Anselmo Nogueira

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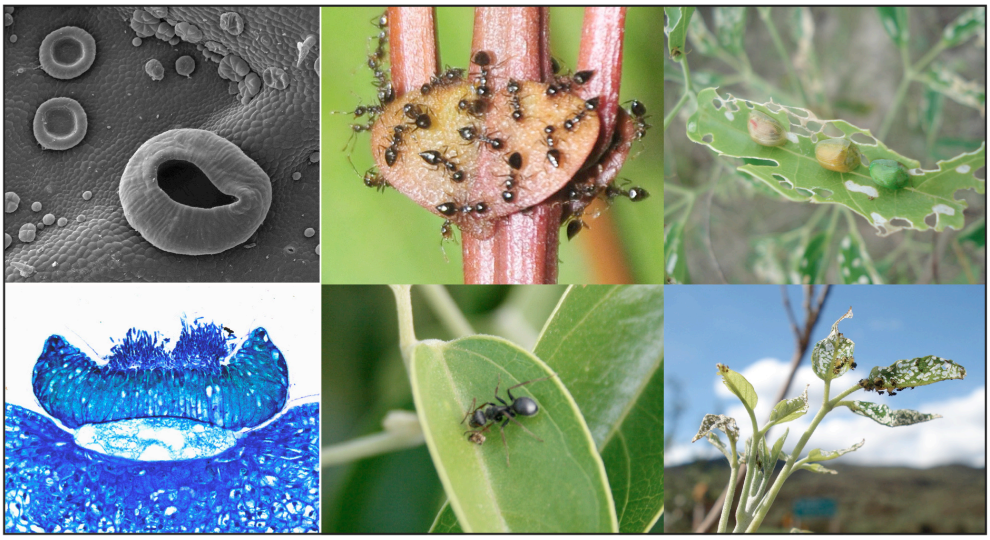

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São Paulo
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ORIENTADORA

Dedico
Aos meus pais
E a minha querida irmã.

"Mas o pobre vê nas estrada

O orvaio beijando as flô

Vê de perto o galo campina

Que quando canta muda de cor

Vai moiando os pés nos riacho

- Que água fresca, nosso Senhor!

Vai oiando coisa a grané

Coisas qui pra mode vê

O cristão tem que andar a pé…"

(Luiz Gonzaga & Humberto Teixeira, 1951)

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SUMÁRIO

Capítulos:

RESUMO

Este trabalho focou nos aspectos evolutivos e ecológicos dos tricomas em espécies da tribo Bignonieae (Bignoniaceae), e no potencial papel defensivo dessas estruturas no aumento da resistência das plantas frente aos herbívoros. Esta tese caracterizou quatro morfotipos de tricomas encontrados nas partes vegetativas das plantas da tribo Bignonieae morfologicamente, estudou os padrões macro-evolutivos destes tricomas e o papel funcional dos tricomas pateliformes secretores de néctar em diferentes escalas de tempo e espaço.

O primeiro capítulo descreveu os quatro tipos de tricomas micro e macro-morfologicamente, e sua relação com a história filogenética das espécies da tribo Bignonieae. Quatro tipos de tricomas distintos foram reconhecidos: tricomas não glandulares (*ng*), tricomas glandulares peltados (*gp*), tricomas glandulares estipitados (*ge*), e tricomas glandulares pateliformes/cupulares (*P/C*). Três destes tricomas provavelmente já estavam presentes no ancestral da tribo Bignonieae (i.e., não glandulares, glandulares peltados e glandulares pateliformes/cupulares) enquanto os tricomas glandulares estipitados surgiram mais recentemente e múltiplas vezes dentro da tribo. Além disso, realizamos uma revisão das terminologias aplicadas a esses tricomas bem como sugerimos uma padronização dos tipos de tricomas para o grupo de forma a diminuir a divergência entre os trabalhos já publicados.

O segundo capítulo testou a eficiência dos tricomas pateliformes secretores de néctar (nectários extraflorais) em duas espécies de *Anemopaegma* das savanas brasileiras. Essas duas espécies atraíram mais formigas que plantas vizinhas, com os indivíduos de *Anemopaegma* com maiores quantidades de nectários sendo visitados por um número maior de formigas que as plantas com um número menor de nectários (variação intra-populacional). No entanto, não foi possível observar um efeito dos nectários e formigas sobre a herbivoria e o desempenho das plantas como esperado pela hipótese de defesa mediada por essas estruturas. Hipóteses alternativas foram utilizadas para discutir os resultados, incluindo uma discussão sobre o possível custo/benefício de tais estruturas em diferentes ambientes (florestas e savanas)

relacionados com a história filogenética do grupo, além da variação das interações esperada entre populações segundo a hipótese de mosaico geográfico.

O terceiro capítulo testou o papel defensivo do sistema planta-formiga mediado pelos nectários extraflorais no contexto filogenético da tribo Bignonieae. Espécies de plantas com um número maior de nectários foram visitadas por um número maior de formigas (teste controlado pelas relações de parentesco entre as espécies). Além disso, espécies mais proximamente relacionadas apresentaram uma diferença na abundância de nectários extraflorais maior do que o esperado pelo modelo neutro de evolução, gerando um padrão de convergência dessas estruturas na tribo Bignonieae. Esse desvio no padrão evolutivo esperado pode ter sido causado por forças direcionais de seleção e momentos de contra-seleção, dado o balanço entre custo-benefício dos nectários extraflorais para as plantas. Neste contexto, dois fatores foram testados para explicar o desvio na evolução da abundância de nectários na tribo Bignonieae: (1) mudança de habitat das florestas para as savannas (fatores extrínsecos); (2) surgimento de novos caracteres morfológicos como outros tipos de tricomas na superfície das plantas (fatores intrínsecos). Ambos fatores podem ter interferido nas interações formiga-planta e no padrão de evolução dos nectários. Dessa forma, a ocupação das savanas levou a uma diminuição do número de nectários (provável contra-seleção dessas estruturas), enquanto o surgimento de tricomas glandulares adesivos teve o mesmo efeito sobre os nectários. Ambos resultados são discutidos considerando a condicionalidade das interações em função da variação biótica (formigas e herbívoros) entre habitats, e também do "trade-off" entre os caracteres de defesa.

O quarto capítulo testou a teoria de coevolução em mosaico geográfico no sistema planta-formiga-herbívoro em 10 populações da espécie de savana *Anemopaegma album*. Não foram encontradas correlações entre os nectários (e variáveis descritoras do néctar), a abundância de formigas visitantes, a herbivoria ou as variáveis de performance das plantas entre as populações. Esse padrão esteve associado principalmente a variação na assembléia de formigas, a qual foi dominada por formigas do gênero *Crematogaster* em uma das populações, mas dominadas por formigas do gênero *Camponotus* na grande maioria das outras populações.

No entanto, 3 das 10 populações estudadas apresentaram um alto número de plantas sem formigas, diminuindo muito as chances dessas populações serem defendidas frente aos herbívoros pelos nectários. A abundância de formigas esteve relacionada negativamente com a herbivoria, e positivamente com as variáveis de performance das plantas entre as populações. Das 10 populações amostradas, 5 delas tiveram os nectários acoplados (do inglês, "matched") com as formigas visitantes, embora o tipo de acoplamento tenha variado entre elas. Dessas 5 populações, somente 3 tiveram uma produção positiva de folhas e baixa herbivoria, no qual duas delas tiveram alta abundância de nectários nas folhas e foram dominadas por formigas do gênero *Camponotus* (maiores em tamanho, mas com baixa capacidade de recrutamento). Já a terceira população teve em média a menor abundância de nectários nas folhas, e a mesma apresentou o maior número de formigas por planta (e maior freqüência), em geral formigas do gênero *Crematogaster* (menores em tamanho mas com grande capacidade de recrutamento). Por serem menores em tamanho, tais formigas utilizaram a secreção dos nectários quase que individualmente. Nem a abundância de nectários, nem as formigas ou mesmo a herbivoria estiveram estruturados espacialmente, corroborando, a hipótese de mosaico geográfico para as interações formiga-planta-herbívoro em *A.album*. Neste contexto, as três populações com as interações formiga-planta "mached" foram consideradas "hot-spots" das interações, nas quais as populações atingiram os valores mais altos das variáveis de performance das plantas, enquanto as demais foram consideradas "cold-spots". A maioria das populações "cold-spots" foi explicada pela falta de formigas suficientes para que as interações com as plantas pudessem se tornar efetivas na defesa frente aos herbívoros, mas outros processos também foram considerados para discutir os resultados apresentados neste trabalho.

ABSTRACT

This thesis focused on the evolutionary-ecology of trichomes in the tribe Bignonieae (Bignoniaceae), and in the potential defensive role of these structures against herbivores. More specifically, we characterized four trichome morphotypes found in vegetative plant parts of representatives of the Bignonieae, as well as studied the macro-evolutionary patterns of these trichomes, and the functional role of patelliforme nectar secreting trichomes in different scales of time and space.

The first chapter describes four different trichome types micro and macro-morphologically, as well as investigates their evolutionary patterns during the history of Bignonieae. The four different trichome types recognized are: non-glandular trichomes (*ng*), glandular peltate trichomes (*gp*), glandular stipitate trichomes (*gst*), and glandular patteliform/cupular trichomes (*P/Cgt*). Our analyses indicated that three of these trichomes were likely already present in the most recent common ancestor of the tribe Bignonieae (i.e., non glandular, glandular peltate, and glandular patteliform/cupular), while the glandular stipitate trichomes evolved more recently and multiple times during the history of the tribe. Results from this study were combined with a literature review in order to revise the trichome terminology and propose standardized names for the various trichome types currently found in the group.

The second chapter tested the efficiency of patelliform nectar-secreting trichomes (extrafloral nectaries, EFNs) in two species of *Anemopaegma* of the Brazilian savannas. These two species attracted more ants than neighboring plants, with individuals that presented higher amounts of EFNs being visited by a higher number of ants than plants with lower amounts of EFNs (intra-population variation). Nonetheless, no effect of EFNs and ants was observed on herbivory nor on the performance of the studied plants, contradicting the expectations of the mediated EFNs defense hypothesis. Alternative hypotheses were also considered including the cost/benefits model to understand the outcomes of ant-plant interaction: (1) phylogenetic inertia hypothesis that connect EFNs-ant interactions with the plant transitions between

different environments (forests to savannas); and (2) geographic mosaic hypothesis that predict differences in the outcomes of ant-plant interactions across populations.

The third chapter tested the defensive role of extrafloral nectaries in the context of the phylogenetic history of Bignonieae. Species of plants with a higher number of EFNs were visited by a higher number of ants (test controlled by phylogeny). In addition, closely related species presented a higher difference in the abundance of EFNs than expected under the neutral model of evolution. Such deviation may have resulted by directional forces of selection and moments of counter-selection, given the costs and benefits of the extrafloral nectaries for the plants. Two specific factors were considered as the major possible determinants of the evolutionary patterns of the EFNs: (1) change of habitat from forests to savannas (extrinsic factors); and (2) emergence of new morphological characters such as other trichome types over the plant's surface (intrinsic factors). Both factors might have altered the ant-plant interactions and the evolution of nectaries. The occupation of the savannas was associated with a decrease in the number of nectaries (likely due to counter-selection of these structures), while the evolution of adhesive glandular trichomes presenting the same effect on the nectaries. Both results are discussed in the light of the biotic variation (ants and herbivores) encountered between habitats, as well as in the light of the trade-off among defensive characters.

The fourth chapter tested the geographic mosaic theory of coevolution in 10 populations of the savanna species *Anemopaegma album*. No correlations were found among extrafloral nectaries (and nectar variables), the abundance of visiting ants, herbivory, and plant performance among populations. This pattern was mainly associated with the variation in the assembly of ants encountered in the various populations. Most populations of *A. album* were dominated by assemblages of *Camponotus* ants, except for one that was dominated by *Crematogaster* ants. However, 3 of 10 populations studied presented a high number of plants without ants, decreasing the chances of defense against herbivores by EFNs. The abundance of ants was negatively associated with herbivory, and positively associated with plant performance variables among populations. Out of the 10 populations sampled, five presented an abundance of EFNs that matched the functional traits of ants. Out of these five populations, only three presented positive leaf production and low herbivory. From these three populations, two presented high abundances of EFNs on the leaves and were dominated by *Camponotus* ants (i.e., bigger in size, but with a low recruiting capacity). The third population presented on average the smallest abundance of EFNs on the leaves; it also presented the highest number of ants per plant (and the highest frequency), which generally were *Crematogaster* ants (smaller in size but with greater recruiting capacity). Because these ants are smaller in size, they used nearly all isolated EFNs encountered over the plant’s surface. Neither the abundance of EFNs, the abundance of ants, and the assemblage of herbivores were structured spatially, corroborating the geographic mosaic hypothesis for the ant-plant-herbivore interactions in *A. album.* In this context, the three populations with "matched" ant-plant interactions were considered 'hot-spots' of interactions, in which the populations reached the highest values of plant performance, while the others were considered 'cold-spots'. The majority of 'cold spot' populations were explained by a lack of sufficient ants to protect the plants effectively.

Introdução Geral

Plantas e herbívoros compreendem grande parte dos organismos sobre a Terra. Diversas teorias foram desenvolvidas para tentar explicar as relações encontradas entre esses organismos. Em particular, a teoria de coevolução de plantas e herbívoros (Ehrlich e Raven, 1964) e a teoria de defesa das plantas (Rhoades e Cates, 1976; Feeny, 1976) assumem que as características das plantas (p. ex., químicas, físicas) estão associadas à diminuição do dano causado por herbívoros. Nos trópicos, a seleção recíproca entre plantas e herbívoros promoveu uma maior especialização nessas interações, dentre as quais as plantas sofrem altas taxas de herbivoria e consequentemente, possuem um maior investimento e diversidade de defesas, quando comparadas às plantas das regiões temperadas (Coley e Aide, 1991). Independentemente do sistema em que essas interações acontecem, acredita-se que nem todas as defesas das plantas sejam fisiologicamente compatíveis (Coley e Aide, 1991), sendo algumas delas redundantes (Agrawal, 2007). Portanto, é possível que a seleção exercida pelos herbívoros sobre as plantas seja direcionada a diferentes síndromes de defesa (p. ex., Agrawal e Fishbein, 2006), na qual diferentes espécies apresentam caracteres distintos distribuídos dentro de um contínuo de escape/defesa frente aos herbívoros (Kursar e Coley, 2003).

Neste contexto, um padrão defensivo comum estaria relacionado à idade das folhas, com plantas aumentando a dureza foliar após a expansão completa das folhas e quase não sofrendo danos por herbívoros após esse período (Coley e Kursar, 1996; Kursar e Coley, 2003; Coley et al., 2005). Assim, as síndromes de defesa das plantas estariam associadas à vulnerabilidade das folhas jovens em duas estratégias distintas: algumas espécies de plantas teriam folhas muito bem defendidas quimicamente desde o início de seu desenvolvimento, com uma taxa de expansão foliar lenta; outras espécies teriam uma quantidade menor de defesas químicas e uma taxa de expansão foliar rápida, diminuindo o tempo de exposição dessas folhas jovens aos herbívoros (Kursar e Coley, 2003). No entanto, essas duas estratégias de escape/defesa não abarcam a quantidade de caracteres reconhecidamente responsáveis pela defesa dos tecidos vegetais. Assim, alguns autores consideraram as síndromes de defesa diferentemente das estratégias de escape e defesa das plantas. Um exemplo pode ser observado em espécies de *Asclepias* (Asclepiadaceae), nas quais três síndromes distintas de defesa foram estabelecidas num estudo que combinou informações ecológicas e filogenéticas do grupo: (1) tolerância/escape, (2) qualidade nutricional baixa, e (3) alta qualidade nutricional e alta defesa direta (Agrawal e Fishbein, 2006).

Existem também trabalhos focados em desvendar o papel funcional de um ou poucos caracteres, dado que alguns deles poderiam ter uma importância maior na defesa contra os herbívoros em espécies particulares. Por exemplo, estruturas morfológicas externas das plantas são as primeiras a entrarem em contato com os herbívoros e poderiam aumentar inicialmente a resistência foliar do tecido vegetal. Em especial, os tricomas estão distribuídos sobre a superfície das plantas e possuem morfologia extremamente diversa (p. ex., uni- a multicelulares, peltados ou ramificados), podendo ser glandulares ou não, variando também a sua densidade e posição nos órgãos vegetais (Levin, 1973). Várias funções são conhecidas para os tricomas (sumarizados em Wagner et al., 2004), dentre elas: (1) redução da oviposição por herbívoros adultos (Coley e Barone, 2001); (2) aumento da resistência foliar, atuando como barreira física contra a herbivoria (Coley e Barone, 2001) e; (3) aumento na tolerância contra seca e raios ultravioletas (Espigares e Peco, 1995). Alguns tricomas glandulares ainda podem assumir papéis diferenciados nas plantas, como por exemplo os tricomas secretores de néctar (um tipo particular de nectário extrafloral; Elias 1983). Funcionalmente, a disponibilização de recursos através da solução açucarada (néctar) produzida pelos nectários atrai insetos, principalmente formigas, que por sua vez defendem as plantas contra os herbívoros (Heil e McKey, 2003). Estas estruturas estão relacionadas com um tipo de defesa indireta frente aos herbívoros, dada a necessidade da presença das formigas para a sua efetividade. Essas estruturas secretoras podem variar na quantidade de substância que secretam, no período em que estão ativas, e no grau de vascularização das mesmas (Elias, 1983).

A seleção natural é o processo microevolutivo responsável por mudanças adaptativas nas populações (Silvertown e Doust, 1993). Acredita-se que esse processo favoreça indivíduos que possuam caracteres tais como tricomas e nectários extraflorais quando esses apresentam variação genética aditiva (herdabilidade), e quando herbívoros selecionam indivíduos fenotipicamente/genotipicamente distintos (Levin, 1973; Heil e McKey, 2003). Dessa forma, quando existe diferença no sucesso reprodutivo associado à presença ou ausência de um determinado gene, a seleção natural é capaz de operar sobre esse gene (Falconer e Mackay, 1996). Neste contexto, três condições são necessárias para que a seleção natural atue: (1) variação do caráter entre indivíduos de uma população (polimorfismo); (2) diferença no desempenho entre os indivíduos que apresentem variação dessa característica e; (3) hereditariedade das diferenças da característica entre os indivíduos (Silvertown e Doust, 1993).

No entanto, no caso particular das interações planta-herbívoro, espera-se que os caracteres de defesa (p. ex., tricomas) das plantas sejam selecionadas somente quando os benefícios de defesa superem os custos de sua produção (Heil e Mckey, 2003; Coley e Barone, 2001). Desta forma, tais estruturas de defesa estariam concentradas sobre partes vegetais mais vulneráveis ou partes em que a perda de tecido vegetal fosse mais custosa à planta (Heil e McKey, 2003). No caso particular das interações mutualísticas planta-formiga (p. ex., Janzen, 1966), uma parte do custo da interação estaria associado a produção da recompensa energética oferecida às formigas por parte da planta (geralmente néctar), enquanto os benefícios estariam relacionados aos serviços prestados pelas formigas sobre as plantas, na defesa frente aos herbívoros, a diminuição do dano foliar (Heil e McKey, 2003). Em geral, as formigas tendem a diminuir a herbivoria e os herbívoros sobre as plantas, e aumentar a performance das plantas (Chamberlain e Holland, 2009; Rosumek et al., 2009; Trager et al. 2010).

Mais recentemente, os caracteres de defesa das plantas e o processo de herbivoria têm incorporado também análises da variação fenotípica das plantas no contexto macroevolutivo (Agrawal, 2007), de forma a considerar as relações de parentesco entre as espécies estudadas (p. ex., Fine et al., 2004; Fine et al., 2006). Dessa forma torna-se possível visualizar o padrão evolutivo dos caracteres nas filogenias, e subsequentemente inferir os diversos processos evolutivos, tais como seleção natural e adaptação (Larson e Losos, 1996) e/ou a evolução neutra dos caracteres (Blomberg et al., 2003). Esses métodos avaliam a divergência fenotípica ao longo do tempo à luz da filogenia (Diniz-Filho, 2000). Quando dispomos de filogenias com informações sobre o comprimento dos ramos, é ainda possível mapear os caracteres de interesse sobre essa filogenia e assim reconstruir os estados ancestrais dos caracteres de interesse. Estas análises permitem uma avaliação da direção e ordem das mudanças morfológicas (Diniz-Filho, 2000), e o teste de hipótese de correlação evolutiva entre esses caracteres (Larson e Losos, 1996). Por exemplo, é possível o teste de hipóteses de "trade-off" entre caracteres (hipótese de correlação negativa), quando tais caracteres são tidos como incompatíveis ou redundantes (Agrawal, 2007); ou ainda a potencial ligação genética entre caracteres (hipóteses de correlação positiva) (Larson e Losos, 1996).

Além disso, esta abordagem permite ainda relacionar as mudanças morfológicas ocorridas dentro de grupos de plantas particulares e as mudanças de regimes de seleção dessas plantas (Larson e Losos, 1996). Determinados padrões podem ser utilizados como evidência de adaptação ou exaptação de determinado estado do caráter a determinado ambiente ou regime de seleção (Larson e Losos, 1996). A reconstrução explícita da evolução de caracteres evidencia o número de vezes que cada estado de caráter surgiu independentemente (Cunningham et al., 1998), e a associação dessas múltiplas evoluções com mudanças em outros caracteres, ou regimes seletivos das plantas. Apesar dos resultados provenientes de análises filogenéticas comparadas serem de suma importância para um melhor entendimento da evolução dos caracteres de defesa das plantas, e para o teste de hipóteses adaptativas, essa abordagem é correlacional, e somente por meio de dados empíricos torna-se possível corroborar os resultados das análises comparadas (Larson e Losos, 1996; Reznick e Travis, 1996).

Desta forma, dados filogenéticos são importantes, por exemplo, para demonstrar se o caráter evoluiu subsequentemente as pressões seletivas. Por outro lado, dados empíricos são necessários para demonstrar a função biológica atual dos caracteres de defesa, a seleção natural exercida pelos herbívoros, e a vantagem conferida por esse caráter para a ocupação de um determinado ambiente. Destes aspectos, a seleção natural é a mais difícil de ser demonstrada (Larson e Losos, 1996; Reznick e Travis, 1996). Assim, a primeira condição necessária para o estudo de seleção natural é a existência de padrões de polimorfismo para as características de interesse (ou potenciais polimorfismos na ausência de dados sobre a base genética de tais características). Quanto maior for o polimorfismo, maior será a amplitude de variação desse caráter na população e maior a chance de se detectar seleção se ela estiver ocorrendo (Ruxton e Colegrave, 2003). No entanto, nos casos em que um determinado fenótipo já tenha sido fixado (sobre o genótipo) e não tenhamos mais polimorfismo na população, manipulações experimentais podem recriar o estado alternativo de ausência do caráter. Através desse procedimento é possível quantificar o valor adaptativo de um caráter particular partindo da hipótese de que ele tenha sido fixado por seleção natural (Wade e Kaliz, 1990).

A seleção pode ainda levar a diferentes situações dependendo dos fenótipos que estão sendo favorecidos na população (Brodie et al., 1995). Por exemplo, a seleção pode preservar características da população favorecendo indivíduos que apresentam fenótipos intermediários (seleção estabilizadora). A seleção pode ainda favorecer indivíduos cujas características encontram-se em apenas um dos extremos da distribuição fenotípica (seleção direcional). Por fim, a seleção pode modificar características da população favorecendo indivíduos cujas características encontram-se em ambos os extremos da distribuição fenotípica (seleção disruptiva) (Brodie et al., 1995). No caso particular dos caracteres de defesa, em especial nos tricomas, a seleção direcional (p. ex., Valverde et al., 2001) e estabilizadora (p. ex., Elle et al. 1999) têm sido invocadas para explicar os padrões de variação encontrados em populações naturais.

Além dos diferentes modelos de seleção aplicáveis as características de defesa das plantas, o processo de seleção pode variar entre populações de uma mesma espécie, dado que as espécies de plantas e animais herbívoros dificilmente se sobrepõem em sua distribuição na paisagem (variação das espécies interagindo entre localidades) (Thompson, 1994). Em outras palavras, as interações planta-animal dependeriam de um contexto específico da comunidade (dependência de contexto; Jones e Callaway, 2007). A Teoria de Coevolução em Mosaico Geográfico incorporou o componente geográfico no estudo das interações planta-animal, reforçando a idéia de que populações diferentes de uma mesma espécie estariam sob diferentes pressões bióticas e abióticas (Thompson, 2005). Dessa forma, as características de defesa das plantas poderiam ser efetivas em determinadas populações, mas não efetivas em outras, ou mesmo desnecessárias em alguns casos (p. ex., na ausência dos herbívoros). Esses padrões seriam dependentes da variação genética das características e do fluxo gênico entre populações, da presença dos herbívoros e outros agentes bióticos relevantes na interação com as plantas, do efeito da interação na resposta adaptativa das plantas (ligação entre os caracteres de defesa e a performance das plantas), dentre outros (Thompson, 1999).

Neste trabalho, procurou-se desvendar os aspectos evolutivos e ecológicos dos tricomas em diferentes escalas temporais e geográficas, enfocando o potencial papel defensivo dessas estruturas na diminuição da herbivoria ou aumento da resistência das plantas. No decorrer do trabalho e de maneira natural deu-se maior ênfase aos tricomas secretores de néctar amplamente associados a visitação de formigas nas espécies de Bignoniaceae, particularmente nas espécies da tribo Bignonieae (*sensu* Lohmann, 2006), dada a ampla variação quantitativa dessas estruturas secretoras dentro e entre espécies em florestas úmidas e nas savanas brasileiras mais secas.

No ***capítulo 1*** descrevemos os diferentes tipos de tricomas distribuídos nas partes vegetativas de espécies da tribo Bignonieae, bem como descrevemos o padrão macroevolutivo dessas estruturas na tribo. Com isso, procuramos revisar a terminologia empregada para tais estruturas, confrontar o conhecimento anatômico, morfológico e funcional das mesmas, e testar se a similaridade morfológica dos diferentes tipos de tricomas foi decorrente da ancestralidade comum.

No ***capitulo 2*** testamos o papel defensivo dos tricomas secretores de néctar (nectários extraflorais) em duas espécies de *Anemopaegma* das savanas neotropicais (*Anemopaegma album* e *Anemopaegma scabriusculum*). Testes inicias foram realizados para quantificar o papel dos nectários na atração da assembléia de formigas e experimentos de exclusão das formigas foram realizados com o objetivo de quantificar o efeito dos nectários (e formigas associadas) no processo de herbivoria, e também na floração e frutificação das plantas. Finalmente foi realizada uma ampla discussão sobre o papel dessas estruturas secretoras, o custo/benefício provido por elas, e a condicionalidade para sua efetividade enquanto defesa. Em especial, duas hipótese foram recuperadas da literatura e discutidas neste capítulo: (1) a hipótese da inércia filogenética, dependente do contexto evolutivo do grupo em que as espécies estão inseridas; (2) e a hipótese de coevolução em mosaico geográfico, que prevê variação nos resultados das interações entre populações, com população defendidas e outras não defendidas pelas formigas.

No ***capítulo 3*** testamos as hipóteses defensivas dos nectários extraflorais e das formigas associadas considerando o maior número de espécies da tribo Bignonieae (Bignoniaceae) no contexto filogenético. Para isso, foram criadas hipóteses sobre o padrão macroevolutivo de variação da abundância de nectários esperado entre espécies em função da mudança de habitat (floresta – savana), e do surgimento de novas características morfológicas (outros tipos de tricomas). Conjuntamente, amostramos as interações formiga-planta em espécies de florestas e savanna da tribo Bignonieae, considerando a abundância de formigas sobre as plantas ("recrutamento") e o número de nectários ativos secretando néctar. Correlações foram realizadas entre a abundância de nectários e a abundância de formigas visitantes, controlando para o efeito da filogenia. Um modelo nulo descrevendo a evolução neutra dos caracteres (evolução browniana) também foi utilizado para confrontar a variação dos nectários em função da filogenia no grupo, bem como para detectar se a evolução desses caracteres desviavam do modelo neutro. Finalmente foram realizados testes da hipóteses relacionando a mudança de habitat (floresta-savanna) e o surgimento de novos caracteres (tricomas em geral) com modificações nas interações planta-formiga neste grupo de plantas em particular. Ambas hipóteses têm grande potencial de serem testadas em outros grupos de plantas dado que padrões semelhantes de ocupação de habitat e variação morfológica já foram descritos na literatura.

No ***capítulo 4*** testamos a hipótese de mosaico geográfico das interações planta-formiga mediadas pelos nectários extraflorais (segundo a Teoria de Coevolução em Mosaico Geográfico; Thompson, 1994, 2005). Nesse contexto, amostramos 10 populações (30 indivíduos cada) da espécie *Anemopaegma album*, um arbusto das savannas brasileiras . Além disso, quantificamos a abundância dos nectários, a concentração de açúcar e volume de néctar, a assembléia de formigas e herbívoros, a herbivoria foliar, e as variáveis que descrevem a performance das plantas (produção foliar entre dois momentos do tempo, e número de plântulas crescendo ao redor do adulto amostrado) em cada uma das populações. Finalmente exploramos os padrões de correlação entre essas variáveis de acordo com as hipóteses adaptativas para os nectários entre as populações. Em uma abordagem correlacional, descrevemos potenciais "hot-spots" e "cold-spots" das interações, os primeiros indícios de que tais interações podem ser vantajosas em algumas populações, mas ineficazes (ou até mesmo ausentes) em outras.

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Capítulo 1

Trichome evolution in Neotropical lianas (Bignonieae, Bignoniaceae)

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ABSTRACT

• *Background and Aims* - Trichomes are epidermal outgrowths that are distributed through various plant parts and are thought to be associated with protection against herbivores, and/or desiccation. These structures are widely distributed from ferns to angiosperms however, little is still known about their general morphology and patterns of topological variation, preventing valid comparisons across taxa. Here, we integrate detailed morpho-anatomical studies of trichomes on representatives of the tribe Bignonieae (Bignoniaceae) with information on the evolutionary history of representatives of this plant group in order to gain a better understanding of current patterns of diversity of trichome types in the tribe.

• *Methods* – We used two sampling schemes to characterize trichome types in Bignonieae: (1) A macro-morphological characterization of trichomes in all 105 species currently included in the phylogeny of Bignonieae; and (2) A micro-morphological characterization of trichomes in 16 carefully selected species of Bignonieae. Trichome morphotypes were coded as binary (presence/absence), while trichome density and size were coded as multistate (discretized from quantitative measurements). Ancestral character state reconstructions of trichomes were conducted using maximum likelihood (ML) assumptions.

• *Key Results* – Two main functional trichome categories were encountered in representatives of Bignonieae: (1) non-glandular trichomes, and (2) glandular trichomes, both of which presented variable sizes and cell numbers. Within glandular trichomes, three morphotypes were recognized: (i) peltate, (ii) stipitate, and (iii) patteliform/cupular trichomes (*P/Cg*). Non-glandular, peltate-glandular, and *P/Cg* trichomes were documented in all 105 species of Bignonieae sampled, although variable trichome densities were encountered. Non-glandular trichomes were uniseriated and varied from uni- to multicellular (up to 25 cells), and simple to branched. Peltate glandular trichomes were multicellular, non-vascularized, stalked (a short stalk), and convex. Patelliform/cupular trichomes were multicellular, non-vascularized, presented a concave surface, and three distinct cell layers: (a) a secretory layer including 30-68 cells without visible pores; (b) an intermediate layer including a single large cell or a single row of cells; and, (c) a multicellular foot. The stipitate glandular trichome, on the other hand, was restricted to 9 of the 105 species sampled. Stiptate glandular trichomes were multicellular, uniseriated, and presented a simple and long-stalked structure. ML ancestral character state reconstructions of each trichome morphotype suggested that the most recent common ancestor of Bignonieae likely presented non-glandular trichomes that were distributed throughout most plant parts. This pattern was also observed for peltate glandular trichomes. Furthermore, the most recent common ancestor of Bignonieae likely presented *P/Cg* trichomes that were restricted to leaflets, but lacked stipitate glandular trichomes.

• *Conclusions* – Ancestral character state reconstructions of trichome types indicated that most trichome morphotypes currently found in representatives of Bignonieae were already present in the most recent common ancestor of all Bignonieae, except for the stipitate glandular trichomes. Such results indicate that the individual trichome types currently found in representatives of Bignonieae are most likely homologous and should thus be treated under the same name, in spite of the plethora of names that has been designated in the literature to these structures in the past. A standardization of trichome terminology greatly facilitates comparations across taxa, allowing inferences on the relationsthips between morphological variants, its eco-evolutionary consequences, and functional properties.

Key-words: Brazil, EFNs, extrafloral nectaries, glandular trichomes, insect-plant interactions, lianas, and morphological evolution.

INTRODUCTION

A good understanding of the evolutionary patterns of individual morphological features represents the basis of comparative studies (MacLeod and Forey 2002). Such studies combine information derived from molecular sequences and phylogenetic analyses with detailed morphological data in order to further understand the patterns of morphological change in the organisms that are distributed through natural systems (e.g. Fougère-Danezan et al. 2010). In those studies, the identification of a common origin for individual morphological features represents a critical step for the establishment of homology hypothesis among morphological structures (Scotland and Pennington 2000, West-Eberhard 2003). Detailed information on the evolution of morphological characters is also critical for valid functional comparisons accross taxa (e.g., Fahn 1986), representing the first step for the test of adaptation hypotheses (Larson and Losos 1996).

Trichomes, for example, are epidermal outgrowths that are widely distributed through the various parts of most plant groups (Levin 1973, Payne 1978). These structures are often associated with protection against herbivores, and/or protection against desiccation (Wagner et al. 2004) thus, being of extreme importance to the maintenance of various plant groups in herbivore-rich environments and in dry areas. Current patterns of trichome variation are thought to have resulted mainly from natural selection, with individuals that present trichomes having lower disadvantages (e.g., leaf damage, loss of water, or high temperature) when compared to individuals lacking those structures (e.g. Levin 1973, Elle et al. 1999, Valverde et al. 2001, Romero et al. 2008, Kaplan et al. 2009, Johnson et al. 2009). Three conditions are necessary for natural selection to act on trichomes in natural populations: (1) trichome characters need to vary among individuals (e.g., polimorphism in trichome characters); (2) trichome morphological differences between plant groups need to be heritable; and, (3) individuals with variable trichome characters need to present differences in fitness (Silvertown and Doust 1993).

Directional selection (Valverde et al. 2001) and stabilizing selection (Elle et al. 1999) have often been used to explain the patterns of trichome variation encountered in natural populations. Such models have gained further support from genetic studies that have characterized DNA regions that are responsible for the patterns of trichome variation observed in model organisms (e.g., QTLs studies by Mauricio 2005, Symonds et al. 2005). Such studies have allowed the use of trichomes in population level quantitative evolutionary studies. Unfortunately, however, little is still known about the general morphology of trichomes and about their patterns of topological variation (Theobald et al. 1979), preventing valid comparisons across taxa. In addition, macroevolutionary patterns of trichome types are still scarce (but see Beilstein et al. 2006 and Chauveau et al. 2011) regarding the mode, timing, and pattern of evolution of trichome morphotypes and trichome densities in plants as a whole.

Trichomes in Bignonieae (Bignoniaceae)

Members of the Bignoniaceae play an important ecological role in Neotropical forests (Gentry 1991), especially representatives of the tribe Bignonieae, the most abundant and diverse clade of Neotropical lianas (Lohmann 2006). This tribe includes 21 monophyletic genera and ca. 400 species (ca. 50 % of all species of Bignoniaceae) (Lohmann 2006). Phylogenetic relationships within representatives of the tribe have been relatively well studied. To date, a well-supported molecular phylogeny based on plastid (*ndh*F) and nuclear (*Pep*C) markers, and including ca. 25% of all species currently recognized in Bignonieae is available (Lohmann 2006) . The wide variation found in the abundance, size, position, and morphology of trichome types encountered in representatives of Bignonieae, makes this tribe an excellent group within which to address trichome evolution in the Neotropics.

Various studies have described the general structure and morphology of trichome types in representatives of the Bignoniaceae (see Table 1). Species of the Bignoniaceae include a variety of glandular and non-glandular trichomes in the stems (especially in the interpetiolar region), prophylls of the axillary buds, petioles, petiolules, adaxial, and abaxial surface of leaflets (e.g., Seibert 1948), as well as in reproductive organs (see Souza et al. 2010). Unfortunately, most of these trichome types have still been poorly studied. In contrast, glandular trichomes have been particularly well documented in some species (Table 1). Despite the fact that trichome morphology has been relatively well studied in representatives of the Bignoniaceae, sampling is still incomplete. In addition, trichome morphology has never been interpreted in an evolutionary context preventing the establishment of homologies and/or meaningful comparisons among trichome morphotypes in this group. Furthermore, trichome studies on the Bignoniaceae have applied a wide array of terms to describe the various types of glandular-trichomes, complicating the distinction among morphotypes and preventing comparative studies. For instance, the patteliform glandular trichomes distributed through the leaf surface, petioles, prohylls of the axillary buds, interpetiolar regions, calices, corollas, and fruits of representatives of Bignonieae have been called "nectaries" or "extrafloral nectaries" (e.g., Elias and Gelband 1976, Oliveira and Leitão-Filho 1987, Nogueira et al. 2011), "scale-like trichomes as morphotype of EFNs" (e.g., Díaz-Castelazo et al. 2005, Machado et al. 2008), and "glands" (e.g., Seibert 1948, Laroche 1974, Lohmann 2006), making a standardization of the trichome terminology extremelly necessary in this group. A unified trichome terminology within Bignonieae would facilitate comparative taxonomic, ecological, and evolutionary research in this morphologically diverse clade of lianas.

In this study we reviewed all studies conducted on the morphology and anatomy of trichomes in representatives of this plant family, as well as conducted additional morphological studies in order to fill in the gaps of information currently encountered in the literature. In particular, we characterized trichome types in representatives of all 21 genera currently recognized in Bignonieae through detailed anatomical studies, macro-morphological descriptions, and quantitative samples of trichome size and density. This information was then used as basis to understand macroevolutionary patterns of non-glandular and glandular trichomes in Bignonieae using a robust molecular phylogeny of the group (Lohmann 2006). The following questions were addressed: (1) What are the trichome morphotypes distributed through vegetative parts of representatives of Bignonieae, and how are they characterized morphologically? (2) Are the morphological variants of each trichome morphotype homologous, or has each trichome morphotype evolved independently within the phylogenetic history of the group? (3) What is the evolutionary pattern of each trichome morphotype in different plant parts, and (4) What is the evolutionary pattern of trichome size and density within Bignonieae?

MATERIALS AND METHODS

Trichome characterization

In order to obtain a clear picture of the patterns of morphological variation encountered in trichome types in representatives of Bignonieae, we first revised all trichome studies available in the literature (Table 1). We then used two complementary sampling schemes to characterize the individual trichome types present in the vegetative portions of representatives of Bignonieae. We first used a stereomicroscope and a broad-scale sampling scheme to characterize the macro-morphology of trichomes in 105 species of all Bignonieae species currently recognized. We then conducted detailed anatomical studies using SEM and light microscopy in a smaller sampling of taxa (16 species of Bignonieae) in order to characterize the micro-morphology of trichomes in Bignonieae. These two sampling schemes led to a careful description of the overall morphology of the individual trichome morphotypes encountered in Bignonieae.

Macro-morphological characterization. We used herbarium specimens to characterize macro-morphological patterns of variation of trichome types in representatives of Bignonieae. For that, three specimens of each of the 104 species of Bignonieae included in the combined molecular phylogeny of Lohmann (2006) plus *Callichlamys latifolia* were sampled. This sampling scheme included representatives of all 21 genera of Bignonieae and taxa distributed through a variety of geographic locations and habitats. Herbarium specimens were analyzed using a stereomicroscope (Olympus SZ60 - Zoom range of 6.3:1). The following variables were recorded: (a) presence/absence of each trichome type, (b) size of each trichome type, and (c) density of each trichome type on the stems, prophylls of the axillary buds, petioles, petiolules, adaxial and abaxial leaf surfaces (basal, middle, and upper portions). Trichome density was sampled in three squares of 1 mm^2 each, located at the basal, middle, and upper portions of leaflets. Larger trichomes such as the patteliform/cupular glandular trichomes were sampled in larger squares with 1 cm^2 instead. The number of patteliform/cupular glandular trichomes (abundance data) was also recorded in the interpetiolar region of stems, as well as in the prophylls of axillary buds, petioles, and petiolules.

Micro-morphological characterization. We used fresh specimens in a micro-morphological characterization of trichome types in 16 species of Bignonieae. The following taxa were sampled: (1) *Adenocalymma pedunculatum* (Vell.) L.G. Lohmann*,* (2) *Amphilophium crucigerum* (L.) L.G. Lohmann*,* (3) *Amphilophium parkeri* (DC.) L.G. Lohmann*,* (4) *Anemopaegma album* Mart. ex DC.*,* (5) *Anemopaegma scabrisculum* Mart. ex DC.*,* (6) *Bignonia prieurei* DC., (7) *Cuspidaria sceptrum* (Cham.) L.G. Lohmann*,* (8) *Dolichandra unguis-cati* (L.) L.G. Lohmann*,* (9) *Fridericia triplinervia* (Mart. ex DC.) L.G. Lohmann*,* (10) *Lundia nitidula* DC.*,* (11) *Mansoa difficilis* (Cham.) Bureau & K.Schum.*,*(12) *Perianthomega vellozoi* Bureau*,* (13) *Pleonotoma albiflora* (Salzm. ex DC.) A.H. Gentry*,* (14) *Pyrostegia venusta* (Ker

Gawl.) Miers*,* (15) *Stizophyllum riparium* (Kunth) Sandwith*,* and (16) *Tanaecium pyramidatum* (Rich.) L.G. Lohmann*.* These species included a broad sample of the evolutionary lineages currently recognized in the tribe, as well as a good representation of the habitats and Biomes occupied by these taxa. Field collections were conducted in areas of savanna (Parque Estadual de Grão Mogol – Minas Gerais State/Brazil and Parque Nacional da Chapada Diamantina – Bahia State/Brazil), and rainforest, including Amazonia (Reserva Florestal Adolpho Ducke – Amazonas State/Brazil), and the Atlantic Rainforest (Reserva Natual Vale – Espirito Santo State/Brazil).

In the field, we fixed mature and young vegetative plant parts in FAA 50% (Formalin-Acetic-Alcohol, Johansen 1940), and then stored those samples in alcohol 70% for subsequent preparation of anatomical cuts for the micro-morphological analyses of trichomes. Sections of 6 µm were conducted for all samples using standard historesin microtechinque. Samples were stained with toluidine blue (O'Brien et al. 1964). For SEM (scanning electron microscopy) studies, samples were dehydrated in an ethyl alcohol series, and specimens were critical point dried and gold coated. The general anatomical descriptions and trichome classification were based on Theobalde et al. (1979). Specific terminology used for the characterization of the patteliform/cupular glandular trichomes followed Elias (1983).

Phylogeny and trichome evolution

Phylogenetic framework. We used a phylogeny of Bignonieae (Lohmann 2006) to represent the breath of morphological and geographical diversity currently encountered in the group. Due to the phylogenetic uncertainty of some nodes in the phylogeny of Bignonieae (Lohmann 2006), five phylogenetic hypotheses were considered in all analyses.

Character coding. Each of the four trichomes morphotypes recorded for Bignonieae were coded as presence/absence (binary coding) according to their occurrence in each plant part considered (stems, prophylls of the axillary buds, petioles, petiolules, adaxial side of leaflets, and abaxial side of leaflets). In addition, continuous measurements of trichome densities were transformed into discrete character states and coded as multi-state characters, as follows: i. **Intermediate density of trichomes**. 1/3 of all species with density values around the median; ii. **Slow density of trichomes**. 1/3 of all species with less trichomes than the intermediate density-class; and iii. **High density of trichomes**. 1/3 of all species with trichome densities with higher trichomes than the intermediate density-class. The same approach was applied to trichome sizes.

Ancestral character state reconstructions. We initially used information on trichome position, structure, and ontogeny (gathered from the literature) to generate hypotheses of homology for the various trichome types following Pinna (1991) and Patterson (1982). We subsequently combined all the morphological information with data on the phylogenetic history of Bignonieae through maximum likelihood ancestral character state reconstructions in order to verify whether the morphological similarity was based on common ancestry.

Maximum likelihood (ML) ancestral character state reconstructions of trichome morphotypes were implemented in Mesquite 2.74 (Maddison and Maddison 2007), with the selection of the most likely condition for each ancestral character state being estimated through a decision threshold T. Whenever the log likelihoods of two states differed by T or more, the state with the lower likelihood (higher negative log likelihood) was rejected (Pagel 1999). In all analyses, trichome characters were equally weighted and considered unordered. For binary traits, two evolutionary models were considered: A Mk1 model with 1 parameter, and in which any particular change is considered equally likely and, a Mk2 model with two parameters that represent forward and backward rates of changes between character states. Models were chosen using the Likelihood Ratio Test (LRT). The evolutionary history of multistate character states (resulting from the discretization of quantitative characters) was only reconstructed for trichomes whose ancestral character state reconstructions based on binary characters (coded as presence/absence) suggested that the individual trait being considered was already present in the most recent common ancestor of the tribe. These analyses separetly considered the presence of each trichome type in each plant part separately. As a result, ancestral state reconstructions of quantitative characters were not conducted for homoplasious trichome types. These analyses were performed exclusively with the Mk1 model with 1 parameter. In addition, ML reconstructions were used to assess the rates of transition of trichome traits in all ancestral character reconstructions.

RESULTS

Morphological characterization of trichome types

Two functional categories of trichomes were observed on vegetative plant parts of representatives of Bignonieae: (1) non-glandular, and (2) glandular trichomes. Both trichome categories presented variable sizes and cell numbers. Within glandular trichomes, three morphotypes were recognized: (a) peltate, (b) stipitate, and (c) patteliform/cupular. All variants of non-glandular trichomes were included under the same category given the similarities of these trichomes. Overall, four trichome morphotypes were recorded on vegetative plant parts of species of Bignonieae (Figure 1): (1) non-glandular trichomes (including unicellular simple, multicellular simple, and multicellular branched – dentritic/stellar); (2) peltate glandular trichomes; (3) stipitate glandular trichomes; and (4) pateliform/cupular glandular trichomes.

Non-glandular trichomes (ng). This trichome morphotype was found in all 105 species and 21 genera of Bignonieae sampled (Figure 2). These trichomes were uni- to multicellular, uniseriate, simple or branched, and formed by 1-25 cells, characterizing short and long trichomes (Figures 2E-F, 2L, 2P, 2U, and 2Z). Trichome cells were covered by a thick, smooth, or rough cuticule. Cuticular warts were present in some cases (e.g., *Anemopaegma*). Approximately 90% of the species sampled (95 out of the 105) presented simple trichomes with one or multiple cells (Figures 2C-D, 2I-K, 2M-P, and 2T-Z); the remaining 10% of the species presented branched trichomes with multiple cells. Multicellular and branched trichomes were encountered in some representatives of six different genera (i.e., *Adenocalymma*, *Amphilophium*, *Callychlamys*, *Fridericia*, *Manaosella*, and *Pleonotoma* (Figures 2A-B, 2G-H, and 2Q-S), not characterizing any particular genus. This trichome type occurred more widely in *Amphilophium* (Figure 3A).

Non-glandular trichomes were encountered in different plant parts (stems, prophylls, petioles, petiolules, and leaflet blades). In 39% of the species (41 out of 105), trichomes were encountered in all vegetative parts, although different trichome densities were observed. In 7.6% of the species (8 out of 105), trichomes were distributed through all plant parts examined, except leaflets. Overall, 82% of the species (86 out of 105) presented trichomes on both sides of leaflets. In plants with trichomes on the abaxial side of leaflets, 19.3% of the species (18 out of the 93) presented glabrous leaf blades, with trichomes growing exclusively over the leaflet veins (Figure 2V). In the remaining 80.7% of the species (75 out of the 93), trichomes were found throughout the abaxial side of the leaflet blade (Figures 2A, 2T, and 2Q). In plants with trichomes on the adaxial side of leaflets, 44.2% of the species (38 out of the 86) presented glabrous leaf blades, with trichomes growing exclusively over the leaflet veins (Figures 2D, 2G-H, and 2M). Approximately one-thrid all species sampled (35 out of 105) presented non-glandular trichomes that were restricted to individual plant parts. In general, these species presented trichomes that were restricted to the leaflet margin (e.g., *Periatomega vellosoi*), and/or to the ridges of the interpetiolar region (e.g., *Mansoa difficilis*), and/or to the apex of the prophylls of the axillary buds (e.g., *Lundia nitidula*). In addition, 27.6% of the species (29 out of the 105) presented high trichome densities (>10 trichomes/mm^2) on the abaxial surface of the leaflet blades. For species with non-glandular trichomes on leaflets, the abaxial surface included approximately 16.5 times more trichomes per mm^2 than the abaxial side (N=63).

Peltate glandular trichomes (pg). This trichome morphotype was documented in all 105 species and 21 genera of the species of Bignonieae sampled (Figure 4). Peltate glandular trichomes were multicellular, non-vascularized, stalked (a short stalk), and convex or rounded at earlier stages of development (sometimes becoming flattened with age). These trichomes presented two distinct cell types: (i) **Head cells.** Head cells were composed of 6-24 thin-walled cells that were covered by a thin and smooth cuticle, but lacked visible pores. Those trichomes also presented a sub-cuticular space that accumulated secretion in many species (Figures 4H2, and 4K2); and, (ii) **Foot cells.** The foot of the trichomes was generally composed of a single cell in most species (Figures 4I3, 4H3, 4F2, and 4J4) but included up to 2-3 cells in some taxa (e.g., *Stizophyllum*).

Peltate glandular trichomes presented a more homogeneous distribution throughout the vegetative plant parts, and a higher variation in the density and size of trichomes. The diameter of the glandular head varied between 0.02 to 0.1 mm^2 (median=0.048, N=103), with larger heads generally presenting a higher number of cells. Species of *Pyrostegia* and *Stizophyllum* presented the largest glandular heads (up to 0.1 mm^2), while species of *Amphilophium* presented the highest trichome densities (up to 216 trichomes/mm^2 in *Amphilophium paniculatum*). Trichomes of *Pyrostegia, Amphilophium*, and *Stizophillum* (14 out of the 105 species sampled) reflected light when observed in the microscope (Figures 4B, and 4E). In some cases, peltate glandular trichomes were difficult to be visualized without magnification lenses either because of its small size (glandular head diameter < 0.028 mm; e.g., *Tynanthus*), or because trichomes were found in low densities (0.6 trichomes/mm^2; e.g., most species of *Adenocalymma*; Figure 4A). In 42% of the species sampled (44 out of 105), the leaflet blade presented high or intermediate densities of peltate glandular trichomes (> 5 trichomes/mm^2) (Figures 4B, 4C, and 4E). Similarly to the patterns found for the non-glandular trichomes, the abaxial side of leaflets presented 5.3 more peltate glandular trichomes by mm^2 on average than the adaxial side (N=97). Furthermore, peltate glandular trichomes were generally easily visualized on the abaxial side of the leaflet blades than in any other vegetative plant part (Figures 4A-E). Trichomes were rarely observed in older portions of the interpetiolar regions, petioles, and petiolules, suggesting that this trichome type is generally lost with age.

Stipitate glandular trichomes (sg). This trichome morphotype was restricted to 9 of the 105 species sampled, and was encountered in 6 of the 21 genera of Bignonieae currently recognized (Figure 6). All species with stipitate glandular trichomes presented a similar anatomical structure that included multicellular, uniseriate, simple, non-vascularized, and stalked (generally long-stalked) trichomes. A group of 8-12 thin-walled cells formed the secretory head, while the trichome body included between 3-12 cells. Stipitate glandular trichomes were rare and restricted to a few species. Plants with *sg* presented high variation in the distribution and density of trichomes among species (Figures 6F-L). In some taxa, these trichomes were associated with the proximity to the inflorescence (e.g., *Lundia densiflora* and *Manaosella cordifolia*). In others, trichomes were dispersed throughout the plant, independently of the inflorescence position (e.g., *Adenocalymma adenophorum*). *Cuspidaria sceptrum* (dry area) and *Adenocalymma adenophorum* (forest) were shown to produce a sticky secretion that retained insects (e.g., Diptera). However, no ants were observed visiting these plants (A. Nogueira, pers. obs.). Developmental analyses of the stipitate glandular trichomes (e.g., *C. sceptrum*; Figures 6A-E) and peltate glandular trichomes corroborated the cathegorization of these trichomes in two distinct morphotypes.

Patelliform/cupular glandular trichomes (P/Cg). This trichome morphotype was present in all 105 species and 21 genera of Bignonieae sampled (Figure 8). The patelliform/cupular trichomes were multicellular, non-vascularized, disc-shaped (patteliform) or cup-shaped (cupular), with a concave surface, and three distinct cell layers: (i) **Secretory layer**: This layer was formed by 30-68 thin-walled columnar cells that were covered by a thin and smooth cuticle, but lacked visible pores. The secretory cells were organized in a palisade-like arrangement and presented a densely stained cytoplasm; (ii) **Intermediate layer**: This layer presented two different morphologies depending on the taxa. While some taxa presented an intermediate layer composed of large, ellipsoid, and vacuolated cells, surrounded by thick and highly suberized anticlinal walls (Figures 8D, 8F, 8I-J, and 8L-M), others presented an intermediate layer that was composed of one row of thick and highly suberized cells that connected the secretory cells to the epidermis (Figures 8G, 8O, and 8P); (iii) **Foot or basal layer:** This layer was multicellular, and consisted of small and quadrangular cells.

Overall, patelliform trichomes are flattened structures (Figures 1J-L, 8E-F, 8H-I, 8M, and 8N-P). This trichome morphotype represented the most common type in Bignonieae. In particular, most species of *Adenocalymma* presented cupular (sometimes flattened patteliform) trichomes. In those cases, the cupular shape was caused by a proliferation and expansion of the lateral cells of the secretory layer, giving rise to a pocket-like structure (Figures 1I, 8A, and 8D). In some cases (e.g., *A. cymbalum*), the expansion of the surrounding epidermis was very marked leading to a "volcano-like" structure (*sensu* Lohmann 2006). A few species (e.g., *Amphilophium parkerii*, *Bignonia prieurei*) presented trichomes with morphologies that were intermediate between the cupular and patteliform variants (Figures 8G, and 8J). These intermediate morphologies were also observed within a single species (e.g., *Adenocalymma pedunculatum*).

The abundance of patteliform/cupular glandular trichomes (*P/Cg*) was highly variable in different plant parts, varying from sparsely distributed in some species to highly aggregated in others. Although all 105 species sampled presented *P/Cg* trichomes, approximately 24.8% of the species sampled (26 out of 105) presented a few *P/Cg* trichomes scattered on the leaflet blades (e.g., *Adenocalymma impressum*, *Manaosella cordifolia*, *Periathomega vellosoi*, *Pyrostegia venusta*, and *Tanaecium pyramidatum*). In contrast, 79 of the species sampled presented aggregated *P/Cg* (> 5 trichomes/mm^2) in different plant parts. In particular, 37.1% of the species (39 out of 105) presented *P/Cg* that were aggregated at the base of leaflets (abaxial side); this feature was particularly common in representatives of *Mansoa* (Figures 8H-I). Furthermore, 35.2% of the species sampled (37 out of 105) presented aggregated *P/Cg* at the interpetiolar region of stems; this feature was particularly common in representatives of *Lundia* and *Fridericia* (Figure 8L). In addition, 22.8% of the species sampled (24 out of 105) presented aggregated *P/Cg* in the prophylls of the axillary buds; this feature was particularly common in representatives of *Adenocalymma, Bignonia*, and *Pleonotoma* (Figures 8A-B). Aggregation of *P/Cg* was rarely documented in the petioles and petiolules, being only encountered in 11 out of the 105 species sampled; this feature was particularly common in representatives of *Pachyptera* and in the monotypic *Callichlamys*. Aggregated *P/Cg* on the adaxial side of the leaflet blades were rare and restricted to *Bignonia corymbosa*. In the field, *P/Cg* trichomes were shown to be yellowish-green and turgid when active, and brownish and dry when inactive. Active trichomes secreted hyaline and viscous nectar. After released, the secretion accumulated in the concave surface of trichomes, from which insects, mostly ants, fed on (A. Nogueira, pers. obs.).

Ancestral character state reconstructions

Maximum likelihood optimizations of the four trichome morphotypes (i.e., non-glandular, peltate glandular, stipitate glandular, and patteliform/cupular glandular) encountered growing in different plant parts (i.e., interpetiolar region of stems, prophylls of the axillary buds, petiole+petiolule, adaxial and abaxial surface of leaflets) are presented in Table 2. Further details on each reconstruction are presented below.

Non-glandular trichomes (ng): ML ancestral character state reconstrutions of non-glandular trichomes indicate that this structure was already present in the most recent common ancestor of Bignonieae (Table 2). In particular, ancestral state reconstructions of *ng* trichomes on the stems (0.84 %), prophylls (0.99%), petioles (0.97%), petiolules (0.94%), and venation of the adaxial side of leaflets (0.86%) led to a single evolution of this trait in each plant part, as well as indicated that *ng* trichomes were already present in these plant parts on the most recent common ancestor of the tribe Bignonieae. On the other hand, even though non-glandular trichomes were likely also present in the adaxial side of the leaflet blade of the most common ancestor of Bignonieae (71%), this feature seems to have been lost at least one time, and gained at least two times. Furthermore, the most recent common ancestor of Bignonieae likely did not present *ng* trichomes on the venation of the abaxial side of leaflets (15.0%) and blade (8.0%), with this trait appearing on the blade of the abaxial side of leaflets within *Adenocalymma* and then re-appering on the venation of the abaxial side of leaflets in the ancestor of the clade *Adenocalymma*+*Neojeobertia* (Figure 3).

The estimated rate of evolution of non-glandular trichomes was unequal, with the rate of gain (mean=0.90%; N=5 reconstructions) being significantly higher than the rate of loss (mean=0.17%; N=5 reconstructions) (Table 2). In other words, the likelihood of gain of *ng* trichomes was significantly higher than their loss. When the two morphological variants of non-glandular trichomes were considered (branched and unbranched), ML reconstructions showed 8 independent evolutions of the branched from unbrached trichomes (Figure 3). Ancestral character state reconstructions of trichome density and size, on the blade of the abaxial side of leaflets suggested that the most recent common ancestor of Bignonieae presented an intermediate density (66.0%) and small trichomes (67.0%) (Table 3).

Peltate & glandular trichomes (pg). Ancestral character state reconstructions of peltate glandular trichomes indicate that the most recent common ancestor of Bignonieae already presented *pg* trichomes in all plant parts (Table 2). In particular, this trichome type was likely already present on the stems (95%), prophylls (99%), petioles (98%), petiolules (98%), and abaxial side of leaflets (99%) of the most recent common ancestor of the tribe Bignonieae. Even though peltate glandular trichomes were likely also present on the adaxial side of leaflets of the most recent common ancestor of Bignonieae (67%), *pg* trichomes seem to have been lost and re-gained at least one time throughout the history of Bignonieae.

The estimated rate of evolution of peltate glandular trichomes was unequal, with the rate of gain (mean=0.34; N=4 reconstructions) being significantly higher than the rate of loss (mean=0.02, N=4 reconstructions) (Table 2). In addition, ancestral character state reconstructions of the density and size of *pg* trichomes suggested that the most recent common ancestor of Bignonieae likely presented an intermediate density on the adaxial side of leaflets (73.0%), but a small size (66.0 %) and high density (66.0%) on the abaxial side of leaflets (Table 3, Figure 5). The largest and denser peltate glandular trichomes were observed in *Amphilophium*, *Pyrostegia*, and *Styzophyllum*.

Stipitate glandular trichomes (stg): The most recent common ancestor of Bignonieae did not present stipitate glandular trichomes in all vegetative plant parts sampled (Table 2). Nine independent gains of stipitate glandular trichomes were hypothetized within Bignonieae (Figure 7), within the following genera: *Adenocalymma* (two gains), *Fridericia* (one gain), *Xylophragma* (one gain), *Cuspidaria* (two gains), *Lundia* (one gain), *Manaosella* (one gain), and *Martinella* (one gain).

The estimated rate of evolution of stipitate glandular trichomes was also unequal. However, contrary to the non-glandular and peltate glandular trichomes, the rate of loss of *stg* was higher (mean=13.22%; N=5 reconstructions) than the rate of gain (mean=1.19%; N=5 reconstructions) (Table 2). Stipitate glandular trichomes were clearly different among taxa, suggesting a structural and genetic independence between these structures. The morphological differences observed in *stg* among taxa and the lack of a historical connection between those trichomes in the various lineages, suggests that the various *stg* trichomes encountered in Bignonieae are not homologous. Thus, the evolution of these trichomes was not explored further.

Patteliform/Cupular glandular trichomes (P/Cg). The most recent common ancestor of Bignonieae likely only presented *P/Cg* trichomes on the abaxial and adaxial side of leaflets (Table 2). In particular, this trichome type was unlikely present on the stems (1%), prophylls (1%), petioles (8%), and petiolules (4%) of the most recent common ancestor of the tribe Bignonieae. In addition, *P/Cg* likely evolved multiple times within each plant part examined (Figure 9). More specifically, at least six independent evolutions of *P/Cg* were hypothesized on the interpetiolar regions of the stems within Bignonieae, including an evolution in the most recent common ancestor of the clade '*Tanaecium+Fridericia+Xylophragma+Cuspidaria +Tynanthus+Lundia+Pachyptera*,' an evolution in the most recent common ancestor of *Dolichandra*, and an evolution in the most recent common ancestor of *Martinella*. Similarly, at least six evolutions of *P/Cg* trichomes were hypothesized on the prophylls of the axillary buds, including one evolution in the most recent common ancestor of *Adenocalymma*, as well as one evolution in the most recent common ancestor of the clade '*Fridericia+Xylophagma*,' and one evolution in the most recent common ancestor of the clade '*Amphilophium+Anemopaegma+Pyrostegia+Mansoa+Bignonia*.'

The estimated rate of evolution of *P/Cg* was also unequal in the various structures analyzed. In the petiole and petiolule, the rate of loss of the *P/Cg* trichomes was higher (mean=0.57%; N=2 reconstructions) than the gain (mean=0.08%; N=2 reconstructions) (Table 2). On the other hand, the rate of gain of *P/Cg* trichomes was higher than the loss in the adaxial (0.37 % vs. 0.09%) and abaxial (13.20 % vs. 0.26 %) side of leaflets (Table 2). Ancestral state reconstructions of the size and density of *P/Cg* trichomes suggested that the most recent common ancestor of Bignonieae presented a high abundance (56.0%) and small glandular heads (63.0%) on the abaxial side of leaflets (Table 3).

DISCUSSION

In this study, we analyzed trichome macro- and micro-morphological data in a phylogenetic context in order to further understand the evolutionary patterns of four trichome morphotypes encountered in vegetative portions of representatives of the tribe Bignonieae (i.e., non-glandular, peltate glandular, stipitate glandular, and patteliform/cuputar glandular). Three of the four trichome morphotypes (i.e., non-glandular, peltate glandular, and patteliform/cuputar glandular) were likely already present in the most recent common ancestor of the tribe Bignonieae, corroborating the hypothesis of a single origin for these individual traits, and suggesting that each of these structures are best treated under the same name.

Integrating morphological data and ancestral character state reconstructions

Trichomes often present variable macro- and micro- morphological structures making it difficult to determine the exact trichome type that is being referred to in the literature (Theobald et al. 1979). In the specific case of the plant family Bignoniaceae, a wide range of terms have been to describe the same trichome type in the literature (Table 1), making it difficult to use such information in comparative studies. Here we intergrated morphological data with a robust phylogenetic framework in order to standardize trichome terminology in the Bignonieae, as well as to gain a more a detailed understanding of the evolutionary patterns of trichome types in this plant group. Below we describe the general morpho-anatomical structure and evolutionary history of each trichome type.

Non-glandular trichomes (ng). Non-glandular trichomes of representatives of Bignonieae have only rarely been described in detail in the literature (e.g., Ogundipe and Wujek 2004). Instead of being described in terms of its structure, *ng* trichomes have been traditionally described in terms of its density. For example, 'vilose leaves' have been described for *Anemopaegma velutinum*, referring to a hairy cover of dense, straight, long, and soft trichomes (Bureau and Schumann 1897). However, no information is available on the anatomy and developmental sequence of non-glandular trichomes in the Bignoniaceae or other plant groups (e.g., Lamiaceae; Naidu and Shah

1981). Even though *ng* trichomes have generally been treated as morphologically homogeneous structures, the variable number of cells (size) and the variable patterns of distribution of this trichome morphotype over different plant parts indicated the need of more detailed morpho-evolutionary studies in representatives of Bignonieae. This kind of information is, among other things, critical for the establishment of accurate hypotheses of homology between different variants of non-glandular trichomes.

Ancestral state reconstructions of *ng* trichomes in Bignonieae indicated that simple and un-branched trichomes were already present in the ancestor of all Bignonieae, from which branching trichomes evolved at least eight times independently. This transition from simple to branched tricomes was similar to that observed in Brassicaceae, in which the pattern of trichome evolution indicated numerous innovations of trichome branching (Beilstein et al. 2006). Furthermore, a hierarchical pattern of occupation of trichomes over different vegetative plant parts was also observed during the evolutionary history of the group. More specifically, the most recent common ancestor of the tribe likely already presented *ng* trichomes on the branches, prophylls of the axillary buds, and petioles. However, it was only later in the history of Bignonieae that trichomes covered the surfaces of leaflet blades. In most species, the density of trichomes remained low and *ng* trichomes remained unbranched. However, a few lineages seem to have recently acquired higher trichome densities (e.g., species of *Amphilophium* and *Tynanthus*) and/or to become branched (e.g., species of *Amphilophium* and *Fridericia*). Unfortunately, no information is available on the genetic basis of trichomes. However, it is possible that putative heterotopic events (as discussed by Baum and Donoghue 2002) may have occurred during the evolutionary history of the tribe Bignonieae.

Peltate glandular trichomes (pg). Peltate glandular trichomes were first recorded in representatives of the Bignoniaceae in the 19th Century in the taxonomic treatments of the family presented in the *Prodromus Systematis Naturalis Regni Vegetabilis* (Candolle 1845) and *Flora Brasiliensis* (Bureau and Schumann 1897). For example, in the *Flora Brasiliensis* (1897), *pg* trichomes were documented in *Cydista aequinoctialis* ("densius lepidota," p. 32), *Pithecoctenium dolichoides* ("conspicue lepidota obsolete pellucide punctulata," p. 22), *Pyrostegia venusta* ("lepidibus minutis inspersa et ope eorum plus minus manifeste pellucide punctate," p. 23), and *Stizophyllum inaequilaterum* ("pellucide punctate," p. 29). Seibert (1948) used two distinct terms to refer to the variants of *pg* trichomes: 'pellucid glands' and 'glandular scales'. This author considered *Amphilophium*, *Pithecoctenium* (= *Amphilophium sensu* Lohmann 2003, in press), *Pyrostegia*, and *Stizophyllum* to have "pellucid glands," and compared these structures to the translucent pellucid dots in leaves of species of Rutaceae. However, in Rutaceae, such structures are present in almost all members of the family and represent secretory cavities (Engler 1931, Kubitzki et al. 2011) that are immersed in the mesophyll, and not epidermal projections like in the Bignoniaceae.

Seibert (1948) considered the majority of the representatives of the Bignoniaceae to present "glandular scales" (and a few "pellucid glands") that he described as "minute scales found on stems, petioles, leaves, calyx, corolla, ovary and fruit, and responsible for the 'lepidote' condition frequently encountered in the Bignoniaceae." Gentry (1980), on the other hand, preferred to call those same structures as "glandular-lepidote trichomes" instead of "glandular scales." More recently, Lohmann (2003) preferred to call those structures as "pellucid glands" instead of "glandular-lepidote trichomes" (Gentry 1980) or "glandular scales" (Siebert 1948). The study of *pg* trichomes conducted here revealed that even though these trichomes are identical in structure, they are much smaller and denser in *Amphilophium*. More specifically, all structures are composed of a glandular convex or rounded head of variable number os cells and foot cells. Furthermore, this structure presents a single origin within Bignonieae, corroborating a hypothesis of homology and indicating that these structures are best treated uniformly under the same terminology. We here propose that these structures are best treated as "peltate-glandular trichomes."

Ancestral character state reconstructions indicate that peltate glandular trichomes were already present in both sides of the leaflet blades and all other vegetative portions of the most recent common ancestor of Bignonieae. However, *pg* trichomes probably originated prior to the origin of Bignonieae, given that this trait was already documented in other representatives of the Bignoniaceae included in three clades defined by Olmstead et al. 2009: the Paleotropical Clade (Ogundipe and Wujek 2004), Tecomeae (Parija and Samal 1936), and Jacarandeae (Zatta et al. 2009). This trichome morphotype is widely distributed through representatives of Bignonieae, but has only been reported in a few representatives of this plant family, likely reflecting a lack of careful trichome studies in other lineages of the Bignoniaceae.

The density and size of the glandular heads represent the most variable features of *pg* trichomes. Ancestral state reconstructions of the individual features of this trichome type indicate that the most recent common ancestor of all Bignonieae likely presented intermediate to high densities of small *pg* trichomes in the abaxial portion of leaflet blades. Even though the most recent common ancestor of the 'Multiples of four' clade and 'Arrabidaea and allies' clade (*sensu* Lohmann 2006) presented peltate glandular trichomes with intermediate sized heads, trichome heads increased in size within the first clade while descreased in size within the second one. However, trichomes with large heads and high densities were relatively rare within Bignonieae, with large heads being restricted to 2 lineages (*Pyrostegia* and *Stizophyllum*), and high densities only being found in 11 lineages, and being particularly common in *Amphilophium*.

Stipitate glandular trichomes (sg). This is the first record of stipitate glandular trichomes on the vegetative portions of representatives of the tribe Bignonieae. This kind of glandular trichomes generally lacks a sticky secretion, and has often been associated with alternative biotic defenses. However, this trichome type has never been associated with ant attraction, which has more generally been associated with nectar-secreating trichomes. For example, adhesive glandular trichomes, a kind of *sg* trichomes have been associated with spider bodyguards that often feed on caracasses of insects that are adhered to these trichomes (Morais-Filho and Romero 2010). Ancestral character state reconstructions of this trichome morphotype indicate that *sg* trichomes were not present in the most recent common ancestor of the group, and suggest that this trichome type has evolved at least nine times independently within the history of the group. These results indicate that even though there is general structural similarity between the *sg* trichomes encountered in the various lineages of Bignonieae, these similarities are not based on a common ancestry. These results also do not corroborate a unified terminology for this trichome morphotype.

Patelliform/Cupular glandular trichomes (P/Cg). Patteliform/Cupular glandular trichomes have been associated with nectar secretion in Bignoniaceae. These trichomes have been generally denominated as extrafloral nectaries (EFNs) and have been observed in different plant parts in nearly all species of the group (Elias 1983). *P/Cg* trichomes have been usually denominated as extrafloral nectaries (EFNs), and have not been thought to be involved in pollination (Elias 1983). However, the specific function of patelliform/cupular glandular trichomes has only been studied in five representatives of the Bignoniaceae, more specifically in: *Campsis radicans* (Elias and Gelband 1975), *Catalpa speciosa* (Stephenson 1982), *Catalpa bignonioides* (Ness 2003), *Anemopaegma album* (Nogueira et al. 2011), and *Anemopaegma scabriusculum* (Nogueira et al. 2011) (Elias and Gelband 1975, Stephenson 1982, Ness 2003, Nogueira et al. 2011 respectively). Nevertheless this terminology (EFNs) was widely used in Bignoniaceae in spite of the lack of functional studies for this structure. That is, the term "EFN" has been used in the literature solely to refer to a particular trichome morphotype, independently of its function.

In Bignonieae, *P/Cg* trichomes were documented in all 105 species sampled in the tribe, and in the 16 species of Bignonieae that were more carefully studied in the lab and in the field. Indeed, this trichome type has been the most widely reported in all previous anatomical studies conducted in the Bignoniaceae (Table 1). However, different terminologies have been used to designate these structures. More specifically, Seibert (1948) generally called those trichomes as "glands," while Elias (1983) named them as "extrafloral nectaries," terminologies that are more directly associated with the function and not with the overall morphological structure. On the other hand, terminologies that are more directly associated with the overall morphological structure of trichomes have also been proposed. In particular, Díaz-Castelazo et al. (2005) treated those trichomes as "scale-like trichomes," while Laroche (1974) treated those structures as "patelliform glands." In addition, Rivera (2000) used the topographical terminology proposed by Fahn (1979) and characterized those trichomes as belonging to "type 12" (i.e., trichomes located in the outer epidermis of the calyx and/or corolla).

Our results corroborate earlier observations that these structures are more often present in the leaflet blades of representatives of the Bignonieae (Elias 1983). However, those structures have also been observed on the petals, sepals, and fruits (Parija and Samal 1936, Seibert 1948, Laroche 1974, Elias and Prance 1978, Stephenson 1982, Subramanian and Inamdar 1989). The variation found in the structure of *P/Cg* trichomes of Bignonieae has been used to distinguish different species (Elias 1983, Elias and Prance 1978). In particular, our study corroborates the earlier suggestion that the large and cupular morphotype of *P/Cg* that is marginally surrounded by the expansion of epidermis is typical of *Adenocalymma* ("volcano" shape gland as proposed by Lohmann 2006). Furthermore, this study also corroborates the observation that the patelliform and cupular trichomes present a uniform anatomical structure (Rivera 2000). Indeed, these trichome variants have been shown to be generally homogenous morphologically but to only vary in terms of their shape, as well as to present a differencial cell proliferation and expantion, which is greater in cupular trichomes. Those results thus suggest that cupular and patteliform trichomes are only superficially different and best treated under the same terminology.

Ancestral character state reconstructions indicated that the most recent common ancestor of Bignonieae already presented *P/Cg* trichomes in both sides of the leaflet blades. However, similarly to what was found for the glandular peltate trichomes, this trait has also been widely documented in representatives of other tribes/clades within Bignoniaceae (Parija and Samal 1936, Seibert 1948), indicating that their origin likely precedes the origin of the tribe. It has been hypothesized that patelliform/cupular glandular trichomes may simply represent an increase in complexity of glandular peltate trichomes (Elias and Newcombe 1979, Parija and Samal 1936). However, this hypothesis remains to be tested through detailed ontogenetic studies of both trichome types, and additional evolutionary studies. Ontogenetic studies conducted with other representatives of the Lamiales demonstrated an ontogenetic resemblance between the patelliform glandular trichomes of the Acanthaceae (McDade and Turner 1997) and those of the Bignoniaceae (Subramanian and Inamdar 1986, Inamdar 1969). In both cases, the trichomes are formed from a single initial protodermic cell, suggesting that the trichomes found in both families might present a common origin, which would imply a much ancient origin for this structure.

Ancestral character state reconstructions of the density of the *P/Cg* trichome morphotype per leaflet indicate that the ancestor of tribe Bignonieae likely presented intermediate densities of *P/Cg* trichomes on the adaxial side of leaflets, but high densities of small trichomes on the abaxial side. Furthermore, ancestral character state reconstructions also indicated that the ancestor of the tribe Bignonieae did not present *P/Cg* trichomes in the interpetiolar portions of stems nor in the prophylls of the axillary buds, with this feature having evolved much more recently in other representatives of the tribe. Since these trichomes have been shown to be associated with ant attraction (Nogueira et al. 2011), the portions of the plant with highest concentrations of these trichomes are thought to be better protected against plant herbivores. The clustering of such structures on particular plant regions, as observed on the prophylls of axillary buds and interpetiolar portions of stems, suggests that these trichomes are protecting plant tissues that are highly costly to the plant (i.e., the meristems of the axillary buds; Heil and McKey 2003).

CONCLUDING REMARKS

Despite the functional importance of trichomes in different plant species and its importance for microevolutionary studies (Levin 1973), little is still known about the macroevolutionary patterns of those structures in different plant families and in different plant organs. Traditionally, trichomes have been thought to have evolved multiple times through the history of the angiosperms. In particular, Theobald et al. (1979, p. 53) noted: “it is evident that the trichomes have had infinite independent origins and as a result no 'phylogenetic' sequence, except on a very limited scale within certain small groups.” However, this does not seem to be the case in the Bignonieae, in which three major trichome morphotypes (i.e., non-glandular, peltate glandular, and patelliform/cupular glandular) seem to have had a single origin in the group. Only the stipitate glandular trichomes seem to have evolved multiple times in vegetative organs of this tribe. Such results indicate that most trichomes found in representatives of the Bignonieae are homologous and best treated under the same name (within each morphotype). A standartization of the trichome terminology greatly facilitates comparations among taxa, as well as allows inferences on the relationsthips between morphological variants and its eco-evolutionary consequences.

Even though the present study contributed important data for a better understanding of the evolution of trichomes in an important clade of angiosperms, it still remains unclear how exactly these trichomes interact genetically, ecologically, and ontogenetically. Further information on the exact interactions among trichome morphotypes would greatly contribute for a better undestanding of the evolutionary patterns of morphological variation and genetic basis of those structures. In addition, further information on the function of the various trichome types studied would also be of extreme importance for the establishment of the role of potential selection agents such as herbivores and abiotic factors (e.g., dessication) for the evolution of those traits. In particular, studies that experimentally test functional hypotheses of trichomes (Chapter 2), and studies that analyze morphological variation in trichomes in the light of a broad phylogenetic sample, including potential biotic and abiotic variables that may have been of importance for the establishment of the patterns of morphological variation currently observed (Chapter 3) are greatly needed. In addition, studies that consider the range of inter-population variation existent in these characters (Chapter 4), are also extremely relevant for a better understanding of the function and eco-evolutionary patterns of the various trichome types encountered in the tribe Bignonieae.

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Table 1. Anatomical studies of glandular and non-glandular trichomes in the Bignoniaceae.

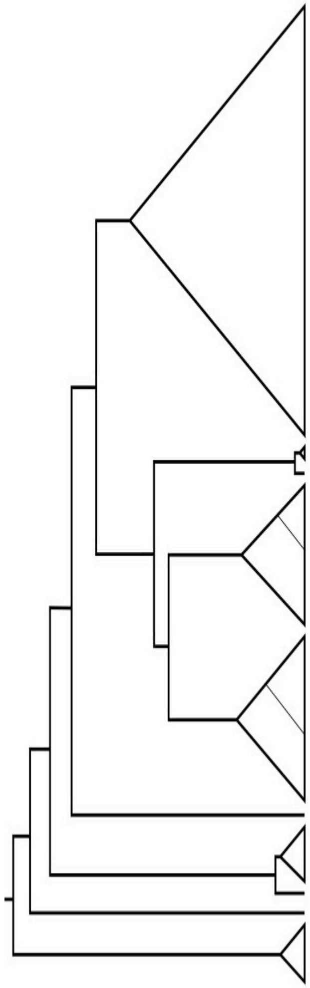
Phylogenetic relationships in Bignoniaceae	Tribe	Species name	Trichome types	References
	BIGNONIEAE	*Lundia corymbifera; Callichlamys latifolia*	Glands.	Seibert (1948)
		Anemopaegma chamberlaynii	Glands and glandular (lepidote) scale/Simple uniseriate trichomes and foliar nectaries.	Seibert (1948); and Inamdar (1969)
		Anemopaegma arvense	Two kinds of glandular trichomes.	Hyakutake & Grotta (1965)
		Adenocalymma comosum	Simple and glandular capitate hairs; and patelliform glands.	Laroche (1974)
		Bignonia capreolata	Scalelike nectaries	Elias (1983)
		Amphilophium paniculatum; and *Mansoa hymenaea*	Scale-like trichomes (morphotype of EFNs).	Díaz-Castelazo et al. (2005)
		Arrabidea brachypoda	Scale-like trichomes and peltate trichomes (extrafloral nectaries)	Machado et al. (2005)
		Memora nodosa	Glandular and non-glandular trichomes	Tresvenzol et al. (2011)
		Adenocalymma marginatum; Amphilophium paniculatum; Anemopaegma flavum; Arrabidaea chica; Arrabidaea corallina; Arrabidaea selloi; Clytostoma binatum; Dolichandra cynanchoides; Macfadyena dentate; Macfadyena unguis-cati; Mansoa difficilis; Melloa quadrivalvis; Parabignonia chodatii; Pithecoctenium crucigerum; Pithecoctenium cynanchoides	Extranupcial nectaries	Rivera (2000)
	TABEBUIA	*Bignonia illicium*	Extrafloral nectaries	Subramanian & Inamdar (1986a)
		Kigelia pinnata	Extrafloral nectaries	Subramanian & Inamdar (1986b)
		Tabebuia serratifolia	Extrafloral nectaries	Thomas & Dave (1992)
		Tabebuia palluda; Parmentiera cerufera; Parmentiera venusta; Crescentia cujete.	*	Ogundipe & Wujek (2004)
		Tabebuia rosea	Scale-like trichomes	Díaz-Castelazo et al. (2005)
	PALEOTROPICAL CLADE	*Spathodea campanulata*	Extrafloral nectaries	Rao (1926)
		Markhamiaa obtussiflora; Markhamia lutea; Markhamia tomentosa; Newbouldia leavis; Stereospermum kunthianum; Stereospermum acuminatissimum; Kigelia africana; Haplophragma adenophylla; Rhodocolea racemosa; Spathodea campanulata.	Simple trichomes (small acicular, subulate, filiform and fasciculate); dendritic trichomes (simple and complex); and two kinds of glandular trichomes (peltate scales and capitate glands). *	Ogundipe & Wujek (2004)
	TECOMEAE	*Tecoma capensis*	Glandular hairs and patelliform glands (extrafloral nectaries) /	Parija & Samal (1936); Subramanian & Inamdar (1989); and Ogungipe & Wujek (2004)
		Campsis radicans; Campsis grandiflora	Extrafloral nectaries	Elias & Gelband (1976)
	JACARANDEAE	*Jacaranda mimosifolia*	Capitate glandular hair; *	Seibert (1948) and Ogundipe & Wujek (2004)
		Jacaranda decurrens	Tricoma glandular escamoso peltado; e tricomas tectores longos;	Zatta et al. (2009)

Table 2. ML ancestral character state reconstructions of different trichome types in the most recent common ancestor of the tribe Bignonieae (Bignoniaceae). Likelihoods are reported as negative logarithms and the ancestral character state is presented in the last column. Estimates of the rates of evolution are also presented. *Non-glandular*=non-glandular trichomes; *pg*=peltate glandular trichomes; *stg*=stipitate glandular trichomes; *P/Cg*=pateliform/cupular glandular trichomes. * Indicates multiple independent gains within the tribe Bignonieae. X=most likely states according to a decision threshold T. The estimated parameters of the evolutionary model favored are presented in bold.

	Trichome characters		**Two-rate model**		**One-rate model**		**Was a two-rate model favored?**	**Estimated ancestral character states for the tribe Bignonieae (proportional likelihood of trichome presence)**	
	Types	**Plant parts**	**Rate of evolution (gain, loss of trichomes)**	**Likelihood**	**Estimated rate of evolution**	**Likelihood**			
1	*non-glandular*	Interpetiolar regions (stems)	**2.150, 0.445**	**48.10**	0.106	56.70	**YES**	Presence (0.84)	
2	*non-glandular*	Prophylls	1.116, 0.012	5.56	**0.005**	**6.96**	NO	Presence (0.99)	×
3	*non-glandular*	Petioles	**0.591, 0.056**	**29.45**	0.043	33.33	**YES**	Presence (0.97)	×
4	*non-glandular*	Petiolules	**0.851, 0.101**	**34.59**	0.055	39.26	**YES**	Presence (0.94)	×
5	*non-glandular*	Leaflets - adaxial (venation)	**0.604, 0.134**	**48.45**	0.106	54.75	**YES**	Presence (0.86)	
6	*non-glandular*	Leaflets - adaxial (blade)	0.221, 0.244	68.52	**0.229**	**68.58**	NO	Presence (0.71)*	
7	*non-glandular*	Leaflets - abaxial (venation)	**0.305, 0.098**	**53.72**	0.117	57.08	**YES**	Absence (0.15)*	
8	*non-glandular*	Leaflets - abaxial (blade)	0.209, 0.138	63.74	**0.173**	**64.33**	NO	Absence (0.08)*	×
9	*pg*	Interpetiolar regions (stems)	**0.510, 0.048**	**29.44**	0.044	33.81	**YES**	Presence (0.95)	×
10	*pg*	Prophylls	15.215, 0.160	5.56	**0.005**	**6.93**	NO	Presence (0.99)	×
11	*pg*	Petioles	**0.271, 0.019**	**19.92**	0.026	22.94	**YES**	Presence (0.98)	×
12	*pg*	Petiolules	**0.272, 0.026**	**22.21**	0.031	26.19	**YES**	Presence (0.98)	×
13	*pg*	Leaflets - adaxial (blade)	**0.306, 0.009**	**18.14**	0.026	22.94	**YES**	Presence (0.67)	
14	*pg*	Leaflets - abaxial (blade)	0.394, 0.010	10.80	**0.010**	**11.90**	NO	Presence (0.99)	×
15	*stg*	Interpetiolar regions (stems)	**1.225, 13.063**	**30.71**	0.048	37.31	**YES**	Absence (0.09)*	×
16	*stg*	Petioles	**1.225, 13.063**	**30.71**	0.048	37.31	**YES**	Absence (0.09)*	×
17	*stg*	Petiolules	**1.225, 13.063**	**30.71**	0.048	37.31	**YES**	Absence (0.09)*	×
18	*stg*	Leaflets - adaxial (blade)	**1.125, 13.063**	**28.28**	0.042	34.25	**YES**	Absence (0.08)*	×

19	*stg*	Leaflets - abaxial (blade)	**1.143, 13.863**	**28.28**	0.042	34.25	**YES**	Absence (0.08)*	×
20	*P/Cg*	Interpetiolar regions (stems)	0.053, 0.132	49.78	**0.091**	**51.26**	NO	Absence (0.01)*	×
21	*P/Cg*	Prophylls	0.053, 0.112	42.17	**0.072**	**43.02**	NO	Absence (0.01)*	×
22	*P/Cg*	Petioles	**0.115, 0.386**	**53.11**	0.136	58.08	**YES**	Absence (0.08)*	×
23	*P/Cg*	Petiolules	**0.046, 0.755**	**21.91**	0.026	24.02	**YES**	Absence (0.04)*	×
24	*P/Cg*	Leaflets - adaxial (blade)	**0.367, 0.087**	**45.40**	0.083	48.21	**YES**	Presence (0.90)	×
25	*P/Cg*	Leaflets - abaxial (blade)	**13.196, 0.261**	**9.86**	0.010	11.91	**YES**	Presence (0.98)	×

Table 3. ML ancestral character state reconstructions of trichome quantitative traits (size and density/abundance) in the most recent common ancestor of the tribe Bignonieae (Bignoniaceae). Likelihoods are reported as negative logarithms and the ancestral character state is presented in the last column, based on a one-rate model of evolution (Mk1). Estimates of the rates of evolution are also presented. *Non-glandular*=non-glandular trichomes; *pg*=peltate glandular trichomes; *P/Cg*=pateliform/cupular glandular trichomes. Size of the glandular head=diameter of the glandular head. X=most likely states according to a decision threshold T.

Trichome characters			**One-rate model**		**Estimated ancestral character state of the tribe Bignonieae (Proportional likelihood)**	
	Types	**Quantitative traits (and position on the plants)**	**Estimated rate of evolution**	**Likelihood**		
1	*non-glandular*	Density (Adaxial side of leaflets)	0.129	102.16	Intermediate (0.66)	×
2	*non-glandular*	Size (Adaxial side of leaflets)	0.231	80.37	Small (0.67)	×
4	*pg*	Density (Abaxial side of leaflets)	0.217	106.46	High (0.66)*	×
5	*pg*	Size of glandular head (Abaxial side of leaflets)	0.202	105.06	Small (0.66)	×
6	*P/Cg*	Number of EFNs (Adaxial side of leaflets)	0.197	109.10	Intermediate (0.67)	×
7	*P/Cg*	Number of EFNs (Abaxial side of leaflets)	0.168	107.30	High (0.56)	×
8	*P/Cg*	Size of the glandular head (Abaxial side of leaflets)	0.199	107.36	Small (0.63)	×

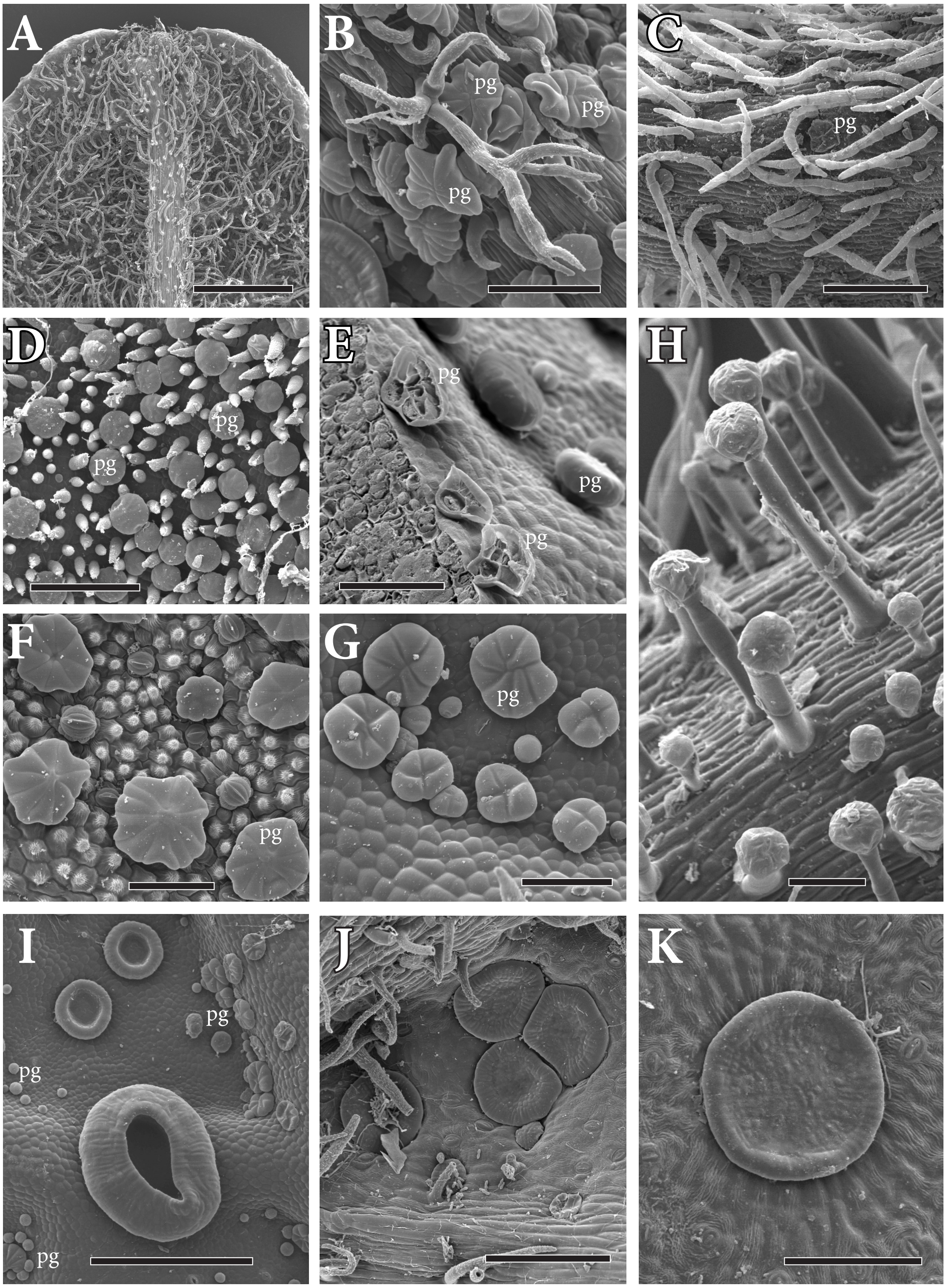


Figure 1 – Trichome morphotypes distributed through vegetative plant parts of representatives of the tribe Bignonieae (Bignoniaceae). A-D. Non-glandular trichomes: A. Simple trichomes on the abaxial side of leaflets of *Anemopaegma scabriusculum* (scale bar=1 mm); B. Branched trichomes and peltate glandular trichomes (*pg*) on the leaflet venation of *Amphilophium crucigerum* (scale bar=100 µm); C. Simple trichomes on the leaflet venation of *Tanaecium pyramidatum* (scale bar=150 µm); D. Simple trichomes and peltate glandular trichomes (*pg*) on the stems of *Periathomega vellosoi* (scale bar=100 µm). E-G. Peltate glandular trichomes. E. *Anemopaegma album* (scale bar=50 µm); F. *Amphilophium crucigerum* (scale bar=50 µm); G. *Adenocalymma pedunculatum* (scale bar=50 µm). H. Stipitate glandular trichomes with adhesive secretion on the petiole of *Cuspidaria sceptrum* (scale bar=50 µm). I. Patteliform/cupular glandular trichomes on the leaflets of *Adenocalymma pedunculatum* with one cupular and two young trichomes on the abaxial side of leaflets (scale bar=250 µm). J. *Anemopaegma album* with aggregated patteliform trichomes on the basal portion of the abaxial side of leaflets (scale bar=200 µm). K. *Periatomega vellosoi* with one patteliform trichome in the abaxial side of the leaflet blade (scale bar=100 µm).

Figure 2 – Variation of non-glandular trichomes in representatives of the tribe Bignonieae. Dashed lines divide the three major evolutionary lineages within the tribe (except *P. vellosoi*). A-B. Branched trichomes in the abaxial side of leaflet blades of *Adenocalymma trichocladum* (scale bar=5 mm and 0.25 mm respectively); C-D. Simple (unbranched) trichomes on the adaxial side of leaflets of *Adenocalymma pubescens* (scale bar=0.25 mm and 5 mm respectively); E. Optical microscopy of simple trichomes (two cells) on the petiole of *Adenocalymma pedunculatum* (scale bar=50 µm); F. Unicellular trichomes on the edge of leaflets of *Periatomega vellosoi* (scale bar=50 µm). G-H. Branched trichomes on the leaf venation of *Amphilophium frutescens* (scale bar=2 mm and 0.25 mm respectively); I-K. Long simple trichomes of *Amphilophium askersonii* on: I. petiole (scale bar=5 mm), J. leaflets (scale ba =0.5 mm), and stems (scale bar = 0.25 mm); L. Optical microscopy of the branched multicellular trichomes on the leaflets of *Amphilophium parkerii* (scale bar=50 µm); M. Simple trichomes on the leaflets and petiolules of *Pyrostegia venusta* (scale bar=1 mm); N. *Anemopaegma scabriusculum* (scale bar=5 mm); O. *Anemopaegma setilobum* (scale bar=0.25 mm); P. Optical microscopy of simple multicellular trichomes on the leaflets of *Anemopaegma scabriusculum* (scale bar=50 µm); Q-T. Branched and unbranched trichomes on the leaflets of *Fridericia*: Q. branched trichomes in *Fridericia cinnamomea* (scale bar=2 mm); R. *Fridericia nigrescens* (scale bar=0.25 mm); S. branched trichomes in *Fridericia dispar* (scale bar=0.25 mm); T. unbranched trichomes in *Fridericia lasiantha* (scale bar=0.25 mm); U. Optical microscopy of simple trichomes from the leaflets of *Fridericia triplinervia* (scale bar =50 µm); V-W. Simple trichomes on the abaxial side of leaflets and prophylls of *Tynanthus polyanthus* (scale bar=0.5 mm and 5 mm respectively); X-Y. Simple trichomes on the leaflets of; X. *Cuspidaria lateriflora* (scale bar=0.25 mm); Y. *Cuspidaria sceptrum* (scale bar=0.25 mm); Z. Optical microscopy of simple multicellular trichomes of *Cuspidaria sceptrum* (scale bar=50 µm).

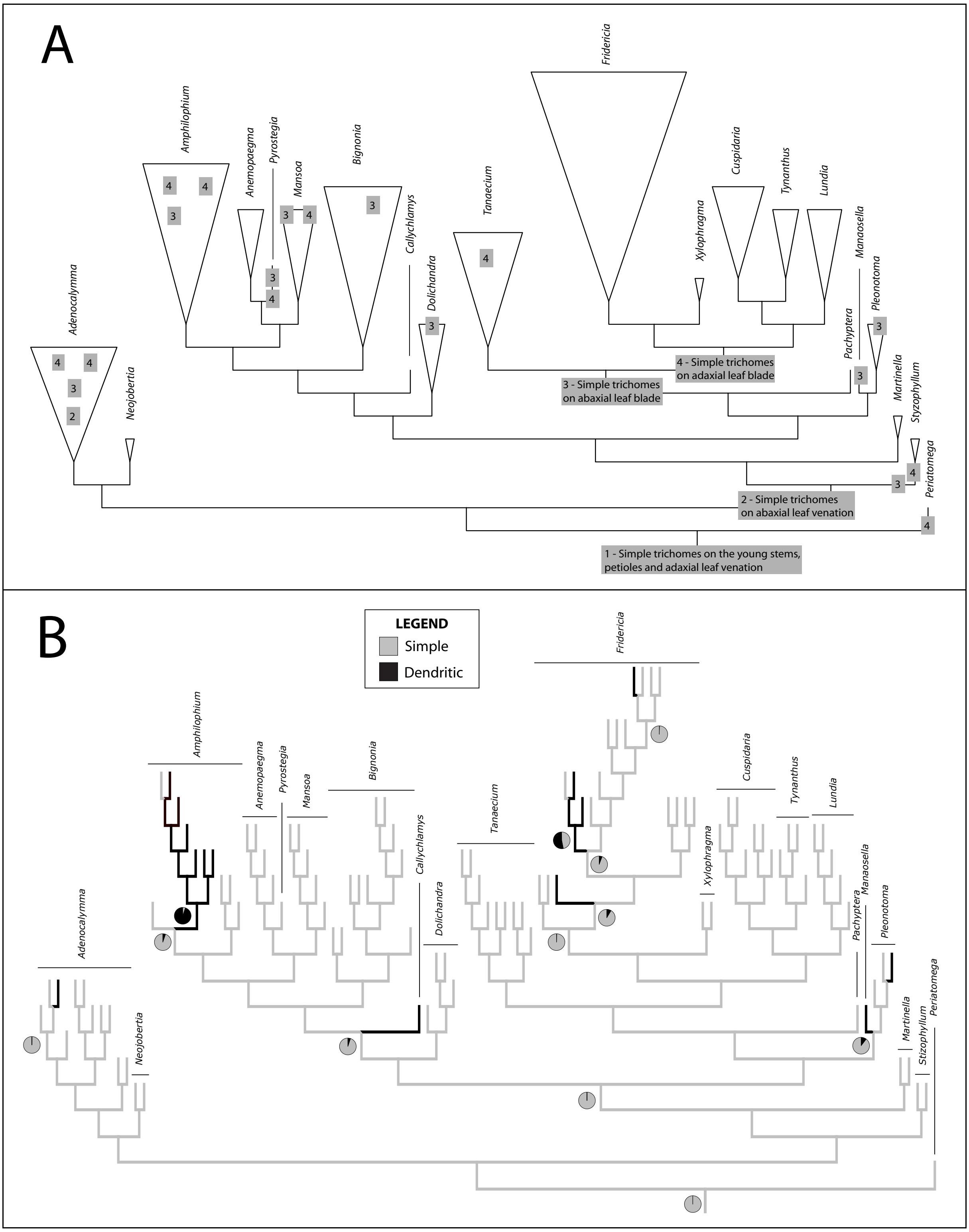


Figure 3 – Evolution of non-glandular trichomes in the tribe Bignonieae. Relationships between taxa follow Lohmann (2006). A. Changes in the location of tector trichomes during the evolutionary history of the tribe Bignonieae. Numbers inside rectangles indicate the number of evolutions of a particular morphology within that lineage. B. Maximum likelihood (ML) ancestral state reconstruction of non-glandular trichomes (simple and dendritic) within the tribe.

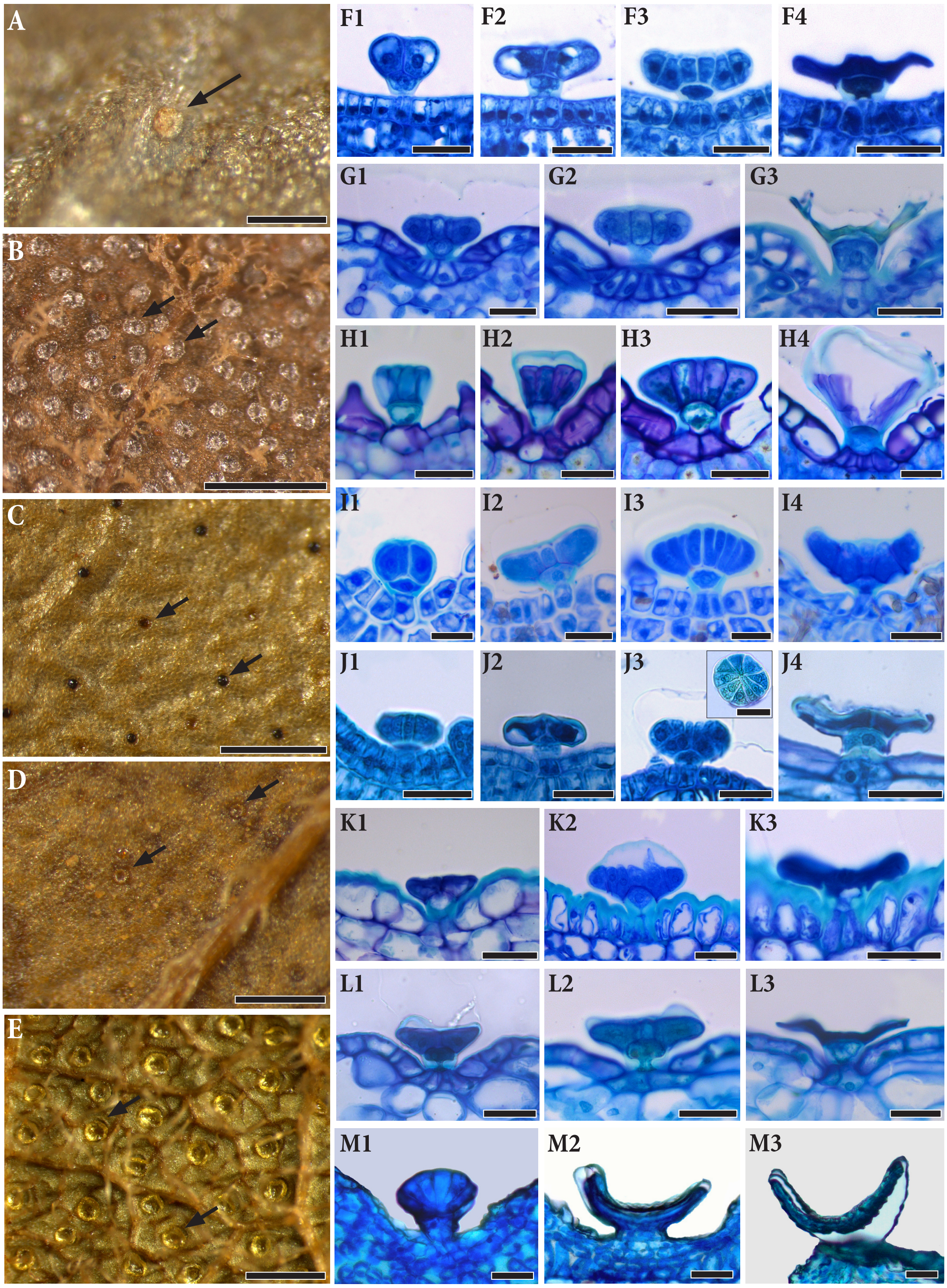


Figure 4 – Variation of peltate glandular trichomes in Bignonieae (Bignoniaceae). A-E. Traditional "lepidote" indumentum in the abaxial side of leaflets of: A. *Adenocalymma cymbalum* (scale bar=0.25 mm); B. *Amphilophium paniculatum* (scale bar=0.50 mm); C. *Anemopaegma chamberlaynii* (scale bar=0.50 mm); D. *Pleonotoma jasminifolia* (scale bar=0.25 mm); E. *Stizophyllum perforatum* (scale bar=0.50 mm); F-M. Developmental stages of peltate glandular trichomes on leaflets: F. *Adenocalymma peduncularum* (scale bar=25 µm); G. *Bignonia prieurei* (scale bar=25 µm); H. *Amphilophium crucigerum* (scale bar=25 µm); I. *Anemopaegma scabriusculum* (I4: *A. album*) (scale bar=25 µm); J. *Fridericia triplinervia* (scale bar=25 µm); K. *Pleonotoma albiflora* (scale bar=25 µm); L. *Tanaecium pyramidatum* (scale bar=25 µm); and M. *Stizophyllum riparium* (scale bar=25 µm).

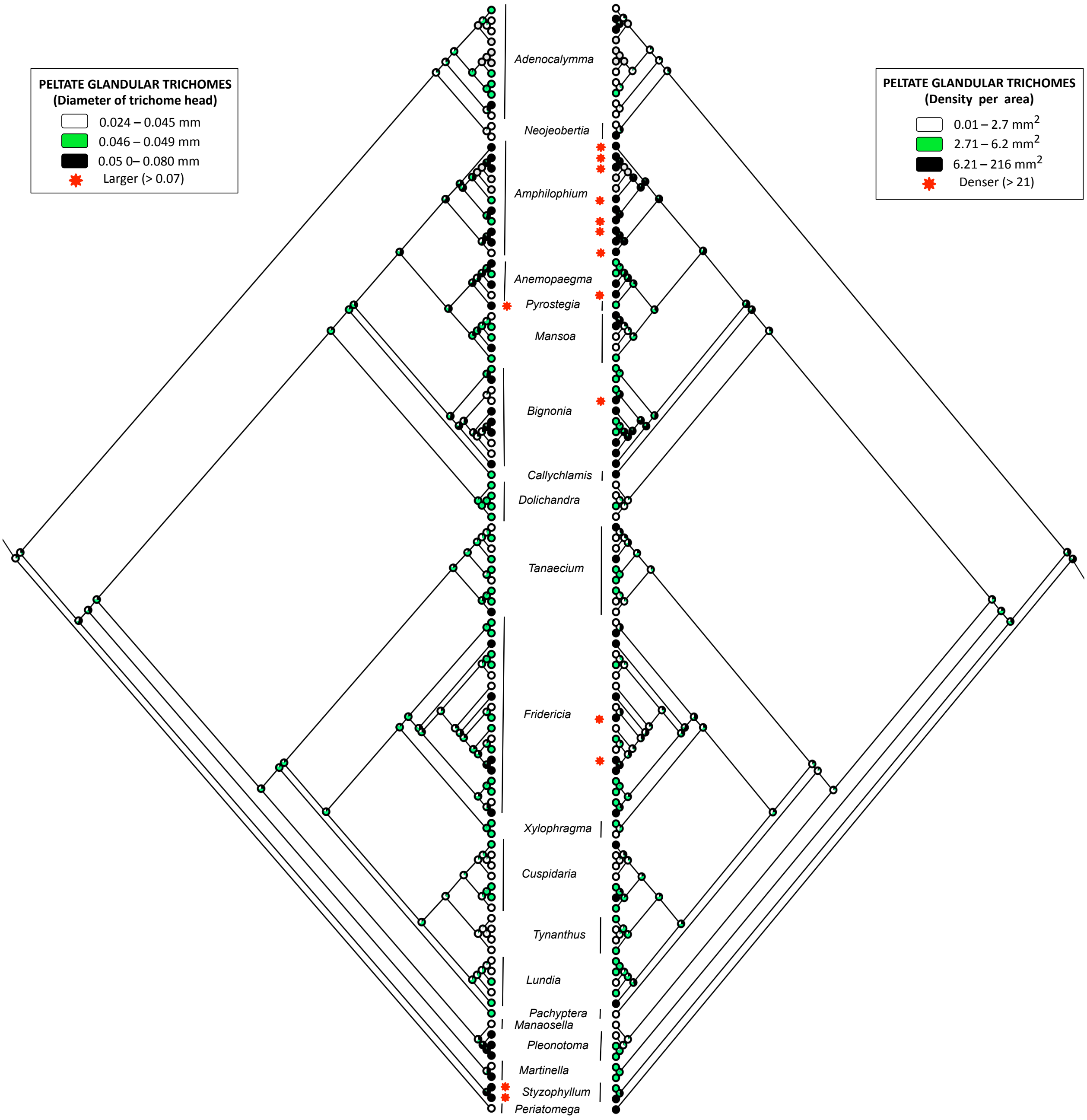


Figure 5 – Evolution of the peltate glandular trichomes in the tribe Bignonieae (Bignoniaceae). On the right, ML ancestral state reconstructions of trichome density. On the left, ML ancestral state reconstructions of the diameter of the trichome glandular heads. Asterisks indicate the lineages in which peltate glandular trichomes are ‘larger’ and ‘denser’ within the tribe.

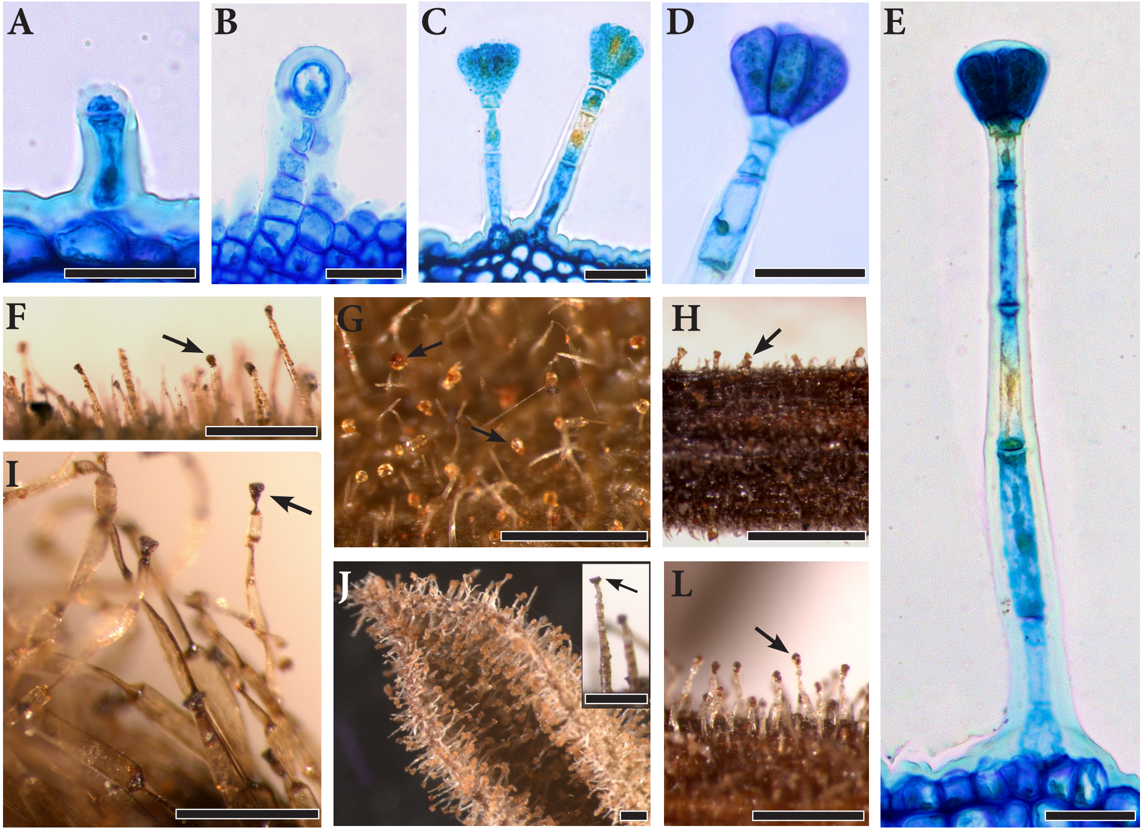


Figure 6 – Variation of stipitate glandular trichomes in Bignonieae (Bignoniaceae). A-E. Developmental stages of stipitate glandular trichomes of *Cuspidaria sceptrum* (scale bar=0.50 µm). F-L. Glandular trichomes on the: F. petiole of *Adenocalymma adenophorum* (scale bar=0.5 mm); G. leaflets of *Fridericia erubescens* (scale bar=0.5 mm); H. Petioles of *Manaosella cordifolia* (scale bar=0.25 mm); I. Stems of *Adenocalymma trichocladum* (scale bar=0.5 mm); J. Young leaves near the inflorescence of *Mansoa hirsuta* (scale bar=0.25 mm); and L. Petioles of *Martinella obovata* (scale bar=0.5 mm).

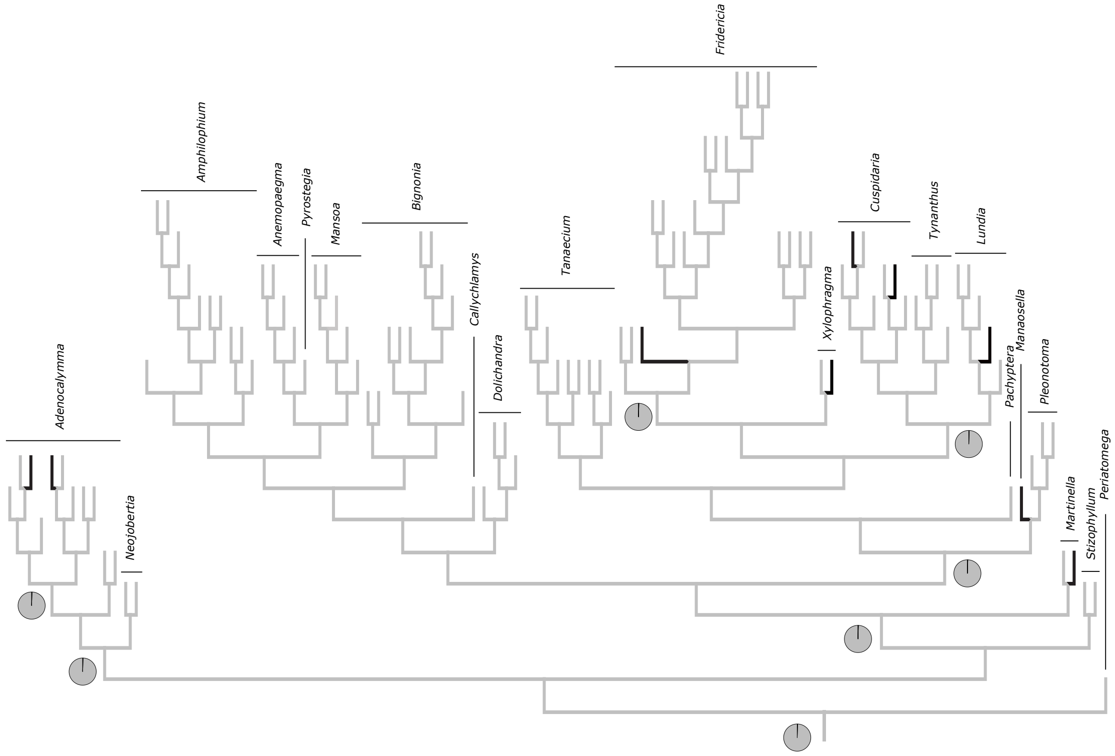


Figure 7 – Evolution of stipitate glandular trichomes in Bignonieae (Bignoniaceae). Maximum likelihood (ML) ancestral state reconstructions indicate nine independent evolutions of this trichome morphotype within that lineage.

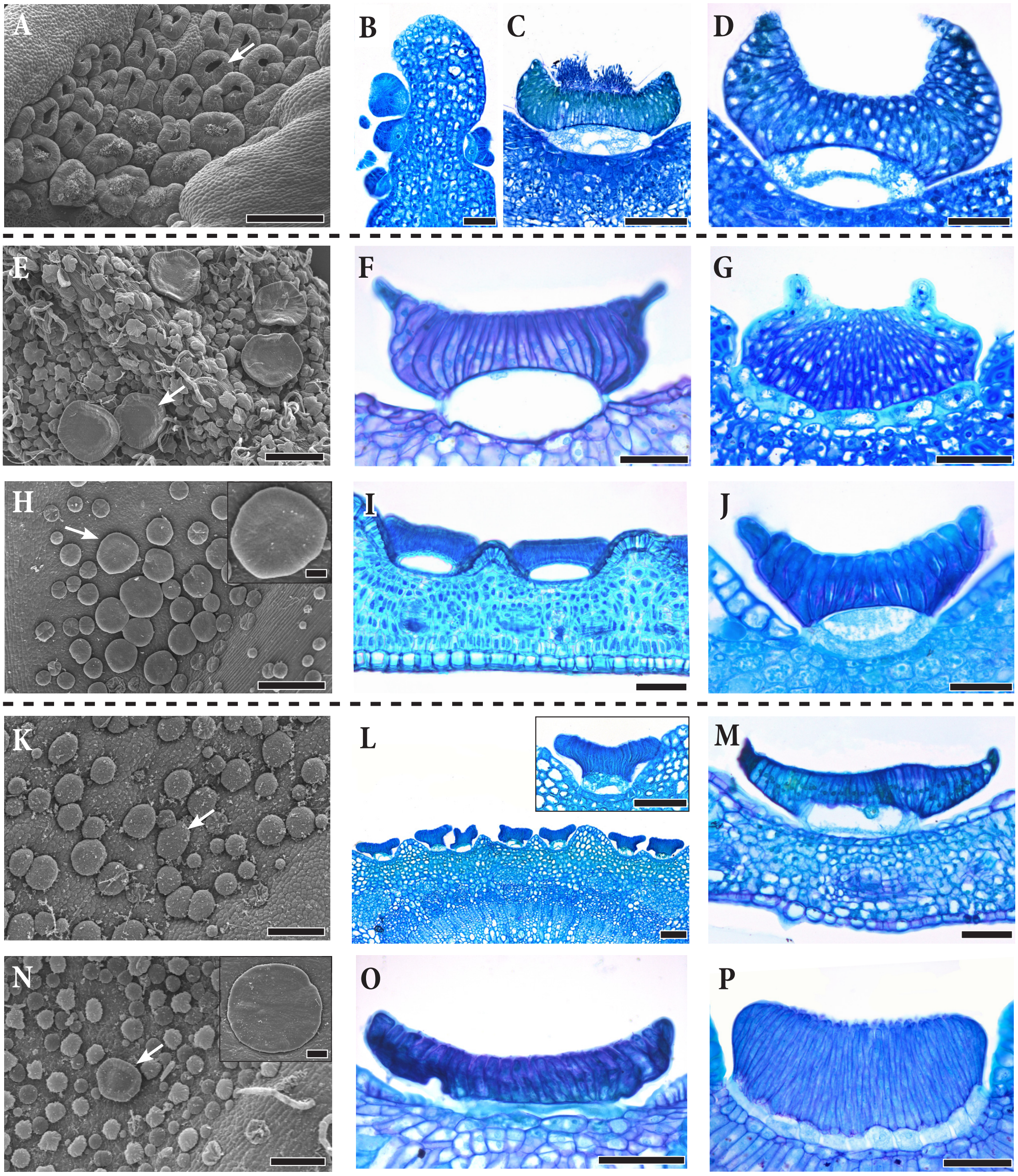


Figure 8 – Variation of patteliform/cupular glandular trichomes in Bignonieae (Bignoniaceae). The dashed line divides the three major evolutionary lineages within the tribe. A-D. Patteliform/cupular trichomes of *Adenocalymma pedunculatum*: A. Mature prophylls with aggregated cupular trichomes (scale bar=350 µm); B. Young prophylls with developing trichomes (scale bar=100 µm); C. Trichomes infected by fungi (scale bar=100 µm); D. Ccupular trichomes on leaflets (scale bar=50 µm). E-G. Patteliform glandular trichomes in *Amphilophium*: E. Abaxial side of young leaflets of *Amphilophium crucigerum* (scale bar=200 µm); and F. Detailed patteliform trichomes on the leaflets of *A. crucigerum* (scale bar=50 µm); G. Trichomes on the leaflets of *Amphilophium parkerii* (scale bar=50 µm). H. Aggregated patteliform trichomes at the base of the abaxial side of leaflets of *Mansoa difficilis* (scale bar=150 µm and 25 µm - top right); I. Trichomes at the base of young leaflets (abaxial side) of *Anemopaegma album* (scale bar=100 µm); J. Patteliform trichomes on the prophylls (adaxial side) of *Bignonia prieurei* (scale bar=50 µm); K. Aggregated trichomes on the young stems (interpetiolar region) of *Lundia nitidula* (scale bar=110 µm); L. Aggregated trichomes on the interpetiolar region of the stems of *Fridericia triplinervia* (scale bar=100 µm); M. Patteliform trichomes on the abaxial side of the leaflets of *Cuspidaria sceptrum* (scale bar=50 µm); N. Trichomes on the abaxial side of the leaflets of *Tanaecium pyramidatum* (scale bar=100 µm and 60 µm - top right); O-P. Trichomes on the adaxial side of the leaflets and prophylls of *Pleonotoma albiflora* (scale bar=50 µm, both).

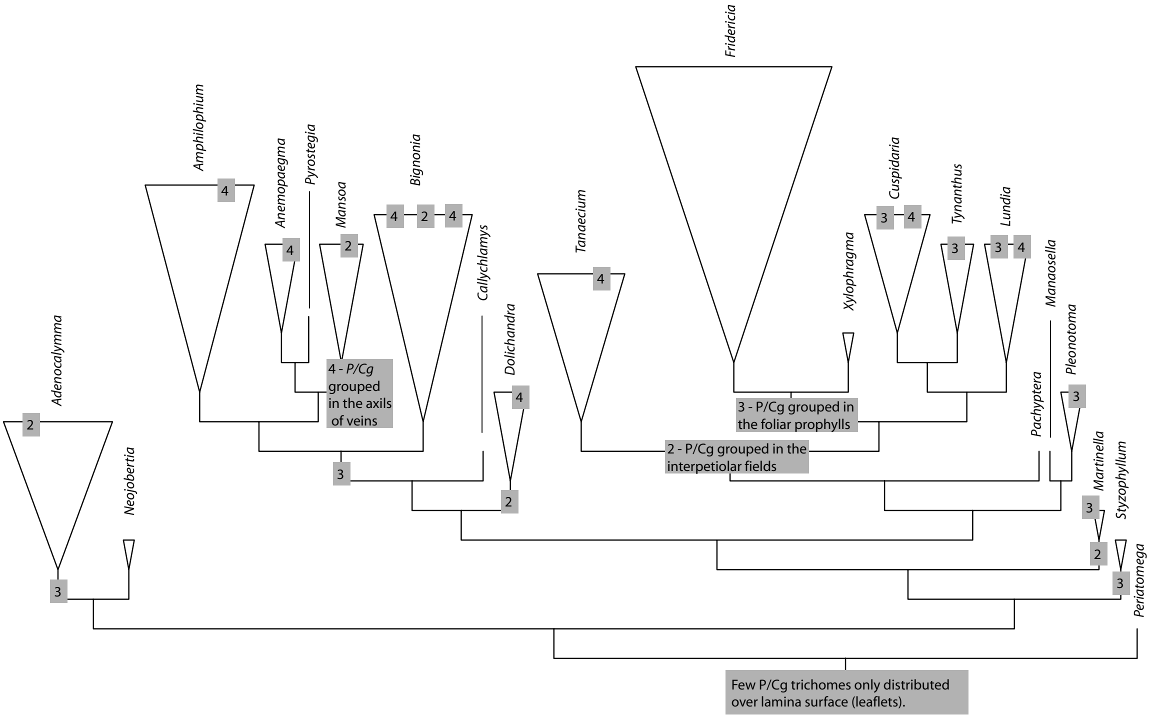


Figure 9 – Evolution of the patteliform/cupular glandular trichomes (*P/Cg*) in the tribe Bignonieae, and changes in the abundance of this trichome morphotype in different plant parts in the tribe Bignonieae (Bignoniaceae). Numbers inside rectangles indicate the number of evolutions of individual morphologies within that lineage.

Capítulo 2

RH: The role of EFNs in two species from savannas

Do extrafloral nectaries present a defensive role against herbivores in two species of the family Bignoniaceae in a Neotropical savanna?

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Abstract

Despite the general belief that the interaction between extra-floral nectaries (EFNs) and ants is mutualistic, the defensive function of EFNs has been poorly documented in South American savannas. In this paper we evaluate the potential impact of EFNs (benefits and costs) on two species of plants from the dry areas of Central Brazil, *Anemopaegma album* and *Anemopaegma scabriusculum* (Bignoniaceae). In particular, we characterize the composition of substances secreted by the EFNs, test whether EFNs attract ants, and whether ants actually present a defensive role, leading to reduced herbivory and increased plant fitness. Histochemical analyses indicated that EFNs from both species of *Anemopaegma* secrete an exudate that is composed of sugars, and potentially lipids and proteins. Furthermore, EFNs from both species were shown to present a significant role in ant attraction. However, contrary to common expectations, ants were not found to protect plants against herbivore attack. No effect was found between ant visitation and flower or fruit production in *A. album*, while the presence of ants led to a significant decrease in flower production in *A. scabriusculum*. These results suggest that EFNs might present a similar cost and benefit in *A. album*, and a higher cost than benefit in *A. scabriusculum*. Since the ancestor of *Anemopaegma* occupied humid forests and already presented EFNs that were maintained in subsequent lineages that occupied savannas, we suggest that phylogenetic inertia might explain the presence of EFNs in the species of *Anemopaegma* in which EFNs lack a defensive function.

Key-words: ant-plant interaction, context-dependency, Brazil, Caatinga, Cerrado, natural selection, nectar glands.

Introduction

Ants and flowering plants interact in a variety of ways (Rico-Gray and Oliveira 2007), and are generally thought to benefit from these interactions (Bronstein 1994). For instance, ants are thought to benefit from the food resources and housing provided by the plants (Beattie 1985), while plants are thought to benefit from ants for pollination (Hickman 1974), seed dispersal (Christianini and Oliveira 2010), and protection against herbivores (Janzen 1966; quantitative review in Rosumek et al. 2009). However, negative impacts of ants to plants have also been recorded. In particular, the negative effect of leaf-cutter ants to plants (Costa et al. 2008), and the negative effect of granivory (Crist and Macmahon 1992) have been well documented.

In general, mutual beneficial interactions are mediated by specific morphological structures (e.g., extrafloral nectaries – EFNs) that are concentrated in more vulnerable plant parts (e.g., sprouts, expanding leaves, apical buds and lateral buds; Heil and McKey 2003). Despite the general belief that EFNs have an important role in plant defense attracting ant bodyguards, it has not been easy to document an increase in plant fitness due to the presence of EFNs (Rico-Gray and Oliveira 2007). It is expected that mutualistic interactions mediated by the EFNs would show "conditionality" or "context-dependency" (Jones and Callaway 2007). Under this assumption, costs and benefits, as well as their respective outcomes, are thought to be affected by current ecological conditions (Bronstein 1994), which would, in turn, explain the potential variations in ant-plant interactions in a predictable way.

In the specific case of ant-plant protection mutualisms, context-dependency does not seem to be a general rule (Chamberlain and Holland 2009), but is thought to be more frequent within species whenever there is variation in local biotic and abiotic conditions (e.g., Tempel 1983; Kersch and Fonseca 2005). In addition, hypotheses that consider the phylogenetic history and geographic context of interactions might also explain the variable outcomes of ant-plant interactions within and between species. For instance, phylogenetic inertia was thought to explain the occurrence of species with EFNs in a Hawaian Island that lacks ants (Keeler 1985). On the other hand, the Geographic Mosaic Theory of coevolution (GMT; Thompson 2005) incorporated a geographical component in biological interaction studies by introducing the idea that different populations from a single species might be under different biotic and abiotic pressures (complex mosaic among populations). Both ideas are directly related to the context-dependency of animal-plant interactions.

The Brazilian savanna covers approximately 2 million km^2 of Central Brazil (Eiten 1972, Ratter et al. 1997), and includes a great diversity of plants with EFNs (approximately 15-25 % of the woody species according to Oliveira and Leitão-Filho 1987, Oliveira and Brandão 1991). In this biome, EFNs present a variety of morphologies (Machado et al. 2008), and are most abundant in representatives of Leguminosae-Mimosoideae, Bignoniaceae, and Vochysiaceae (Oliveira and Freitas 2004). The defensive hypothesis of EFNs was tested in eight savanna species (Oliveira and Freitas 2004, Rico-Gray and Oliveira 2007, Korndorfer and Del-Claro 2006, Nascimento and Del-Claro 2010, Byk and Del-Claro 2010), however only half of the studied taxa (*Caryocar brasiliense*, *Chamaecrista debilis*, *Ouratea spectabilis* and *Qualea multiflora*) presented a defensive role of nectaries, including a negative effect of the EFNs (and associated ants) on herbivory and a positive effect on fitness components (e.g., number of flowers, fruit set or seeds per fruits).

In the specific case of the plant family Bignoniaceae, the function of EFNs has only been studied in three North American representatives of the Bignoniaceae: *Campsis radicans* (L.) Seem., *Catalpa speciosa* (Warder) Engelm., and *Catalpa bignonioides* Walter (Elias and Gelband 1975, Stephenson 1982 and Ness 2003, respectively), leaving a large gap in our current understanding on the role of these structures in the Neotropics. In the present study, we used two species of *Anemopaegma* (Bignoniaceae) to test whether EFNs present a defensive function against herbivores. Specifically, we study: (1) The features of the nectar secreted by these two species of *Anemopaegma* and its relationship to substances known to represent rewards to ants; (2) Whether EFNs of *A. album* and *A. scabriusculum* present a role in ant attraction; and, (3) Whether ants defend individuals of *Anemopaegma* against herbivore attack and/or whether ants have any impact on flower/fruit production in those species.

Material and Methods

STUDY AREA AND PLANTS

This study was conducted at the State Park of Grão Mogol and neighboring areas located 550 km north of Belo Horizonte (Minas Gerais, Brazil). The region presents a seasonal climate with temperatures between 20º-30ºC in the rainy season and between 13º-27 ºC in the dry season (http://www.inmet.gov.br/). The mean annual rainfall is around 1,100 mm, with a dry season between May and September. The soil is composed of remnants of ancient crystalline surfaces (Proterozoic) and is predominantly shallow, acidic and poor in nutrients and organic matter (Pirani et al. 2003).

The genus *Anemopaegma* Mart. ex Meisn (Bignonieae, Bignoniaceae) is composed by forty-one species distributed through forests (N = 30) and savannas (N=11) from Mexico to Argentina (Lohmann 2003, 2011); and highly supported (100%) as monophyletic (Lohmann 2006). Two species of *Anemopaegma* with great levels of variation in the abundance of foliar EFNs were selected for this study: *A. album* and *A. scabriusculum*.

Anemopaegma album Mart. ex DC. is a shrub distributed throughout Brazilian savannas ('cerrados', 'caatingas' and transitional areas called 'carrascos') of Minas Gerais and Bahia (Brazil), with glabrous to puberulous leaves, and triangular prophylls of the axillary buds. The leaves are trifoliate with EFNs grouped at the axils of veins in the abaxial side of leaflets, and sparsely distributed over the adaxial side of the blade. *Anemopaegma scabriusculum* Mart. ex DC. is a shrub and also occurs throughout the savannas of eastern Brazil, from Minas Gerais to Piauí, reaching the state of Goiás, with pubescent leaves and orbicular prophylls of the axillary buds. The leaves are also trifoliate, but without EFNs grouped. In this species the EFNs are scattered over the adaxial side of the blade, rarely on the abaxial side. To characterize the morphology and exudates of the EFNs in both *Anemopaegma* species we used optical and electronic microscopy, histochemical analyses, urine test strips and a portable refractometer (see Appendix 1S in Supporting Information for these detailed methods).

CHARACTERIZATION OF ANTS AND HERBIVORE ASSEMBLAGES

Ants and herbivores found in *A. album* and *A. scabriusculum* were collected during field-work and subsequently identified in the laboratory by insect specialist. The behavior of ants found on individuals of *A. album* and *A. scabriusculum* was characterized in the field by describing the degree of use of EFNs, and a description of interactions with herbivores (antagonistic or neutral relationship). Furthermore, the behavior of herbivores was characterized by describing the feeding habit, including in some cases a description of the larval stage in which the herbivores also fed on the plant.

ANT ATRACTION BY THE EXTRAFLORAL NECTARIES

Observational data were collected on natural populations of *A. album* and *A. scabriusculum* from August 2008 to June 2009. Experimental populations belonging to both species were located in areas of savanna with low human impact, and separated by approximately 30 km. All plants sampled were taller than 50 cm and included only adult individuals (potentially reproductive according to our observations). Two experiments were conducted to test whether individuals of *A. album* and *A. scabriusculum* attracted more ants than neighboring species, and whether ant abundance found on individuals of *A. album* and *A. scabriusculum* was correlated to the amount of EFNs. These tests were carried out separately on each species.

For the first field experiment, 14 pairs of individuals that included one individual of *A. album* and its nearest heterospecific neighbor, were tagged. Selected heterospecific neighbors were at non-reproductive stage and presented a similar number of leaves than its respective *Anemopaegma* couple. Similarly, 18 pairs of individuals that included one individual of *A. scabriusculum* and its nearest heterospecific neighbor were also tagged. For all tagged individuals of each pair, we recorded the number of ants (abundance) on each experimental plant. We did not exclude neighboring plants with extrafloral nectaries or honeydew-producing homopterans as we wanted to quantify ant visitation in *A. album* and *A. scabriusculum* under natural conditions (i.e., which also includes situations in which the nearby plants present EFNs). Paired t-tests were applied to assess whether the average difference in number of ants between paired plants (*Anemopaegma* and nearest neighbor) was higher than expected by chance.

For the second set of observations, another 14 individuals of *A. album* and 24 new individuals of *A. scabriusculum* were tagged in each sampling locality. For each individual, we recorded the average number of EFNs per leaflet (4-6 leaflets sampled without detaching the leaves), the number of leaflets with active EFNs per plant, and the number of ants per plant. We used multiple linear regressions to test whether the variables representing EFNs were associated with the number of ants encountered on each plant. This analysis also included the covariate 'time of the day when ants were collected' in order to control for this source of variation in the data.

EFFECT OF ANT-VISITATION ON HERBIVORY AND PLANT REPRODUCTION

Ant-exclusion experiments were carried out to evaluate the defensive role of ant visits against herbivores and its influence on flower and fruit production in *A. album* and *A. scabriusculum*. Due to differences on the spatial arrangement of plants of the two species of *Anemopaegma*, we used a paired experiment to *A. scabriusculum* (more clumped distribution) and non-paired experiment to *A. album* (more scattered distribution). Specifically, we used 48 paired plants (24 sample units) of *A. scabriusculum* and 25 scattered plants of *A. album.* For each plant pair, one randomly selected plant was used as control, while the other was used for the ant exclusion experiment. Ants were prevented from reaching treated plants with Tanglefoot® (Tanglefoot Co., MI, USA), a sticky resin that was applied as a barrier at the base of each individual. All ants found on treated plants were removed at the time of resin application. In addition, plants that could serve as bridges of ants to treated plants were pruned. None of these procedures were applied to the control individuals so that ants could have free access to those individuals.

Total leaf area and area of damage were measured on previously marked leaves in the field using visual assessment with the aid of an acrylic sheet that included a 0.3 cm^2 grid. The leaves that were opposite to the undamaged leaves selected for the experiments were marked with thin colored wires. For this experiment, we did not use the marked leaves for herbivory measurements in order to prevent the marking from having any effect on insect movement. Herbivory was estimated as a proportion of the leaf area that was removed during the first 15 days (first period), and during the following 55 days (second period; lasted between the 16th and the 70th experimental days). The leaf herbivory generated in the first sampling period was subtracted from the accumulated herbivory during all experimental days in order to assess herbivory in two different periods. To quantify herbivory in the first 15 days (first period), two kinds of marked leaves based on the leaf edge were used: 4-6 young leaflets and 4-6 mature leaflets per plant. Young leaves were marked when they were still unexpanded buds (or in the process of expansion), while mature leaves were marked after they were already fully expanded (and completely undamaged). Mature leaves were not followed during the second period (after the first 15 days of experiment), because most leaves showed signs of senescence.

Two separate analyses were performed with the data collected. The first analysis was a two-way ANOVA that was employed to test for the effect of leaf age (young vs. mature leaves) and ant presence (absence vs. presence) on the amount of herbivory accumulated in the first period. In this analysis, leaf age was included as an intrinsic factor of plants in order to control for this source of variation given that this factor has been previously associated with herbivory (i.e., higher herbivory levels were found in younger leaves; Coley 1980). The second analysis was employed to test for the effect of ants on herbivory through time. This analysis was a repeated-measures ANOVA with one variable between subject effects (ant exclusion) and one variable within subject effect (herbivory time period). Similarly, a repeated-measures ANOVA was also used to evaluate the impact of ants on the number of flowers produced per plant during the two sampling periods. Furthermore, the impact of ants on fruit set was evaluated through the difference in the number of fruits produced between control plants and plants in which ants had been removed. All analyses included the covariate "number of leaves of each plant" in order to control for the potential effect of plant size on herbivory.

Results

Extrafloral nectaries (EFNs) in *A. album* and *A. scabriusculum* are usually in depressions that are located in both surfaces of leaflets (see Appendix 2S in Supporting Information for a morphological description of EFNs). In *A. scabriusculum* these nectaries are more predominant in the adaxial surface, where they are scattered and present an average of 11.3 EFNs (SE=0.8, *n*=24 plants) per leaflet (Figure 1B), with the abaxial surface with less than 1 EFN per leaflet. In *A. album,* plants presented an average of 3.6 scattered EFNs per leaflet (SE=0.8, *n*=14 plants) and 29.6 aggregated EFNs per leaflet. These EFNs were concentrated in the abaxial surface (SE=4.3, *n*=14 plants), forming clusters near the central vein at the base of the blade (Figure 1D).

Histochemical analyses indicated that the secretory tissues of the EFNs from both species of *Anemopaegma* presented sugars, proteins and lipids (Figure 1E). However, no pectin/mucilage, phenolic compounds, or alkaloids were detected. Direct measurements on the exudates conducted with a portable refractometer (and test strips) detected a concentration of 71.0 % (SE=7.4, n=10 plants with 37 leaflets in each one) sugar on 0.8 µl of the total exudate collected in *A. album* and 30.6 % (SE=3.6, n=21 plants with 52 leaflets in each one) sugar on 27.3 µl of the total exudate in *A.*

scabriusculum. The volume of nectar secreted by the EFNs was lower (and more concentrated) in *A. album* than in *A. scabriusculum*.

Four genera of ants dominated the interaction with EFNs (Table 1), with ants belonging to the genus *Camponotus* being the most abundant of the two species of *Anemopaegma*. On average, *A. album* had 12.4 ants/plants (SE= ±3.3; N=14) from which 20.5 % were *Camponotus*, while *A. scabriusculum* had 23.3 ants/plants (SE= ±5.5; N=14) from which 97 % were *Camponotus*. The most common herbivores (Table 2) were the following: *Charidotis* sp. (Chrysomelidae, Coleoptera), *Aerenicopsis* sp. (Cerambycidae, Coleoptera), *Sumitrosis* sp. (Chrysomelidae, Coleoptera), and *Xestotrachelus robustus* (Romaleidae, Orthoptera). Observations of the interactions (physical contact) between ants and herbivores showed that all four common herbivores were indifferent to the most common ants of the genera *Camponotus* and *Cephalotes*.

No significant difference was encountered between the abundance of ants on individuals of *A. album* and neighboring species (Table 3). However, individuals of *A. scabriusculum* presented 11.7 more ants than their closest neighbors on average. Both *A. album* and *A. scabriusculum* presented a higher abundance of ants on plants with higher number of leaves with active EFNs (Figures 3A e C). In *A. album*, every 30 leaflets (10 leaves) with active EFNs led to an increase of two ants per experimental plant. No ants were found in individuals without active EFNs (e.g., with senescent leaves or leaves at very young stages). Similarly, every 30 leaflets (10 leaves) with active EFNs of *A. scabriusculum* led to an increase of 1.8 ants per experimental plant. Furthermore, every five EFNs per leaflet of *A. album* led to an increase of 1.4 ants per plant on average (Figure 3B). In *A. scabriusculum*, on the other hand, ant visitation did not vary with the average number of EFNs (Figure 3D).

Significant differences were found between the damage of leaflets of young and mature leaflets of *A. album* ($F_{(1,\ 22)}$=18.9, P<0.001) and *A. scabriusculum* ($F_{(1,\ 21)}$=5.4, P=0.030) during the first 15 experimental days. During this period, control individuals of *A. album* presented an average leaflet damage of 21.1 % (SE=7.1, n=12) on young leaflets, and an average leaflet damage of 1.1 % (SE=0.4, n=12) on mature leaflets. Leaflets of *A. scabriusculum* were significantly less damaged during the same period; on average, 0.003 % (SE=$6.8*10^{-4}$; n=23) of the total area of young leaflets, and 0.0002 % (SE=$6.8*10^{-5}$, n=23) of the total area of mature leaflets were consumed. Considering the total foliar damage accumulated over the 70 experimental days (using only data from young marked leaves) the plants of *A. album* presented an average leaflet damage of 58.6 % (SE=11.3, *n*=12), while *A. scabriusculum* presented an average leaflet damage of 39.4 % (SE=7.0, *n*=23) of its area during the same period.

No difference was found for leaf damage between individuals with or without ants of *A. album* ($F_{(1,22)}$=0.12, *P*=0.73) and *A. scabriusculum* ($F_{(1, 21)}$=1.61, *P*=0.22) in the two sampling periods (Figure 4). Behavioral observations of the ants that were visiting EFNs (mostly *Camponotus*) in both species of *Anemopaegma* studied did not indicate any antagonistic interactions with the species of herbivores that may have contributed to a decrease in leaf damage (Table 3). The feeding behavior of the common herbivores, three species of beetles on *A. album* and a species of cricket on *A. scabriusculum*, showed a potential preference for feeding on individuals of *Anemopaegma* versus other plants in the field (A. Nogueira, pers. obs.). This result was stronger in three out of four herbivore species, in which both the larvae and adults fed on individuals of *Anemopaegma* (Figure S1, Table 4). Throughout the development of this study, only crickets (rarely) were seen feeding on plant species other than *Anemopaegma*.

Apart from the lack of an effect on herbivory in both species of *Anemopaegma* studied, ant visitation had no impact on flower ($F_{(1, 22)}$=0.86, *P*=0.36) and fruit production ($F_{(1, 22)}$=0.03, *P*=0.86) in *A. album*, suggesting a lack of a defensive role by the ants that visited this species (Figures 5A). Conversely, ants led to a decrease in flower production ($F_{(1, 21)}$=11.03, *P*=0.003) in *A. scabriusculum*, suggesting a potential cost associated with the ant-EFN interaction in this species (Figure 5B). Unfortunately, we were unable to evaluate the impact of ant visitation to fruit production in *A. scabriusculum* because only one individual (out of the 48 individuals sampled) produced fruits.

Discussion

This study evaluated the potential impact of EFNs and associated ants to prevent leaf damage, and consequently, to improve flower production and fruit-set in two species of *Anemopaegma* (Bignonieae, Bignoniaceae). We found that EFNs produce a secretion composed of sugars, and potentially proteins and lipids, all of which represent important components of the ant diet (Carroll and Janzen 1973). This nectar resource is especially important to ant groups with some specialized anatomy (e.g., proventriculus adapted to benefit from the availability of liquid foods, Davidson

1997), such as that encountered in *Camponotus*, the most abundant group of ants sampled on the EFNs of the two species of *Anemopaegma* studied. *Camponotus* ants were also seen dominating the interaction with plants in the savannas (Oliveira et al 1987, Del-Claro et al 1996, Oliveira 1997, Korndörfer and Del-Claro 2006, Nascimento and Del-Claro 2010, Byk and Del-Claro 2010). In those studies, *Camponotus* ants were also the most abundant, as well as showed potentially protective patterns against herbivores (mostly Lepidoptera). On the other hand, ants belonging to the genus *Cephalotes* (the second most abundant in our study) did not present any defensive role for plants and were likely commensal or parasitic (see Bik and Del-Claro 2010). Studies addressing the role of each ant group in facultative ant-plant interactions mediated by EFNs only started to be conducted experimentally very recently (e.g., Bik and Del Claro 2010). In the particular case of neotropical savannas, it is reasonable to assume that *Camponotus* might play an important role in these interactions.

In this study, EFNs led to an increase in ant visitation in both species of *Anemopaegma*. However, contrary to expectations under traditional plant-defense hypotheses, none of these plant species were defended by ants (Beattie 1985, Heil and McKey 2003 and Rico-Gray and Oliveira 2007). One possible scenario is that the EFNs were not able to attract the minimum amount of ants that is necessary to reduce leaf herbivory. Studies conducted in savannas documented an average of 4.42 (± 4.3) ants/plant in *Qualea grandiflora* (Oliveira et al 1987), and an average of 1.97 (± 1.6; daytime) and 1.80 (± 1.6; nighttime) ants/plant in *Caryocar brasiliense* (Oliveira 1997), both of which were shown to represent sufficient amounts of ants to protect those taxa against herbivores. In our study, an average of 12 ants/plant was documented in *A. album*, and an average of 23 ants/plant in *A. scabriusculum*. Even if the studied individuals were controlled for plant size, the studied species of *Anemopaegma* would still present a higher number of ants per plant than *Q. grandiflora* and *C. brasiliense* suggesting that factors other than ant number (e.g., the identity of the ants or herbivores), would be responsible for the 'inefficiency' of the nectaries.

The cost-benefit model can be applied for the study of traits that are associated with the ant-plant-herbivore system (Heil and McKey 2003). Under this model, the production of EFNs and associated secretions are costly to the plant, however this cost is outweighed by the benefits provided by ant visitors, leading to an increase in plant-fitness (Heil and McKey 2003). However, other scenarios are also possible for these interactions (e.g. commensalism or parasitism with ants; context-dependency by Jones and Callaway 2007). More specifically, ants may not represent effective defenses to the plants, and/or herbivores may be more efficient than expected at feeding the plants (e.g., Tempel 1983; Kersch and Fonseca 2005). Following this reasoning, it is possible that the community of ants and herbivores found in the present study might explain the ineffective antiherbivore defense of the nectaries. In fact, further information in 10 populations of *A. album*, suggests that both ant and herbivore assemblage substantially vary across population, as well vary the EFNs abundance and the leaf damage by herbivores (Nogueira et al. unpublished). Moreover, we did not find a defensive role of ants, nor we found a positive impact to plant reproduction in both species of *Anemopaegma*. In *A. scabriusculum* in particular, we actually found a negative impact of ants for plant reproduction. These results suggest that the costs and benefits of EFNs might be equivalent in *A. album* (ants as commensal) but more costly than beneficial in *A. scabriusculum* (ants as parasites).

Apart from the cost-benefit model, the hypothesis of phylogenetic inertia might also explain the lack of trait functionality in some species or populations. In particular, phylogenetic inertia might play an important role in lineages in which the history of habitat occupation by taxa varied between different selective regimes (e.g., groups of closely related species in which some species occur in forests and others in savannas). Under this scenario, traits found in the ancestors of particular lineages would be maintained in their descendants due to phylogenetic inertia (see Orzack and Sober 2001 and Blomberg and Garland 2002). In the specific case of EFNs, phylogenetic inertia would imply the maintenance of EFNs in some taxa simply because EFNs were present in the ancestor of the lineage and not necessarily because EFNs would confer higher fitness to these taxa presently or in new habitats. This hypothesis was initially used to explain the occurrence of plants with EFNs in an island of the Hawaiian archipelago that lacks ants (Keeler 1985).

In the particular case of *Anemopaegma*, the evolutionary history and morphological characteristics of the studied species might help explain the presence of EFNs in spite of its inefficient defensive role. Indeed, EFNs were already present in the ancestors of *Anemopaegma* (A. Nogueira et al. *unpublished*; Figure 02). Furthermore, EFNs were likely more abundant in the ancestors than in the studied species (A. Nogueira et al. *unpublished*), those of which occupied forest habitats

(Lohmann 2003, Lohmann et al. 2011 *in press*). EFNs were subsequently kept in all species of *Anemopaegma* even after lineages occupied drier areas such as the Brazilian savannas. It is possible that these structures might have presented a defensive role in the past but that this role was lost after the transition to dry areas, where these species might have started to interact with a new assemblage of ants and herbivores. Under the new selective regime, species with EFNs might only experience the cost associated with the production of EFNs but not the benefits (defensive role), leading to an overall decrease in fitness surrogates such as flower and fruit production. Indeed, the average abundance of EFNs is higher in species of *Anemopaegma* that are currently distributed through forests than in species that are currently distributed through savannas (see Appendix 3S in Supporting Information), suggesting a decrease in the number of EFNs after the invasion of savannas. However, this study still lacks information on the role of the EFNs in forest species, which would be very important in order to test the phylogenetic inertia hypothesis.

Another non-mutually exclusive explanation for the lack of defensive role of EFNs in the studied species falls within the framework of the Geographic Mosaic Theory of coevolution (GMT; Thompson 2005). The GMT has incorporated a geographical component for the study of interactions by remarking that different populations from a single species might be under different biotic and abiotic pressures (context-dependency of the interaction outputs differently in each population). Within the context of our study, GMT would predict local differences among populations (and species in expanded temporal scale), which might lead to variation in the ant-plant interaction (e.g.; Alonso 1998, Manzaneda and Rey 2008), with some populations not being protected by the ants. The geographic variation of ant-plant interactions on savannas were observed among populations of the ant-plant *Tococa guianensis*, in which some populations were found to persist in some areas without these obligate ants on the plants (Moraes and Vasconcelos 2009). The lack of a defensive role of EFNs in both species of *Anemopaegma* studied suggests that the ants available in our study sites were not sufficiently aggressive to protect the visited plants against herbivores (very efficient herbivores). Indeed, none of the ants observed visiting populations of *A. album* presented an aggressive behavior against the most common herbivores encountered in these species. Among the herbivores observed in *A. album*, adults and larvae of *Charidotis* sp. (Cassidini, Chrysomelidae, Coleoptera) were the ones that contributed for the highest amount of leaf damage. Field observations indicated that common ants of the genus *Camponotus* and secondarily *Cephalotes* did not interfere in the reproduction or oviposition of adults, nor in the movement of larvae of the herbivore *Charidotis* sp. A similar pattern was observed in the other two most common herbivores that visited *A. album*. In *A. scabriusculum*, on the other hand, the highest amount of leaf damage was caused by a single herbivore, the cricket *Xestotrachelus robustus*. Similar to the patterns observed in *A. album*, the ants observed in *A. scabriusculum* (mostly *Camponotus*) did not seem to protect plants from cricket damage.

Overall, our results suggest that plants with higher densities of EFNs might not present an advantage over individuals with lower densities of EFNs in populations of *A. album* and *A. scabriusculum*. However, it remains unclear why exactly nectaries remained present in these populations. It is possible, for instance, that EFNs might have remained as a result of some specific predictions associated with the GMT (Thompson 1999) such as: (1) source populations efficiently defended by ants (i.e.,"hotspots") might be supplying the genetic variation necessary for the presence and abundance of EFNs through gene flow in populations not efficiently defended by ants (i.e.,"coldspots "), (2) momentarily inefficient populations might be defended from time to time by ants (temporal variation of the system), leading to a maintenance of EFNs even during the periods in which these structures were inefficient, (3) the cost of EFNs and its respective secretion would be very low in savanna-like environments (usually with abundant light and a large reserve of carbohydrates in plants), maintaining the variation of EFNs even though these structures would be inefficient or ineffective in reducing herbivory under such conditions (almost like a "neutral" trait).

Although the temporal and spatial scales of this study are restrictive to unfold the exact causes of the patterns encountered, it is reasonable to assume that the conditionality of the interactions is associated with the local community of ants and herbivores, and indirectly with the variation of abiotic factors. Furthermore, it is also reasonable to hypothesize that the pattern encountered might have resulted from a loss of the functionality of EFNs that likely happened following the transition of taxa from humid forests to savannas. In order to fully test this hypothesis, it would be important to conduct studies within a broad geographic context. More specifically, it would be important to gain a deeper understanding of the patterns of variation of EFNs (and the role of them) found in taxa that presently occur in forests and savannas, using other representatives of the plant family Bignoniaceae in which similar habitat transitions and morphological structures are present. Only with a broader sampling, including detailed studies with multiple lineages and populations will we be able to gain a more thorough understanding of the ecological and evolutionary processes that shaped the evolution of EFNs in those species.

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TABLES

Table 1 – Percentage of plants occupied by the ants (per genus) that visited the EFNs of species of *Anemopaegma* and their respective behavior.

Ant genus using nectar from EFNs	***A.album*** (N=14)	***A.scabriusculum*** (N=18)	**Observed ant behavior**
Camponotus	64.3 %	100 %	Individuals that move quickly, with intermediate abundance and recruitment, chemical defense.
Cephalotes	35.7 %	5.5 %	Individuals that move slowly, with low abundance and recruitment, without chemical defense.
Crematogaster	7.1 %	0 %	Individuals that move slowly, with high abundance and recruitment, chemical defense.
Pseudomyrmex	*50 %	0 %	Individuals that move quickly, with low abundance and recruitment, without chemical defense.

* In general present with only one individual on the whole plant.

Table 2 – Herbivores most commonly seen feeding on vegetative portions (mainly leaves) of *A. album* and *A. scabriusculum*. Figure 1S available online in the Supporting Information.

Herbivore species	**Species of *Anemopaegma***	**Observed behavior of herbivores and damage to the plant**
Beetle *Charidotis* sp. (Figure 1S: A,B and C)	*A. album* (rarely in *A. scabriusculum*)	All life stages feed on the plant; adults were never seen feeding on or laying eggs on other plant species.
Beetle *Aerenicopsis* sp. (Figure 1S: D and E)	*A. album* (exclusively)	Only adult were observed feeding on plants; adults were exclusively seen feeding On the younger branches and midrib of the expanding leaflets.
Beetle *Sumitrosis* sp. (Figure 1S: F)	*A. album* (exclusively)	All life stages feed on the plant; larval stages were herbivore miners, and adults were never seen feeding on other plant species.
Cricket *Xestotrachelus robustus* (Figure 1S: G and H)	*A. scabriusculum* (rarely in *A. album*)	Young nymphs and adults were observed feeding on leaves; the younger the crickets, the higher the number of individuals observed on the plant. It was the only species seen also feeding on other plant species.

Table 3 – Difference in ant abundance between individuals of *A. album* (or *A. scabriusculum*) and their nearest-neighbors. Experiments were carried out on *A. album* (n=14) and *A. scabriusculum* (n=18) separately.

Paired experimental plants	**Mean difference in ant abundance (± SD)**	**t-test**
A. album x neighbors	3.8 ± 21.0	$t$paired $_{(1,13)}$ = 0.65; p= 0.25
A. scabriusculum x neighbors	11.7 ± 18.7	$t$paired $_{(1,17)}$ = 2.66; $\boldsymbol{p}$**= 0.01**

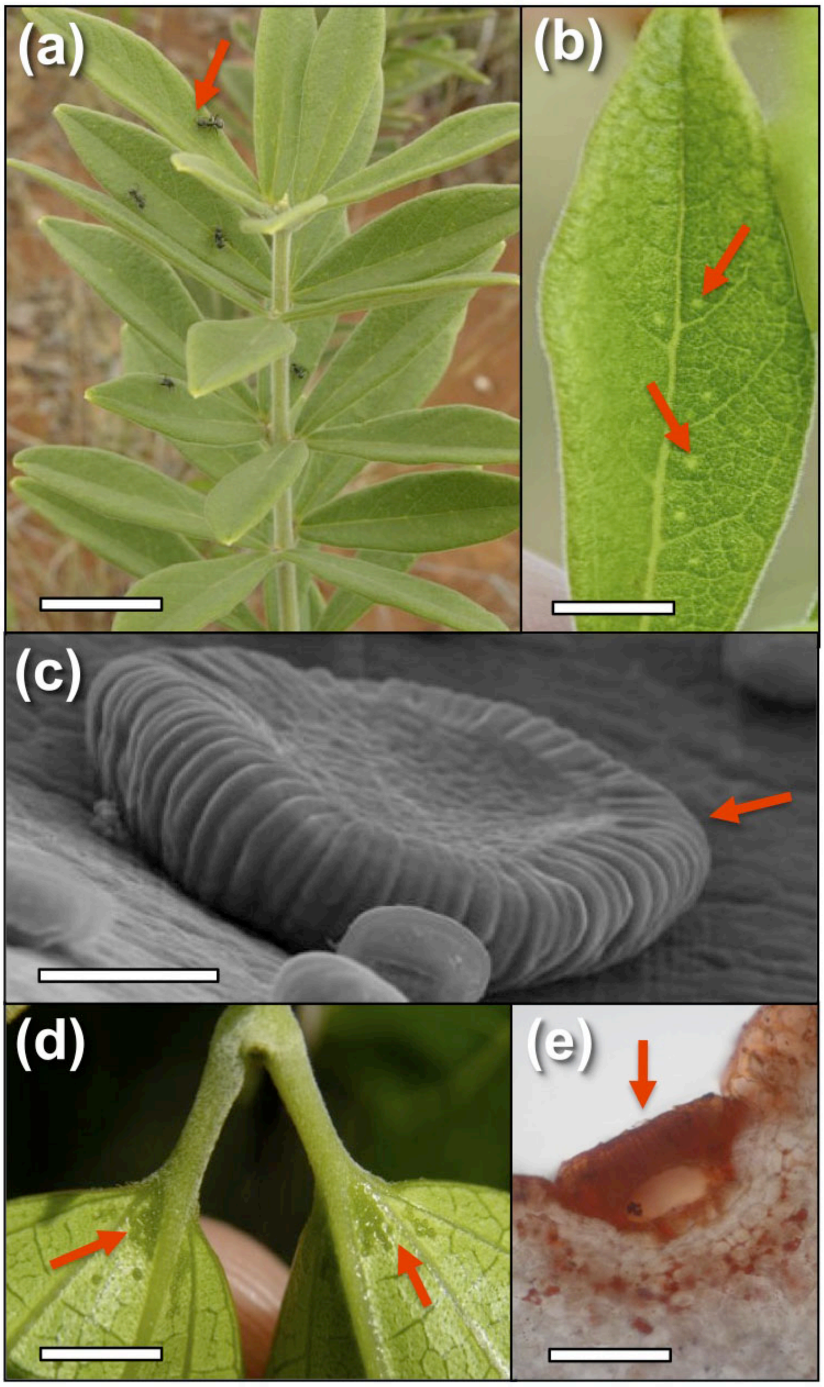

Figure 1 – Extrafloral nectaries of the *Anemopaegma* species. (a) Ants of the genus *Camponotus* visiting EFNs of *A. scabriusculum* (scale bar = 40 mm); (b) EFNs scattered on the adaxial side of the leaflets of *A. scabriusculum* (scale bar = 15 mm); (c) Detailed morphology of EFN of *Anemopaegma album* by SEM (scale bar = 50 µm); (d) EFNs grouped at the axils of veins on the base of the abaxial side of leaflets of *A. album* (scale bar = 10 mm); and (e) light microscopy of EFNs showing the positive reaction of total lipids of *A. album* (scale bar = 140 µm).

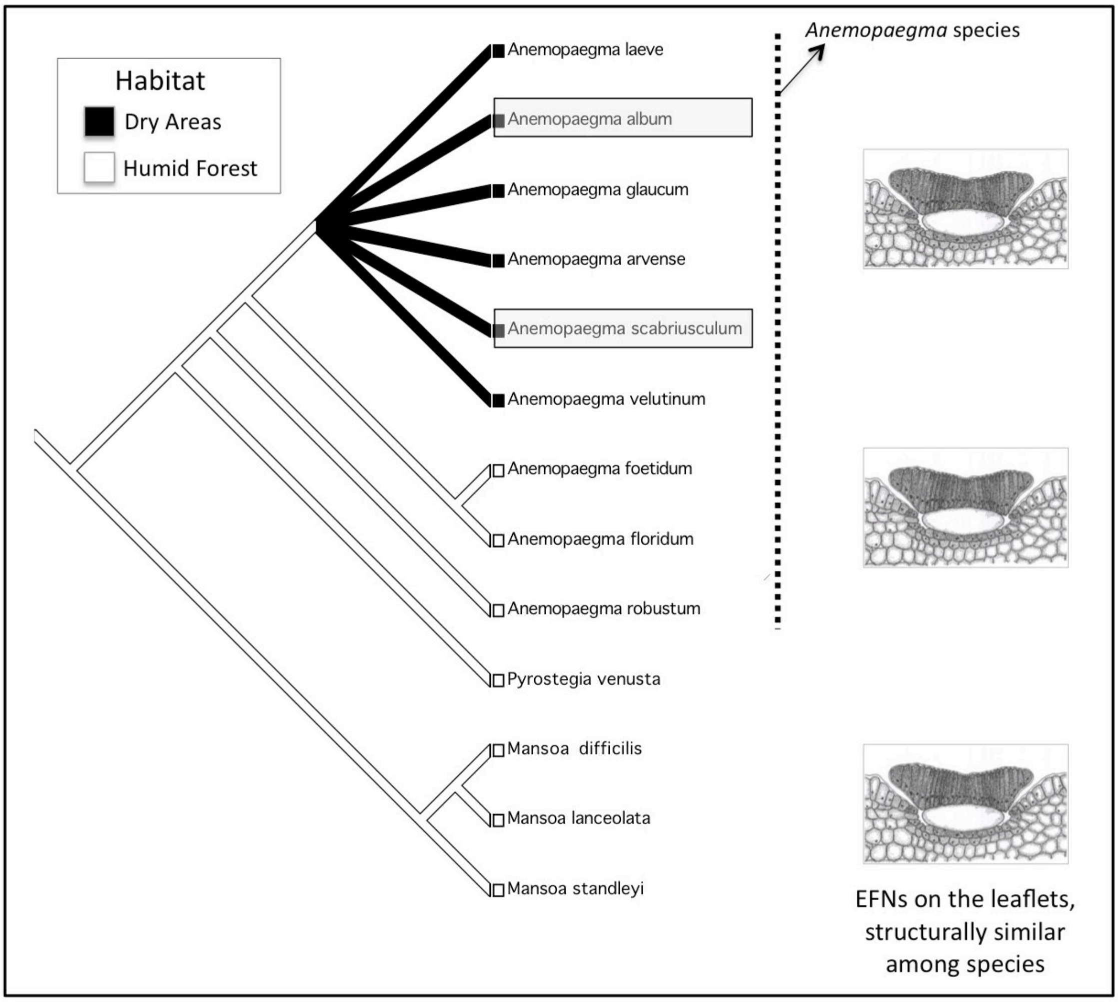

Figure 2 - Phylogenetic relationships between species of *Anemopaegma* (modified from Lohmann 2006 and Lohmann et al. *unpublished*) indicating that the ancestor of *Anemopaegma* occupied a humid environment and already presented extrafloral nectaries (EFNs); subsequent lineages moved into dry areas and maintained the EFNs.

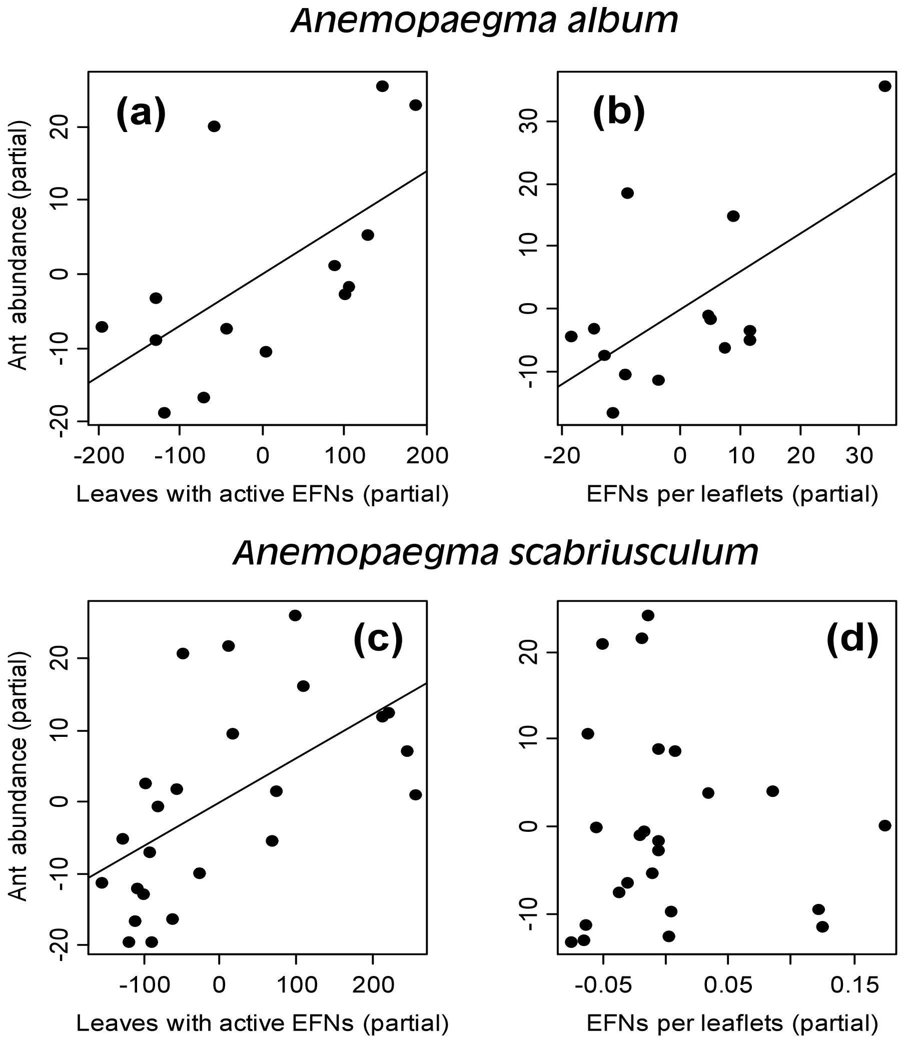

Figure 3. Linear partial regressions of ant abundance and two different descriptors of EFNs in *A. album* ($F_{(2,11)}$=4.58; R^2 = 0.35; p<0.04) and *A. scabriuculum* ($F_{(2,21)}$=5.29; R^2 = 0.27; p<0.02). In *A. album,* ant abundance was positively correlated with the number of leaflets with active EFNs (p = 0.02), and the average number of EFNs per leaflet (p = 0.02). In *A. scabriusculum*, ant abundance was correlated only with the leaflets with active EFNs (p = 0.01), and not with the average number of EFNs per leaflet (p = 0.68).

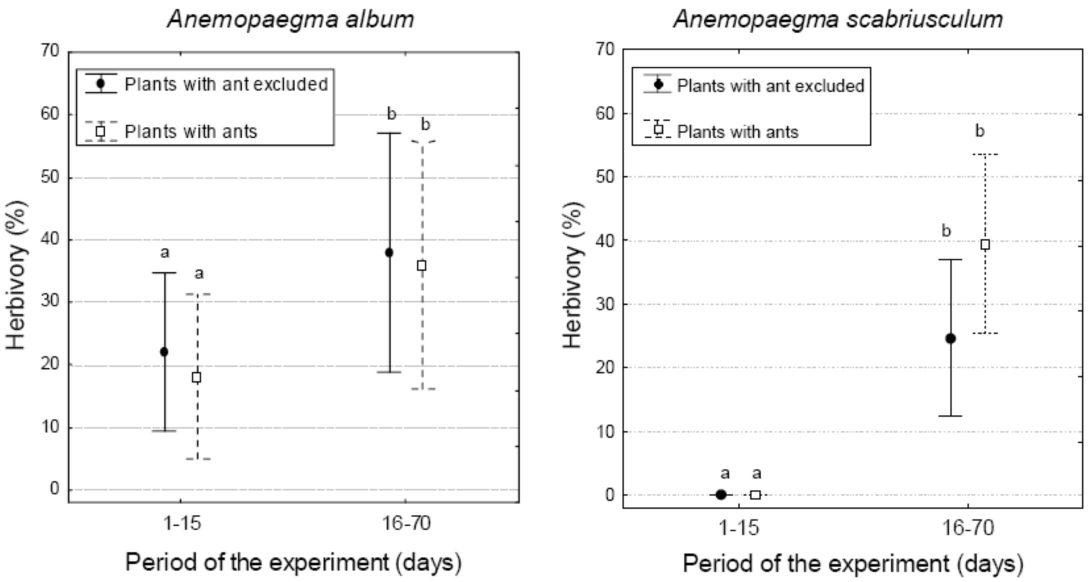

Figure 4. Leaflet damage during the two sampling periods (1-15 days and 16-70 days). No effect of ant visitation was observed on leaf damage of *A. album* or *A. scabriuculum*. No significant interaction between factors was found, indicating that the effect of ant exclusion on the level of herbivory was consistent between sampling periods. Similar letters on vertical bars (0.95 confidence intervals) indicate no significant difference within each sampling period.

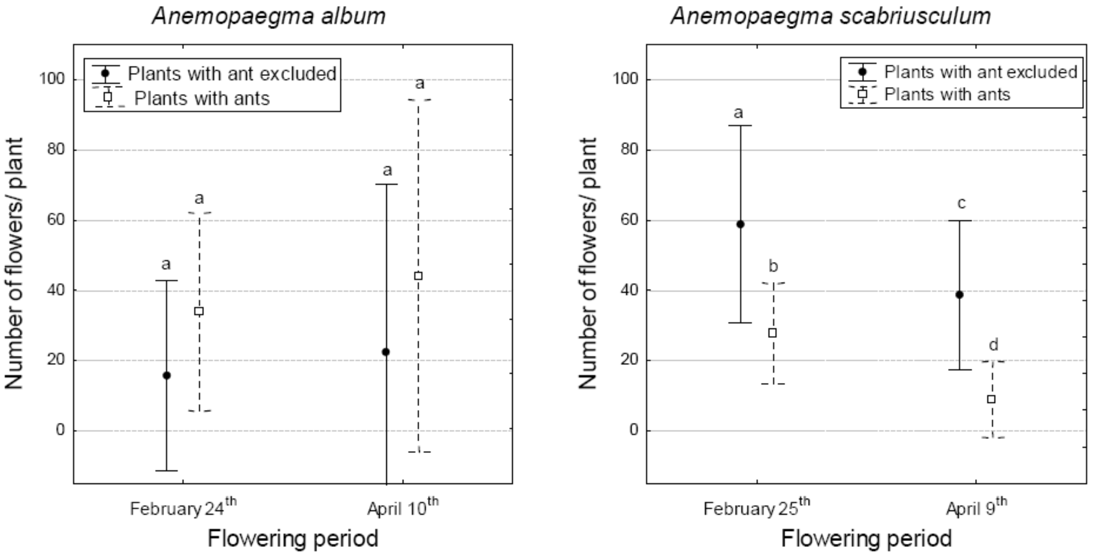

Figure 5. Flower production in *A. album* (left) and *A. scabriusculum* (right). Flower production did not vary with ant visitation in *A. album*. In *A. scabriusculum,* flower production decreased with ant visitation. Results for each species were similar in the two sampling periods (February and April, 2009; that is the interaction between factors was not significant). Different letters on vertical bars (0.95 confidence intervals) indicate a significant difference within each sampling period.

On-line supplementary materials

Appendix 1S – Detailed methods to describe the structure and secretion of the extrafloral nectaries

To characterize the general organization of leaf glands, mature leaves were fixed in FAA (Formalin-Acetic-Alcohol, Johansen 1940), embedded and sectioned into slices of 6 µm using standard historesin microtechnique, and stained with toluidine blue (O'Brien et al. 1964). For SEM (scanning electron microscopy) studies, samples were fixed in FAA (Johansen 1940), post-fixed in osmium tetroxide 1% and dehydrated in an ethyl alcohol series. Specimens were critical point dried and gold coated. To characterize the main classes of substances encountered in the EFNs of *A. album* and *A. scabriusculum*, histochemical analyses were performed. Fresh hand-cut sections were subjected to eight different histochemical tests: (a) Fehling's solution to detect reducing sugars (Purvis et al. 1964); (b) Sudan IV to detect total lipids (Johansen, 1940); (c) α-naphthol dimethyl-paraphenylenediamine (NADI) to detect terpenes (David and Carde 1964); (d) 0.02% ruthenium red aqueous solution to detect mucilage/pectin (Johansen 1940); (e) 10% ferric trichloride aqueous solution to label phenolic compounds (Johansen 1940); (f) Dragendorff reagent to detect alkaloids (Svendsen and Verpoorte 1983), and (g) mercuric bromophenol blue to detect total proteins (Mazia et al. 1953). Standard control procedures were carried out simultaneously, following the recommended protocols.

Fehling's qualitative test was further complemented with additional tests to determine the presence of glucose. Complementary analyses were conducted by collecting a sample of the secretion produced by the EFNs using 1µl and 5 µl microcaps obtained from Drummond (USA) and Merck (Germany), respectively. We used urine test strips (Combut-Test, England) to detect the presence of sugar. In addition, the sugar concentration (and volume) of the secretions were quantified with a portable Eclipse refractometer (Standley, England).

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Appendix 2S - Morphological description and development of foliar EFNs

The EFNs encountered in both species of *Anemopaegma* (Bignonieae, Bignoniaceae) are circular, disc-shaped, and present a concave surface, with three distinct cell layers: (i) **Secretory layer**: Formed by 32-40 columnar cells in a palisade-like arrangement that is densely cytoplasmic and thin-walled. Cells are covered by a thin and smooth cuticle and lack visible pores; (ii) **Intermediate cell layer**: Consists of one large, ellipsoid and vacuolated cell, with anticlinal walls that are thick and highly suberized; (iii) **Foot or basal layer**: Pluricellular, consisting of smaller and more quadrangular in shape than the adjacent epidermal cells. These cells present thickened anticlinal walls that are covered by cuticle. The nectaries lack vascularization.

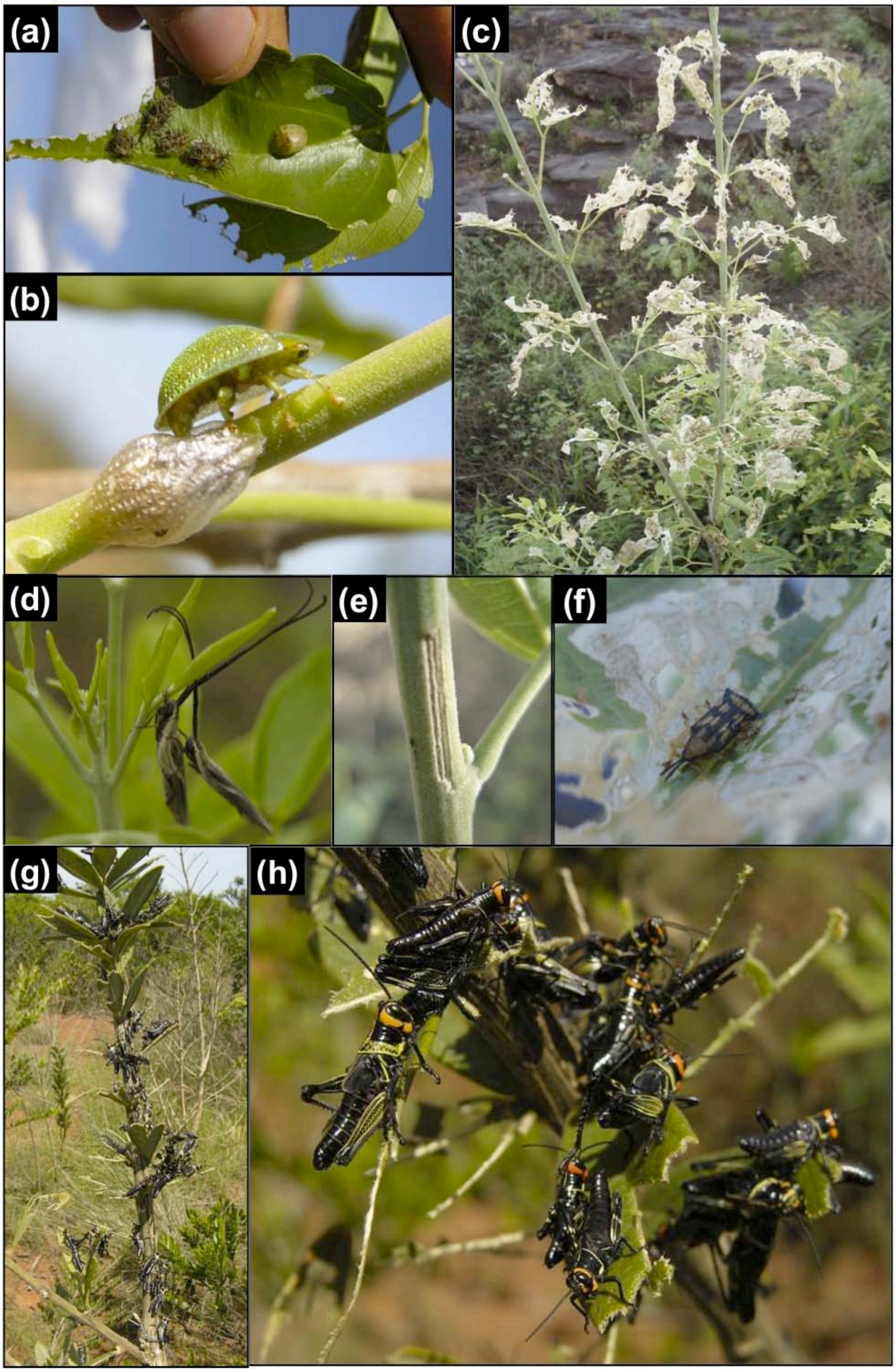
Although these glands are already visible in very young leaflets (17-25 mm), they just start secreting in the intermediate stage of leaflet expansion (>50 mm); the secretory phase is, however, active until the senescence of the leaflet. Active nectaries are yellowish-green and turgid, while inactive nectaries are brownish and dry. The secretion is hyaline and somewhat viscous. After released, the secretion accumulates on the concave EFNs surface, from where it can be gathered by insects, mostly *Camponotus* ants.

Appendix 3S – Abundance of EFNs on 17 species of *Anemopaegma* from different habitats (rainforest N=10; and savannas N=7).

In the vegetative plant parts of *Anemopaegma* species, EFNs are distributed on leaflets (six leaflets considered on each count) and prophylls (four prophylls considered on each count), but absent on stems and petioles. In general, the abundance of EFNs was greater in forest species than in savanna species. Asterisks indicate species with many non-glandular trichomes (denser on abaxial side of leaflets). Bold indicates species studied in savannas.

Habitat	Species	Abundance of EFNs			
		Leaflets[1]	Prophylls[2]	Total (per node)	Average EFNs per habitat (SE; N)
Forest	*A. chamberlaynii*	164	64	228	292.9 EFNs/node (± 72.8; N=10)
	A. chrysoleucum	234	0	234	
	A. citrinum	322	6	328	
	A. foetidum	142	0	142	
	A. floridum	178	0	178	
	A. hylarianum (*)	72	0	72	
	A. paraense	735	84	819	
	A. parkerii	345	52	397	
	A. robustum	404	79	483	
	A. setilobum (*)	48	0	48	
Savanna	***A. album***	**177**	**0**	**177**	101.0 EFNs/node (± 27.1; N=7)
	A. arvense	2	0	2	
	A. glaucum	92	0	92	
	A. laeve	116	41	157	
	A. scabriusculum (*)	**114**	**0**	**114**	
	A. velutinum (*)	8	0	8	
	A. sp1 (PI, Brazil) (*)	40	117	157	

Figure 1S – Most common herbivores found in *A. album and A. scabriusculum*.



Capítulo 3

RH: Extrafloral nectaries, trichomes and selective regimes changes

Evolution of Extrafloral Nectaries: Interactions between trichome types and selective regimes

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Abstract

Considerable attention has been paid to the evolution of functional traits. In particular, much effort has been devoted to a better understanding of the role of extrafloral nectaries (EFNs) for ant-plant-herbivore interactions. Here, we measured quantitative EFN traits in 105 plant species included in a well-supported phylogeny of the tribe Bignonieae (Bignoniaceae), and collected field data on ant-EFN interactions in 32 species. This data allowed us to determine the evolutionary patterns of EFNs in the group, as well as to identify an association between the abundance/aggregation of EFNs in selected vegetative plant portions with ant visitation (a surrogate of ant guarding). We further detected an association between the evolution of EFNs and habitat shifts (transitions from forest to savannas), as well as identified trade-offs between the evolution of EFNs and other defensive traits (i.e., trichomes). Overall, the neutral evolution of EFNs seems to have been associated with the evolution of new traits and environmental transitions, leading to changes of the cost-benefit of these structures to plants thus, changing the direction of natural selection in the group.

Key-words: ant-plant interaction, Brazil, Cerrado, defense traits, phylogenetic signal, herbivory, macroevolution, savannas, trade-off.

Introduction

Extrafloral nectaries (EFNs) are morphological structures that secrete nectar but are unrelated to pollination (Elias, 1983). These structures indirectly increase plant resistance against herbivores by attracting insects (mainly ants) that protect plant tissues (e.g., Oliveira & Freitas, 2004). Although the output of facultative ant-plant interactions is context specific (Bronstein, 1994), i.e., dependent on biotic and abiotic factors in a population scale (Kersch & Fonseca, 2005), ants generally protect plants and decrease herbivory, contributing to a positive effect on fitness (Rosumek et al., 2009). The interaction between ants and EFNs represent a great model for microevolutionary studies focused on the ecological and evolutionary outputs of ant-plant-herbivore interactions (Heil & McKey, 2003).

Plant defensive characters result from the accumulation of evolutionary responses to different selection agents (e.g., ants and herbivores on nectaries; Rutter & Rausher, 2004). Such evolutionary accumulation of responses may lead to a pattern of staggered characters in the phylogeny (Agrawal et al., 2009), with some functionally redundant characters (Agrawal, 2007). When several characters are involved in a similar function, interactions between them are commonly expected, which may lead to evolutionary trade-offs between characters (negative correlation). As a result, macroevolutionary studies on the quantitative variation of traits generally test two main hypotheses: (1) phylogenetic conservatism and (2) trade-offs among co-occurring traits within single taxa (Agrawal, 2007). The phylogenetic consevartism hypothesis predicts that the variation on the evolution of individual characters is proportional to the branch lengths of a phylogenetic tree (phylogenetic signal *sensu* Blomberg et al., 2003). In this context, significant phylogenetic signal does not imply inertia or constraint (Blomberg & Garland, 2002).

In studies in which individual traits are shown not to follow neutral models of evolution (e.g., after deviation of Brownian model of evolution), researchers seek to understand the exact factors that are responsible for the evolutionary deviations from the neutral model. Hypotheses on the potential agents of selection associated with these characters in the microevolutinary scales could direct the hypothesis for the macroevolutionary scale. The importance of this theorical framework is bigger when the studied characters are related to a specific function and/or relevant processes in the plant life cycle, like pollination (e.g., Alcantara & Lohmann, 2011), seed dispersal (e.g. Lorts, Briggeman, & Sang, 2008) and plant defense against herbivores (e.g. Agrawal, 2007). However, the detailed ecological data that is necessary to answer questions of macroevolutionary pattern of these traits generally lacking. For EFNs in particular, very few studies have explored their evolutionary history and/or examined their function in detail (but see Marazzi & Sanderson, 2010).

Ecological, genetic, and developmental factors are thought to contribute to deviations from the Brownian mode of evolution of EFNs. More specifically, individual EFN characters are expected to interact more with characters located in the same plant portion (or organ), than with characters located in different plant parts (within and between different modules; Eble, 2005). Such interactions may be direct (i.e., genetic or ontogenetic, envolving rearrangement of a character in the same region where they develop), or indirect (e.g., involving ecological factors). For example, the occurrence of certain trichome types (non glandular or adhesive glandular trichomes), and/or its increase in density may complicate ant movement over plant surfaces, influencing the evolution of nectaries in response to ants. Such pattern has been observed in four species of myrmecophytes, in which the distance between the individual trichomes located on the shoot seem to select for ant visitors by worker body length (Davidson et al., 1989). Thus, the influence of trichomes on the occupation of different plant parts by ants may cause a trade-off between these trichomes and morphological structures involved in the attraction and maintenance of ants. In those cases, trichomes indirectly affect the evolution of EFNs, with EFNs often becoming more costly than beneficial to plants because of a lack or reduction of the number of ant visitors, thus becoming selected against during the course of plant evolution.

Extrinsic factors such as, habitat change can promote a change in the assemblage of ants and herbivores that interact with plants. Such change in the ant assemblage may, in turn, modify the cost/benefit of EFNs for plants, leading to deviations in the evolution of these structures. The occurrence of highly efficient herbivores (harmful to plants and indifferent to ants), or the occupation of EFNs by non-protective ants (or the lack of ants in some situations; e.g., Moraes & Vasconcelos, 2009) may lead to a high cost for the production of EFNs, with the strength of selection against EFNs depending on the cost associated with these structures. Changes in the environments/habitats (e.g., invasions) occupied by individual plants over time may lead to a replacement of the associated fauna and a decoupling of interactions, shifting the evolutionary direction of the morphological structures that mediate these interactions. In Amazonia, for example, researchers have documented a turnover in ant assemblages between forests and savannas (Vasconcelos & Vilhena, 2006). A similar change in the assemblage of ants was recorded between contrasting habitats in Bolivia (Verhaag & Rosciszewski, 1994), which would justify our hypothesis considering the decoupling of interactions caused by habitat shifts that occurred in the past.

In sum, two main factors seem to significantly impact the evolution of EFNs, ant-guarding and herbivory: (1) intrinsic factors such as the emergence of new morphological structures in the same organ in which the nectaries are distributed (e.g., glandular and/or non glandular trichomes), and (2) extrinsic factors such as biotic or abiotic changes associated with the geographic distribution of plants (e.g., habitat shifts or colonization from forest to xeric environments such as the brazilian cerrados and caatingas).

The objective of this study was to determine the pattern of evolution of quantitative EFN traits in the tribe Bignonieae (Bignoniaceae). We tested whether the evolution of EFNs represented a defense mediated by ant guard in close association with herbivory level and with the ant guarding services. Under this hypothesis, we predict that geographic, habitat, and other environmental changes associated with ant protection and risk of herbivory are associated with changes in character states. The alternative hypothesis was that the current distribution of EFNs in representatives of Bignonieae might have resulted from the occurrence of EFNs in the most recent common ancestor of the tribe Bignonieae, with phylogenetic inertia and trade-offs with other defensive characters impeding subsequent evolutionary responses to the variations of ant guarding.

In order to test these hypotheses, we first controlled for phylogeny and tested for an association between the abundance of EFNs and ant visitation (a surrogate for ant guarding services). We then used phylogenetic methods to determine the pattern of evolution of EFN aggregation on selected vegetative parts given that this feature seems to be very important for the establishment of variation in ant visits among plants (A. Nogueira, pers. obs.). We subsequently evaluated whether EFNs tend to be more similar than expected by neutral evolutionary model in closely related species (i.e., phylogenetic signal; Blomberg et al., 2003), and whether changes in selective regimes (habitat transition in lineages from a more humid forest to drier savannahs) interfere in the function and/or in the evolutionary history of EFNs (extrinsic factors). Lastly, we evaluated whether the evolution of other morphological features on the plants' surface (glandular and non glandular trichomes) interfere on the functional pattern and evolutionary history of nectaries (intrinsic factors).

Materials and methods

Study system, phylogeny, and data collection

The tribe Bignonieae (Bignoniaceae) mainly includes lianas that present an important ecological role in dry and wet neotropical forests (Gentry, 1991). The tribe includes 396 species (ca. 50 % of all Bignoniaceae species) that are distributed through 21 monophyletic genera (Lohmann, 2006). A well-supported molecular phylogeny including 105 species of the tribe based on plastid (*ndh*F) and nuclear (*Pep*C) sequences for 104 taxa is currently available for the group (Lohmann 2006). This molecular phylogeny was combined with fossil data to estimate the time of divergence of all nodes in Bignonieae (L.G. Lohmann et al., unpubl.). This analysis used a penalized likelihood approach (Sanderson, 2002) and two fossils as calibration points (L.G. Lohmann et al., unpubl.). The penalized likelihood tree with branch lengths proportional to time (supplementary material available in Alcantara & Lohmann 2011) was used here to study the evolutionary pattern of quantitative descriptors of the extrafloral nectaries (EFNs) in Bignonieae. As we did not have a priori expectations for the patterns of evolution of EFN traits, branch lengths were untransformed.

The tribe Bignonieae is notable for the high variation in the abundance and location of EFNs among species. In Bignonieae, EFNs are distributed on the nodes of stems (interpetiolar fields), prophylls of the axillar buds, petioles, petiolules, adaxial and abaxial sides of leaflet blades. We sampled at least three specimens of each of the 104 species of Bignonieae included in the combined molecular phylogeny of Lohmann (2006), plus *Callichlamys latifolia* in order to describe the wide variation of EFN found in this group. Extrafloral nectaries (one kind of nectar secreting trichome in this group) and two other types of trichomes (sticky glandular and non-glandular) were counted in vegetative parts of dried herbarium specimens using a stereomicroscope and fresh materials collected in the field for some species. The exact sampling scheme adopted in different vegetative plant parts is described in detail in Appendix 1S (supplementary material).

Relationship between ants and nectaries

To study the relationship between the abundance of EFNs and ant visitation, twenty species of Bignonieae from three different Brazilian sites were sampled with at least four individuals of each species. The sites sampled were Reserva Florestal Adolpho Ducke - AM (rainforest), Parque Nacional do Viruá - RR (rainforest), and Parque Estadual do Morro do Chapéu - BA (savanna). The species sampled are available in the Appendix 2S (supplementary material).

Since the active EFNs of Bignonieae are restricted to young branches and newly expanded leaves (e.g., Nogueira et al., 2011), only plants with newly expanded branches were sampled in an attempt to describe ant-plant interactions in the various species sampled. In order to standardize the sampling effort across taxa: a single node of each plant was sampled in all cases. Individual nodes include an interpetiolar branch region (both sides), two prophylls, and two pairs of leaves, with variable numbers of leaflets. The abundance of EFNs was quantified using the following variables: (1) number of EFNs in the region of greatest aggregation in each node, and (2) total number of EFNs per node (two opposite leaves, prophylls, and interpetiolar region).

Ants were sampled on EFNs. Initially, ants were counted on the newly expanded branches of the plant and then standardized by a single node for comparisons between species. The main region for ant attraction in each species (EFN aggregation region) was documented. Ants were collected using special flat-tipped tweezers and fixed in 90% alcohol for subsequent identification. Ant collection was always conducted between 9 A.M. to 5 P.M. for all taxa. On the other hand, ant visitation was also monitored between 8 P.M. to 10 P.M in order to assess whether there was any replacement of ant species visiting the plants between these time periods.

Linear regressions testing by permutation were performed to examine the relationships between the abundance of EFNs and ant visitors across species. In order to fix the non-independence of plants species, considering the phylogenetic relationships between them, Simple and Partial Mantel test were conducted on distance matrices. For this, a third matrix representing the phylogenetic distances between species (patristic distance) was calculated using the molecular phylogeny of the group (Lohmann, 2006). Linear regressions and Mantel tests were performed with the R package lmPerm (Wheeler, 2010) and Vegan (Oksanen et al., 2011), respectively.

Phylogenetic pattern of EFN aggregation

We determined habitat transitions and the pattern of evolution of the aggregation of EFNs in Bignonieae. EFN aggregation was coded as a binary character state in different plant parts, with 0 representing 5 or less EFNs, and 1 representing more than 5 EFNs in a single location. Habitats were coded as multistate (forests, savannas, and ambiguous). We investigated the pattern of evolution of EFN aggregation in abaxial and adaxial side of leaflets, in prophylls of the axillary buds, and in the node region of stems. Ancestral character state reconstructions were conducted using maximum likelihood assumptions and the software Mesquite 2.74 (Maddison & Maddison, 2007).

Phylogenetic signal of EFN traits

To test the null hypothesis of phylogenetic conservatism of EFNs (through a Brownian mode of evolution), we considered seven distinct characters to represent the quantitative variation of the abundance of EFNs between the 105 species of the tribe sampled. For this, three dried herbarium specimens were sampled and the average data was used in the analysis for each taxon. The abundance descriptors of EFNs used were: (1) EFNs in the interpetiolar region of branches (nodes), (2) EFNs in the prophylls of the axillary buds, (3) EFNs in the petioles and petiolules, (4) EFNs scattered on the adaxial side of leaflets, (5) EFNs aggregated on the adaxial side of leaflets, (6) EFNs scattered on the abaxial side of leaflets, and (7) EFNs aggregated on the abaxial side of leaflets. A multivariate analysis (Principal Components Analysis) was used to reduce the dimensionality of the descriptors of EFNs and concatenate the variation in abundance of EFNs into a smaller number of uncorrelated dimensions (main axis). Three principal components were used in subsequent hypotheses testing.

According to the Brownian model of evolution, phenotypic divergence is proportional to time of divergence between species. We thus tested if the descriptors of EFNs followed the Brownian mode of evolution through K statistic (Blomberg et al., 2003). This test was applied to the seven descriptors of EFNs and to the three principal components derived from the original variables. K-values not significantly different from zero indicate a lack of phylogenetic signal. Whenever $K > 1$, EFNs were interpreted as being more similar in closely related species than expected by the Brownian mode of evolution. Whenever $K < 1$, EFNs were interpreted as being less similar in closely related species than expected by the Brownian mode of evolution. All analyses were conducted in the R package Picante (Kembel et al., 2010)

Trichomes and selective regime changes

In order to determine the factors that could be associated to changes in the evolution of EFN abundance, we tested the potential effect of habitat transition (forest-savanna) and trichome types (glandular and non-glandular) on the number and distribution of EFNs on the plants examined. Three classes of habitats were considered: specialist in rainforest, specialist in savanna, and generalist (when the species was found in both classes or presented an ambiguous classification). Three classes of trichome density were considered: functionally glabrous (when trichomes are absent or scarce, not impeding ant movement), with non-glandular trichomes (only providing a physical barrier to ant movement), and adhesive glandular trichomes (providing both chemical and physical barriers to ants). Specimens were classified as having non-glandular trichomes whenever trichomes with sizes that were equal or greater than 0.3 mm were found on twigs and leaves, and/or whenever trichome density was equal or greater than 10 trichomes per mm^2. Trichomes that were smaller or only present in densities that were smaller than those were considered functionally glabrous.

The effect of (1) habitat transition and (2) trichome appearance was separately tested on the principal components (three axis), with each axis representing the variation of EFNs among taxa. Scores of species on each factor of the trait variation were used as dependent variables, while habitat and trichome appearance were used as categorical variables in a MANOVA (more than one dependent variable) and in an ANOVA (one dependent variable). Both tests were conducted in a phylogenetic context. The null distribution of this statistical test was obtained by simulating new sets of dependent variables on the phylogenetic tree under a Brownian mode of evolution. Whenever significant differences were found in the univariate analyses, post-hoc tests were performed repeating the same procedure for each possible pair of habitat and trichome type. Standard and phylogenetic MANOVAs/ANOVAs were performed with the functions phy.manova and phy.anova of the R package Geiger (Harmon et al., 2009).

Results

EFNs abundance and aggregation and ant activity

Extrafloral nectaries were found in all species of Bignonieae studied in herbarium sheets (N=105) and during fieldwork (N=20). High variability on the abundance and position of the EFNs were observed among species (Figure 1; Chapter 1). The higher the abundance of active nectaries on vegetative plant parts (young nodes in expansion or newly expanded nodes), the greater was the number of ants found on the nectaries (Figure 2). The positive relationship between ant abundance and the abundance of EFNs was maintained when the analysis was controlled for phylogeny (Table 1), considering only the abundance of aggregated nectaries in particular positions per node. These results suggest that the differences observed in the number of ant visitors between species is mainly due to the presence of EFN clusters, and not to the variation in EFNs scattered over plants. In general, EFN clusters (> 5 EFNs) were observed in the same position for each species, mostly in the prophylls (22.8 % of the species sampled), interpetiolar regions (36.2 % of the species sampled), and at the base of the abaxial side of leaflets (37.1 % of the species sampled). A fez taxa presented two EFN cluster locations but this was unfrequent and, in these cases, the clusters appeared on the prophylls of the axillary buds, and at the abaxial side of leaflets (10.5 % of species; e.g., *Anemopaegma robustum* and *Bignonia* spp.). EFN clusters were also occasionally found at the base of the petioles (e.g., *Callichlamys latifolia*), petiolules (e.g., *Pachyptera kerere*), or at the apex of the abaxial side of leaflets (e.g., *Bignonia corymbosa*). EFN did not present any aggregation pattern in 23 species out of 105 species sampled (21.9 % of the species sampled).

A single species of ants generally monopolized the available EFN resources in a given plant, regardless of whether the plant was found in a forest or a savanna. In forests, the same ant species that used EFN resources during the day, were also observed visiting the EFNs during the period. The average abundance of ants visiting the plants in forests was 6.25 ants per node (SE= ±0.93), with more than 50% of all interactions being observed in forests monopolized by ants of the genus *Crematogaster* (N=25 plant species). In contrast, a turnover of ant species was observed between day and night periods in the savannas. In addition, a lower abundance of ants was observed interacting with EFNs in savannas, with an average of 0.53 ants per node (SE= ± 0.12). Differently from forest species, the most frequent and abundant group of ants on savanna plants was *Camponotus* (N = 7 plant species). Further details on the abundance of ants are presented as supplementary materials.

Ancestral character state and phylogenetic conservatism of EFNs

The macroevolutionary patterns of nectaries estimated by ML ancestral character state reconstructions indicated that the most recent common ancestor of the tribe did not present aggregated EFN in any plant parts (Figure 3). On the other hand, the most recent common ancestor of the tribe likely presented low amounts of EFNs scattered over the leaf blade. In addition, EFNs were probably lacking from the interpetiolar region, petioles, and petiolules. However, the first evolution of clusters of EFNs in the tribe Bignonieae (> 5 EFNs in the same position of the plant) seems to have happened early in the evolution of the group, before the first incursion of plants from Neotropical rainforest to drier environments. The first plant part that presented a large amount of aggregated EFNs was the prophyll of the axillary bud. The majority of the evolutions of clusters of EFNs (regardless of the position in the plant) seems to have occurred in lineages that occupied forests (> 90%).

The first three first axes of the PCA conducted with seven descriptors of the variation of EFNs explained more than 60% of the variation in the data (Table 2). While the first axis mainly expressed the variation of the abundance of EFNs in the prophylls of the axillary buds and leaflets (EFNs scattered on the adaxial and abaxial sides of leaflets), the second axis expressed the variation in the abundance of EFNs in the interpetiolar portions, and the third axis expressed the variation at the base of leaflets.

K statistics were significantly different from zero for four EFNs characters and for the first two axes of the PCA (Table 3). This indicates that the evolution of these characters was not random during the history of the group. All values of K were not significantly lower than 1, indicating that the divergence in the abundance of EFNs among closely related taxa was greater than expected by the shared history among these species, (Table 3). In other words, those characters presented a low phylogenetic conservatism.

Habitat shifts, trade-offs, and the evolution of EFNs

In order to explore which factors might be associated with the low values of K (i.e., higher divergence than expected by a neutral mode of evolution), we tested whether habitat shifts (forest » savanna) presented a negative impact on the evolution of EFNs (e.g., decrease in abundance of nectaries), causing deviation from the Brownian mode of evolution (Table 4 and Figure 4). The first PCA axis considered the variation of aggregated EFNs in the prophylls of the axillary buds and the variation of scattered EFNs in the leaflets, and was associated with change in plant habitat. More specifically savanna species presented a decrease in the number of nectaries when compared to the forest taxa. While habitat generalists and forest specialists did not differ in the first axis of the PCA, savanna species were much different from those groups. However, this pattern was not as clear in the second axis of the PCA, the axis that represented the aggregated EFNs in the interpetiolar regions. In this axis, forest specialists and savanna specialists were not statistically different from each other, although the savanna taxa on average presented fewer EFNs in the interpetiolar regions when compared with forest specialists and habitat generalists. In particular, habitat generalists presented a higher number of EFNs when compared with the other groups of plants.

As far as the evolution of glandular and non glandular trichomes is concerned, only the emergence of glandular trichomes presented a clear effect on the evolution of the abundance of EFNs (Table 4 and Figure 4), probably leading to a drastic reduction of these structures. The effect of the evolution of glandular trichomes on the reduction of EFNs was clear in the tribe Bignonieae despite the small sample size (N = 9 species considered).

Discussion

This study used the hypothesis of defense mediated by ants and ‘ant guarding’ (Heil & McKey, 2003) as our basic theoretical framework and tested a series of specific hypotheses associated with the evolution of extrafloral nectaries in the tribe Bignonieae (Bignoniaceae). Emphasis was given to factors that may have affected the evolution of ant-plant associations (e.g., habitat change) and to the cost-benefit of nectaries to the plants. Detailed experimental studies with natural populations of representatives of the tribe Bignonieae indicated that ants can act as agents of selection on individual EFN traits (Rudgers, 2004). Indeed, it has been shown that plants with higher rates of ant visitation are less visited by herbivores and suffer less leaf damage which leads to a greater production of flowers and seeds (Rosumek et al. 2009, Trager et al. 2010). In this context, the frequency of nectaries has been shown to be positively correlated with seed production (Rosumek et al. 2009, Trager et al. 2010). These evidences corroborate the idea that facultative and generalist ant-plant interactions may have played important roles for the evolution of nectaries (REF). In this context, temporal and spatial variation of the interaction between biotic agents (ants and herbivores) are thought to have been involved in the evolution of EFNs between species over long periods of time. However, such microevolutionary patterns had not been previously linked to expected patterns of the evolution of EFNs in plants. In this study, we encountered a connection between the abundance and aggregation patterns of EFN in vegetative plant parts with ant visit activity (a surrogate of ant guarding). In addition, we documented the macroevolutionary pattern of EFNs abundance, which was negatively associated to habitat shifts and influenced by trade-offs with other defensive traits (i.e., trichomes).

Relationship ant visit between the abundance and aggregation of EFNs and ant activity

The nectaries of representative of the tribe Bignonieae differ from those from other plant groups (e.g., *Inga*, *Senna* - Marazzi & Sanderson, 2010; *Chamaecrista* - Rutter & Rausher, 2004) in their small size. More specifically, those nectaries are patelliform and glandular (Elias, 1983), and are distributed in various arrangements and patterns of abundance over various plant parts (Nogueira et al., pers. obs.). In this study, the patterns of quantitative variation of EFNs among species was positively correlated with the number of ant visitors (i.e., ant visits reached their maximum in plants with the higher amounts of EFNs). In addition, species with higher ant abundance also seemed to be less damaged by herbivory (A. Nogueira, pers. obs.). Nonetheless, a careful examination of such trend would require periodic plant surveys for a high number of species.

EFNs presented their highest concentration in the prophylls of axillary buds and interpetiolar regions of branches. These plant parts were critical for the maintenance of ant attraction in plants. This was the first study to record such correlation, representing an important step towards a better understanding of the patterns of ant attraction to different types of EFNs (Díaz-Castelazo et al. 2005). In two species of *Anemopaegma*, the quantitative variation of EFNs was associated with ant visitation (Nogueira et al., 2011), in which the higher the average number of nectaries on the leaves (and/or the number of leaves with active nectaries) the greater the abundance of ants on them. Field studies have often investigated the relationship between the number of visitors to EFNs and the identity of the species of ants that visited those EFNs (e.g., Rico-Gray & Oliveira 2007). However, studies that provide information about the abundance of the ant visitors (or the most ant visitors) are still rare in plants. The last reviews on the subject did not detail the relationship between EFNs and ants, and did not provide either the average number of ants per plant (or plant part) in particular species (Bronstein 1998, Rosumek et al 2009, Chamberlain & Holland 2009, Trager et al 2010).

It is widely accepted that herbivore protection is directly proportional do the degree of ant-related investments by plants (Stanton & Palmer, 2011). Despite the importance of “ant abundance” to plant protection (Bronstein, 1998), this variable had been often neglected (Trager et al., 2010). Although the diversity of ants is extremely important for ant-plant interactions, the actual number of ant visitors is also critical to reduce herbivory (e.g., Rudgers & Strauss, 2004). In other words, not only we need to know “who are the ant visitors?” but also "how many ants are there?" (Bronstein, 1998) in order to measure the success of an interaction. In the tribe Bignonieae, forest plant species were shown to attract a higher number of ants than the savanna species. While the savanna species mostly attracted *Camponotus* ants, forest species mainly attracted *Crematogaster* ants, (see Appendix 2S), the most dominant ant-type in terms of resource use in forest environments (Baccaro et al., 2010). The differential ant identity and abundance found in the different environments studied here was unexpected pattern since a high turnover of ant species has been described in nearby savannas and forests (e.g., Verhaag & Rosciszewski, 1994; Vasconcelos & Vilhena, 2006).

EFN phylogenetic conservatism

Extrafloral nectaries are known to be evolutionarily labile (Heil, 2008; Heil, 2011; Rudgers & Gardener, 2004). However, very few studied have actually addressed the evolution of EFNs using a robust phylogenetic framework. In this study, K values for EFN descriptors (and principal components representing them) were shown to be lower than 1. These results indicated a greater phenotypic divergence between species than expected according to a neutral mode of evolution (i.e., Brownian mode of evolution), corroborating the high evolutionary lability of nectaries expected. Likewise, a $K > 1$ also indicates the evolution of similar morphologies (e.g., aggregation of EFNs) in distantly related taxa (Blomberg et al., 2003; Revell et al., 2008). This pattern also suggests that common ecological and evolutionary forces may have driven the diversification of distantly related lineages. Evolutionary lability (or low phylogenetic signal) implies rapid evolutionary change but does not specify a biological process (e.g., genetic drift, natural selection; Revell et al., 2008), those of which could be determined through further functional, ecological, and evolutionary studies.

Habitat shifts, trade-offs, and the evolutionary lability of EFNs

In this study, multiple evolutions of clusters of EFNs were documented in different plant parts of representatives of the tribe Bignonieae, suggesting a recurrent selective force on these structures in this clade. Such lability may have been caused by the beneficial role of the interaction with ants, leading to an increase in plant resistance against herbivores. In general, studies considering the conditionality of ant-plant interactions have shown that these interactions are mostly beneficial, leading to a significant reduction in herbivory and a significant increase in characteristics that are associated with plant fitness (Chamberlain & Holland, 2009). However, comparisons most of the studies on ant-plant interactions did not include phylogenetically controlled experiments, limiting the value of such studies. Here, we considered a series of testable hypotheses about factors that could potentially modify the cost-benefit of EFNs in plants during evolutionary time. In particular, any reduction of the benefits (e.g., absence or reduction of protective ants), and increase in costs (e.g., undesirable water loss) could induce a counter-selection of EFNs. In turn, any reduction in costs and/or increase in benefits of EFNs could generate selection towards an increase in the abundance of EFNs on the presence of genetic variation within populations thus, leading to the establishing of these characters in evolutionary lineages.

Tests on the effect of change in habitat (from forest to savanna) and emergence of trichomes (glabrous-pubescent glandular) on the evolution of the abundance of EFNs indicated that both factors may alter the evolutionary pattern of EFNs expected by the null model (neutral evolution). It is reasonable to assume that the benefits of EFNs on drier environments would be considerably decreased in Bignonieae species, given that changes of environment/habitat have an impact on the relationship between plants ant ant visitors. For example, we observed quantitative (i.e., a decrease in the number of ants per plant node) and qualitative (i.e., an increase in interactions with *Camponotus* ants) changes in ant visitation to EFNs after transitions from forests to savannas. Indeed, the appearance of very aggressive herbivores in savannas was documented in savanna species of Bignonieae, whose immediate ancestors occupied forest environments (Nogueira et al., 2011). Moreover, changes associated with the water consumption by EFNs, would likely make these structures more costly in savannas than in the original rainforest environments. More specifically, a reduction in the number of EFNs would be expected in order to balance the cost-benefit of EFNs in lineages of plants that invaded drier environments.

The first evolutionary event and most of the other evolutions of EFN clusters within the tribe Bignonieae took place in forest environments, perhaps because there has not been sufficient time since the first occupations of savannas for these traits to evolve within this environment. It is possible that the cost-benefit balance between ants and EFNs may have been positive to plants in multiple lineages independently (i.e., those interactions would present a low cost and be highly protective against herbivores). However, experimental tests on the biological function of EFNs in forest species of the tribe Bignonieae would be necessary in order to test this hypothesis. Functional data for EFNs is extremely rare and are currently only available for three

North American species belonging to *Campsis* and *Catalpa* (Elias & Gelband, 1976; Stephenson, 1982; Ness, 2003) and two savanna species from the genus *Anemopaegma* (Nogueira et al., 2011). Further functional studies are greatly needed for EFNs in savanna and forest environments.

Similarly to what has happened with habitat shifts, the evolution of adhesive glandular trichomes seems to have also contributed to a deviation from a neutral pattern of evolution of EFNs. It is possible that the evolution of these trichomes may have altered the pattern of EFN use by ants, explaining the reduction of EFNs in plants presenting these structures. In *Adenocalymma adenophorum* (Amazon forest) and *Cuspidaria sceptrum* (savannas), the two species of Bignonieae that presented adhesive trichomes, no ant visitors were observed. A similar pattern was seen in the Legume genus *Chamaecrista*, in which the loss of EFNs occurred concurrently with the evolution of adhesive glandular trichomes (Conceição et al., 2009). The patterns observed in *Chamaecrista* and Bignonieae suggest a disruption of the ant-plant relationship, with the glandular trichomes making the movement of ants more difficult over the plants. It is possible that plants with adhesive glandular trichomes might have been associated with the arrival of new bodyguards who could, in turn, be more effective for plants than ants. Spider-plant interactions have been documented in *Trichogoniopsis adenantha*, in which spiders from genus *Peucetia* effectively defended those plants against herbivores (Romero et al., 2008). In the lack of live preys, the spiders fed on insect carcasses that were attached to glandular trichomes. This pattern was observed in another plant species, in *Rhynchanthera dichotoma* (e.g., Morais-Filho & Romero, 2010).

In the tribe Bignonieae, the decrease in EFN abundance did not lead to a complete disappearance of such structures in the group. In fact, EFNs were observed in all 105 sampled species sampled. However, a drastic decrease in the abundance of such structures was observed in some lineages (i.e., some species of the genus *Anemopaegma*, *Cuspidaria* and *Fridericia*). This pattern suggests that the presence of a few scattered (not clustered) nectaries over the plants might not represent a significant cost for the plants. In the contrary, the maintenance of few scattered nectaries could predispose lineages with few nectaries to retrieve the number of "lost" nectaries in the presence of additive genetic variability of this feature, and selective forces in favor of this mechanism of anti-herbivory defense.

Additional studies on the evolutionary ecology of EFNs in plant groups with discrete and/or quantitative variation of EFNs would be extremely important to determine whether the patterns here encountered can be generalizable to other plant lineages. Even though a series of transitions from forests to savannas have been encountered in the tribe Bignonieae (Lohmann et al., umpubl.) and other plant groups (Simon et al., 2009), the opposite scenario has also been documented in *Rhipsalis* (Cactaceae), in which the invasions probably occurred from drier areas to humid forests (Calvente et al., 2011). Lineages that have historically shifted habitats are particularly for a better understanding of the patterns of evolution of plant-animal interactions in a macroevolutionary scale.

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TABLES

Table 1. Relationship between ant abundance, density of EFNs, and phylogenetic distance (phy-distance) among 20 species of Bignonieae. Phylogenetic distance estimation (patristic distance) was used to incorporate phylogenetic relationships among species in the analysis. Variables in all Mantel tests were converted to Euclidean distances. EFN1 represents the abundance of aggregated nectaries in particular positions per node; EFN2 represents the total abundance of nectaries per node.

	Relationship among variables	Statistical test	r	*P*
1	Ant abundance to phy-distance	Simple Mantel	0.02	0.37
2	EFN1 to phy-distance	Simple Mantel	-0.04	0.61
3	EFN2 to phy-distance	Simple Mantel	0.01	0.45
4	Ant abundance to EFN1	Simple Regression	**0.65**	**0.001**
5	Ant abundance to EFN2	Simple Regression	**0.47**	**0.020**
6	Ant abundance to EFN1 controlling for phy-distance	Partial Mantel	**0.35**	**0.004**
7	Ant abundance to EFN2 controlling for phy-distance	Partial Mantel	0.12	0.21

Table 2. PC1, PC2 and PC3 loadings from principal components analyses of variables representing the abundance of EFNs on different plant parts among 104 Bignonieae species. Bold indicates loadings higher than 0.6. The proportion of variation explained by the original data of each principal component is presented in parentesis. * EFNs clustered on leaflet blades represent the sum of EFNs on the apex and base of leaflets, where these aggregations were common on the abaxial surface of leaflets.

EFNs on different plant parts	Principal Components		
	PC1 loadings (26 %)	PC2 loadings (24.1 %)	PC3 loadings (16.2 %)
Stem (node)	0.263	**-0.792**	0.034
Petiole and petiolule	0.054	-0.566	0.465
Prophylls of the axillary buds	**-0.739**	0.488	-0.091
Adaxial side (scattered EFNs)	**-0.663**	-0.378	-0.315
Adaxial side (rarely clustered EFNs*)	-0.315	-0.568	**-0.620**
Abaxial side (scattered EFNs)	**-0.699**	-0.155	0.425
Abaxial side (clustered EFNs*)	-0.426	-0.146	0.488

Table 3. Phylogenetic signal of the abundance of EFNs distributed on different plant parts of 105 species of Bignonieae (Bignoniaceae). Traits whose evolutionary patterns were significantly associated with phylogenetic history ($P < 0.05$) are presented in bold. K-statistics is shown for each trait separately and for each principal component extracted from multivariate analyses of the trait variability (Table 2). All significant values of K were less than 1, indicating that EFNs were less similar in closely related species than expected by the Brownian model of evolution. *Aggregated EFNs on the leaflet blades represent the sum of EFNs on the leaflet apices and bases, where the aggregations were most common.

EFNs on different plant parts	K statistic	*p* value
Stem (node)	0.64	**0.012**
Petiole and petiolule	0.27	0.915
Prophylls of the axillary buds (total EFNs)	0.67	**0.002**
Adaxial side (scattered EFNs)	0.68	**0.001**
Adaxial side (rarely clustered EFNs*)	0.35	0.690
Abaxial side (scattered EFNs)	0.37	0.747
Abaxial side (clustered EFNs*)	0.68	**0.009**
PCA1	0.70	**0.001**
PCA2	0.57	**0.002**
PCA3	0.46	0.242

Table 4. Impact of habitat change (extrinsic factors) and of the evolution of trichomes (intrinsic factors) on the evolutionary history of EFNs based on Phylogenetic Manova/Anova Tests. The variation of EFNs was represented by scores on principal-component analyses conducted on variables representing the abundance of EFNs on different plant parts (Table 2). Habitats of Bignonieae species were classified as tropical rain forest, dry areas, or both (generalist behavior); trichomes were classified as functionally absent, with non-glandular trichomes (physical barriers only), or adhesive glandular trichomes (physical and chemical barriers). Effects with $P < 0.05$ level of significance are presented in bold. Marginally significant effects are also noted (+).

EFN variables	F statistic	Traditional-P value	P-value corrected by phylogeny (P*)
HABITAT TRANSITION EFFECT Manova test ($F_{(2,97)} = 3.18$; P = 0.005; P* = 0.001)			
PCA1 (EFNs in prophylls and leaflet blades)	4.92	0.066	**0.058** (+)
PCA2 (EFNs in stems)	7.90	0.008	**0.007**
PCA3	1.87	0.193	0.243
APPEARANCE TRICHOMES EFFECT Manova test ($F_{(2,97)}$ =1.09; p=0.365; P* = 0.287)			
PCA1 (EFNs in prophylls and leaflet blades)	2.50	0.087	**0.066** (+)
PCA2 (EFNs in stems)	0.02	0.982	0.980
PCA3	0.98	0.379	0.324

FIGURES

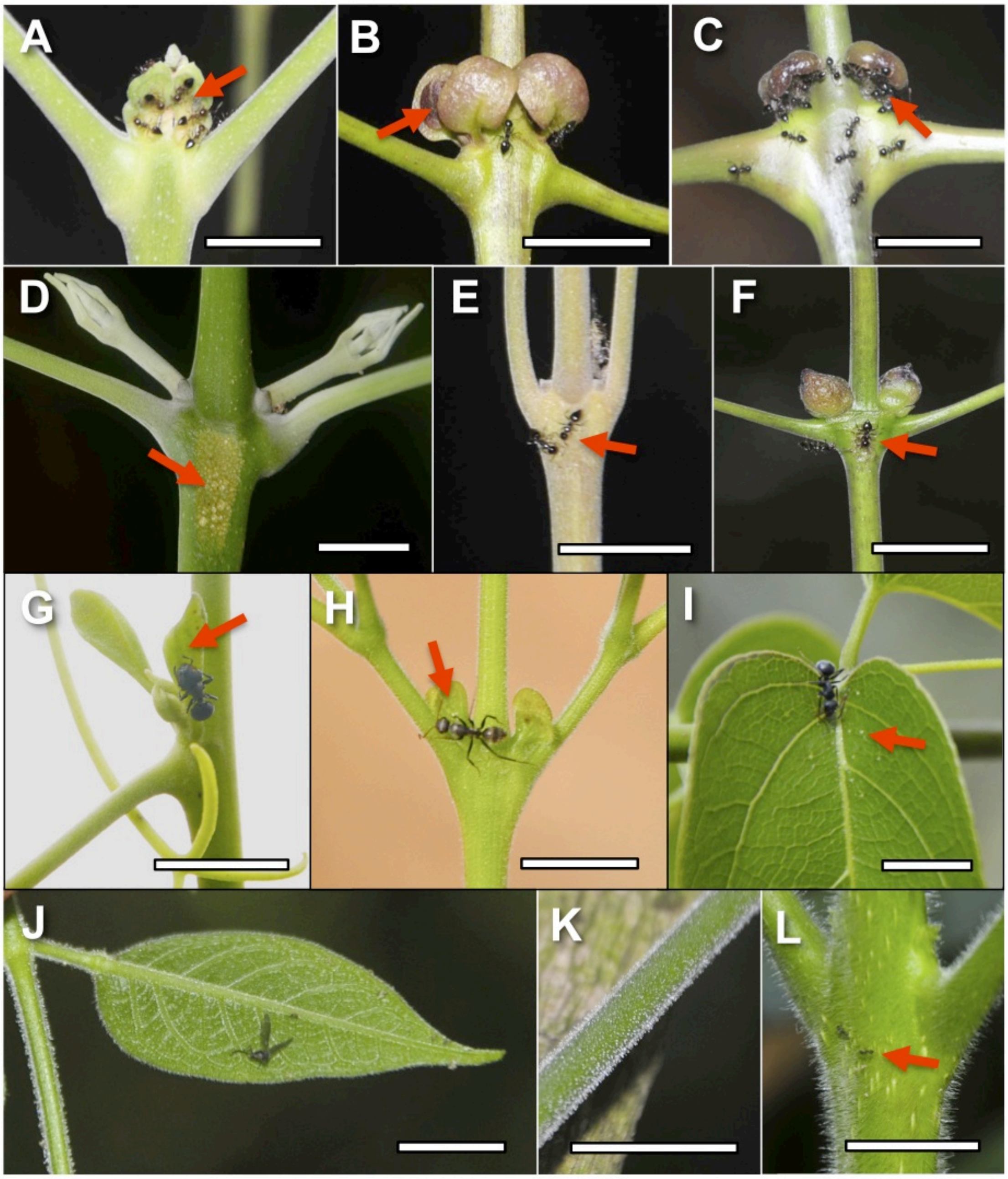


Figure 1. Ant-plant interactions in species of Bignonieae from Neotropical rainforests and savannas. Red arrows indicate the location of nectar secreting EFNs. A-F. Amazonian taxa with *Crematogaster* ants visiting EFNs from the prophylls of axillary buds (A-C) and interpetiolar portions of stems (D-F). A. *Adenocalymma flaviflorum*, scale bar = 15 mm; B. *Bignonia aequinoctialis*, scale bar = 12 mm; C. *Anemopaegma robustum*, scale bar = 11 mm; D. *Fridericia trailli* (secreting nectar), scale bar = 10 mm; E. *Tynanthus panurensis,* scale bar = 10 mm; and, F. *Pachyptera aromatica,* scale bar = 16 mm. G-I. Savanna species with ants visiting EFNs of the prophylls of the axillary buds (G-H), and the adaxial side of leaflets (I). G. *Amphilophium elongatum* with *Cephalotes* ants*,* scale bar = 9 mm; H. *Adenocalymma pedunculatum* with *Camponotus* ants*,* scale bar = 13 mm; and *Cuspidaria convoluta,* scale bar = 11 mm. J-M. Trichomes on the plant surface: J. Diptera stuck in glandular trichomes (with sticky secretion) on the leaflets of *Adenocalymma adenophorum*, scale = 8 mm; K. glandular trichomes on the petioles in plants without ants*,* scale bar = 8 mm; and L. non-glandular trichomes and scarce ants of the genus *Asteca* in *Fridericia* sp., scale bar = 21 mm.

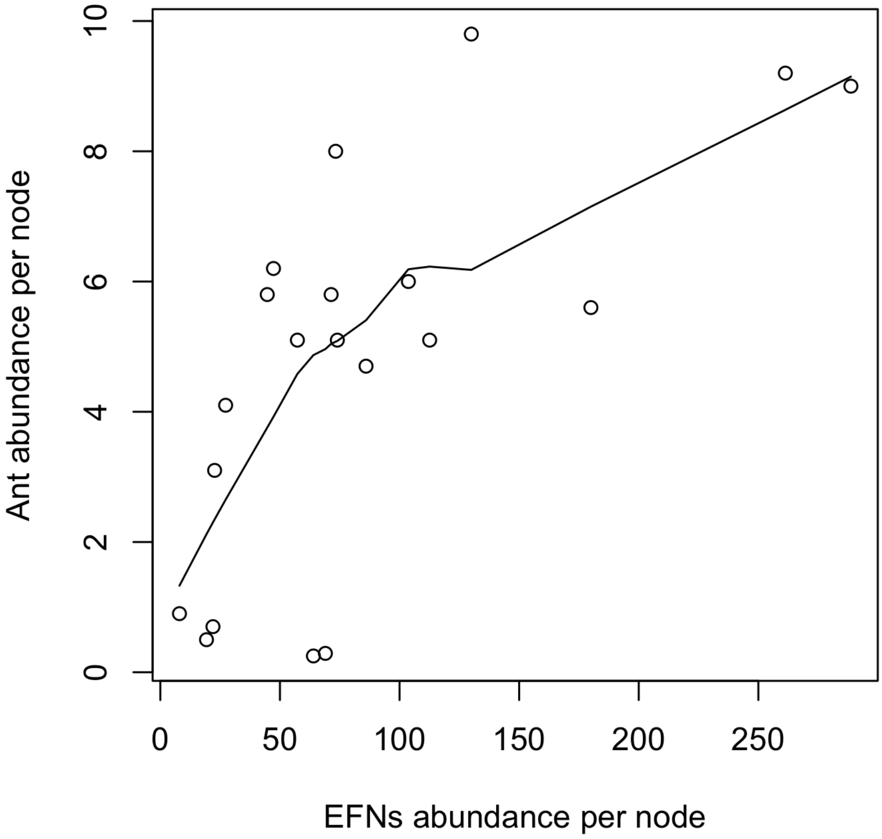


Figure 2. Relationship between ant abundance and the number of clusters of EFNs in particular plant parts of rainforests (N=17) and savanna (N=3) species of Bignonieae. Linear statistical results are presented in Table 1 (line 4). The trend line describing a visual relationship between the two variables was based on the lowest smoother using a locally weighted polynomial regression. The three species with fewer ants per node were savanna species. For this analysis, all 105 species of Bignonieae included in the phylogeny of the group were sampled. A complete list of the species sampled during fieldwork is available in the supplementary material.

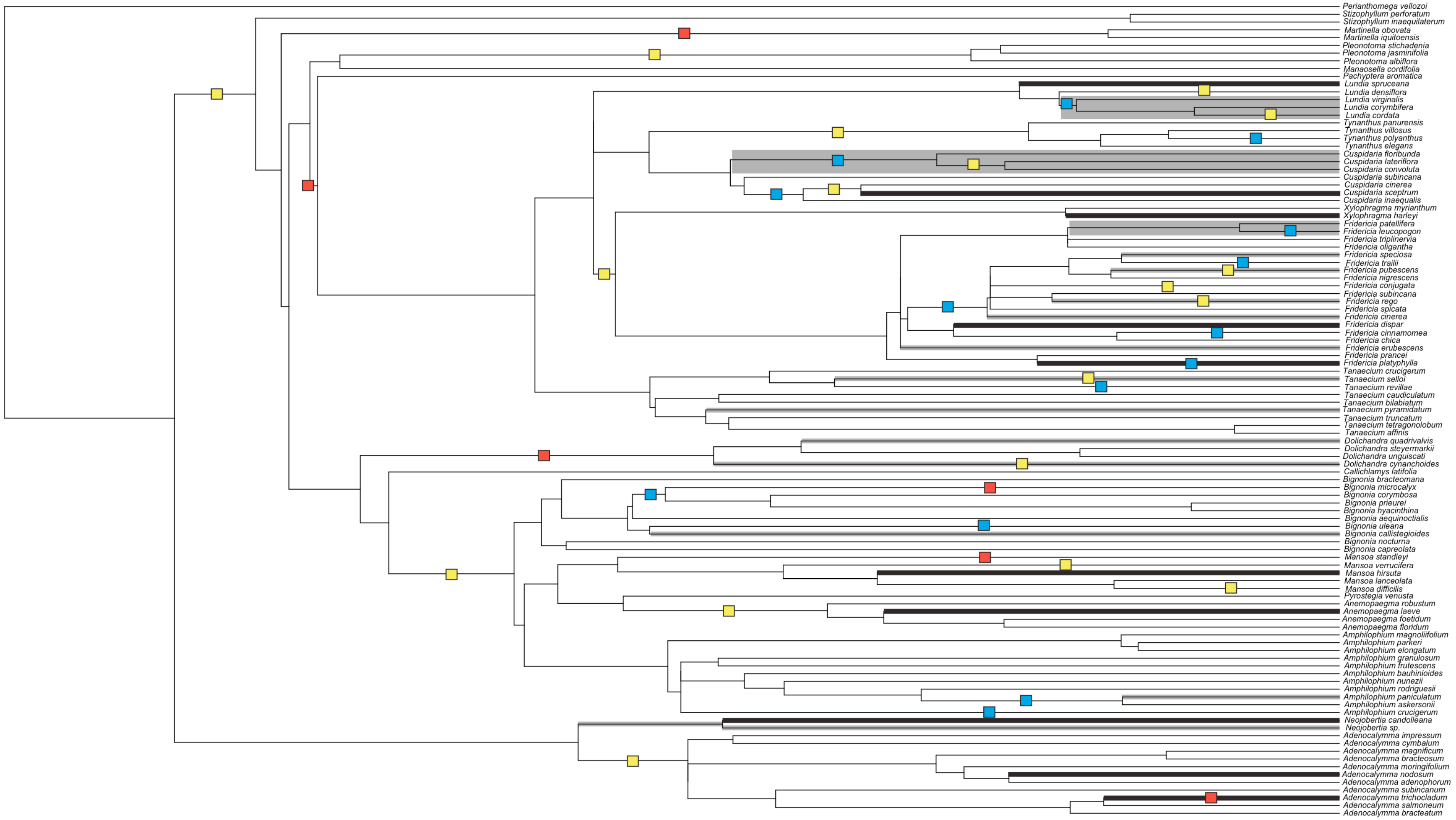


Figure 3. Macroevolutionary patterns of aggregation of extrafloral nectaries (> 5 EFN) with branch lengths proportional to time. Evolution of EFN aggregation in interpetiolar regions (red), prophylls of the axillary buds (yellow), and leaflet base (blue) in species of Bignonieae (Bignoniaceae). Species that invaded savanna environments are presented in black. Generalist species (i.e., species that occcupy forest and savannas) are presented in gray. Losses of these characters are not shown.

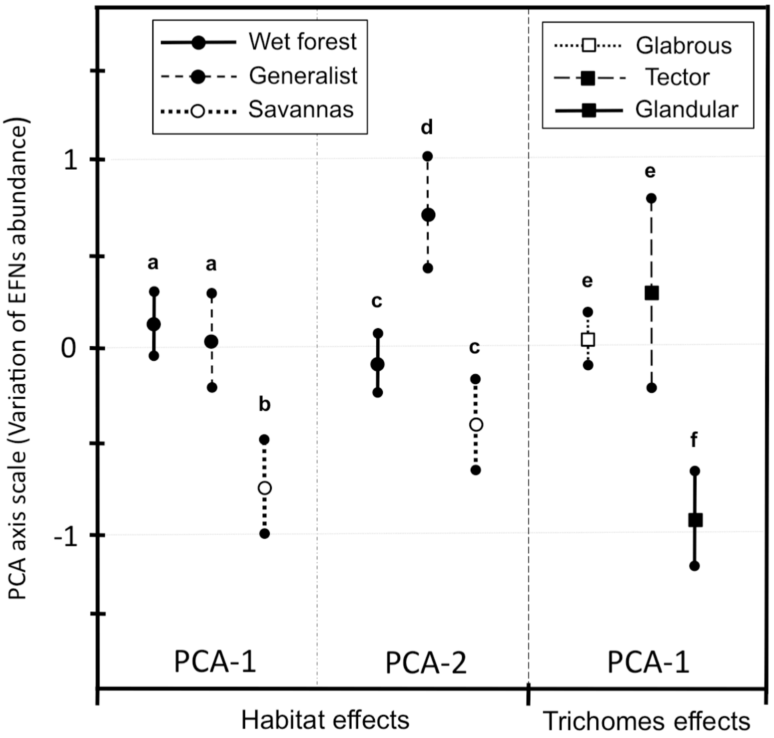
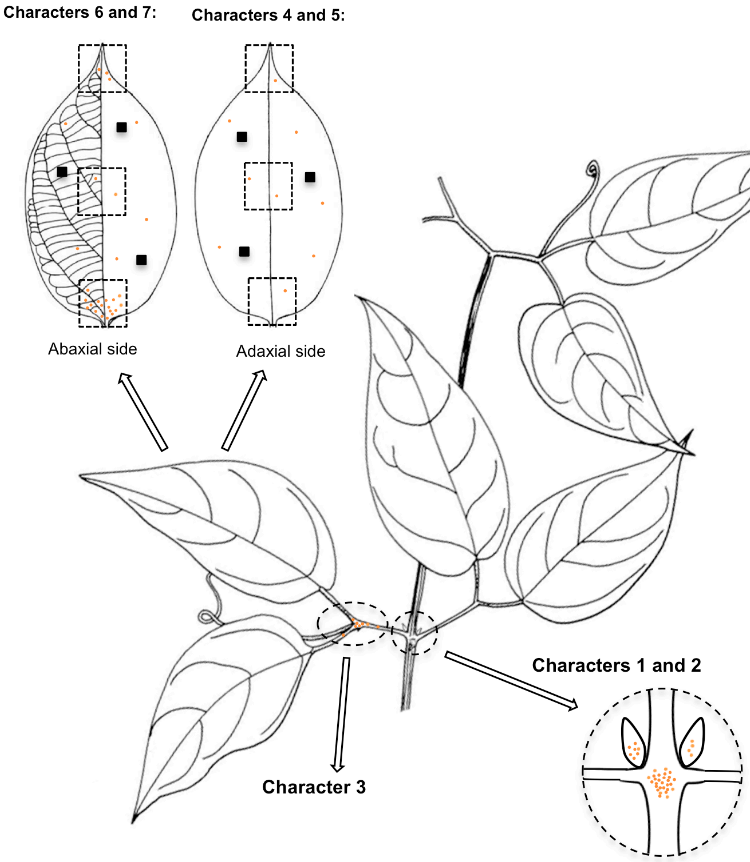


Figure 4. Post-hoc tests performed repeating the procedure for each possible habitat pair (forest specialists, habitat generalists, and savanna specialists) and trichome types (glabrous, tector, and glandular) after the significant differences were found in the univariate analyses (PCA-1 and PCA-2 for habitat changes; and only PCA-1 for the evolution of trichomes). Three independent analyses were performed for each PCA axis. Different letters are used to indicate significant differences between treatments ($p<0.05$) in each analysis.

On-line supplementary materials

Appendix 1S. Sampling scheme of EFNs and trichomes in different vegetative plant parts. EFNs are represented by orange points. Detailed descriptions on the procedures used to measure EFNs are presented in the table below. Areas marked by dashed squares on leaflets (1 cm^2) were used to sample EFNs. Black squares (1 mm^2) were used to sample trichomes. Quantitative variation of the number of EFNs on plants was used to calculate seven quantitative characters describing EFNs among species of Bignonieae (Bignoniaceae).



Characters	EFNs position on the plants	Methodological description of EFNs measurements
1	Stem (node)	Average number of EFNs on the interpetiolar region (midpoint), including 1 centimeter of height on the stem.
2	Prophylls of the axillary buds	Average number of EFNs on the prophylls, including total area of adaxial and abaxial side of prophylls.
3	Petiole + petiolule	Average number of EFNs on the adaxial side of petiole and petiolule.
4	Adaxial side (scattered EFNs)	Average number of EFNs on the intermediate dashed square (1 cm^2 of area) on the leaflets. Big leaflets had two squares sampled on the intermediate zone of leaflets.
5	Adaxial side (rarely aggregated EFNs)	Amount of average number of EFNs on the apex and base of leaflets marked with a dashed square (1 cm of height).
6	Abaxial side (scattered EFNs)	Average number of EFNs on the intermediate dashed square (1 cm^2 of area) on the leaflets. Big leaflets had two squares sampled on the intermediate zone of leaflets.
7	Abaxial side (aggregated EFNs)	Amount of average number of EFNs on the apex and base of leaflets marked with a dashed square (1 cm of height). Aggregated EFNs on the blade of leaflets were the sum of EFNs on the apex and the base of leaflets, where these aggregations were common.

Appendix 2S. Ant abundance sampled during fielwork on Bignonieae, including species from savanna (State Park of Grão Mogol - MG), and wet forests (National Park of Viruá – RR, and Ducke Reserve – AM). Species marked by asterisks are included in the phylogeny of Bignonieae (Lohmann, 2006), and were used to test the relationship between ant abundance and abundance of EFNs per node. SE = standandar error, N = number of plant specimens examined, NA = non-applicable (data was unavailable).

species	**habitat**	**ant abundance (mean)**	**SE**	**N**	**Dominant ant genus**	**% ant dominant**	**Attractive EFNs region**
Adenocalymma pedunculatum	Savanna	1.00	NA	1.0	*Camponotus*	NA	prophylls+leaflets
Adenocalymma sp.	Savanna	0.40	0.21	4.0	NA	NA	prophylls+leaflets
Anemopaegma album	Savanna	0.35	0.10	14.0	*Camponotus*	0.64	leaflets
Anemopaegma scabriusculum	Savanna	0.94	0.11	24.0	*Camponotus*	1.00	leaflets
*Cuspidaria convoluta**	Savanna	0.29	0.11	4.0	*Camponotus*	NA	leaflets
*Cuspidaria sceptrum**	Savanna	0.25	NA	2.0	NA	NA	leaflets
*Fridericia platyphylla**	Savanna	0.50	NA	2.0	*Camponotus*	NA	leaflets
*Adenocalymma adenophorum**	Wet Forest	0.90	0.80	9.0	NA	NA	prophylls
Adenocalymma flaviflorum	Wet Forest	0.20	0.10	4.0	NA	NA	prophylls
Adenocalymma longilinium	Wet Forest	7.00	2.10	21.0	*Crematogaster*	0.71	prophylls+leaflets
*Adenocalymma moringifolium**	Wet Forest	5.80	1.50	9.0	*Crematogaster*	0.54	prophylls+leaflets
Adenocalymma neoflavidum	Wet Forest	17.40	4.20	12.0	*Crematogaster*	0.58	prophylls+leaflets
*Adenocalymma subincanum**	Wet Forest	5.10	2.70	4.0	*Crematogaster*	0.75	prophylls+leaflets
Adenocalymma tanaeciicarpum	Wet Forest	6.80	3.00	13.0	*Crematogaster*	0.77	prophylls+leaflets
*Amphilophium magnoliifolium**	Wet Forest	4.70	0.80	4.0	*Crematogaster*	0.50	prophylls+leaflets
*Anemopaegma floridum**	Wet Forest	3.10	1.30	8.0	*Crematogaster*	0.43	prophylls
*Anemopaegma robustum**	Wet Forest	9.20	4.00	14.0	*Crematogaster*	0.64	prophylls+leaflets
*Bignonia aequinoctialis**	Wet Forest	6.00	2.80	4.0	*Crematogaster*	0.75	prophylls
*Bignonia prieurei**	Wet Forest	4.10	2.70	8.0	*Crematogaster*	0.43	prophylls
Bignonia sp.	Wet Forest	20.30	6.00	4.0	*Crematogaster*	0.75	prophylls
*Callichlamys latifolia**	Wet Forest	6.20	2.20	4.0	*Crematogaster*	0.50	prophylls+leaflets
*Fridericia nigrescens**	Wet Forest	0.70	0.50	8.0	*Asteca*	2/4=0.50	stem node (interpetiolar region)
*Fridericia prancei**	Wet Forest	5.10	1.10	4.0	*Crematogaster*	0.75	stem node (interpetiolar region)
*Fridericia trailii**	Wet Forest	9.00	4.30	8.0	*Crematogaster*	0.62	stem node (interpetiolar region)
*Fridericia triplinervia**	Wet Forest	5.60	3.30	7.0	*Crematogaster*	0.50	stem node (interpetiolar region)

*Lundia densiflora**	Wet Forest	5.10	3.60	7.0	*Crematogaster*	0.43	stem node (interpetiolar region)
*Pachyptera aromatica**	Wet Forest	5.80	1.30	12.0	*Crematogaster*	0.70	stem node (interpetiolar region)+leaflets
Pachyptera kerere	Wet Forest	7.40	4.50	9.0	*Crematogaster*	0.30	stem node (interpetiolar region)+petioles
*Pleonotoma albiflora**	Wet Forest	9.80	3.70	5.0	*Crematogaster*	1.00	prophylls+tendrils
Pleonotoma melioides	Wet Forest	2.40	0.90	4.0	*Crematogaster*	0.60	prophylls+tendrils
Styzophillum riparium	Wet Forest	0.50	NA	2.0	NA	NA	NA
*Tynanthus panurensis**	Wet Forest	8.00	4.50	8.0	*Crematogaster*	0.37	stem node (interpetiolar field)

Capítulo 4

Geographic mosaic of an ant-plant guard system: *Anemopaegma album* and its ants

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Abstract

1. Most species are collections of genetically differentiated populations, with each population representing a separate evolutionary unit with the potential for diverging from each other in response to specific environmental cues. More specifically, variation of plant-animal interactions across the geographic range of the plants species is known to generate different patterns of selection on specific plant traits, influencing the evolutionary dynamics of such interactions. In this paper, we examined the geographical variation of plant anti-herbivore defense mediated by ant guarding (plants offering nectar to ants in extrafloral nectaries, and ants protecting the plants against herbivores) under the general framework of the geographic mosaic theory of coevolution (GMT).
2. We measured extrafloral nectarie (EFNs) traits, ant and herbivore assemblages, herbivory intensity and plant performance in 10 populations of the shrub *Anemopaegma album* (Bignoniaceae) from Neotropical savanna. We hypothesize that if herbivory is a strong pressure for EFNs-bearing plants, then differences among habitat and/or localities in herbivore guild and ant visitor assemblage (composition and abundance) will have evolutionary influences, determining differences among populations in the abundance and function of EFNs. Two alternative evolutionary outcomes were tested.: (1) correlation across populations in EFNs traits, ant assemblage composition and function, herbivore damage and plant performance; (2) a mosaic of matched and mismatched populations. Matching would represent local fits among EFN traits and ant visitor assemblages, frequently resulting in low herbivory and high performance (evolutionary hotspot), while mismatching would mean lack of fit and more frequently high herbivory and low plant performance (coldspots).
3. The ant visits to EFNs correlated across populations negatively with herbivory and positively with plant performance, meaning that local variation in ant visits was fundamental for variation in plant defense and performance. However, no significant values of correlations across populations were found between EFNs traits and abundance of ants, herbivory or plant performance, thus refuting the correlational pattern. In contrast we could identify several local situations of matching as well as several mismatched populations. We corroborated that several matched populations reached the highest plant performance and low herbivory intensity, while commonly the mismatched ones (associated to low occurrence of ants in EFNs) have low performance, although it did not always mean high herbivory. The matched and mismatched

populations pattern was not geographically determined, with several matched populations being widely separate of each other.

4. This study represents the first analysis for the geographic structure of antihervore defense mediated by ant-guard system. Our results are consistent with a GMT prediction of a geographic structure of biotic interactions in a mosaic of matched and mismatched populations with hotspots and coldspots, and provide insights for a better understanding of the evolution of this type of plant-animal interaction.

Key-words: adaptive landscape, ant-plant interaction, Brazil, defense traits, geographic variation, herbivory, natural selection.

Introduction

The intraspecific variation in animal-plant interactions and the evolutionary divergence of these interactions among populations are the productive engine of much of the morphological diversity that exists between species (Herrera et al., 2006). This is particularly important since macroevolutionary patterns result from cumulative outcomes from microevolutionary processes that act at the intraspecific level, leading to morphological variability and to an adaptive potential to traits (Thompson, 1994). More specifically, the variation of biotic interactions that are essential for the life cycle of plants (e.g., pollination, seed dispersal, herbivory and defense) should generate different patterns of selection on specific plant traits between populations of the same species.

Plants and their animal interactors commonly differ in their geographic ranges. The community context where these interactions occur vary across these ranges (e.g., mutualism: Herrera et al., 2006; Rey et al., 2006; Rey & Manzaneda, 2007; antagonisms: Benkman, 1999; Thompson, 1997, 1999; Stinchcombe & Rausher, 2002; Zangerl & Berenbaum, 2003; Mezquida & Benkman, 2005), affecting the outcome of individual interactions. However, only recently has the geographical context been explicitly incorporated into the ecological and evolutionary studies of biotic interactions (reviewed in Thompson, 1994, 2005; Herrera et al., 2006; Rey et al., 2009). Indeed, it is possible that the local variations of animal-plant interactions (e.g., pollination review by Herrera et al., 2006), may have been responsible for discrepant results among studies (Thompson, 1994).

Ants commonly interact with plants in very different ways. These interactions can be positive (e.g., pollination, seed dispersal, and defense against herbivores) or negative (e.g., herbivory and seed predation) (Beattie, 1985; Beattie & Hughes, 2002; Rico-Gray & Oliveira, 2007). However, the ecological and evolutionary consequences of the geographical variation in these interactions are still poorly known. The assemblages of ant visitors of plants that rely in mutualisms with ants vary with the plant species range due to the effect of habitat and climatic variation in ant distribution. For example, ant visitor assemblages in myrmecophytic systems has been shown to be highly variable across localities both in terms of their species' composition and species' function (Manzaneda et al., 2007; Manzaneda & Rey, 2009a, 2009b). Similarly, the assemblage of ants that visit EFNs of a single species, vary among localities (Kersch & Fonseca ,2005). Whenever differences in the species composition of ant assemblages are translated into functional differences between assemblages (i.e., a different representation of functional groups such as legitame dispersers, facultative dispersers, and elaiosome consumers, in the case of myrmecochore systems), spatial and geographic variations in the ant visitor assemblage leads to shifts in selective pressures on plants with potential for evolutionary divergence among populations (Rey & Manzaneda, 2007; Alcántara et al., 2007).

On the other hand, mutualistic interactions may show “conditionality” or “context-dependency” (Jones & Callaway, 2007) determined by current ecological conditions (Bronstein, 1994). Such conditional outcomes of the interactions are associated with the variation of costs and benefits (Heil & McKey, 2003) that are associated with the partners and with the particular abiotic and community context where the interaction develops. In many cases, such context dependency explains the spatial and temporal variation in ant-plant interactions in a predictable way (Bronstein, 1994). The context-dependency in ant-plant mutualisms seems to be more frequent within species than between taxa (Chamberlain & Holland, 2009 for ant-plant protection mutualisms; Manzaneda & Rey, 2011 for ant dispersal systems), especially in the presence of local variation in biotic and abiotic conditions (e.g., Tempel, 1983; Kersch & Fonseca, 2005; Manzaneda & Rey, 2011). In the case of plant defenses mediated by ants, it is expected that the outcome of the interaction will be conditional to the type of herbivores found and to the presence of alternative plant sources encountered for the herbivores in each locality (Stinchcombe & Rausher 2002; Zangerl & Berenbaum, 2003). Indeed, herbivory intensity varies across populations due to the variation in the guild of insect herbivores found in each locality (e.g., Løe et al., 2007).

Geographic variation in the outcome of animal-plant interactions has been considered a fundamental component of the coevolutionary processes acting among interacting organisms. This idea has lead to the proposition of the Geographic Mosaic Theory of Coevolution (GMT; Thompson, 1994, 2005). This theory has been extended to the evolutionary outcomes of plant-animal interactions, postulating that animal partners might influence plant evolution in some communities (hotspots in which the interactions lead to significant selection on plant traits) but not in others (coldspots in which no significant selection mediated by animal interactors occur, or in which the outcome of local selective pressures is continuously disrupted by gene flow). Such geographic variation in the outcome of the interactions might originate a mosaic of matching and mismatching phenotypes across the geographic range of plant taxa (Løe et al., 2007; Thompson, 2005; Garrido et al., 2002 for ant-plant seed dispersal mutualisms).

All of these arguments strongly suggest that the incorporation of geographic variation to the study of the outcome of animal-plant interactions is crucial for a better understanding of the evolution of plant traits in response to animal selective pressures (Alcántara et al., 2007). In this paper, we incorporate a geographic context in the study of interactions between ant guarders and a myrmecophyte plant species that bears extrafloral nectaries (EFNs). We hypothesize that herbivory is a strong pressure for EFNs-bearing plants, with differences in herbivore guilds and ant visitor assemblages (composition and abundance) among habitats and/or localities influencing evolution at local-scales (population scale), and establishing differences in the abundance and function of EFNs among populations, and/or across species ranges. According to this hypothesis, herbivore damage across-localities is expected to be correlated with herbivore damage, plant defensive structure (i.e., abundance of EFNs and nectar production), and ant guard services (i.e., number of ant visits or defensive attacks). Alternatively, the evolution of plant defenses (EFNs) mediated by ants is expected to perform in a way that is congruent with the GMT. Differently to previous hypotheses, GMT does not necessarily imply correlations among herbivore damage, EFNs, and ant guard defenses across localities. Instead, a mosaic with matched, and mismatched populations is expected. In matched population, EFNs are expected to function according to herbivore pressures and ant services, with high mean population fitness. On the other hand, the function of EFNs is unrelated to herbivory level and ant service, with low mean population fitness in mismatched populations.

This study aimed to test whether the eco-evolutionary predictions of the GMT framework were in accordance with the patterns of variation observed in the interactions among the myrmecochore shrub *A. album* (Bignoniaceae), its ants, and insect herbivores across ten populations from a Neotropical savanna. First, we characterized the inter-populational variation of EFNs (abundance and size of nectaries), and assessed the variation in nectar volume and sugar concentration. We then described the patterns of variation encountered among populations in the ant assemblages that visited the EFNs, in the herbivore assemblages, and in the intensity of herbivory suffered by the individual plants. Finally, we tested the existence of across population correlations for nectary abundance, size, and function (nectar production and concentration) with: (i) functional properties of the ant assemblages, (ii) abundance of herbivores and foliar damage, and (iii) estimates of mean population plant performance or fitness. A lack of significant correlations are consistent with the geographic mosaic of matching and mismatching populations between ants and plant EFNs (see for example, Garrido et al. (2002), in case of a myrmecochory interaction). As a consequence, we also searched for populations in which EFNs fit local ant assemblages and verified whether the defense provided by EFNs were particularly efficient in those populations. That is, we determined whether lower mean herbivore damage led to high mean fitness or population performance (measured as low reduction or increase in foliage, and the density of plant saplings around the adult plants). This same pattern would not be expected whenever EFNs did not match local ant assemblages.

Methods

Study system and plant populations

This study was conducted in 10 different sites distributed over 600 km in areas of Neotropical savannas between the states of Minas Gerais and Bahia. These localities presented a seasonal climate with maximum and minimum temperatures between 20.3º-31ºC in the rainy season (October to March) and between 15.3º-30.5 ºC in the dry season (http://www.inmet.gov.br/). The mean annual rainfall ranged among localities between 650 and 1.100 mm (north to south), with accumulated mean rainfall in the rainy season around 710 mm (November-February), and around 40 mm (June-August) in the dry season.

The 10 localities were chosen according to the occurrence of populations of *Anemopaegma album* Mart. ex DC. (Bignoniaceae). In addition, the areas were selected in order to encompass most of the geographic range of this species (Figure 1). *Anemopaegma album* is a shrub distributed throughout the 'cerrados,' 'caatingas,' and transitional areas called 'carrascos.' These plants are bee-pollinated (Gentry, 1974) and have two main floral/fruit herbivores, both of which are caterpillars (Lepidoptera; A. Nogueira, pers. obs.). The leaves of *A. album* are trifoliated with great variation in the abundance of EFNs. EFNs of this species actively attract ants (Nogueira et al., 2011). The majority of EFNs are grouped at the base of the abaxial side of leaflets, where their abundance is positively related to the ant visitor abundance (Nogueira et al., 2011). Likewise, few nectaries are sparsely distributed over the abaxial and adaxial sides of the leaflet blades. At least three insect herbivores, belonging to Coleoptera and Orthoptera, fed on leaves (Nogueira et al., 2011), and frequently causing extreme foliage loss.

We tagged 30 specimens of *A. album* in each of the 10 sites (N= 300 individuals distributed in N=10 populations) from October to November, 2009. We visited these plants at two different time periods: (t_0) onset of the rainy season (November 2009) when most of the individuals of *A. album* had produced new leaves; and (t_1) end of the rainy season (March-April 2010), coinciding with the fruiting period of those plants. Several ecological variables were measured in these two moments. At t_0, plants were marked and geo-referenced in the field. Concurrently, the total number of leaves and visitors (ants and herbivores) were sampled on each plant in each of the 10 populations sampled. At t_1, we re-sampled the same variables previously measured at t_0, and collected 20% of the leaves of each plant. These leaves were held in the laboratory and used to quantify the levels of herbivory and some descriptive variables of extrafloral nectaries (size and abundance of EFNs) per plant. At t_1 we also sampled the exudates (nectar secreted by EFNs) of half of the plants found at each population (N = 15 per population), and calculated the volume and concentration of total sugar content in the secretion. At this time, we also sampled the total number of fruits per plant, and the number of seedlings around each adult plant sampled. Further methodological details of data collection for each ecological variable are presented in the sections below.

Data collection

Characterization of ant and herbivore assemblages

All ants using the EFNs of *A. album* and all herbivores feeding on vegetative plant parts were counted on the plants in the two sampling periods (t_0 and t_1). Initially, both ants and herbivores were counted on all leaves and branches of the sampled plants. One specimen of each ant and herbivore morphotype was collected with special flat-tipped tweezers and fixed in 90% alcohol for subsequent identification. The data on behavior of *A. album* herbivores described in Nogueira et al. (2011) (and some new ones), were used to identify and quantify the main herbivores, including larval and adults stages. Since these herbivores are mainly active during daytime (between 8 A.M. and 6 P.M.), samples of ants and herbivores were only made during this period. Three variables were used to describe the variation of ants and herbivore communities: relative frequency of plants occupied (N=30 plants/population), average number of individuals per plant, and species composition (variation in the abundance of the more frequent genera).

EFNs traits

The extrafloral nectaries of *A. album* (patteliform glandular trichomes generally associated with nectar secretion; Chapter 1) were quantified at t_1 in five leaflets per plant (30 plants in each population) using a stereomicroscope. Since the highest abundance of nectaries is found on clusters at the base of the abaxial surface of leaflets (Nogueira et al., 2011), we considered three different variables in order to describe the abundance of EFNs on leaflets: (1) the number of EFNs clustered at the base of abaxial surface of leaflets (1 cm^2); (2) the number of EFNs scattered on the abaxial surface of leaflets (excluding the base), and; (3) the number of EFNs scattered on the adaxial surface of leaflets. In addition, we quantified the diameter of the secretory head of the largest nectary at the EFN clusters located at the base of the abaxial side of leaflets. We then collected samples of the secretion produced by the EFNs using 1µl and 5 µl microcaps obtained from Drummond (USA) and Merck (Germany) in order to characterize the exudates of the EFNs in *A. album*. The sugar concentration (and volume) of the secretions was quantified with a portable Eclipse refractometer (Standley, England).

Herbivory and fitness

At t_1, herbivory was estimated as a proportion of the total leaf area that was removed from the leaflets sampled. At that time, 15-20% of the leaflets were collected, including leaves from different plant parts (three leaves in each branch, including leaves from the apex, middle portion, and base of each branch) to quantify the percentage of herbivory (%) on each plant. In the laboratory, we quantified the total leaf area and the damaged area on each leaflet using visual assessment with the aid of an acrylic sheet that included 0.3 and 0.4 mm^2 grids.

Three fitness variables were measured: (1) production of leaves; (2) number of produced fruits in t_1; and (3) number of seedlings around each adult plant measured. The production of leaves was calculated by the difference of leaves encountered at t_1 and t_0. For samples admited that some plants could have a negative production (less leaves than the number of leaves in the first sampling time). The other two fitness variables were obtained at t_1. For this measurement, we counted all fruits and all seedlings around the individual adult plants (4-meters diameter circle around the plant = 12.6 m^2) in each population. Seedlings were defined as plants with 50 cm or lower.

Data analyses

To explore the variation in EFN traits and herbivory damage, we conducted variance partitioning analyses with Restricted Maximum likelihood estimation.

To characterize the spatial patterns of variation in ant (genus level) and herbivore (species level) assemblage composition, we used the coefficient of Steinhaus (Legendre & Legendre, 1998). This coefficient is also known as the proportional similarity index (*PS*): $\sum \min (p_{ai}, p_{bi})$, in which for *n* species, p_{ai} is the relative abundance of species *i* in a population *a*, and p_{bi} is the relative abundance of species *i* in population *b*. The *PS* index ranges from 0 (maximum dissimilarity; i.e., no common taxa between *a* and *b*) to 1 (maximum resemblance, i.e., identical generic composition between *a* and *b*). Following Manzaneda et al. (2007), we tested the null hypothesis of “dissimilar assemblage composition” (*PS*=0), and assessed the statistical significance for each coefficient of similarity by determining the 95% confidence limits through bootstraping (Manly, 1998). These analyses were conducted in R environment (R Development Core Team, 2011). Observed *PS* values were compared to a distribution of randomly generated *PS* values, calculated for each of 10000 paired draws with replacement from the samples being compared (Jordano, 1994). The proportional similarity index was calculated with the R package ‘asbio’ (function *get.dist*) and the 1- *steinhaus* distance (Aho, 2011).

Linear regressions were performed to examine the relationship of EFN traits to the abundance and frequency of ants, and the relationship of EFN traits to four fitness variables (i.e., herbivore damage, production of leaves, and sapling abundance). In addition, we explored whether the similarity of ant and herbivore assemblages, as well as the population mean values of EFN traits and fitness depended on geographical distance. We built two dissimilarity matrices, one of which was built from pairwise dissimilarity coefficients (1 - PS) between populations, and the other included pairwise geographical distance between populations. The distance dependence in the similarity of the ecological variables was tested using a Mantel test (Legendre & Legendre, 1998). The significance of the Mantel statistics was determined with 5000 permutations. These statistical analyses were run with the Vegan package for R environment (R Development Core Team, 2011).

Results

Variation on EFN traits

Extrafloral nectaries were found throughout the whole leaf blade (abaxial and adaxial surfaces), with a large number of EFNs concentrated at the base of the abaxial side of the leaflet blades (except for one population, 'Mirangaba'). EFN traits (size and abundance) varied significantly within and between populations (Figure 2). On average, plants presented 29.3 ± 3.9 EFNs per leaflet (Leaflet base at the abaxial side) considering all populations sampled (Table 1). In particular, the populations of 'Mirangaba' and 'Caetité' presented the smallest abundances of EFNs per leaflet (1.6 and 17.1 EFN's/leaflet respectively), while the populations of 'Palmeiras and 'Morro do Chapéu' presented the highest values (42.7 and 39.9 EFN's/leaflet, respectively). Furthermore, the highest sizes of EFNs varied from 0.117 mm ('Palmeiras' and 'Rio das Contas') to 0.176 mm ('Morro do Chapéu') between populations. The volume of nectar secreted varied from 0.01 to 5.41 µl/plant (50 leaflets sampled per plant, in 15 plants per population), and the sugar concentration varied from 13.7 to 55.6% of sugar (Table 1).

Ant variation across populations: frequency, abundance, and assemblage composition

We detected great geographical variation in the abundance of ants and in the composition of ants that fed on the EFNs (Figure 1). A positive correlation across populations was found between the frequency of plants occupied by ants and the average number of ants found per plant, in the first sampling period at $t_{(0)}$ (r=0.83, p=0.003, N=10), and in the second sampling period at $t_{(1)}$ (r=0.80, p=0.005; N=10). In general, the higher frequency of plants occupied by ants (range = 31.7 – 88.3 %), in a given population, the higher the number of ants per plant (range= 0.5 – 12.2. ants/plant). The most visited populations by ants were the 'Mirangaba' population (88% of the plants occupied, with 12.2 ants per plant on average) and the 'Morro do Chapéu' population (88% of the plants occupied, 10.2 ants per plant). On the other hand, the 'Caetité' (31.7% of plants occupied, with 1.9 ants/plant) and 'Palmeiras' populations (40% of plants occupied, with 0.5 ants per plant), represented the least visited populations. The populations with higher average abundance of ants per plant were in the north of the state of Bahia (Brazil), showing a dependency on the geographic distance (r=0.36; p=0.044; Table 4). This pattern was not observed for the frequency of plants occupied by ants.

The composition of ants was very similar between populations when pair-wise compared (average PS = 0.60; Table 2). These results were different from those obtained for the frequency of plants occupied by ants and the average abundance of ants per plant. The similarity in the composition of the ant assemblage between populations was due to the high abundance of *Camponotus* ants, in association with the low abundance of *Pseudomirmex* and *Cephalotes* ants in seven of the 10 populations sampled (Figure 1). Overall, three populations presented ant assemblage compositions that were different from the most common pattern: 'Mirangaba', 'Cristália', and 'Grão Mogol'. 'Mirangaba' presented almost exclusively *Crematogaster* ants, differing from all the other populations. On the other hand, "Cristália" was the only population with a high number of individuals of *Cephalotes* ants although many *Camponotus* ants were also found in this community. Finally, the population of "Grão Mogol" had many *Camponotus* ants, as well as other groups of ants that were only rarely observed in the other populations (e.g., *Pheidole*). Differently from the average abundance of ants per plant (dependent on geographic distance), the similarity of the assembly of ants (PS) was independent from geographic distance (r = -0.316, p = 0.938; Table 5).

In terms of function, the four most frequent ant genera encountered in plants of *A. album* presented different behaviors. While *Crematogaster* has the most actively recruiting behavior (Appendix 1S), followed by the *Camponotus* and *Cephalotes, Pseudomirmex* did not have a recruiting behavior. *Cephalotes* was quite passive in the presence of other insects on the plants, while *Camponotus* ants were generally very agile and fast.

Herbivores and herbivory

Ten different species of herbivores were detected on plants of *A. album*, some of them were observed feeding on leaves and branches, while others were detected only through their traces/marks on plants (e.g., galling and some sucker insects). The most common herbivores (Table 3) were: *Charidotis* sp. (Chrysomelidae, Coleoptera), *Sumitrosis* sp. (Chrysomelidae, Coleoptera), and *Xestotrachelus robustus*

(Romaleidae, Orthoptera). These herbivores were responsible for the greatest proportion of leaf damage observed in the field (see Nogueira et al., 2011). On average, the pair-wise similarity in the composition of the most common herbivores was smaller (average PS=0.51; Table 2) than that observed in ant assemblages. Apart from the similarity on the average abundance of herbivores, the composition of the herbivore assemblages was not dependent on the geographic distance ($r = -0.129$, $p = 0.799$, and $r = -0.012$, $p = 0.573$, respectively; Table 5).

The average herbivory was of 30.2 ± 6.5 % (N=10 populations). The populations with the smallest herbivory levels per plant were 'Mirangaba' (9.3%) and 'Morro do Chapeu' (16.7 %), while the highest herbivory levels were found in 'Caetité' (82.5 %) and 'Cristália' (42.4 %). Herbivory was also marginally correlated with the total abundance of the main herbivores sampled ($r = 0.593$; $p = 0.07$).

Correlation among traits, functional properties of ants, herbivory, and fitness

There was no significant correlation across populations between EFN traits (size, abundance, and nectar descriptors) and the characters used to describe ant assemblages (frequency and abundance of ants) (Figure 3, Table 4). Similarly, there was no significant correlation between EFN traits and the measurements of plant performance or fitness across populations (Table 4). In contrast, a positive relationship was found between the average abundance of ants in $t_{(0)}$ and the variables describing plant performance. Similarly, a marginally significant negative relationship ($p = 0.06$) was detected between the mean abundance of ants and the mean percentage of leaf herbivory per plant (Figure 4, Table 4). Overall, the higher the mean abundance of ants per plant, the lower the leaf damage per plant and the higher the production of leaves and saplings around adult plants (Figure 4). In addition, the two variables describing the performance of plants were also correlated to each other positively (Table 4): the higher the production of leaves, the higher the number saplings around the adult plants.

Matched and mismatched populations

The joint consideration of mean population EFN traits and functional ant assemblage traits was used to detect matched populations (those with an apparent match between EFNs and the functional behaviour of the ant visitor assemblage), and mismatched populations (those with an apparent mismatch between EFNs and the ant visitor assemblage). Among the ten populations considered here, five represent cases of matching, while the other five represent mismatched populations. The five cases of matching were in the following populations: ‘Mirangaba,’ ‘Morro do Chapéu,’ ‘Grão Mogol,’ ‘Mato Verde,’ and ‘Rio de Contas’ (Figure 5). In ‘Mirangaba,’ ant assemblages were dominated by small ants (*Crematogaster*), with high recruiting behaviours. These ants were able to operate adequately with the scattered and relatively low productive EFNs found; in this population the number of EFNs clustered at the base of leaflets was minimum. The other four cases of matching (‘Morro de Chapéu,’ ‘Grão Mogol,’ ‘Mato Verde,’ and ‘Rio de Contas’) were characterized by larger ants (especially *Camponotus*) with a lower recruiting ability, that was favoured by highly productive and clustered EFNs (*ca.* 40 EFNs were clustered at the base of leaflets in both populations; Figure 5). It is worth noting that three of these populations presented high levels of and visitation, low herbivory, and a high production of leaves (Figure 5). In those cases, matching between EFN traits and the functional traits of the ant visitor assemblages translated positively to plant performance. Moreover these three populations represented the only cases in which positive values of leaf production were observed. The two matched population in which matching did not translate positively to plant performance were ‘Mato Verde’ and ‘Rio de Contas,’ with negative leaf production in spite of a low level of herbivory, and low abundance of herbivores (Figure 5, Table 3).

Among the mismatched populations, the most common pattern was a low abundance of visitors on plants and a density of EFN aggregation that was lower than the levels found for the matched populations. This was the case of the ‘Caetité,’ ‘Abaira,’ ‘Cristália,’ and ‘Mucugê’ populations (Figure 5). Except from the ‘Abaira’ population, all other populations included a high density of herbivores, as well as received severe foliage damage (Table 3). Those populations also presented a strongly negative leaf production between the two dates in which leaves were record. Interestingly, ‘even though the ‘Abaira’ population presented a negative leaf production, this population presented a much better performance than the other mismatched populations. Finally, the last mismatched population, ‘Palmeiras,’ was characterized by high aggregation of relatively small EFNs (although strongly variable among plants within the population). However, this population also presented extremely low frequencies of visits from a diverse assemblage of ant visitors. In this case, the mismatching was more associated with the paucity of visits; this observation was consistent between the two recording dates. In any case, it apparently renders low plant performance as estimated by a clearly negative leaf production of these populations.

Discussion

Recently the plant-animal interactions have been studied in a wider geographic scale, incorporating explicitly the variation in the interespecific interactions and their outcomes among populations (Geographic Mosaic Theory of coevolution - GMT, Thompson 2005). In this study we evaluated the geographic pattern of an ant-plant interaction and its outcome across ten populations of the myrmecochore shrub *A. album* (Bignoniaceae) from the Neotropical savannas. This constitutes the first attempt to analyze the geographic structure, and its evolutionary implications, of an ant-guard system. We found a geographic structure of the interaction outcome which was in agreement with the ecological-evolutionary predictions of the GMT framework. Ant visits to EFNs correlated across populations negatively with herbivory and positively with plant performance, meaning that local variation in ant visits was fundamental for variation in plant defense and performance. However, no significant values of correlations across populations were found between EFNs traits and abundance of ants, herbivory or plant performance, Such lack of correlations was apparently a consequence of a geographic mosaic of local matchings and mismatchings between functional properties of ants and plant EFNs traits. In particular, matching occurred only in populations where the defense provided by ants and EFNs prove to be particularly efficient (i.e., lower mean herbivore damage and high mean performance in the population measured as low reduction or increase in foliage and enhanced density of juveniles around the adult plants), while the same did not occur where EFNs were not matched with the local ant assemblages.

EFNs pattern of variation in Anemopaegma album

Little information is available about the function of extrafloral nectaries in the majority of plant groups, spite of the wide distribution and morphological diversity of these organs (Theobald, Krahulik, & Rollins 1979; Elias 1983). In general, larger EFNs, with more complex structure and vascular supply (e.g. elevated EFNs) secreted more nectar than glandular trichomes non-vascularized (Díaz-Castelazo et al., 2005). However, large numbers of small EFNs, as the patelliform glandular trichomes on Bignoniaceae species (e.g. *A.album*), with lower structural costs, could be as effective as large vascularized secretory structures (Díaz-Castelazo et al. 2005). Especially in Bignoniaceae, the function of these small secretory structures has been rarely tested in relation to its capacity of attracting ants (two species, Nogueira et al. 2011), decreasing herbivory or herbivores (four species; Stephenson 1982, Ness 2003 and Nogueira et al. 2011), or increasing plant performance (three species, Ness 2003 and Nogueira et al. 2011).

In the ant-plant systems mediated by the nectar secretion of EFNs it would be expected that the variation of interactions within and between populations would be determined in a great part by the variation in morphology/function of these secretory structures. In this study we observed that the variation of the abundance of extrafloral nectaries of *Anemopaegma album* was not clearly related to the nectar traits (volume and sugar concentration) and ant abundance across populations. The opposite pattern was observed within two population of the same species (A.album, Nogueira et al. 2011), and between species in a related plant group. At the macrocevolutionary level, strongly positive correlation has been detected between the number of EFNs and the number of ant visitors within the Bignonieae tribe, after controlling for the phylogenetic relationship among them (Nogueira et al. in prep.; Chapter 03). These previous results might suggest an adaptive value for the EFNs traits during the evolution of these plants.

The existence of quantitative variation in the number of nectaries between plants and populations is a great opportunity for exploring small variations in the resource offer to ants (differently to the qualitative variation of EFNs in other groups - e.g. elevated nectaries in Leguminosae, Machado et al. 2008). In our case, however, samplings of secretion in minute glandular trichomes always gather error (adding noise in the data) given the low quantity of secretion available in each nectary (e.g. scale-like trichomes sensu Díaz-Castelazo et al. 2005). This limitation arises because the secretion sampling in the field is based on the use of capillaries and dilution methods when the secretion is very sticky (Elias 1983). Nonetheless, even without significant across populations correlation between abundance of EFNs and nectar traits, differences between populations were observed, with populations with few nectaries in the leaflet base secreting on average small quantities of nectar (e.g

‘Mirangaba’).

Although the genetic bases for EFN attributes are almost unknown (Mitchell 2004), some information on the heritability of EFNs is available: Rudgers (2004) found that, for wild cotton, the proportion of leaves with EFNs and the size of the glands had genetic variation and significant heritabilities. The fact that, at least for some plant species, the EFNs traits are inheritable has important evolutionary implications since it can be subject to natural selection. In the specific case of Bignoniaceae species, the heritability of EFN traits is yet unknown. The absence of this information is a great limitation to our understanding of evolutionary consequences of the ant-plant interactions, and their variable outcomes, among populations of *Anemopaegma album*.

Variation in ant visitor and insect herbivore assemblage

Ant visitor assemblages showed relatively high similarity among localities. Although the four main ant genera visitors of *A. album* (*Camponotus*, *Crematogaster*, *Cephalotes* and *Pseudomyrmex, all* common ants on Neotropical savannas plants; Schoereder et al. 2010) changed their frequencies among populations, in most populations, the plants were preponderantly visited by *Camponotus* genus, what explains the high similarity found in the ant assemblage between populations. The importance of the plant-*Camponotus* association for the protection of plant tissues against herbivores has been previously described in the savannas (e.g. Del-claro, Berto, & Réu 1996; Del-Claro et al. 1996; Oliveira 1997; Korndorfer and Del-Claro 2006; Nascimento and Del- Claro 2010). Furthermore, the presence of *Camponotus* ants is known to be recognized visually by some herbivores, which are in this way repelled , reducing herbivory (e.g. Sendoya, Freitas, & Oliveira 2011) . For example, ovipositing female butterflies are known to use visual cues to discover host plants conferring greater larval survival (Rausher 1978). Furthermore, the ant assemblages are strongly shaped by competitive hierarchies (Bluthgen & Fiedler 2004), and *Camponotus* could outcompete other ants under different conditions within savanna’s environment. Nevertheless, one population, 'Mirangaba', singularly differed from the common pattern of ant visitor assemblage dominated by *Camponotus.*In this population the ant assemblage was dominated by *Crematogaster* genus The small body size of this genus could favor (or at least, make possible) the utilization of plants with few nectaries on the leaves.

We consider ant body size as an important functional trait for the interaction with EFNs. Ant body size could be related with liquid consumption, aggression, recruiting and speed abilities,among other life history variables (Nowbahari et al. 1999; Davidson et al. 2004; Hurlbert et al. 2008; Chamberlain & Holland 2009). The four most frequent ant genera behaved differently on the plants of *A. album*. While *Crematogaster* has the most actively recruiting behaviour, followed by *Camponotus*, *Cephalotes* and *Pseudomirmex* apparently did not recruit towards EFN sources. *Cephalotes* prove to be passive in presence of other insects on the plants (e.g. Byk & Del-Claro 2010), while *Camponotus* was, in general, very agile. The behavior of *Crematogaster* ants, higher capacity of ant recruitment, explains the pattern found in the population 'Mirangaba' with higher average number of ants on plants with fewer and scattered nectaries (and contributes to the lack of correlational between EFNs abundance and ant visits.

An aggressive behavior of ant species against the most common herbivores was not directly observed in this study, but it has been previously described in Nogueira (et al 2011), where the ant aggressive behavior was observed against the adult as well as larval stages of some herbivores (e.g. as in the case of the beetle *Charidotis* sp.). It is probably that the defense mediated by ant attacks to herbivores would be more effective on less damaging herbivores, while repelling effects against other most damaging herbivores act through visual cues (Sendoya, Freitas, & Oliveira 2009). On the other hand, ant-plant mutualisms are often characterized by great variation in the benefits provided by different partner species due to differences in the quality and quantity of their interactions. In this way, ant services could vary among ant species or genus in terms of frequency of use EFNs, their abundance on the plants ('recruitment' availability) and the defensive effect against herbivores, resulting in different effects on plant performance (Ness et al 2006).

The link between the variation of EFNs, ant and herbivore assemblage, herbivory and plant performance

A clear link across populations between nectaries and ant visitor and herbivore assemblages, intensity of herbivory and plant performance did not emerge from this study. The relatively high similarity among populations detected in the composition of the ant visitor assemblages may contribute to explain the lack of correlation between the average number of EFNs and ant service. However, the ant services (estimated by ant visits) explained well the foliar damage by herbivores, and specially the average performance of plants at the population level. This pattern may reflect the balance between costs and benefits of ant-plant interactions (Bronstein 1994; Heil & McKey 2003), with higher values of average performance per population as a proxy of higher benefits than costs of interaction. Since the mutualistic interactions are conditioned by the biotic and abiotic context (Bronstein 1994), it would be expected different outcomes from these interactions between populations of the same species (Thompson 2005). In particular, the costs and benefits in protection mutualisms would depend on the local abundance of (i) the protector, (ii) the beneficiary of protection, and (iii) the natural enemy of the beneficiary (Bronstein 1998), all of which substantially varied across populations in this study (context dependence or conditionality - Jones & Callaway, 2007). Moreover, we have also shown in previous section that variation in functional traits of the ant assemblage also conditioned differences in the outcome of the interaction at different populations. Variable outcomes in the ant-plant mutualisms depending of the abundance of ants and level of herbivore damage (Rudgers & Strauss, 2004) has been for example observed for three populations of wild cotton (*Gossypium thurberi*), and some studies have shown that abiotic factors are also important in the output of ant-plant mutualisms (Kersch & Fonseca 2005, with *Inga vera*).

On the other hand, EFNs function has been previously tested in two species of *Anemopaegma* , but results did not show any reduction of herbivore damage or increase in plant reproductive outcome (Nogueira et al. 2011). We have further shown a macroevolutionary pattern of decrease in the abundance of extrafloral nectaries in the tribe Bignonieae resulting from a shift from tropical forest to drier savanna habitats (Nogueira et al. unpublished - chapter 03 of this thesis). Both results together could suggest that the 'inefficiency' of nectaries and its ants, described above, could be a reflection of the establishment of new biotic interactions in the savannas (e.g. modification of the ant and/or herbivores assemblages). In this context it would be expected that the EFNs of *A. album* (and others savanna species of this plant group) would not be 'efficient' in the majority of the sampled populations, if not in all. Our results are in accordance with these predictions, and indicate the majority of populations were not protected efficiently by the ants (seven out of ten populations).

In short, we refuted the first scenario for the macro-ecological and evolutionary outcome of the interaction that we formulated in the Introduction, which stated that if herbivory is a strong pressure for EFNs-bearing plants, then differences among habitat and/or localities in herbivore guild and ant visitor assemblage (composition and abundance) will determine differences among populations in the abundance and function of EFNs. In contrast, we did detect that a higher number of ants (average population abundance) was associated with a decrease in herbivory and an increase in mean population plant performance, suggesting that although not efficient in many populations there is an advantage of being visited by ant protectors. It seems that the variation across populations in plants, ants and herbivores (abundances, identities and ecological/functional services) generated a geographic mosaic of the ant-plant interactions. We next discuss this geographic mosaic and its macro-ecological and evolutionary implications.

The geographic mosaic in the interaction of A.album and its ant-guarders

The degree of fit between the average value of EFNs traits (abundance) and functional traits of the ant assemblage (size and recruitment) in each population revelled at least five matched populations of *A.album* (and five mismatched). Matching might arise from adaptive response to the selective pressure on EFNs exerted by efficient ant protection against herbivores, but it may also arise randomly or by drift, that is, not being adaptive. In absence of other information, local adjustments between ant functional traits and EFNs may be tentatively considered to be adaptive wherever a matched population has considerable higher performance than mismatched populations and, in any case, accounts for minimum values of herbivore damage among the populations studied. Such populations where local adjustment of ant assemblages and EFNs functional traits is conducive to enhanced performance may be considered evolutionary hotspots to distinguish them from matched populations but with low plant performance in which matching was not adaptive (coldspots). Mismatched populations are indicative also from evolutionary coldspots, especially where connected to low performance values.

In our ant-plant interaction system, 3 out of 5 matched populations may be considered hotspots since they were the most visited by ants, the less damaged by herbivores and had positive leaf production and the highest abundance of juveniles ('Mirangaba', 'Morro do Chapéu' and 'Grão Mogol').Among these three 'hot-spots', one case was in the south extreme of the *A. album* distribution (‘Grão Mogol’), while the other two cases were in the most northern localities. In these last localities (‘Mirangaba’ and ‘Morro do Chapéu’) close to each other, there was a noticeably discrepancy between them in the nature of the matching: while in ‘Mirangaba’ the EFNs were rare and scattered in the leaflets and attracted to small ants *Crematogaster* with a recruiting behaviour, in 'Morro do Chapéu' they were highly abundant and aggregated in basal part of leaflets and attracted *Camponotus* ants, with low recruitment and bigger body size than *Crematogaster*, which was also the case of the extremely distant population of ‘Grão Mogol’. These two circumstances clearly reflect a geographic mosaic of matched and mismatched populations, with hot and coldspots appearing independently of the geographic distance. Similar patterns were found for a myrmecochore system by Garrido et al. (2002).

The rest of populations (two cases of matching and 5 cases of mismatching) may be interpreted as 'cold-spots'. Many factors, others than the evolutionary/ biogeographic history of the group (i.e. the shift from tropical forest to drier savannas environments), could explain the absence of adjustment between the nectaries and ant assemblages (that is the mismatching), high herbivore damage and the average low plant performance. For example, in the case of the 'Cristália' population, the majority of nectaries were monopolized by ants of genus *Camponotus,* sharing with the ants of genus *Cephalotes*. *Cephalotes* ants are known as commensals or parasites (Byk & Del-Claro, 2010), and they may be interpreted as costs rather than benefits for the interaction. On the other hand, it may be argued that the cost of EFNs and its secretion would be very low in savanna-like environments (usually with abundant light and a large reserve of carbohydrates in plants), maintaining the variation of EFNs even though these structures would be inefficient or ineffective in reducing herbivory under such conditions (almost like a ‘‘neutral’’ trait).

Another important driver for mismatching and evolutionary cold-spots occurrence in this interaction was the type of herbivores. The occurrence of herbivores ‘immunes’ to ant presence that depauperated plant foliage could explain the inefficiency of the ants in many sites. Three out of 4 more frequent herbivores on the plants of *A. album* were indifferent to the interaction with the most frequent ants. This indifference was observed in herbivores and *Camponotus* encounters. Therefore, the populations of 'Caetité', 'Mucugê', 'Palmeiras' and 'Ponte' were intensely attacked by 4 herviores, differently to other populations. This strong effect of the ‘almost immune' herbivores to the ants, if prolonged through time, may have prevented an adaptive fit between the ants and the extrafloral nectaries in these populations. In any case, the coldspots nature of these populations may reside also in the overall low abundance of ants visiting plants in the populations. This was the case of 'Caetité', 'Cristália' and 'Mucugê' (Figure 5). All these populations had high presence of herbivores and received severe foliage damage, having also negative leaf production.

Little is known about the causes of the high leaf deficit in the populations of 'Rio de Contas', 'Mato Verde (two matching but coldspots localities) and "Abaíra'. All 3 populations had great quantity of nectaries, intermediate abundance of ants, and at the same time presented a deficit in the production of leaves. Other environmental, or even unknown physiological factors could have interfered in the plant-ant interaction in these populations more than what was possible to observe. The abiotic factor could affect the interactions in different ways (Kersch & Fonseca 2005). Moreover, the high number of EFNs in theses populations could arise by gene flow from other populations. Source populations efficiently defended by ants (i.e., hotspots) might be supplying the genetic variation necessary for the presence and abundance of EFNs through gene flow in populations not efficiently defended by ants (i.e., coldspots) (Thompson, 2005). Similarly, temporal variation of the system could generate momentarily inefficient populations defended from time to time by ants, leading to maintenance of EFNs even during the periods in which these structures were inefficient.

In short, our findings were in accordance with the expectances of the GMT. Geographic variation in the partners involved in this plant-animal interaction originated a macroecological and evolutionary outcome which conforms a mosaic of matching and mismatching between functional traits of the ant assemblages and EFNs of the plants, with evolutionary hot and coldspots. Many different mechanisms, beside the geographic variation of partners, contribute to generate such a mosaic, including the biotic and abiotic conditioning of the outcome of the interaction.

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TABLES

Table 1. Variation of EFN traits (population average ± SE) in each of the 10 populations of *A. album* (Bignonieae, Bignoniaceae) sampled.

Population sampled	EFN traits					
	Size* (mm)	Abundance (N= 27-32)			Nectar (N=15)	
		Abaxial (base aggregated)	Abaxial (scattered)	Adaxial (scattered)	Volume	% Sugar
Abaira	0.148 ± 0.009	23.6 ± 4.0	0	0.05 ± 0.02	0.13 ± 0.08	55.6 ± 5.7
Caetité	0.122 ± 0.006	17.1 ± 2.0	0.07 ± 0.03	1.28 ± 0.25	0.74 ± 0.32	27.1 ± 3.0
Cristália	0.170 ± 0.010	28.3 ± 3.3	0.28 ± 0.06	0.84 ± 0.12	1.82 ± 0.64	21.3 ± 4.2
Grão Mogol	0.132 ± 0.006	38.3 ± 2.3	0.64 ± 0.20	0.78 ± 0.14	1.17 ± 0.43	33.1 ± 8.2
Mato Verde	0.149 ± 0.006	37.4 ± 3.5	1.89 ± 0.35	1.46 ± 0.17	4.67 ± 2.15	13.7 ± 1.3
Mirangaba	0.145 ± 0.005	1.6 ± 0.2	0.09 ± 0.08	0.22 ± 0.03	$0.01 \pm 1*10^{-3}$	15.0 ± 0.1
Morro do Chapéu	0.176 ± 0.004	39.9 ± 2.1	0.01 ± 0.004	0.21 ± 0.03	1.46 ± 0.51	24.2 ± 1.9
Mucugê	0.154 ± 0.005	31.8 ± 4.6	0.92 ± 0.30	0.25 ± 0.05	0.59 ± 0.29	36.1 ± 4.5
Palmeiras	0.117 ± 0.003	42.7 ± 3.8	1.40 ± 0.29	0.44 ± 0.04	1.06 ± 1.05	37.5 ± 7.2
Rio de Contas	0.117 ± 0.003	32.2 ± 2.6	1.18 ± 0.17	0.48 ± 0.05	5.41 ± 1.69	18.8 ± 2.2

Size* = diameter of the largest EFNs at the base of the abaxial surface of the leaflet blade (region of nectary clustering in the leaflets of *A. album*).

Table 2. Proportional similarity (PS) between ants and herbivorous assemblages of *A. album*. Above the diagonal: ant assemblages (identified at the generic level). Below the diagonal: herbivorous assemblages (identified to the species level). A bootstrap resampling procedure was used to assess if the PS-values shown differed significantly from zero ($P \leq 0.05$). PS-values different from zero are presented in bold.

	Similarity									
Populations	MUC	ABA	MIR	MCH	PAL	CAE	RCO	MVE	GMO	CRIS
Mucugê (MUC)	-	**0.848**	0.133	**0.849**	**0.838**	**0.778**	**0.881**	**0.831**	**0.410**	**0.618**
Abaira (ABA)	0.194	-	0.164	**0.934**	**0.726**	**0.850**	**0.900**	**0.948**	**0.427**	**0.661**
Mirangaba (MIR)	**0.810**	0.191	-	0.147	0.164	0.068	0.209	0.153	**0.705**	0.164
Morro do Chapéu (MCH)	0.097	0.333	0.081	-	**0.700**	**0.915**	**0.840**	**0.969**	**0.423**	**0.596**
Palmeiras (PAL)	0.109	0.405	0.114	**0.749**	-	**0.615**	**0.781**	**0.709**	**0.427**	**0.658**
Caetité (CAE)	**0.457**	**0.451**	**0.474**	**0.587**	**0.640**	-	**0.755**	**0.902**	**0.364**	**0.511**
Rio de Contas (RCO)	0.085	0.360	0.092	**0.914**	**0.787**	**0.607**	-	**0.854**	**0.472**	**0.693**
Mato Verde (MVE)	0.328	**0.438**	**0.369**	**0.699**	**0.676**	**0.871**	**0.711**	-	**0.427**	**0.609**
Grão Mogol (GMO)	0.120	0.413	0.151	**0.825**	**0.848**	**0.661**	**0.890**	**0.778**	**-**	**0.427**
Cristália (CRIS)	0.189	**0.446**	0.229	**0.834**	**0.686**	**0.727**	**0.805**	**0.854**	**0.803**	**-**

Table 3. Herbivores responsible for major damage in plants of *A. album* in each population. Whenever hebivores presented an increase in abundance at $t_{(1)}$, data at $t_{(0)}$ was considered. Herbivore abundance is described by the total number of organisms sampled from each population. The % of ant occupation is described as the number of plants occupied/total number of plants sampled (in parentheses). Herbivory was estimated by the amount of leaf tissue consumed.

Populations	Beetle *Charidotis* sp. (Coleoptera)			Beetle *Sumitrosis* sp. (Coleoptera)	Cricket *Xestotrachelus robustus* (Orthoptera)	Herbivory (%)	
	Adults	Larvae (variable instars)	Oviposition			Mean	SE
Abaíra	1 (3%)	5 (3%)	2 (7%)	0 (0)	0 (0)	23.4	6.0
Caetité	15 (17%)	289 (17%)	87 (40%)	0 (0)	278 (17%)	82.5	5.9
Cristália	63 (57%)	606 (53%)	428 (90%)	7 (10%)	190 (17%)	42.4	5.3
Grão Mogol	68 (53%)	516 (63%)	172 (90%)	2 (3%)	49 (7%)	27.3	3.2
Mato Verde	8 (20%)	109 (20%)	46 (50%)	0 (0)	70 (13%)	19.1	2.7
Mirangaba	2 (7%)	0 (0)	10 (13%)	2 (7%)	168 (17%)	9.3	1.1
Morro do Chapéu	5 (17%)	87 (27%)	50 (70%)	8 (23%)	0 (0)	16.7	1.5
Mucugê	7 (17%)	1 (3%)	1 (3%)	53 (20%)	281 (33%)	32.0	3.7
Palmeiras	20 (33%)	343 (30%)	59 (53%)	0 (0)	20 (7%)	22.0	2.4
Rio de Contas	9 (17%)	57 (10%)	27 (27%)	2 (7%)	1 (3%)	26.6	3.8

Table 4. Pearson's product-moment correlation of the abundance and size of EFNs with functional properties of ant assemblages as well as with the mean population fitness, abundance of herbivores and leaflet damage.

Variables	EFNs traits		Ant traits (t_0)		Herbivore abundance (t_0)	Herbivory (%)	Fitness variables	
	Size (mm)	Abundance at the leaflet base	Frequency	Abundance			Production of leaves	Number of saplings
EFN size	-	0.021	0.488	0.300	0.134	-0.260	0.121	-0.131
EFN abundance		-	-0.332	-0.400	-0.374	-0.177	-0.274	-0.049
Ant frequency			-	**0.829***	-0.134	-0.396	0.403	0.468
Ant abundance				-	-0.179	-0.415$^+$	**0.712***	**0.624***
Herbivore abundance					-	0.593**	-0.262	-0.443
Herbivory						-	**-0.640***	**-0.723***
Production of leaves							-	**0.711***
Number of saplings								-

*Significant correlations values are shown in bold ($P \leq 0.05$); **p=0.07; $^+$Significant values ($p<0.05$) only excluding the population 'Caetité' with amounts of herbivory considered as outliers.

Table 5. Simple Mantel statistics (*r*) and the associated probabilities (*p*) for tests of spatial dependency of all variables used to describe ant-plant interactions and fitness components among populations.

Statistics	**EFN traits**		**Ant traits (t_0)**			**Herbivore traits**		Herbivory (%)	Production of leaves	Number of saplings
	Size (mm)	Abundance at the leaflet bases	Frequency	Abundance	PS (composition of ants)	Abundance (t_0)	PS (composition of herbivores)			
R	-0.097	0.153	0.108	0.361	-0.316	-0.129	-0.012	0.032	0.236	-0.002
P	0.716	0.221	0.221	**0.044**	0.938	0.799	0.573	0.401	0.105	0.489

FIGURES

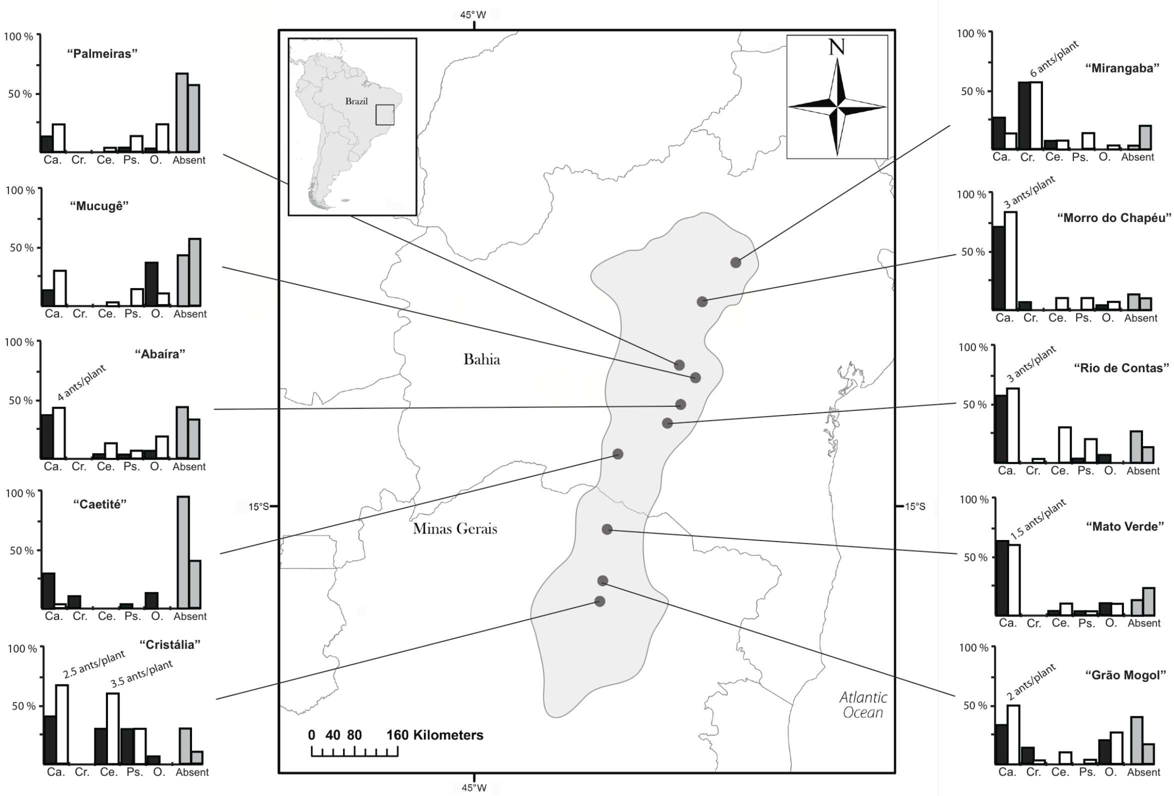

Figure 1. Map with the geographic range of *A. album* (translucent gray). The location of the 10 experimental populations under study is presented. The populations were distributed over 600 Km within areas of Neotropical savannas. Frequency graphs around the map show the ant visitor assemblage at each population, depicted as the percentage of plants occupied by different ant genera: "Ca." = *Camponotus*; "Cr." = *Crematogaster*; "Ce." = *Cephalotes*; "Ps." = *Pseudomirmex*; "O." = others ants (rare species); and "Absent" = plants without ants (unoccupied). Black columns represent the frequency of ants at $t_{(0)}$. White columns represent the frequency of ants at $t_{(1)}$. Gray columns represent the frequency of unoccupied plants in each population ($t_{(0)}$ and $t_{(1)}$ respectively). The average number of ants per plant in each population is presented only to the ant genera with more than 1.5 ants/plant. Populations with the frequency chart on the right side were the most occupied, presenting on average more ants than the populations presented on the left side of the Figure.

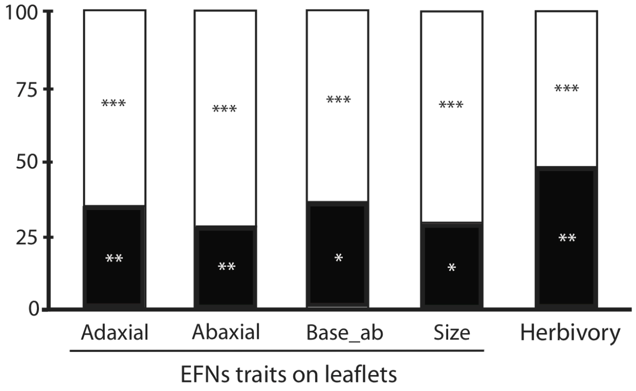

Figure 2. Variance partitioning between populations (black) and within populations (white) in EFN traits and herbivory in *A. album*. All analyses were conducted with the Restricted Maximum likelihood estimation. ***P < 0.001; **P < 0.05; * p = 0.051 (both above).

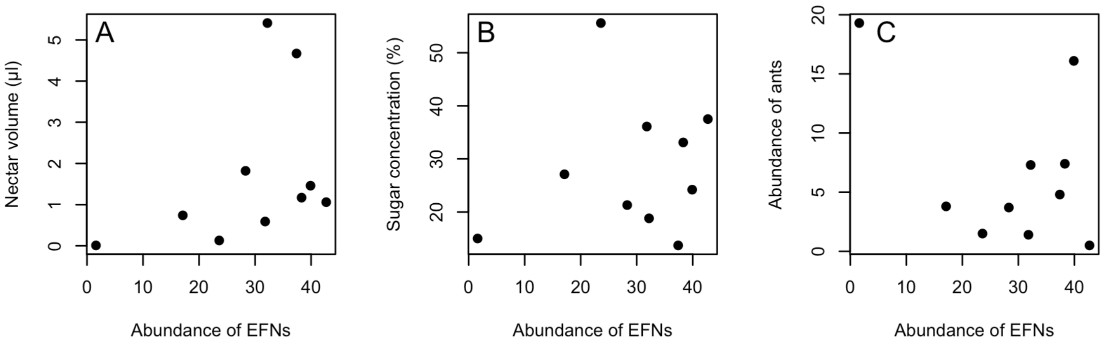

Figure 3. Relationships between EFN traits (A and B: volume, sugar concentration and average abundance of EFNs at the base of leaflets), and (C) abundance of EFNs with the abundance of ants among populations (N=10). No positive relationships across population were observed between EFN traits, and between the abundance of ants, as expected under the functional hypothesis generally accepted for EFN traits.

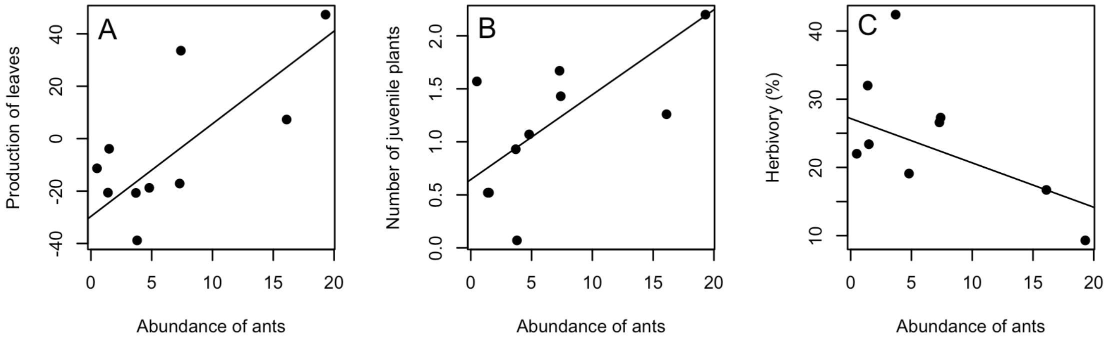

Figure 4. Relationships between ant abundance (t_0) and (A) production of leaves ($F_{(1,8)}$=8.2; *p*=0.02), (B) number of juvenile plants of *A. album* ($F_{(1,8)}$=5.1; *p*=0.05) and (C) foliar damage ($F_{(1,7)}$=4.9; *p*=0.06). In the last graph, the population "Caetité", with herbivory mean of 84 % was removed as it represents an outlier. However, the original results without exclusion of the outlier are presented in Table 5.

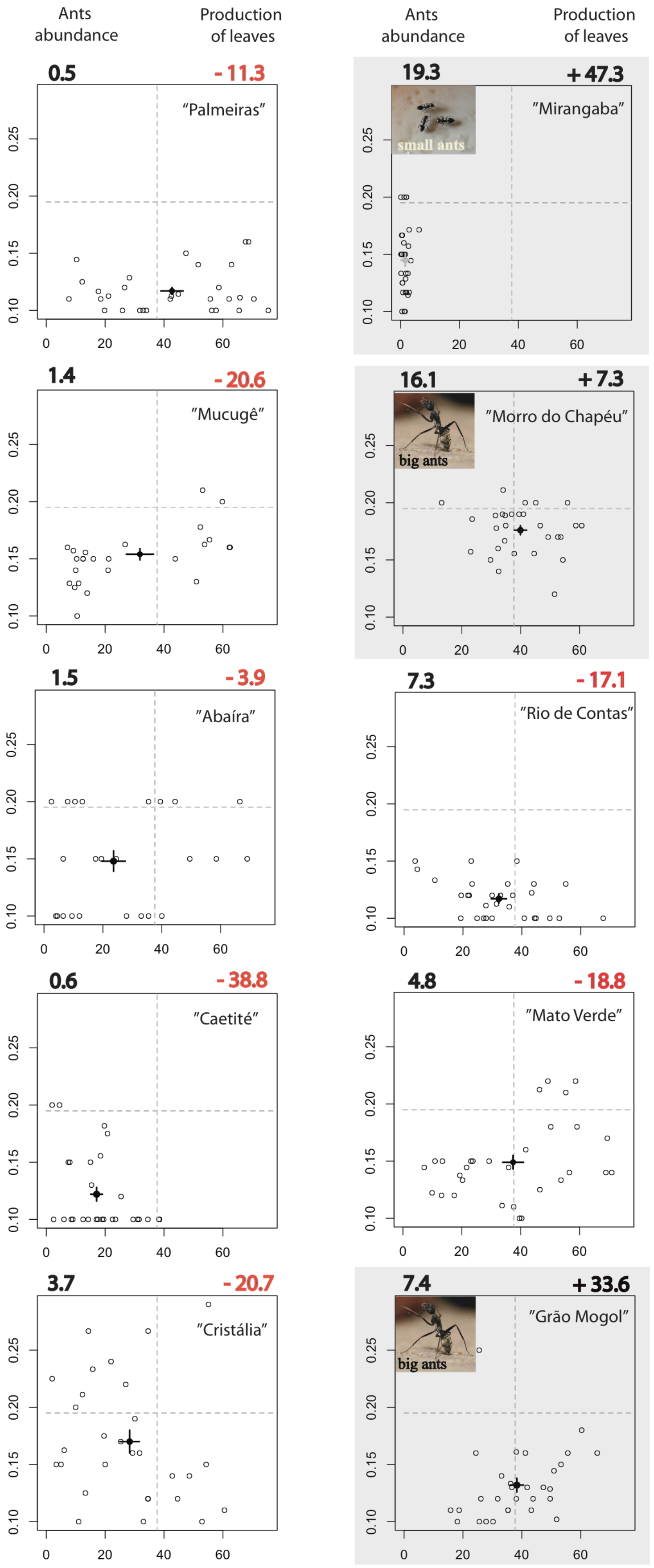
Ants abundance
Production of leaves
Ants abundance
Production of leaves
0.5
- 11.3
"Palmeiras"
19.3
+ 47.3
"Mirangaba"
small ants
1.4
- 20.6
"Mucugê"
16.1
+ 7.3
"Morro do Chapéu"
big ants
1.5
- 3.9
"Abaíra"
7.3
- 17.1
"Rio de Contas"
0.6
- 38.8
"Caetité"
4.8
- 18.8
"Mato Verde"
3.7
- 20.7
"Cristália"
7.4
+ 33.6
"Grão Mogol"
big ants
0.10
0.15
0.20
0.25
0
20
40
60

Figura 5. The mosaic of matched and mismatched populations. Scatterplots characterize the variation of EFNs within and among populations of *A. album*. EFN size (y-axis) and number of EFNs clustered at the leaflets bases (x) are represented for individual plants (a dot in the panel). Each panel corresponds to a single population. Local production of leaves and ant abundance (a functional property of ants denoting recruitment toward a food source) are shown for each locality. Highly negative values in leaf production denote important herbivore damage whereas positive values represent high plant performance. Sites considered as hotspots of interactions, in which ants and EFNs are well-matched, rendering in high plant performance, are shaded. Ants that monopolized the EFNs in each hotspot are presented as inset photographs.

On-line supplementary materials

Appendix 1S. Proposed functional properties of the main groups of ants visiting EFNs. Last column represent some previous ecological examples to which this proposed functional properties of ants are referred to. Quantitative variables are expressed as the mean ± standard error, and range.

Ant characters	Ant genus				References (most relevant examples)
	Camponotus	*Crematogaster*	*Pseudomirmex*	*Cephalotes*	
Ant size (mm)	4.1 ± 0.8 (n=50)	1.2 ± 0.2 (n=10)	5.2 ± 0.4 (n=30)	3.8 ± 0.3 (n=30)	Chamberlain and Holland, 2009; Sendoya et al., 2009.
Ant recruitment (abundance per plant)	3.7 ± 0.3 (n=131) range (1-25)	9.9 ± 3.1 (n=19) range (1-63)	1.3 ± 0.1 (n=34) range (1-3)	3.1 ± 0.5 (n=44) range (1-16)	Bronstein, 1998; Rudgers & Strauss, 2004.

Considerações finais e perspectivas

Neste trabalho procuramos explorar diversos aspectos da evolução e ecologia dos tricomas distribuídos na superfícies das plantas nas partes vegetativas de espécies da tribo Bignonieae (Bignoniaceae).

Quatro morfotipos de tricomas foram identificados nas 105 espécies da tribo Bignonieae amostradas: tricomas não-glandulares, glandular peltados, glandular estipitados e glandular pateliforme/cupulares. O detalhamento morfológico e análises filogenéticas comparativas revelaram que a maior parte destes tricomas já estavam presentes no ancestral da tribo, exceto os tricoma glandular estipitado o qual evoluiu posteriormente. Tais resultados favoreceram o estabelecimento de uma terminologia unificada para os diversos tipos de tricomas, de forma a facilitar estudos funcionais, ecológicos, e evolutivos dos tricomas na tribo Bignonieae.

Os tricomas pateliformes ou nectários extraflorais de duas espécies de *Anemopaegma* (*A. album* e *A. scabriusculum*) atraíram formigas nas savanas do norte de Minas Gerais, mas não foram efetivos no aumento da resistência foliar frente aos herbívoros. Neste caso em particular, os herbívoros foram muito danosos as plantas e pareceram especializados nas espécies de *Anemopaegma* (observações de 2 anos). Dentro deste cenário, é possível que a invasão dos ancestrais de *Anemopaegma* das florestas úmidas para savanas tenham modificado as interações formiga-planta-herbívoro, de forma que o balanço entre o custo-benefício desses nectários tivesse sido alterado, tornando tais estruturas geralmente 'ineficientes' em áreas de savanas.

O fato de múltiplas 'invasões' independentes de plantas das florestas úmidas terem ocorrido para as savanas durante a história de diversificação das espécies de Bignonieae favoreceu o teste da hipótese de modificação do número de tricomas pateliformes (nectários extraflorais) em paralelo às transições de ambientes florestais para savanas, e o teste de hipóteses de "trade-off" entre os nectários e o surgimento de outros tipos de tricomas. De uma forma geral, quanto maior o número de nectários, maior o número de formigas encontradas visitando as plantas. Além disso, a evolução dessas estruturas desviou do modelo neutro de evolução (movimento browniano), com dois fatores principais explicando esse desvio: a 'ocupação/invasão' das plantas de floresta para as savanas, e o surgimento de tricomas glandulares adesivos, os quais tiveram um efeito semelhante na diminuição do número de nectários nas plantas. Esses resultados sugerem que mudanças de habitat e o surgimento de novos tipos de tricomas podem ter "quebrado" interações que foram efetivas no aumento da resistência das plantas no passado.

Populações distintas de *Anemopaegma album* distribuídas do norte de Minas Gerais até a região central da Bahia foram muito variáveis em termos das interações formiga-planta-herbívoro e das características dos nectários extraflorais entre as 10 populações amostradas. Não foram encontradas correlações entre os descritores dos nectários, as formigas, a herbivoria e as variáveis de performance das plantas entre populações. Por outro lado, a abundância de formigas esteve negativamente relacionada com a herbivoria, e positivamente relacionada com as variáveis de performance. Nem a abundância de nectários, nem as formigas ou mesmo a herbivoria estiveram estruturados espacialmente. Ambos resultados corroboram a hipótese de mosaico geográfico entre formigas e plantas. Cinco populações das dez amostradas tiveram os nectários acoplados (do inglês, "matched") com as formigas visitantes. Destas cinco populações, somente três tiveram uma produção positiva de folhas e baixa herbivoria. Duas dessas populações tiveram alta abundância de nectários nas folhas e foram dominadas por formigas do gênero *Camponotus* (maiores em tamanho, e com baixa capacidade de recrutamento). Já a terceira população apresentou a menor abundância de nectários nas folhas, e o maior número de formigas por planta (e maior freqüência), as quais pertenciam predominantemente ao gênero *Crematogaster* (menores em tamanho mas com grande capacidade de recrutamento). Neste contexto, as três populações cujas interações formiga-planta eram acopladas e cujas populações atingiram os valores mais altos das variáveis de performance foram consideradas "hot-spots" das interações, enquanto as demais foram consideradas "cold-spots".

Todos esses resultados sugerem que as interações formiga-planta, e o papel funcional dos nectários extraflorais, tenham sido efetivos por diversas vezes nas florestas mais úmidas no passado (e também hoje), mas pouco efetivos (ou raramente) nas savanas deste grupo de plantas em particular. É provável que o ancestral da tribo Bignonieae tivesse um número baixo de nectários, os quais não apresentavam distribuição agregada e estavam distribuídos sobre os folíolos exclusivamente (Capítulo 1). A presença de apenas poucos nectários dispersos no limbo foliar geralmente leva a uma baixa atração de formigas no ambiente florestal (Capítulo 3), e provavelmente não conferiram um papel defensivo no ancestral da tribo Bignonieae. No entanto, essa estrutura inicial provavelmente representou uma pré-adaptação para uma morfologia subsequente no grupo que realmente tornou-se efetiva contra a herbivoria no ambiente florestal, nas regiões de agregação de nectários nos profilos da gema axilar, na região interpeciolar dos ramos, e na base da face abaxial dos folíolos (mais raramente nos pecíolos e peciólulos na tribo Bignonieae). No entanto, a falta de testes experimentais sobre o papel defensivo dos nectários extraflorais e das formigas visitantes em espécies de plantas florestais da tribo Bignonieae ainda deixa essa questão em aberto no ambiente florestal mais úmido.

Além disso, as interações formiga-planta das florestas foram dominadas (> 50% das interações por espécie de Bignonieae) essencialmente por formigas do gênero *Crematogaster* (Capítulo 3). Coincidentemente, na abordagem "entre populações" da espécie *Anemopaegma* *album* de savana (Capítulo 4), a população com maiores valores de performance e menor herbivoria foi a população em que as interações formiga-planta foram dominadas também por formigas do gênero *Crematogaster* (população 'Mirangaba'). Ambos resultados levantam a questão sobre o papel particular destas formigas nas interações com as plantas da tribo Bignoineae. Testes experimentais sobre o efeito particular das *Crematogaster* sobre a herbivoria, separadamente das demais formigas, contribuiriam com dados importantes para o esclarecimento do aspecto coincidente revelado em nossos resultados.

Todas essas novas questões tornaram o sistema formiga-planta-herbívoro mediado pelos nectários extraflorais (tricomas peteliformes/cupulares glandulares) em Bignoniaceae, e em especial em Bignonieae, extremamente interessantes para os estudos futuros. O conjunto de informações levantados nessa tese, torna possível a formulação de experimentos e perguntas mais refinadas dentro desse sistema tropical em particular. Espera-se assim, que essa tese seja somente o primeiro passo no estudo evolutivo das interações formiga-planta em Bignoniaceae como um todo, e seu papel frente aos herbívoros, e outros processos relevantes no ciclo de vida das plantas.