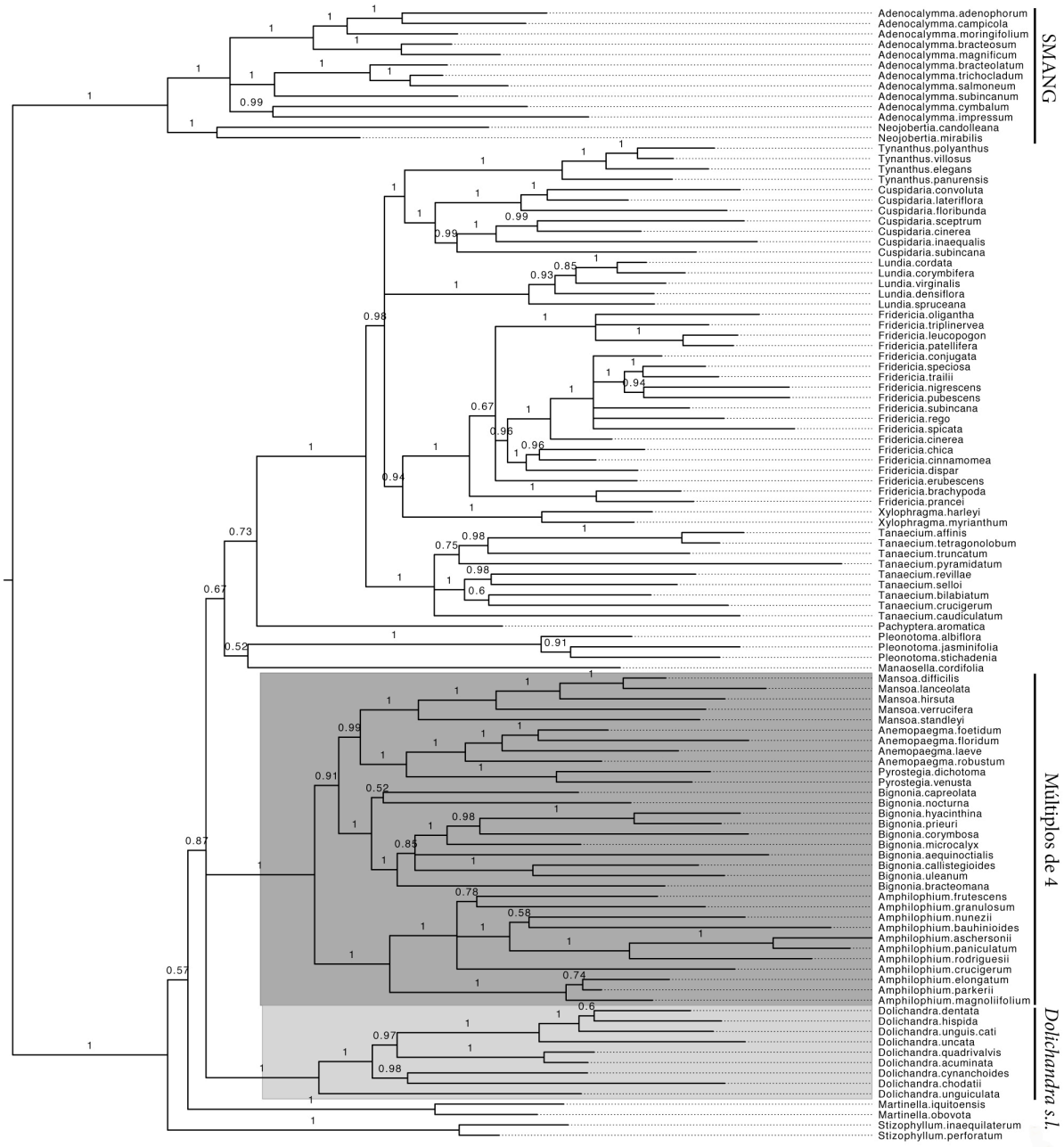

Fig. 1. Filogenia da tribo Bignonieae obtida apartir dos marcadores moleculares *ndh*F e *Pep*C. O clado Múltiplos de Quatro está marcado em cinza escuro. O gênero *Dolichandra s.l.* está marcado em cinza claro.

Anexo 2: Teste de marcadores moleculares para *Dolichandra s.l.*

MARCADORES PPR

Para a amplificação dos marcadores PPR utilizamos inicialmente as condições de PCR descritas por Yuan *et al.* (2009) obtendo como resultado bandas em gel de agarose fracas para as regiões AT1G09680 e AT5G39980 e nenhuma banda para as regiões AT2G37230 e AT3G09060. Fizemos alterações no protocolo original e chegamos às seguintes condições de PCR para os marcadores AT3G09060 e AT5G39980: 4 min a 94 °C, 4 ciclos de 15 seg a 94 °C, 10 seg a 53 °C, e 2 min a 72 °C, seguidos por 30 ciclos de 15 seg a 94 °C, 15 seg a 51 °C, e 1.5 min a 72 °C, com um passo final de extensão de 10 min a 72 °C. Como resultado da amplificação obtivemos bandas simples em gel de agarose, com tamanho esperado em Verbenaceae e quantidade de DNA suficientes para que um sequenciamento direto fosse conduzido, como realizado por Yuan *et al.* (2009). Enquanto o marcador AT1G09680 manteve bandas em gel de agarose fracas após os ajustes no protocolo de amplificação, nenhuma banda em gel de agarose foi obtida para o marcador AT2G37230, de forma que estes marcadores foram descartados.

Na etapa seguinte do trabalho verificamos o grau de variabilidade genética e informação filogenética de cada um dos marcadores restantes, reconstruindo a filogenia do gênero utilizando o critério de parcimônia, implementado no programa PAUP4.0Beta10 (Swofford, 2002). Observamos que o marcador AT3G09060 apresentou 29 sítios informativos e baixa resolução para a reconstrução filogenética com nove amostras. O marcador AT5G39980, por sua vez, apresentou 59 sítios informativos para sete amostras de *Dolichandra s.l.*, representando quatro espécies diferentes. Esse número de bases informativas é considerado bom, contudo os clados obtidos da reconstrução filogenética possuem baixa sustentação (Fig. 1). Dessa forma, ambos os marcadores (AT3G09060 e AT5G39980) foram descartados.

MARCADORES COSII

Para os marcadores COSII testamos as condições de PCR descritas por Tepe e Bohs (2010), além de

alterarmos a temperatura de anelamento e tempo de anelamento e realizarmos "Nested PCR." No entanto, não conseguimos amplificar nenhuma das três regiões. Os ortólogos de cópia simples (COS) foram utilizados em filogenias para as famílias Solanaceae e Rubiaceae e potencialmente poderiam ser aplicados para todo o clado das Euasterídeas (Wu et al. 2006), contudo não obtivemos sucesso em sua amplificação e por isso os três marcadores testados foram descartados. Para maiores detalhes sobre a concentração de reagentes nas reações de PCR, vide Tabela 1. O marcador *trn*Q-*rps*16 (Shaw, 2007) foi descartado devido à presença de um indel de aproximadamente 500 pb.

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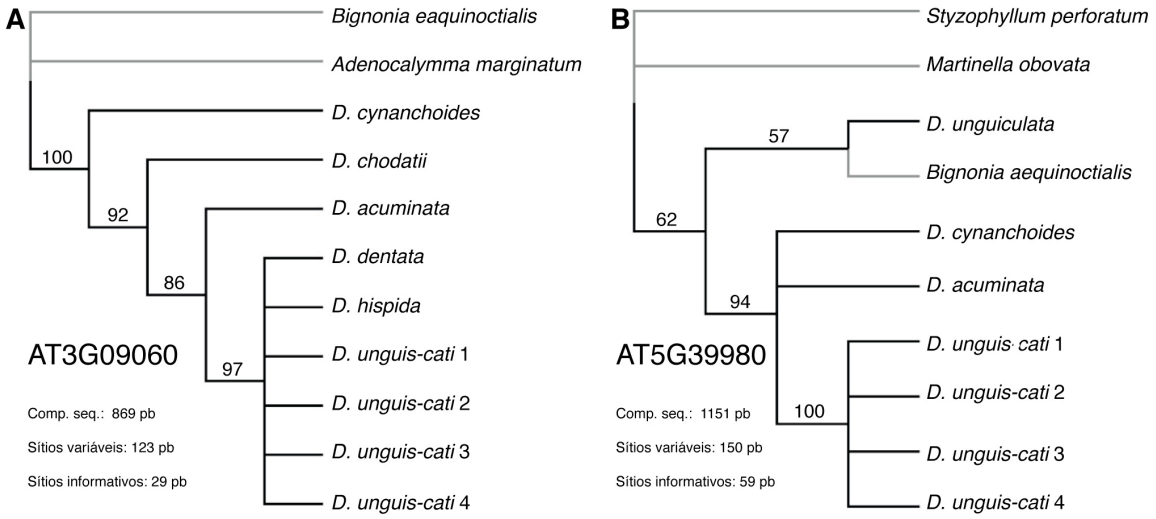

Fig. 1. **A,** Árvore de consenso estrito de um total de 4 árvores mais parcimoniosas para o marcador molecular AT3G09060. **B**, Árvore de consenso estrito de parcimônia de um total de 30 árvores mais parcimoniosas para o marcador AT5G39980. Valores de sustentação de bootstrap estão acima dos ramos em ambas topologias.

Tabela 1. Condições das reações de amplificação dos marcadores PPR e COSII (volume de 20µl).

Reagentes	Quantidade
Tampão de reação 5x	4 µl
MgCl2 (25mM)	2 µl
dNTP (10mM)	0.8 µl
Primer F (10mM)	0.4 µl
Primer R (10mM)	0.4 µl
Albumina Bovina (BSA)	0.4 µl
DMSO (Fragmentos Nucleares)	0.2 µl
Taq polymerase	0.16 µl
DNA molde (20-80ng)	1 µl
H2O	q.s.p 20 µl

Anexo 3: Scripts utilizados na reconstrução filogenética.

PARCIMÔNIA

```
Begin PAUP;
log start=yes file=combpar.log replace=yes;
outgroup Adenocalymma.marginatum;
set outroot=mono;
set increase=auto;
exclude constant;
exclude uninf;
Hsearch addseq=random nreps=10000;
        savetrees file=combparsearch.tre replace=yes;
        contree / strict=yes treefile=combcconst.tre;
        contree / majrule=yes treefile=combconmajr.tre;
        describetrees;
bootstrap nreps=1000 treeFile=combparboot.tre replace=yes search=heuristic/ addseq=random nreps=1000;
        savetrees file=ndhFcombMajRule.tree from=1 to=1 savebootp=nodelabels;
log stop;
end;
```

BAYESIANA

```
Begin mrbayes;

        charset pepC = 1-737;

        charset ndhF = 738-2733;

        charset trnL = 2734-3634;

        charset gap = 3635-3667;

partition favored = 4: pepC, ndhF, trnL, gap;

set partition = favored;

set autoclose=yes nowarn=yes;

        Lset applyto=(1) nst=6 rates = invgamma;

        Lset applyto=(2) nst=6 rates = gamma;

        Lset applyto=(3) nst=6;

        unlink statefreq=(all) revmat=(all) shape=(all) pinvar=(all);

        mcmcp ngen= 10000000 relburnin=yes burninfrac=0.25  printfreq=2000  samplefreq=1000 nruns=4
        nchains=4 savebrlens=yes;

        mcmc;

        sumt burnin=2500;

        sump burnin=2500;

end;
```


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