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Interações evolutivas entre borboletas da tribo Troidini (Papilionidae, Papilioninae) e suas plantas hospedeiras no gênero *Aristolochia* (Aristolochiaceae)

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"The scientist does not study nature because it is useful to do so. He studies it because he takes pleasure in it; and he takes pleasure in it because it is beautiful. If nature were not beautiful, it would not be worth knowing and life would not be worth living".

Henri Poincaré, *The value of Science*

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"O que tem que ser tem muita força".

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Introdução Geral

É reconhecidamente imensa a contribuição dos insetos em qualquer levantamento sobre diversidade animal, e dentre estes, a dos insetos fitófagos. Estima-se que 70% dos insetos sejam parasitóides ou parasitas, dos quais cerca de 50% parasitas de plantas, a maioria em um ou poucos hospedeiros relacionados (Price, 1980). Esta proporção pode ser explicada pela diferenciação dos insetos fitófagos sobre uma imensa diversidade de recursos gerados pela radiação das angiospermas, de modo que teria sido através de adaptações às plantas hospedeiras que as populações de fitófagos se diferenciaram e por fim especiaram (Gillot, 1995). Estudos filogenéticos de linhagens de insetos confirmam que grupos de insetos herbívoros são significativamente mais diversos do que seus grupos irmãos não-fitófagos, confirmando a importância das plantas na sua diversificação (Mitter *et al.*, 1988).

Interações entre insetos herbívoros e suas plantas hospedeiras têm sido estudadas por um longo tempo. Muitos estudos enfocam principalmente a evolução destas interações, considerando aspectos como o conservantismo taxonômico na utilização de hospedeiros (Bernays, 1998; Janz *et al.*, 2001), e se um "ponto final" desta evolução seria uma especialização total, levando a um "beco sem saída" evolutivo (Futuyma e Moreno, 1988). Espécies de insetos relacionadas taxonomicamente freqüentemente se alimentam em plantas hospedeiras relacionadas, embora uma concordância precisa entre a filogenia dos insetos e das plantas seja rara (Ehrlich e Raven, 1964; Holloway e Hebert, 1979; Mitter *et al.*, 1991; Janz e Nylin, 1998; Lopez-Vaamonde *et al.*, 2003). Este conservantismo é influenciado basicamente pelos compostos secundários encontrados nas plantas (Jaenike, 1990; Futuyma *et al.*, 1993; Bernays, 1998), e Fraenkel (1959) inclusive afirma que o uso discriminatório de certas plantas pelos insetos seria a razão de ser destas substâncias, que agem induzindo ou prevenindo a obtenção de nutrientes por insetos herbívoros. Embora os

compostos secundários sejam definitivamente importantes em limitar o uso de certas plantas como hospedeiros, não é claro se este papel é desempenhado pela presença de compostos que impedem a alimentação em plantas não-hospedeiras ou pela ausência de estimulantes específicos encontrados no hospedeiro (Bernays, 1998). No entanto, algumas evidências sugerem que a função positiva dos químicos é que é importante (Bernays, 1998).

Outros fatores intrínsecos e extrínsecos também podem limitar o uso de certas plantas como hospedeiros para insetos herbívoros (Ronquist e Nylin, 1990), como, por exemplo, preferências de oviposição das fêmeas (Chew e Robbins, 1984) e a sobrevivência das larvas em diferentes plantas. As lagartas de muitas espécies de borboletas, por exemplo, crescem e sobrevivem melhor em um número limitado de espécies hospedeiras, e como elas não se deslocam a grandes distâncias, são dependentes da acuidade da fêmea na escolha do local de oviposição para sobreviverem (Feeny *et al.*, 1983). A distribuição geográfica das espécies é outro fator que pode limitar o uso de plantas como hospedeiros, pois um herbívoro não pode comer uma espécie de planta que ele nunca encontra (Pasteels e Rowell-Rahier, 1991; Dobler *et al.*, 1996; Kelley e Farrell, 1998). Além disto, os nutrientes da planta podem desempenhar um papel importante nesta escolha (Bernays, 1998).

Ehrlich e Raven (1964) discutem o conservantismo na utilização de plantas hospedeiras por insetos herbívoros, e propõem a hipótese de coevolução para explicar o passo-a-passo da evolução desta interação, onde plantas teriam desenvolvido resistência em relação a possíveis predadores e estes, por sua vez, teriam desenvolvido adaptações para tolerância dessas defesas. Segundo estes autores, as plantas teriam desenvolvido compostos secundários como conseqüência de pressões seletivas e os insetos herbívoros, ocasionalmente, desenvolveram adaptações a grupos específicos de compostos secundários que lhes permitiram ultrapassar estas barreiras. Defesas químicas conferidas por estes compostos secundários são superadas pelos insetos através de mecanismos fisiológicos e comportamentais, tais como uma alta sensibilidade às características específicas do hospedeiro

(Bernays, 1998). Ehrlich e Raven (1964) propõem ainda que através do modelo de coevolução, os insetos podem ser capazes de desintoxicar certas substâncias químicas de defesa da planta e, eventualmente, os mesmos compostos que impedem o uso da planta podem se tornar atrativos para alimentação.

A definição estrita de coevolução implica em uma resposta adaptativa de duas ou poucas espécies interagindo a mudanças evolutivas entre si (Janzen, 1980). No caso de insetos fitófagos, as interações parecem ser o produto de coevolução difusa, que seria um processo evolutivo em resposta a uma série de pressões seletivas (Thompson, 1990). Na coevolução difusa, as defesas vegetais teriam surgido para impedir ataques tanto de insetos fitófagos quanto de outros inimigos naturais, e não apenas de uma espécie de inseto. Da mesma forma, as adaptações dos insetos não podem ser atribuídas à sua interação com uma única espécie de planta.

Além da hipótese de coevolução, outras hipóteses foram propostas para explicar a evolução da interação entre herbívoros e plantas hospedeiras. Ronquist e Nylin (1990) propõem outros modelos além daquele proposto por Ehrlich e Raven (1964). No modelo *Arms Race tipo I* (com mudanças ocorrendo em um tempo microevolutivo), os efeitos dos mecanismos de defesa das plantas e de contra-defesa dos herbívoros é menos drástico do que no modelo proposto por Ehrlich e Raven (1964) (com mudanças ocorrendo em um tempo macroevolutivo), ou seja, a defesa nas plantas pode não excluir totalmente os insetos que as usam. No modelo de *Especialização*, a tendência geral em direção à especialização nos insetos teria ocorrido independentemente das mudanças evolutivas nas plantas. No modelo de *Colonização Tardia* (Jermy, 1984, 1993; Percy *et al.*, 2004), a colonização teria ocorrido em grupos de plantas já existentes, de forma que os grupos de insetos seriam muito mais jovens do que os grupos de plantas dos quais eles se alimentam. No modelo *Oportunista*, os insetos teriam se mudado tão freqüentemente para novas plantas hospedeiras, algumas vezes não relacionadas, que o padrão histórico se perdeu.

O conceito de que a especialização pode ser um "beco sem saída" evolutivo tem sido central em estudos sobre a evolução de interações entre plantas hospedeiras e inseto herbívoros. Vários fatores podem promover especialização, incluindo diferenças de desempenho em diferentes hábitats, competição por recursos, resistência a predadores, alto custo no processamento de informações, encontro de parceiros, baixos custos na procura por hábitats favoráveis e mutações deletérias com expressão em hábitats específicos (Nosil, 2002). Em contraste, hábitats raros ou imprevisíveis, dificuldades em encontrar hábitats favoráveis e maior disponibilidade de recursos podem favorecer um hábito generalista. A especialização pode ser vantajosa de várias maneiras. Por exemplo, herbívoros especialistas podem desenvolver uma simplificação na cadeia biossintética de sinais químicos (Bernays, 1996). Da mesma maneira, insetos especialistas são capazes de investir menos em enzimas de desintoxicação para lidar com um número menor de compostos químicos das suas plantas hospedeiras (Futuyma e Moreno, 1988) e, em um ambiente competitivo, especialistas podem ser capazes de excluir generalistas se os primeiros forem consumidores mais eficientes (Futuyma e Moreno, 1988). Além disto, especialistas são capazes de responder mais prontamente aos químicos encontrados nas plantas que impedem a alimentação ou são repelentes (Bernays, 1998). Em adição, ser um especialista pode permitir a um herbívoro selecionar entre vários compostos secundários da planta, e fazer uso deles como pistas para a escolha da planta hospedeira (Bernays, 1996).

A interação entre borboletas da tribo Troidini (Papilionidae, Papilioninae) e as plantas do gênero *Aristolochia* (Aristolochiaceae, Aristolochioideae) tem sido amplamente usada na literatura como exemplo de coevolução entre insetos herbívoros e suas plantas hospedeiras. Brown *et al.* (1995) inclusive demonstram uma evidência de que as borboletas Troidini filogeneticamente basais usam preferencialmente as espécies de *Aristolochia* basais, enquanto espécies de borboletas derivadas usam espécies de hospedeiros derivados, sugerindo assim uma ligação coevolutiva entre os dois grupos. As características desta associação concordam com muitas das premissas da

hipótese coevolutiva: lagartas de borboletas Troidini se alimentam quase exclusivamente de plantas hospedeiras do gênero *Aristolochia*, com forte preferência de certas espécies de Troidini por espécies particulares de *Aristolochia*, e são conhecidas por seqüestrarem compostos secundários presentes nas plantas hospedeiras (Klitzke e Brown, 2000). Estes compostos químicos tornam as borboletas impalatáveis para predadores potenciais (Brower e Brower, 1964; Rothschild *et al.*, 1970) e alguns autores sugerem que os ácidos aristolóquicos seqüestrados das plantas hospedeiras protegem quimicamente estas lagartas do ataque de predadores e parasitóides (Nishida e Fukami, 1989a; Sime, 2002). Os compostos químicos seqüestrados pelas larvas são mantidos até a fase adulta, e as borboletas adultas, assim como as lagartas, advertem sua impalatabilidade através de coloração de advertência, o que as tornam modelos em anéis miméticos (Tyler *et al.*, 1994; Sime *et al.*, 2000). Estas borboletas são mimetizadas por um grande número de outras borboletas Papilionidae, e também por borboletas Pieridae, Satyrinae, Nymphalidae e grupos de mariposas com comportamento diurno (Brown *et al.*, 1991). Estes mesmos compostos químicos podem ainda funcionar como estimulantes para alimentação em borboletas Troidini (Chew e Robbins, 1984; Nishida e Fukami, 1989b). A íntima associação entre Troidini e *Aristolochia* faz desta interação um modelo bastante apropriado para estudos sobre a evolução da interação entre herbívoros e plantas hospedeiras.

As borboletas Papilionidae

As borboletas Papilionidae (swallowtail butterflies) possuem distribuição cosmopolita e a família é composta por aproximadamente 580 espécies divididas em três sub-famílias: Baroniinae, com um único gênero e espécie ocorrendo no México (*Baronia brevicornis*), Parnassiinae (com distribuição Holártica) e a cosmopolita Papilioninae (Scriber, 1995). A maioria das espécies é tropical e algumas possuem importância econômica como pragas de culturas cítricas e de

umbelíferas, além de serem estudadas quanto à sua ecologia (Brown *et al.*, 1981; Spade *et al.*, 1988; Morais e Brown, 1991), evolução (Aubert *et al.*, 1999; Caterino e Sperling, 1999; Morinaka *et al.*, 1999; Reed e Sperling, 1999; Morinaka *et al.*, 2000; Caterino *et al.*, 2001; Kondo e Shinkawa, 2003; Zakharov *et al.*, 2004a; Zakharov *et al.*, 2004b), comportamento (Rausher, 1978; Stamp, 1986), genética (Sperling, 1993; Bossart, 1998) e ecologia química (Rothschild *et al.*, 1970; Urzúa e Priestap, 1985; Nishida *et al.*, 1993; Honda e Hayashi, 1995; Klitzke e Brown, 2000; Sime *et al.*, 2000).

Lagartas de papilionídeos possuem uma glândula epidérmica eversível localizada próximo à cabeça chamada osmetério, que é evertida sempre que a lagarta é perturbada (Stamp, 1986). Produtos voláteis presentes na secreção da glândula podem repelir alguns predadores, sendo particularmente efetivos contra predadores invertebrados, como formigas e mantídeos. Secreções do osmetério incluem uma diversidade de terpenóides que têm um forte aroma (Bowers, 1993).

Nos papilionídeos, a fêmea escolhe com grande cuidado a planta em que vai ovipositar e a posição exata dos ovos (Tyler *et al.*, 1994). Nestas borboletas, visão e respostas olfatórias às plantas hospedeiras estão envolvidos no comportamento de oviposição (Rausher, 1978; Sachdev-Gupta *et al.*, 1993) e, de fato, muitas espécies hospedeiras de papilionídeos são proximamente relacionadas, taxonômica e quimicamente. Os compostos químicos que estimulam oviposição são complexos, consistindo de diferentes classes de substâncias tais como flavonóides, ácidos carboxílicos e compostos básicos (Nishida, 1995).

A maior sub-família de Papilionidae é Papilioninae, com cerca de 485 espécies (Haüser *et al.*, 2002), que subdivide-se por sua vez em três tribos: Troidini, Graphiini e Papilionini.

Troidini e Aristolochia

A tribo Troidini é predominantemente tropical, com muitas espécies concentradas nas florestas das Américas Central e do Sul, e na região IndoAustraliana (Weintraub, 1995). Os Troidini incluem 130 espécies divididas em 12 gêneros, três deles com ocorrência neotropical: *Battus* (11 espécies), *Euryades* (2 espécies) e *Parides* (34 espécies) (Tyler *et al.*, 1994). Borboletas destes três gêneros são os únicos Troidini encontrados no Brasil. As borboletas Troidini adultas voam lentamente, e apresentam marcas vermelhas ou amarelas em suas asas e corpos (Brown *et al.*, 1991). Troidini é longamente reconhecido como um grupo natural, principalmente devido à morfologia bastante homogênea das suas larvas e pupas (Miller, 1987).

A família Aristolochiaceae (composta por sete gêneros e aproximadamente 460 espécies) distribui-se principalmente pelos trópicos e regiões temperadas (Judd *et al.*, 2002) e as plantas são ervas, lianas ou ocasionalmente arbustos, com folhas alternas, simples e pecioladas, com formas muito variadas. O gênero *Aristolochia* é o mais importante dentro da família Aristolochiaceae, e conta com aproximadamente 400 espécies (Kelly e Gonzáles, 2003), com cerca de 90 ocorrendo no Brasil (Leitão e Kaplan, 1992). As plantas neste gênero são sub-arbustos ou lianas, e contêm caracteristicamente ácidos aristolóquicos (nitrofenantrenos) que têm efeitos farmacológicos em vertebrados (Brown et al. 1981). As plantas também contêm alcalóides benzilisoquinolínicos e mono-, sesqui-, di e triterpenos (Teresa *et al.*, 1983; Lopes *et al.*, 1987; Lopes e Bolzani, 1988; Lopes *et al.*, 1990; Luiz *et al.*, 1990; Leitão e Kaplan, 1992; Leitão *et al.*, 1992; Vila *et al.*, 1997; Bomm *et al.*, 1999; Palmeira *et al.*, 2001; Wu *et al.*, 2001; Priestap *et al.*, 2002; Tsuruta *et al.*, 2002; Priestap *et al.*, 2003; Francisco *et al.*, 2004; Shi *et al.*, 2004; Wu *et al.*, 2004).

Klitzke (1992) separou *Aristolochia* em três grupos, de acordo com suas características químicas: a) espécies com ácidos aristolóquicos nas folhas (*A. sessilifolia*, *A. melastoma*, *A. rumicifolia*, *A. arcuata*, *A. macroura* e *A. triangularis*), b) espécies sem ácidos aristolóquicos nem

ácidos labdanóicos nas folhas (*A. odora* e *A. elegans*) e c) espécies sem ácidos aristolóquicos e com ácidos labdanóicos nas folhas (*A. esperanzae*, *A. cymbifera* e *A. galeata*). A presença ou ausência destes compostos pode ser um dos fatores que influenciam a utilização das diferentes espécies de *Aristolochia* pelas espécies de Troidini (Klitzke, 1992).

Pela análise química de 17 espécies de Troidini, Klitzke (1992) mostrou a existência de ácidos aristolóquicos em todas as espécies, em concentrações muito variáveis. A análise de imagos de seis espécies destas borboletas da região de Campinas demonstrou que estes organismos apresentam alcalóides, além dos ácidos aristolóquicos, sendo que a concentração de alcalóides é muito superior à de ácidos aristolóquicos.

A química de *Aristolochia* pode influenciar os padrões de exploração por Troidini, permitindo a identificação das plantas hospedeiras adequadas (Brown *et al.*, 1981). Testes com cinco espécies destas borboletas mostraram que as fêmeas não ovipõem igualmente em todas as espécies de planta hospedeira disponíveis (Brown *et al.*, 1981). Além disto, existem evidências de que as lagartas podem apresentar diferentes reações às várias plantas escolhidas pelas fêmeas (Morais e Brown, 1991; Klitzke, 1992).

As relações das borboletas da tribo Troidini com suas plantas hospedeiras são extremamente importantes na consideração da evolução do grupo. O desenvolvimento de métodos filogenéticos, principalmente para estudos sobre padrões e taxas de evolução de caracteres, tem fornecido novas ferramentas para investigar estes e outros aspectos da interação entre plantas hospedeiras e insetos herbívoros (Mitter *et al.*, 1991; Janz *et al.*, 2001). A análise de filogenias permite inferir padrões de radiação dos insetos e plantas, ainda que não ocorra, necessariamente, uma cladogênese paralela (Futuyma e Keese, 1992). Estas análises fornecem evidências das taxas e padrões de diversificação e especialização, permitindo inferências sobre processos ecológicos e evolutivos. Além disto,

permitem também diferenciar os caracteres que surgiram por convergência adaptativa daqueles que são compartilhados por origem comum (Maddison e Maddison, 1999).

Além da análise filogenética propriamente dita, uma série de dados podem ser submetidos à análise fenética. Através de matrizes de distância e similaridade, pode-se construir dendrogramas que refletem relações fenéticas, de forma que é possível inferir até que ponto estas relações são congruentes com as relações filogenéticas dos grupos em questão (Becerra, 1997; Becerra e Venable, 1999). Matrizes de similaridade podem ser elaboradas com dados genéticos, morfológicos, bioquímicos, ecológicos, geográficos, entre outros. Estas matrizes podem ser construídas tanto para os insetos como para as plantas e comparadas estatisticamente, assim como se podem buscar correlações entre eles.

Sendo assim, este trabalho primeiramente apresenta uma filogenia molecular proposta para as borboletas Troidini do Novo Mundo dos gêneros *Battus*, *Euryades* e *Parides* (Figura 1), baseada em três genes, dois mitocondriais (citocromo oxidase I e II) e um nuclear (Fator de Elongação-1α) (Capítulo 1). Sobre esta hipótese filogenética, estudou-se a evolução de quatro caracteres morfológicos e ecológicos, com ênfase na utilização de plantas hospedeiras do gênero *Aristolochia*, procurando-se mostrar uma possível tendência em direção a uma maior especialização no uso de hospedeiros. O Capítulo 2 estuda as interações químicas e filogenéticas entre plantas do gênero *Aristolochia* que ocorrem no sudeste do Brasil e são utilizadas como hospedeiros por borboletas Troidini simpátricas (Figura 2). Estudou-se o padrão de sesquiterpenos destas plantas com o objetivo de se gerar um dendrograma de similaridade química, e as análises filogenéticas foram propostas com base no gene cloroplástico *mat*K e de uma região não-codificadora entre os genes *trn*L-*trn*F. Por fim, o Capítulo 3 estuda a história evolutiva da interação entre Troidini e *Aristolochia* e o padrão atual de utilização de hospedeiros, com enfoque para as espécies de borboletas com ocorrência no sudeste do Brasil, para as quais se têm dados mais completos sobre a

utilização de plantas hospedeiras, bem como sobre os compostos químicos destas plantas e sua distribuição geográfica. Neste capítulo, as filogenias de Troidini e *Aristolochia* foram comparadas, além de comparações entre a filogenia das borboletas e dendrogramas baseados na similaridade química e distribuição geográfica das espécies de *Aristolochia* usadas como hospedeiros.

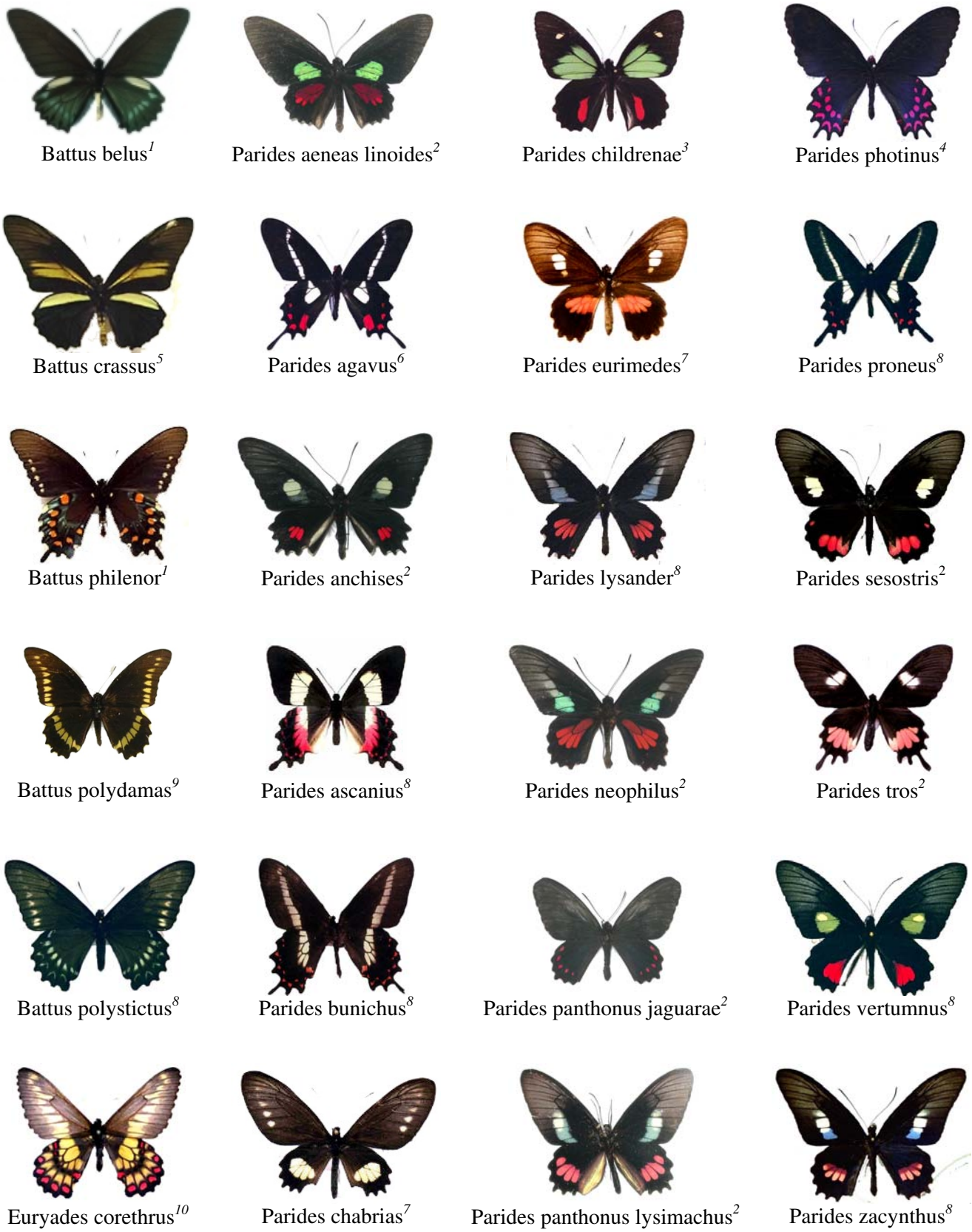
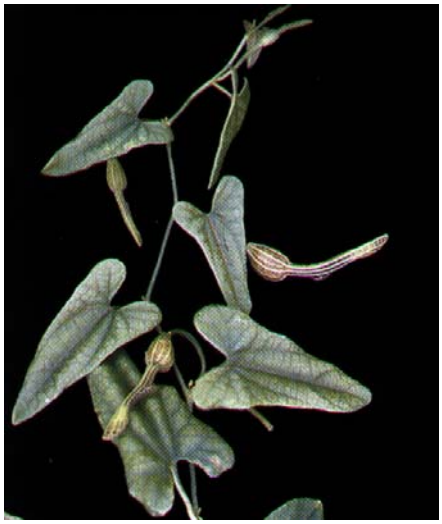


Figura 1. Espécies de borboletas Troidini neotropicais estudadas neste trabalho.



A. arcuata

A. chamissonis[1]

A. cymbifera[2]

A. cynanchifolia[2]

A. elegans[2]

A. esperanzae[2]

Figura 2. Espécies de *Aristolochia* do sudeste do Brasil estudadas neste trabalho.

A. galeata[3]

A. gigantea[2]

A. macroura[3]

A. melastoma[2]

A. paulis

A. triangularis[3]

Figura 2. Continuação

Fotos figura 1: 1 – modificadas de http://papilionidae.chat.ru; 2 – cedidas por A. V. L. Freitas; 3 – modificadas de http://www.hondurasecotours.com; 4 – modificadas de http://www.butterflies.org; 5 – modificadas de http://insects-online-de; 6 – modificadas de http://www.jabeca.or.jp; 7 – modificadas de Tyler *et al.* (1994); 8 – modificadas de http://home.att.net; 9 – modificadas de http://nearctica.com; 10 – modificadas de http://www.pteron-world.com; 10 – modificadas de http://www.ornithoptera.net.

Fotos figura 2: 1 – cedida por F. C. Campos Neto; 2 – K. L. Silva-Brandão; 3 – modificadas de http://mpeixoto.sites.uol.com.br.

Objetivos

O objetivo geral deste trabalho foi estudar a evolução da interação entre borboletas da tribo Troidini e as suas plantas hospedeiras do gênero *Aristolochia* usando o que Ronquist e Nylin (1990) chamam de "enfoque de padrão", ou seja, o uso das filogenias de grupos associados para reconstruir a história coevolutiva da interação.

Os objetivos específicos foram:

1. Propor uma filogenia molecular para as borboletas Troidini neotropicais baseada em dados moleculares;

2. Estudar a evolução de caracteres morfológicos e ecológicos sobre a hipótese filogenética gerada para os Troidini;

3. Estudar as relações químicas e filogenéticas das plantas do gênero *Aristolochia* do sudeste do Brasil;

4. Comparar a filogenia obtida para os Troidini com a filogenia das *Aristolochia* para testar se a filogenia das plantas hospedeiras determinou a mudança de hospedeiros ao longo da filogenia das borboletas;

5. Comparar a filogenia dos Troidini com dendrogramas obtidos com a similaridade química das *Aristolochia* e com a sua distribuição geográfica.

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Capítulo 1

Phylogenetic relationships of the New World Troidini swallowtails (Lepidoptera: Papilionidae) based on COI, COII, and EF-1α genes

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Abstract

A phylogeny of the Neotropical members of the tribe Troidini (Lepidoptera: Papilionidae) was obtained with sequences of three protein-coding genes: two mitochondrial (COI and COII) and one nuclear (EF-1α). Parsimony and Bayesian analyses of 33 taxa resulted in very similar trees regardless of method used, with the 27 troidines always forming a monophyletic clade. The genus *Battus* is sister group to the remaining troidines, followed by a clade formed by the Paleotropical taxa (here represented by three specimens). The genus *Euryades* is the next branch, and sister group of *Parides*. The genus *Parides* is monophyletic, and is divided into four main groups by Maximum Parsimony analysis, with the most basal group composed of tailed species restricted to SE Brazil. Character optimization of ecological and morphological traits over the phylogeny proposed for troidines indicated that the use of several species of *Aristolochia* is ancestral over the use of few or a single host-plant. For the other three characters, the ancestral states were the absence of long tails, forest as the primary habitat and oviposition solitary or in loose group of several eggs.

Key words – Lepidoptera, molecular phylogeny, *Parides*, swallowtail butterflies, Troidini

Resumo

Uma filogenia dos membros Neotropicais da tribo Troidini (Lepidoptera: Papilionidae) foi obtida a partir da seqüência de três genes codificadores de proteínas: dois mitocondriais (COI e COII) e um nuclear (EF-1α). Análises de Parcimônia e Bayesiana de 33 taxa resultaram em árvores bastante similares, independentemente do método utilizado, com os 27 Troidini sempre formando um ramo monofilético. O gênero *Battus* é grupo irmão dos demais Troidini, seguido pelo ramo formado pelos taxa Paleotropicais (aqui representados por três espécimes). O gênero *Euryades* é o próximo ramo, e grupo irmão dos *Parides*. O gênero *Parides* é monofilético, e está dividido em quatro grupos principais pela análise de Máxima Parcimônia, com o grupo mais basal composto das espécies com cauda do SE do Brasil. Otimizações de Caráter de dados ecológicos e morfológicos sobre a filogenia proposta para os troidines indicaram que o uso de várias espécies de *Aristolochia* é o caráter ancestral, ao invés do uso de poucas ou de uma única planta hospedeira. Para os outros três caracteres, os estados ancestrais foram ausência de uma cauda longa, floresta como habitat primário e oviposição de ovos solitários ou em grupos dispersos de vários ovos.

Palavras chave – Lepidoptera, filogenia molecular, *Parides*, Troidini

Introduction

Swallowtail butterflies (Papilionidae) are among the most popular insect taxa, and have greatly contributed to studies of ecology, behaviour and evolution in insects (Scriber, 1995). Many studies have been published with this group, including on ecology (Brown *et al.*, 1981; Spade *et al.*, 1988; Morais and Brown, 1991), behaviour (Rausher, 1978; Stamp, 1986), and chemistry (Rothschild *et al.*, 1970; Urzúa and Priestap, 1985; Nishida *et al.*, 1993; Honda and Hayashi, 1995; Klitzke and Brown, 2000; Sime *et al.*, 2000).

Papilionid butterflies are divided into three subfamilies: Baroniinae, with a single genus and species occurring in Mexico [*Baronia brevicornis*, believed to be the most basal taxon (Tyler *et al.*, 1994; Scriber, 1995; Caterino *et al*., 2001)], Parnassiinae (broad Holarctic distribution), and the cosmopolitan Papilioninae (Scriber, 1995). According to Haüser *et al.* (2002), the subfamily Papilioninae has 485 species, divided into three tribes: Papilionini, Graphiini, and Troidini. The tribe Troidini is predominantly tropical, with most species concentrated in the lowland forests of Central and South America and in the IndoAustralian region (Weintraub, 1995). The tribe includes 130 species divided into 12 genera, three of which occur in the Neotropics: *Battus* (11 species), *Euryades* (2 species), and *Parides* (34 species) (Tyler *et al.*, 1994). The genus *Parides s. str.* is exclusively neotropical, and includes several species on official lists of endangered species (MMA, 2003). These include the Southeast Brazilian *Parides ascanius*, which is considered endangered due to the destruction of habitat and host plants (Otero and Brown, 1986; Tyler *et al.*, 1994), and other sensitive species such as *P. tros*, which is rare on the coastal slopes of the Atlantic Forest, and deserving of attention and monitoring now and in the future (Tyler *et al.*, 1994).

Troidines are frequently cited in the literature as classic examples of coevolution with their host-plants *Aristolochia* (Aristolochiaceae) (Weintraub, 1995), earning them the name "*Aristolochia*

swallowtails" (Brown *et al.*, 1981). The features of this association agree with most of the premises of the coevolutionary hypothesis (Ehrlich and Raven, 1964). The larvae of Troidini feed almost exclusively on *Aristolochia* species, and sequester the major secondary metabolites of these plants, aristolochic acids (Klitzke and Brown, 2000). These compounds are thought to make the butterflies unpalatable to potential predators (Brower and Brower, 1964; Rothschild *et al.*, 1970; Nishida and Fukami, 1989; Sime, 2002). Larvae and adults of many species advertise their unpalatability through aposematic coloration, making them notable in their roles as unpalatable models in mimicry rings (Tyler *et al.*, 1994; Sime *et al.*, 2000).

Based upon the classificatory groundwork of Haase (1892) and Rothschild and Jordan (1906), morphological studies investigating the phylogenetic relationships among Trodini butterflies include those of Munroe and Ehrlich (1960), Munroe (1961), Hancock (1983), and Miller (1987). Recently, several molecular studies have been added to this list (Aubert *et al.*, 1999; Caterino and Sperling, 1999; Morinaka *et al.*, 1999; Reed and Sperling, 1999; Morinaka *et al.*, 2000; Caterino *et al.*, 2001; Kondo and Shinkawa, 2003; Zakharov *et al.*, 2004a; Zakharov *et al.*, 2004b). Most of these studies have suggested that Troidini + Papilionini form a clade, with Graphiini basal to both (Hancock, 1983; Miller, 1987; Caterino *et al.*, 2001; Kondo and Shinkawa, 2003; Zakharov *et al.*, 2004a). However, the internal relationships among members of Troidini remain controversial (Vane-Wright, 2003).

Morphological classifications (e. g., Munroe, 1961; Hancock, 1983; Miller, 1987) have divided Troidini into two subtribes: Battina, including only the genus *Battus*, and Troidina, including Southeast Asian *Cressida*, *Troides*, *Ornithoptera*, *Trogonoptera*, *Pachliopta*, *Losaria*, *Pharmacophagus* and *Atrophaneura* (including *Panosmia*) and Neotropical *Euryades* and *Parides*. The genus *Parides* is sometimes circumscribed to include both the Neotropical representatives addressed here and members of *Atrophaneura*. Morinaka *et al.* (1999) and Morinaka *et al.* (2000)

studied the molecular phylogenetic relationships among Asian *Ornithoptera* butterflies, including some species in other genera of Troidini, using the mitochondrial gene ND5. Kondo and Shinkawa (2003) also used the ND5 sequences to propose a molecular phylogeny for three genera of birdwing butterflies, *Trogonoptera*, *Troides* and *Ornithoptera*, and Kato and Yagi (2004) have studied the phylogeny of geographical races of *Atrophaneura alcinous* butterflies from Asia with the same gene. Tyler *et al.* (1994) presented the only published phylogenetic hypothesis of the species-level relationships of the New World troidines, based on adult and larval morphological characters, adult behavior and chemistry. In addition, the phylogeny of the genus *Battus* was studied by Racheli and Oliverio (1993) using adult morphological characters.

There are few studies focusing on the internal relationships of the genus *Parides* (see Tyler *et al.*, 1994), and the only phylogeny published so far includes only four species in this genus. Considering the diversity and ecological importance of this group in the Neotropics, a phylogenetic hypothesis is necessary to help understand the biogeography, behaviour, chemical ecology and evolution of host-plant use among *Parides* and other troidine species. The aims of this study are: (1) to infer a molecular phylogeny of the New World Troidini butterflies of the genera *Battus, Euryades* and especially *Parides s. str.*, based on DNA sequences of mitochondrial and nuclear genes, in order to propose a hypothesis about their evolutionary history; and (2) investigate the evolution of four ecological and morphological traits within the genus *Parides*.

Materials and methods

Specimens

Individual butterflies representing approximately half of the species of the Neotropical Troidini genera, *Parides* (17 of 34 species, with representatives of all subgeneric groups recognized by Tyler *et al*., 1994), *Battus* (5 of 11 species), and *Euryades* (1 of 2 species) were collected in the field (Table 1.1, Fig. 1.1). Upon collection, the wings were separated from the body and stored in glassine envelopes, and the bodies were preserved in a freezer at -70°C. In some cases, DNA was extracted from older, dried specimens from the collection of K. S. Brown. Vouchers of all samples have been deposited in the Museu de História Natural of UNICAMP. Previously published sequences of five species of Troidini, two species of Graphiini, two species of Papilionini, one of Parnassiinae, and *Baronia brevicornis* (Baroniinae) were obtained from GenBank (Caterino *et al.*, 2001). The final matrix has 47 terminals representing 33 species, including 27 Troidini and six non-troidine papilionids as outgroups (Table 1.1).

Table 1.1. Species of Troidini sampled, with localities and GenBank accession number. In species with more than one individual sampled, the order in the table corresponds to the sequential number in the trees.

Species	Locality	GenBank Accession Numbers	
		COI / COII	EF-1α
Baroniinae			
Baronia brevicornis Salvin, 1893	Mexico*	AF170865	AF173405
Parnassiinae			
Parnassius phoebus (Fabricius, 1973)	AB, Canada*	AF170872	AF173412
Papilioninae			
Papilionini			
Papilio glaucus Linnaeus, 1758	MD, USA*	AF044013	AF044826
P. machaon Linnaeus, 1758	Coudoux, France*	AF044006	AF044819
Graphiini			
Graphium agamemnon (Linnaeus, 1758)	SE Asia*	AF170874	AF173414
Protographium (*Eurytides*) *marcellus* (Cramer, [1777])	FL, USA*	AF044022	AF044815
Troidini			
Byasa (*Atrophaneura*) *alcinous* (Klug, 1836)	Okura, Japan*	AF170876	AF173416
Losaria (*Pachliopta*) *neptunus* (Guérin-Méneville, 1840)	Malaysia*	AF044023	AF044829
Troides helena (Linnaeus, 1758)	Malaysia*	AF170878	AF173418
Battus belus (Cramer, [1777])	Alta Floresta, MT, Brazil	AY804350 / AY804386	AY804422
Battus crassus (Cramer, [1777])	Alta Floresta, MT, Brazil	AY804351 / AY804387	AY804423
Battus philenor (Linnaues, 1771)	VA, USA*	AF170875	AF173415
Battus polydamas (Linnaeus, 1758) (two specimens)	Campinas, SP, Brazil	AY804352 / AY804388	AY804424
	Campinas, SP, Brazil	AY804353 / AY804389	AY804425
Battus polystictus (Butler, 1874) (two specimens)	Atibaia, SP, Brazil	AY804354 / AY804390	AY804426
	Atibaia, SP, Brazil	AY804355 / AY804391	AY804427
Euryades corethrus (Boisduval, 1836)	Barra do Quarai, RS, Brazil	AY804356 / AY804392	AY804428
Parides aeneas linoides K. Brown & Lamas, 1994 (two specimens)	Alta Floresta, MT, Brazil	AY804357 / AY804393	AY804429
	Alta Floresta, MT, Brazil	AY804358 / AY804394	AY804430
Parides agavus (Drury, 1782) (two specimens)	Campinas, SP, Brazil	AY804359 / AY804395	AY804431
	Campinas, SP, Brazil	AY804360 / AY804396	AY804432
Parides anchises (Linnaeus, 1758) (three specimens)	Campinas, SP, Brazil	AY804361 / AY804397	AY804433
	Campinas, SP, Brazil	AY804362 / AY804398	AY804434
	Belo Horizonte, MG, Brazil	AY804363 / AY804399	AY804435
Parides ascanius (Cramer, [1775]) (two specimens)	Seropédica, RJ, Brazil	AY804364 / AY804400	AY804436
	Seropédica, RJ, Brazil	AY804365 / AY804401	AY804437
Parides bunichus (Hübner, 1821) (two specimens)	Santa Maria, RS, Brazil	AY804366 / AY804402	AY804438
	Santa Maria, RS, Brazil	AY804367 / AY804403	AY804439
Parides chabrias (Hewitson, 1852)	Alta Floresta, MT, Brazil	AY804368 / AY804404	AY804440
Parides childrenae (Gray, 1832)	French Guiana	AY804369 / AY804405	AY804441
Parides eurimedes (Stoll, 1782) (two specimens)	Panama	AY804370 / AY804406	AY804442
	Panama	AY804371 / AY804407	AY804443
Parides lysander (Cramer, [1775])	Costa Rica	AY804372 / AY804408	AY804444
Parides neophilus (Geyer, 1837) (three specimens)	Campinas, SP, Brazil	AY804373 / AY804409	AY804445
	Campinas, SP, Brazil	AY804374 / AY804410	AY804446
	Campinas, SP, Brazil	AY804375 / AY804411	AY804447
Parides panthonus jaguarae (Foetterle, 1902)	Belo Horizonte, MG, Brazil	AY804376 / AY804412	AY804448
Parides panthonus lysimachus (Honrath, 1888)	Rio Teles Pires, MT, Brazil	AY804377 / AY804413	AY804449
Parides photinus (Doubleday, 1844)	Costa Rica*	AF170877	AF173417
Parides proneus (Hübner, 1831) (three specimens)	Cotia, SP, Brazil	AY804378 / AY804414	AY804450
	Cotia, SP, Brazil	AY804379 / AY804415	AY804451
	Rio Claro, SP, Brazil	AY804380 / AY804416	AY804452
Parides sesostris (Cramer, [1779])	Panama	AY804381 / AY804417	AY804453
Parides tros (Fabricius, 1793)	Picinguaba, SP, Brazil	AY804382 / AY804418	AY804454
Parides vertumnus cutora (Gray, 1853)	Alta Floresta, MT, Brazil	AY804383 / AY804419	AY804455
Parides zacynthus (Fabricius, 1793) (two specimens)	Picinguaba, SP, Brazil	AY804384 / AY804420	AY804456
	Cananéia, SP, Brazil	AY804385 / AY804421	AY804457

* Reference: Caterino *et al.* (2001)

Molecular techniques

Total genomic DNA was extracted following the protocol of Genomic Prep™ Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech) or DNeasy® Tissue Kit (Qiagen) from thorax/abdominal tissue of frozen individuals. The DNA of three species of Troidini, *Battus crassus*, *Parides chabrias* and *P. panthonus lysimachus*, was extracted from the legs of dried museum specimens. For these materials, we used the protocol of DNeasy® Tissue Kit modified for ancient material, that is, samples were lysed overnight and recovered with 50 μL of dilution buffer, as suggested by the manufacturer's technical support. This protocol has been widely used by research groups studying butterfly systematics, and have been shown to be reliable (Wahlberg *et al.*, 2003). Purified DNA was stored in TE buffer at -20°C. For each of the specimens we sequenced the entire mitochondrial *cytochrome oxidase I* and *II* genes (COI and COII) and the nuclear gene *elongation factor-1α* (EF-1α) using the primer combinations listed in Table 1.2. When possible, we sequenced at least two individuals of each species. In Papilionidae, these genes, and especially COI, are suitable to elucidate relationships at species and generic levels (Caterino and Sperling, 1999; Caterino *et al.*, 2001; Zakharov *et al.*, 2004a) and Sperling (2003) has recommended the use of EF-1α in combination with COI/COII mitochondrial genes to study phylogenetic relationships among butterflies in general. Both genes have advantages and disadvantages: mtDNA is easier to amplify, does not have non-coding regions (introns), has no recombination, and evolves at higher rates. However, mtDNA may present high levels of homoplasy because of an extreme A/T bias in third positions (Harrison, 1989). Nuclear genes can be advantageous due to the less biased base composition, and generally evolve more slowly than mitochondrial genes, making them better markers for deep divergences (Lin and Danforth, 2004). The choice of these genes in our study is due mainly to their use in other studies of Papilionidae (Caterino and Sperling, 1999; Reed and

Sperling, 1999; Caterino *et al.*, 2001; Zakharov *et al.*, 2004a) since studying comparable gene regions contributes synergistically to a more comprehensive picture of the evolution of all butterfly groups (Caterino *et al.*, 2000).

Amplification of DNA was performed using two methods. We used a direct method for COI and COII, using primers that amplified a sequence of about 500 bp (COI) or 700 bp (COII) in length. For EF-1α we used a nested method, a sequence of primers that first amplified around 1200 bp, and then amplified a smaller 500-700 bp fragment from each half of the larger piece. All fragments were amplified in a total volume of 25 μL. The following thermal cycling protocol was used for COI and COII: 96°C for 2 min, 35 cycles of 94°C for 1 min, 45°C for 1:30 min, 72°C for 1:50 min, and a final extension period of 72°C for 4 min. The cycling profile for EF-1α was 95°C for 2 min, 30 cycles of 95°C for 1 min, 45°C for 1 min, 72°C for 1:50 min, and a final extension period of 72°C for 4 min.

PCR products were cleaned by using a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) or a Gel Purification Kit (Qiagen), and then amplified for sequencing using the protocol of ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit. PCR fragments were sequenced in ABI 373 or ABI 377 automated sequencers. All fragments were sequenced in both directions, using the same primers listed in Table 1.2. Sequences were analyzed with the program SeqEd™ version 1.0.3 (Applied Biosystems, Inc.), and aligned manually by the Se.Al program (Rambaut, 1996) using the translated amino acid sequences and the *Drosophila yakuba* sequence for COI and COII (Clary and Wolstenholme, 1985). All sequences were deposited in GenBank (accession numbers in Table 1.1).

Table 1.2. Primers used in this study.

Gene	Name	F/R	Location[a, b] (3'end)	Sequence (5'→3')
COI	K698	F	1460	TACAATTTATCGCCTAAACTTCAGCC
	K699	R	1840	AGGAGGATAAACAGTTCACCC
	Ron-mod	F	1751	GGTTCACCTGATATAGCATTCCC
	Nancy-mod	R	2192	CCTGGTAAAATTAAAATATAAACTTC
	Jerry	F	2183	CAACATTTATTTTGATTTTTTGG
	Mila	R	2659	GCTAATCCAGTGAATAATGG
	BrianXV	F	2495	CATCAATTCTATGAAGATTAGG
	PatII	R	3014	TCCATTACATATAATCTGCCATATTAG
COII	Patrick	F	3038	CTAATATGGCAGATTATATGTAATGGA
	Eva	R	3782	GAGACCATTACTTGCTTTCGATCATCT
EF-1α	Hillary	F	2103	CACATYAACATTGTCGTSATYGG
	Monica	R	2645	CATRTTGTCKCCGTGCCAKTCC
	Al	F	2582	GAGGAAATYAARAAGGAAG
	Tipper	R	3344	ACAGCVACKGTYTGYCTCATRTC

[a] Position relative to *Drosophila yakuba* (Clary and Wolstenholme, 1985) for COI and COII primers. Primers obtained from Caterino and Sperling (1999)

[b] Position relative to *Drosophila melanogaster* to EF-1α primers. Primers obtained from Cho *et al.* (1995)

Y=C/T; S=C/G; R=G/A; K=G/T; V=C/G/A

Phylogenetic analyses

The phylogenetic analyses were performed with PAUP* 4.0b10 (Swofford, 2002), using Maximum Parsimony. Bayesian analysis was carried out with MrBayes 3.08v (Huelsenbeck and Ronquist, 2001). The purpose of doing Bayesian analysis was to investigate the effects on the results under the most restrictive assumptions of data analysis.

The Partition Homogeneity Test of PAUP* 4.0b10 (Swofford, 2002) was used to assess congruence among molecular data sets. This test is equivalent to the ILD test[1] of Farris *et al.* (1994), which has been employed as a method for determining whether separated data sets should be combined in a single parsimony analysis (Yoder *et al.*, 2001). In the present study, this test was used as a measure of heterogeneity among the data sets (as in Freitas and Brown, 2004), and not as a way to validate or invalidate the combined analysis [see also Brower *et al.* (1996) and DeSalle and Brower (1997)]. We performed the test under parsimony, using the following parameters: heuristic search, TBR branch-swapping, with 100 random addition sequences, and 500 replicates to generate the null hypothesis. The transition/transversion ratio was estimated in MEGA, version 2.1 (Kumar *et al.*, 2001).

Maximum Parsimony analyses (MP) were performed on the entire data set, as well as for each gene separately, using heuristic search with 500 random taxon addition replicates, TBR branch-swapping, gaps scored as missing data, and all characters equally weighted. A strict consensus tree was computed whenever multiple equally parsimonious trees were obtained. The consistency index (CI) and the retention index (RI) were calculated by the PAUP "tree scores" option. CI is a

[1] The ILD test works by first finding the sum of the lengths of the most parsimonious trees of the partitions in question. Partitions of sizes equal to the original partitions are then generated by random sampling (without replacement) from the entire data set. By summing tree lengths from multiple random partitions, a null distribution of tree length sums may be generated. By comparing the original tree length sum to the null distribution, it is possible to determine if the data partitions in question are significantly incongruent (Reed and Sperling, 1999).

measurement of how the data fit to the cladogram. The maximum is 1, which corresponds to a complete fit; no homoplasy is involved, and changes in any particular character (position) appear only once on the cladogram, so that no parallelisms or reversals are needed (Bremer, 1994). The robustness of each branch was determined using the non-parametric bootstrap test (Felsenstein, 1985), with 1000 replicates and 10 random taxon additions[2]. Bremer support and Partitioned Bremer support values (to obtain the contribution of each data set to the Bremer support values of the combined analysis by calculating how many extra steps are needed to lose a branch) (Bremer, 1988; Bremer, 1994; Baker and DeSalle, 1997; Baker *et al.*, 1998) were calculated using TreeRot (Sorensen, 1999), in conjunction with PAUP* 4.0b10 (Swofford, 2002). The analysis was conducted with 100 random taxon addition replicates, TBR branch-swapping and 100 trees held in each replicate. Following Wahlberg and Nylin (2003) and Wahlberg *et al.* (2003), we will refer to the support values as either giving weak, moderate, good or strong support when discussing our results. We define "weak support" as Bremer support values of 1-2 (mostly corresponding to bootstrap values of 50%-61%), "moderate support" as values between 3-4 (bootstrap values 62%-74%), "good support" as values between 5-8 (bootstrap values 75%-88%) and "strong support" as values >8 (bootstrap values 89%-100%).

We used the program MODELTEST 3.06 (Posada and Crandall, 1998) to determine the available substitution model with the best fit to each partitioned data set. Bayesian analyses (Huelsenbeck and Ronquist, 2001; Huelsenbeck *et al.*, 2001; Huelsenbeck *et al.*, 2002) were carried out for the combined data set under the model GTR+G+I [General Time-Reversible model (Rodrígues *et al.*, 1990), with gamma distribution (Γ) and with proportion of invariable sites (I)].

[2] Bootstrap is a method of resampling one's own data to infer the variability of the estimate. Each bootstrap sample consists of a new data table with the same set of species, but with some of the original characters duplicated and others dropped by the process of sampling *n* characters from the original set with replacement (Felsenstein, 1985). The bootstrap *P* value is the proportion of trees containing a particular phylogenetic group.

According to Nylander *et al.* (2004), analysis of combined data by Bayesian methods permits partition-specific substitution models and parameters. For that reason, all substitution model parameters (gamma shape parameter, proportion of invariable sites, character state frequencies, substitution rates of GTR model) were allowed to vary across partitions (=genes). We conducted six simultaneous chains for $1.0x10^6$ generations, sampling trees every 100 cycles. Stability of the process was assessed by plotting the likelihood scores against generation time (Lin and Danforth, 2004). The first 1000 trees were discarded as "burn in". For all analyses, *Baronia brevicornis* (Baroniinae) was used as outgroup to root the tree.

Analyses of character evolution

We investigated the evolution of some ecological and morphological traits superimposed onto the phylogenetic hypothesis proposed for Troidini butterflies:

Character 1. "Use of host-plants", using data obtained from several literature and field sources (Rausher, 1980; Brown *et al.*, 1981; Otero and Brown, 1986; Papaj, 1986; Stamp, 1986; DeVries, 1987; Rausher and Odendaal, 1987; Spade *et al.*, 1988; Morais and Brown, 1991; Tyler *et al.*, 1994; Brown *et al.*, 1995; Weintraub, 1995; Klitzke and Brown, 2000; Freitas and Ramos, 2001). Janz *et al.* (2001) and Wahlberg (2001) discuss the difficulty in coding the use of host-plants in analyses of character optimization. Here we chose to use the number of *Aristolochia* species used as host by each Troidini species as a multistate character (Table 1.3); the results were the same if compared with a binary character coded as "generalist" or "specialist" (using the same definition of Janz *et al.*, 2001). We followed the approach of Janz *et al.* (2001) to test the hypothesis of specialization as a "dead end" (Futuyma and Moreno, 1988), i.e., we compared the number of host-plant gains (colonizations) and losses (specializations) in our analyses of character evolution. According to Janz *et al.* (2001), more host-plant losses than gains indicate a trend toward increasing

specialization;

Character 2. "Presence or absence of long tail on the hindwing" (we considered a "long" tail a hindwing projection three times longer than any other projection);

Character 3. "Primary habitat": Forest (dominant tall trees with closed canopy); Scrub (open woody forest, including chaparral, semi arid formations and Brazilian "cerrado" and "restinga") and Open (dominant open, mainly grassland or small herbs, including south Brazilian "pampas");

Character 4. "Gregarious immatures", based mainly on oviposition patterns [following Tyler *et al.* (1994), defining two kinds of oviposition: tight bunch of many ordered eggs vs. solitary or loose group of several eggs].

Character states were treated as unordered and of equal weights, and were optimized on the MP phylogeny, using MacClade 3.08 program (Maddison and Maddison, 1999). We performed the analysis over the MP tree due to the nature of optimizations algorithms, which are based on parsimony. "Host-plant use" could be analysed asymmetrically, with gains costing more than losses (as in Wahlberg, 2001), but we considered that within a single genus, the costs of gains and losses of a host-plant are similar, since the biological, behavioural and ecological apparatus of oviposition and larval feeding do not have to experience significant changes. Analyses of character evolution used the same outgroups as the phylogenetic analyses (the results were the same if only Papilioninae outgroups were used).

To test whether there is a phylogenetic signal in the characters traced, we used the methodology proposed by Wahlberg (2001), modified from the PTP test described by Faith and Cranston (1991). The test consists in comparing the number of steps of the tree constructed with the actual data with the number of steps obtained for each random reshuffling of the states of each separated character. We performed 300 random reshufflings of character states among the fixed terminal taxa, with the equally weighted data set, using the option "shuffle" from the utilities menu

in the MacClade program (Maddison and Maddison, 1999). The probability (*P*) that the observed pattern does not differ randomly is given by the number of replications as short as or shorter than the tree obtained with the actual data, plus one, divided by the number of replications. Following Faith and Cranston (1991), a significant phylogenetic signal is observed when *P* is less than 0.05, and here, the minimal value should be 0.003 (number of trees as short or shorter than the original tree + 1/300).

Table 1.3. Host plants used by Troidini butterflies and by the outgroups used in phylogenetic analyses.

Species	Host plant family	Host plant species	References
Baronia brevicornis	Leguminosae		(Brown *et al.*, 1995)
Parnassius phoebus	Crassulaceae, Fumariaceae, Scrophulariaceae		11
Papilio glaucus	Aceraceae, Betulaceae, Bignoniaceae, Carpinaceae, Fagaceae, Juglandaceae, Lauraceae, Magnoliaceae, Oleaceae, Platanaceae, Rhamnaceae, Rosaceae, Rutaceae, Salicaceae, Tiliaceae		11
P. machaon	Compositae, Rutaceae, Umbellifera		10, 11
Graphium agamemnon	Annonaceae		
Protographium marcellus	Annonaceae		11
Byasa alcinous	Aristolochiaceae		12
Losaria neptunos	Aristolochiaceae		12
Troides helena	Aristolochiaceae		12
Battus belus	Aristolochiaceae	Many *Aristolochia* species	10
Battus crassus	Aristolochiaceae	*Aristolochia cymbifera; A. elegans; A. esperanzae; A. macroura; A.veraguensis*	3, 6, 11
Battus philenor	Aristolochiaceae	*A. acontophilla; A. asclepiadifolia; A. californica; A. erecta; A. macrophylla;A. micrantha; A. odoratissima; A. orbicularis; A. pilosa; A. pringlei; A. reticulata; A. serpentaria; A. tentaculata*	1, 4, 5, 7, 8, 10
Battus polydamas	Aristolochiaceae	*A. arcuata; A. acontophylla; A. anguicida; A. argentina; A. asclepiadifolia; A. bilabiata; A. chilensis, A. conversiae, A. cymbifera; A. deltoide; A. elegans*; *A. esperanzae*; *A. foetida; A. galeata*; *A. gigantea*; *A. grandiflora; A. littoralis, A. macroura*; *A. melastoma*; *A. micrantha; A. montana; A. odoratissima; A. orbicularis; A. paulistana; A. tagala, A. tentaculata; A. trilobata; A. triangularis; A. veraguensis*	2, 3, 6, 8, 9, 10, 11, 13
Battus polystictus	Aristolochiaceae	*A. arcuata*; *A. galeata; A. gigantea*; *A. melastoma*; *A. triangularis*	10, 11
Euryades corethrus	Aristolochiaceae	*A. fimbriata; A. sessilifolia*	10, 13
Parides aeneas linoides	Aristolochiaceae	Many *Aristolochia* species	10
Parides agavus	Aristolochiaceae	*A. arcuata*; *A. elegans*; *A. esperanzae*; *A. gigantea; A. littoralis; A. melastoma*; *A. rumicifolia; A. triangularis*	2, 9, 11, 13

Table 1.3. Extended.

Parides anchises	Aristolochiaceae	*A. arcuata*; *A. brasiliensis; A. cymbifera*; *A. elegans*; *A. esperanzae*; *A. galeata; A. littoralis; A. macroura; A. melastoma*; *A. odora; A. paulistana; A. rumicifolia; A. triangularis; A. trilobata*	2, 9, 10, 11, 13, 14
Parides ascanius	Aristolochiaceae	*A. macroura*	3
Parides bunichus	Aristolochiaceae	*A. arcuata*; *A. elegans*; *A. esperanzae*; *A. littoralis; A. melastoma*; *A. triangularis*	2, 9, 13
Parides chabrias	Aristolochiaceae	*A. acutifolia; A. barbata; A. bicolor; A. burchelli; A. didyma; A. stomachoides*	10
Parides childrenae	Aristolochiaceae	*A. maxima; A. tonduzii*	6, 10
Parides eurimedes	Aristolochiaceae	*A. maxima; A. odoratissima; A. pilosa; A tonduzii*	10
Parides lysander	Aristolochiaceae	*A. acutifolia; A. barbata; A. bicolor; A. burchelli; A. didyma; A. stomachoides*	10
Parides neophilus	Aristolochiaceae	*A. arcuata*; *A. elegans*; *A. esperanzae*; *A. melastoma; A. ruiziana; A. Trilobata*	9, 10
Parides panthonus jaguarae	Aristolochiaceae	*A. chamissonis*	15
Parides panthonus lysimachus	Aristolochiaceae	Many *Aristolochia* species	10
Parides photinus	Aristolochiaceae	Many *Aristolochia* species	10
Parides proneus	Aristolochiaceae	*A. arcuata; A. elegans; A. esperanzae*; *A. melastoma*	9, 13
Parides sesostris	Aristolochiaceae	*A. acutifolia; A. barbata; A. bicolor; A. burchelli; A. didyma; A. stomachoides*	10
Parides tros	Aristolochiaceae	*A. arcuata; A. cynanchifolia; A. rumicifolia*	11, 13
Parides vertumnus cutora	Aristolochiaceae	Unknown	
Parides zacynthus	Aristolochiaceae	*A. macroura; A. odora; A. paulistana*; *A. triangularis*	11, 13

References: (1) Rausher (1980); (2) Brown *et al.* (1981); (3) Otero and Brown (1986); (4) Papaj (1986); (5) Stamp (1986); (6) DeVries (1987); (7) Rausher and Odendaal (1987); (8) Spade *et al.* (1988); (9) Morais and Brown (1991); (10) Tyler *et al.* (1994); (11) Brown *et al.* (1995); (12) Weintraub (1995); (13) Klitzke and Brown (2000); (14) Freitas and Ramos (2001); (15) Correa, F. F. C. (pers. comm.).

Results

The full data set contained 3330 nucleotides, 2169 from the mitochondrial DNA and 1161 from EF-1α. Single-codon gaps were found both in COI and COII. Two taxa, *Parides chabrias* and *P. photinus*, had gaps at the position 1984-1986 of COI in relation to *Drosophila yakuba* sequence, and *Battus crassus* and *P. chabrias* had gaps at the position 3423-3425 of COII. All *Parides* species showed a gap at the position 3458-3460, while only one *Battus* species (*B. polydamas*) showed a gap at that position. The alignment of EF-1α did not show indels.

No differences were found in base composition among sequences within each of the partitioned genes (Table 1.4). However, the transition/transversion ratio among the three genes was quite different among codon positions (Table 1.4), and in the third codon position of the mitochondrial genes COI and COII this ratio was 0.7 and 0.9, respectively, suggesting transition saturation at this position. EF-1α sequence did not show this problem of saturation at third positions, although the difference among codons was strong (Table 1.4).

Table 1.4. Summary of the sequence statistics over gene partitions.

	All genes	COI	COII	EF-1α
Number of analyzed characters	3330	1527	642	1161
Number of invariant characters	2072	928	336	802
Number of variable characters	1258	599	306	359
Parsimony-informative sites	1000	480	233	287
CI	0.4	0.348	0.425	0.521
RI	0.655	0.598	0.667	0.773
Ti/Tv ratio				
All positions	1.2	0.9	1.2	2.3
1st codon position	2.3	2.5	2.4	1.4
2nd codon position	1.2	1.6	1.1	0.7
3rd codon position	1.0	0.7	0.9	2.5
Frequencie of A, C, G, T		0.30, 0.15, 0.13, 0.42	0.34, 0.13, 0.10, 0.42	0.27, 0.26, 0.24, 0.23
Base Frequencies		χ^2_{138}=44.69; P=1.0	χ^2_{138}=55.44; P=0.99	χ^2_{138}=36.94; P=1.0

Phylogenetic analyses

Partitioned data

Parsimony analyses of the three genes separately resulted in different topologies (Fig. 1.1). The COI sequences resulted in five equally parsimonious trees, with 2416 steps (CI=0.348; RI=0.598). The strict consensus tree is shown in Fig. 1.1a. The analyses of COII resulted in one most parsimonious tree (Fig. 1.1b), with 978 steps (CI=0.425; RI=0.667), and EF-1α analyses resulted in 144 equally parsimonious trees (941 steps, CI=0.521; RI=0.773), with strict consensus shown in Fig. 1.1c. The homoplasy in the partitions was greater in the COI sequence (CI=0.348) than in the COII sequence, and EF-1α showed the smallest homoplasy. Both COI and COII gave well resolved trees with strong support at the tips of the tree (Fig. 1.1a, b), and EF-1α presented strong support in the internal nodes also (Fig. 1.1c).

A

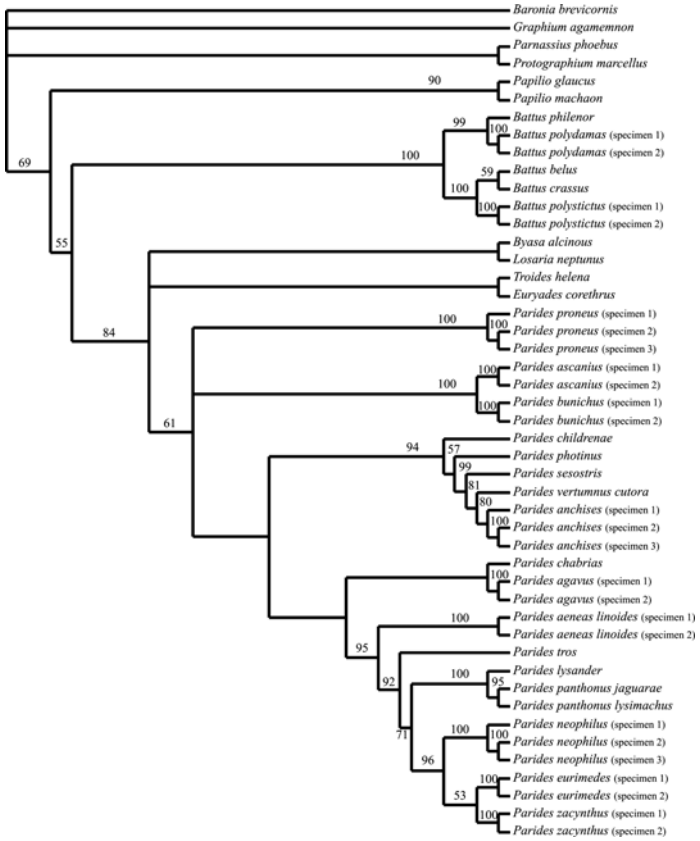
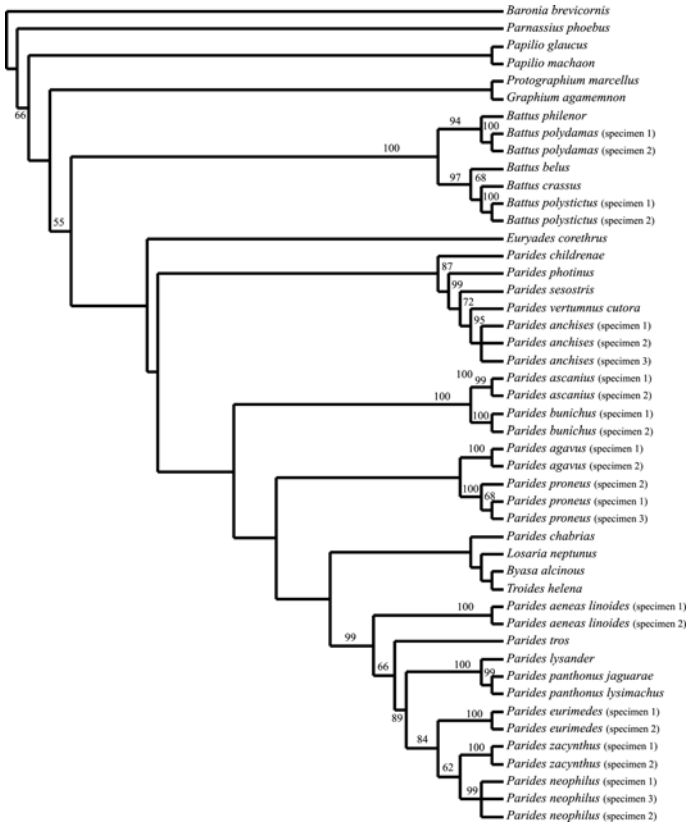


Figure 1.1. Most parsimonious trees obtained by each gene separately. (a) strict consensus tree of five equally parsimonious trees found in parsimony analysis using COI nucleotide sequence (length=2416 steps); (b) most parsimonious tree found in parsimony analysis using COII nucleotide sequence (length=978 steps); (c) strict consensus tree of 144 equally parsimonious trees found in parsimony analysis using EF-1α nucleotide sequence (length=941 steps). Numbers above the branches indicate the bootstrap support obtained with 1000 replicates (where exceeds 50%).

B



C

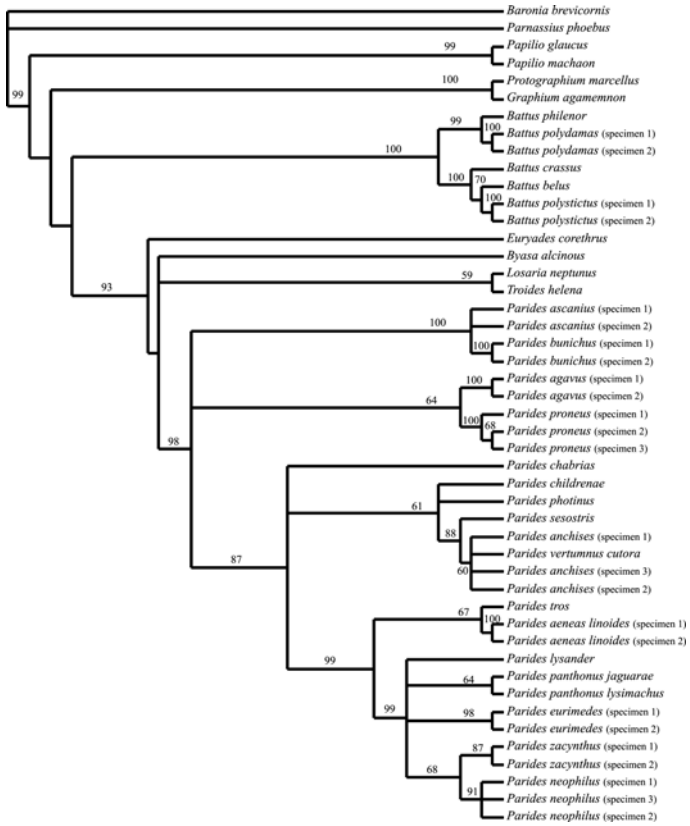


Figure 1.1. Extended.

Combined data

The ILD test results suggest that partitions of the data into COI+COII (mtDNA) and EF-1α were not incongruent (P=0.708).

The total number of parsimony-informative sites in the full combined data set was 1000 (30%). COII showed the highest proportion of parsimony-informative sites, followed by COI and EF-1α (Table 1.4).

Parsimony searches over the equally weighted combined data set resulted in one most parsimonious tree, with 4368 steps (CI=0.4; RI=0.655) (Fig. 1.2). The tribe Troidini appeared as a monophyletic group. The genus *Battus* appeared as monophyletic and sister group to all remaining Troidini, supported by strong bootstrap and good Bremer values. *Battus* is divided into two groups (with strong bootstrap and Bremer values), one of them containing *B. polystictus*, *B. belus*, and *B. crassus*, and the other containing *B. polydamas* and *B. philenor*. The clade with the three paleotropical genera *Troides* + *Byasa* + *Losaria* is the sister of the remaining neotropical taxa (with strong bootstrap and Bremer support), but the relationships among these genera are still unclear. The genus *Euryades* is sister to the monophyletic genus *Parides* (with strong bootstrap and Bremer support). The *Parides* clade is in turn divided in four groups: *Group 1*. *ascanius* + *bunichus*, with strong bootstrap and Bremer support; *Group 2*. *agavus* + *proneus*, with weak bootstrap and Bremer support; *Group 3*. *chabrias* + *childrenae* + *photinus* + *sesostris* + *anchises* + *vertumnus*, with no bootstrap support and weak Bremer support; and *Group 4*. *aeneas* + *tros* + *eurimedes* + *neophilus* + *zacynthus* + *lysander* + *panthonus*, supported by strong bootstrap and Bremer values. Based on our sampling, groups 1 and 2 comprise species of *Parides* with tails on the hindwing that are restricted to Southern South America (the only exception is the tailed *P. tros*, which appears in group 4). In addition, all species represented by more than one exemplar appeared as monophyletic entities.

The contribution of each gene to the combined tree, assessed by partitioned Bremer support, shows that there are few conflicting nodes. The COI data provide the greatest source of conflict, as found by Wahlberg *et al.* (2003) for the family Nymphalidae (Lepidoptera). COI sequences showed conflict in seven of 44 nodes, COII in five, and EF-1α in only one node.

Bayesian analyses for the combined data set became stationary well before generation 100,000. The topology of the consensus tree was quite similar to that obtained by MP (Fig. 1.3), with the Bayesian tree differing in the relationships mainly among the species in group 4. In the Bayesian analysis, *P. eurimedes* is the sister taxon to *P. zacynthus*, with a low Posterior Probability (PP)[3] (67%), conflicting with the sister relationship of *P. neophilus* + *P. zacynthus* found in the MP analysis. In both MP and Bayesian analyses, these three species formed a monophyletic clade with strong support (100% of bootstrap and PP support). Also different from MP, Bayesian analyses implied three groups within *Parides*, joining the groups 1 and 2 in a single clade (88% PP support). Clades which did not agree with MP trees showed weak or moderate PP support in the Bayesian analysis, such as *P. proneus* as the sister species of *P. ascanius* + *P. bunichus* (51% PP support) and *Troides helena* as the sister group of *Euryades* + *Parides* + *Battus* (59% of PP).

Analyses of character evolution

Character optimization of Troidini ecological and morphological traits over the inferred MP molecular phylogeny suggests that the ancestral states of these characters were: 1 - the use of many *Aristolochia* species as host-plant, 2 - absence of long tail, 3 - forest as main habitat, and 4 - oviposition solitary or in loose group of several eggs (Fig. 1.4). The ancestral states for the *Parides* clade were similar, except for character 2 – "presence or absence of long tail", which showed an equivocal result (Fig. 1.4).

[3] PP represents the probability that the corresponding clade is true given the model, the priors and the data (Huelsenbeck *et al*., 2002)

We found that the number of host-plant losses along the evolution of Troidini was higher than the number of host-plant gains, with four unambiguous events of host-plant losses and none of host-plant gain (Fig. 1.4). DELTRAN (which maximizes parallel changes) and ACCTRAN (which maximizes early gains) tracings were used to resolve the only ambiguity found (Maddison and Maddison, 1999). Both reconstructions resulted in five events of host-plant losses.

The presence of a long tail may have arisen four times along the evolutionary history of the Troidini. DELTRAN tracing indicated five gains of a long tail, and ACCTRAN tracing pointed out four gains and one loss. The shift of primary habitat from forest to scrub or to open habitat has unambiguously occurred three times and once, respectively (Fig. 1.4), and the oviposition of tight bunches of many ordered eggs is inferred to have arisen once along the evolution of troidine butterflies.

The phylogenetic signals for “use of host plants” and “gregariousness of the immatures” were both strong ($P = 0.003$ for both characters), suggesting that the distribution of these traits among taxa can be explained by their phylogenetic relationships. The phylogenetic signals of the characters “presence or absence of long tail”, and “primary habitat”, were both marginally significant ($P = 0.053$ and $P = 0.056$, respectively).

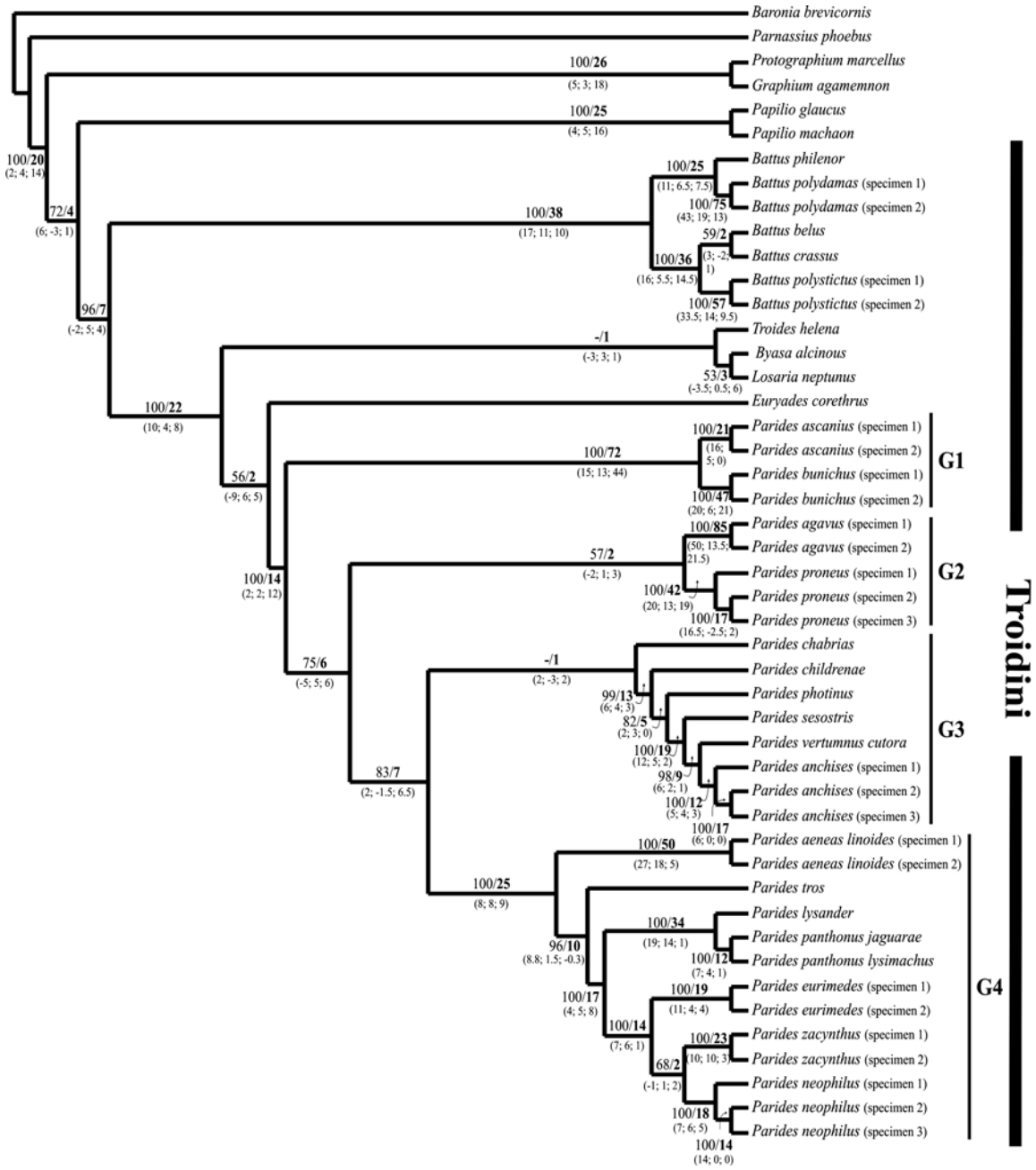


Figure 1.2. Most parsimonious tree based on combined data analysis. Values above the branches indicate bootstrap values from 1000 replications (where exceeds 50%) (regular font) and Bremer support (bold), respectively, for the node to the right of the numbers. Numbers in parentheses are partitioned Bremer support values, which indicate the contribution of COI, COII and EF-1α, respectively, to the Bremer support value of the combined analysis. The sum of the three values of the partitions gives the Bremer support for that branch.

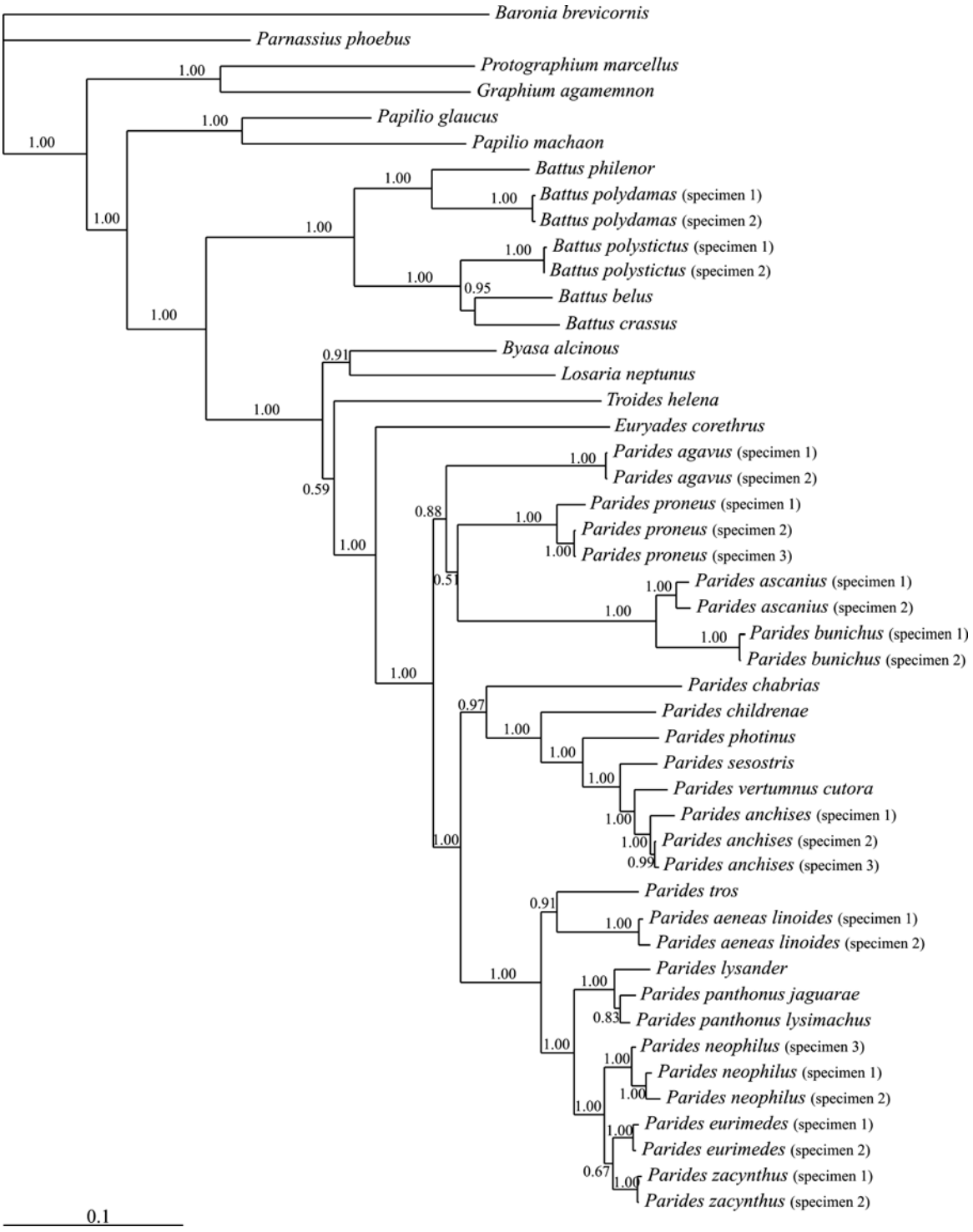


Figure 1.3. Phylogenetic tree inferred by Bayesian analysis from combined data set. Values above the branches indicate Bayesian Posterior Probability.

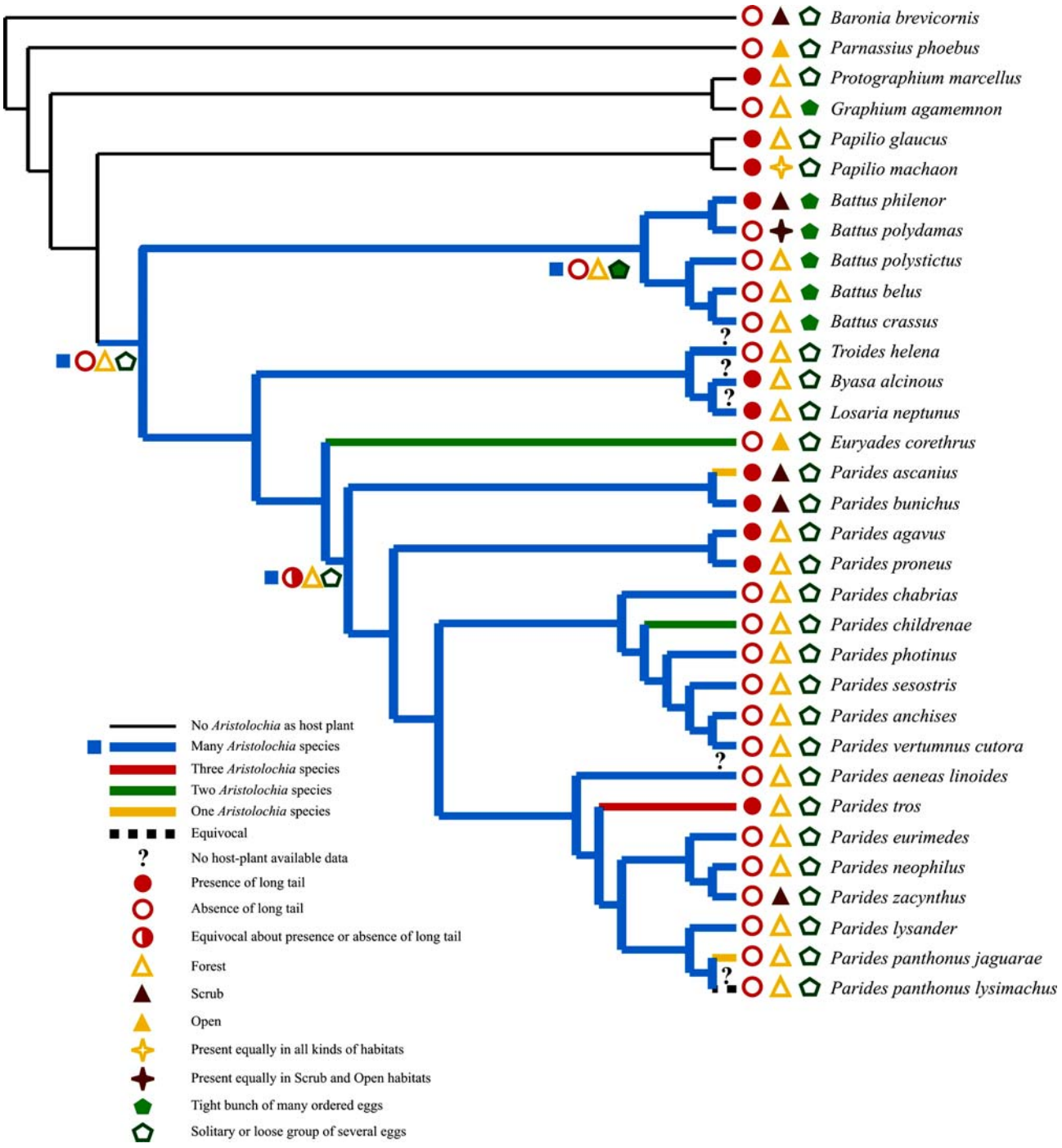


Figure 1.4. Optimization of ecological and morphological traits onto the MP phylogeny of Troidini.

Discussion

The basal Troidini

Our results for the intergeneric relationships among the Troidini largely corroborate those obtained by prior morphological researchers (Munroe, 1961; Hancock, 1983; Miller, 1987) and the molecular hypothesis of Caterino *et al.* (2001). The general topology is maintained even with the inclusion of additional taxa in some clades (in our case, *Euryades*, *Parides*, and *Battus*). However, this topology has some weakly supported branches in both this study and in Caterino *et al.* (2001), especially the branch of the IndoAutralian species, which is poorly sampled in both studies. A complete sampling of all Troidini genera, including representatives of all major lineages, will be necessary before we can fully understand the relationships among the Troidini genera, considered to be of the greatest uncertainty within the Papilionidae (Vane-Wright, 2003).

The position of *Battus* as basal to all remaining Troidini agrees with Munroe (1961), Hancock (1983) and Miller (1987), each of whom placed this genus in the subtribe Battina, sister of the subtribe Troidina (which includes all troidine genera except *Battus*), corresponding also with Caterino's (2001) hypothesis. Munroe and Ehrlich (1960), in addition, considered *Battus* the most distinctive genus in the tribe based on several singular morphological features, but their study leaves unclear if *Battus* or *Euryades* + *Cressida* should be the most basal Troidini [neither included in Caterino *et al.* (2001)]. Those results disagree with those of Morinaka *et al.* (1999), based on the ND5 gene, that showed *Battus* closer to the Graphiini, far from all remaining Troidini. That result is weakly supported, however, and is likely due to insufficient taxonomic and character sampling, given that they sequenced only a short mitochondrial region. The internal relationships among *Battus* reported here agree completely with Racheli and Oliverio (1993, including all species in the

genus), who also found the genus *Battus* divided into two major groups.

We found that *Euryades* occupies a sister group position with *Parides*, differing from Miller (1987), who placed it with a group of Paleotropical genera. This close association agrees with Hancock's (1983) hypothesis, who suggested a sister relationship among (*Cressida* + *Euryades*) + *Parides*. The support in the clade *Euryades* + *Parides* could eventually be improved by the inclusion of *Cressida* and the other species of *Euryades*, as well as better representation of other Old World troidine genera.

The genus Parides

This paper presents the first detailed phylogenetic hypothesis for the genus *Parides*, including 17 of the 34 recognized species representing all of the subgeneric groups recognized by Tyler *et al*. (1994) [all other studies published to date have examined only 1 to 4 species (Tyler *et al.*, 1994; Morinaka *et al.*, 1999; Morinaka *et al.*, 2000; Caterino *et al.*, 2001)]. Our more intensive sampling makes possible the identification of the major lineages (groups 1 to 4) within the genus.

The basal position of group 1 (*P. bunichus* + *P. ascanius*) was in part suggested by Tyler *et al*. (1994: 179), who argue that the restriction to a single host-plant species and the presence of tails in the hindwings of *Parides ascanius* (a species not included in the cladograms in that work) suggest that this is the most basal species in the genus. The strongly supported sister relationships between *P. ascanius* and *P. bunichus* is sustained both by their preference for scrub habitats over forest (Tyler *et al.*, 1994), and the possibility of hybridization in the field and laboratory (Otero and Brown, 1986). *Parides ascanius* is very similar morphologically in adult features to *P. bunichus*, especially *P. bunichus chamissonia*, including genitalia and minor elements of wing color-pattern (Otero and Brown, 1986; Tyler *et al.*, 1994). A complete study on the relationships of all subspecies of the *P. bunichus* - *P. ascanius* group could help to clarify the patterns of diversity within this

clade.

Group 2 is now composed of *P. proneus* and *P. agavus*, two species with tails on the hindwings also restricted to the southern Neotropics. The results of Bayesian analysis shows groups 1 and 2 forming a single clade basal to all remaining *Parides*, with all species sharing the presence of tails on the hindwings and geographic distribution restricted to the southern Neotropics. This group could also include some additional tailed species such as *P. phalaecus* (Ecuador), *P. montezuma* (Mexico), *P. gundlachianus* (Cuba) and *P. alopius* (NW Mexico), according to Tyler *et al.* (1994).

Groups 3 and 4 include all remaining *Parides*, many with wide distributions in Amazonia and Central America. The clade *eurimedes* + *zacynthus* + *neophilus* in group 4 is the only point where different analyses disagreed. This still unresolved clade includes one broadly distributed species (*P. neophilus*) and two restricted species; *P. zacynthus* from the coastal Atlantic sand forests, and *P. eurimedes* with Transandean distribution. Future work with multiple populations of these three species could add important information about the patterns of colonization of different habitats by *Parides* in the Neotropics, and the relevance of this to formation of new subspecies.

In our results, *P. chabrias* is included in group 3, with no bootstrap support and just a weak Bremer support in MP analyses, and a strong PP value in Bayesian analyses. Tyler *et al.* (1994) included *P. chabrias* in a group of species with unusual wing-shape in males, including also *P. hahneli* (tailed), *P. quadratus*, *P. pizarro*, *P. vercingetorix* and *P. klagesi*. In our results, *P. chabrias* could be placed in group 3, but a more basal position, forming a separate species group, cannot be ruled out. It is intriguing that *P. chabrias* and putative relatives belong to a separate mimicry complex that includes the ithomiines *Methona* and *Thyridia*, rather than the black with pink-and-green or white-spots-pattern typical of most of the "derived" *Parides* species.

Even if there are many questions still open, the present study clarifies the major internal

relationships of the genus *Parides*, and provides a useful hypothesis to test ecological and biogeographic theories in the evolution of this group.

Future work that could further reveal the internal relationships within the genus *Parides* should include: 1. more species of *Parides*, covering some of the lacunae indicated above [especially additional taxa in the *chabrias* group of Tyler *et al.* (1994) and "tailed *Parides*" from N and S sectors], and 2. new sources of information, including different genes (such as *wingless* and 28S) and morphological characters of both adults and immatures.

Analyses of character evolution

This section is mostly based on the two characters with a strong phylogenetic signal, "use of host plants" and "gregariousness of the immatures". The characters "presence or absence of long tail", and "primary habitat", both with *P* values above 0.05, are only briefly discussed.

Use of host plants - The concept that specialization can be an evolutionary dead end has been central in host-plant/herbivore evolutionary studies. Many factors can constrain the use of plants as food, such as secondary compounds found in them (Jaenike, 1990; Futuyma *et al.*, 1993; Bernays, 1998), female oviposition preferences (Ronquist and Nylin, 1990) and the geographical distribution of species (Pasteels and Rowell-Rahier, 1991; Dobler *et al.*, 1996; Kelley and Farrell, 1998). However, it seems that over evolutionary time, diet breath in insects (Colwell and Futuyma, 1971) has both increased and decreased (Bernays, 1998), sometimes leading to specialization (Moran, 1988; Ronquist and Nylin, 1990; Kelley and Farrell, 1998; Nosil, 2002), and others leading to generalism (Armbruster and Baldwin, 1998; Scheffer and Wiegmann, 2000; Janz *et al.*, 2001).

Based upon the available data for host-plant use, the ancestral state of host plant use for both Troidini and *Parides* is the use of many *Aristolochia* species (Fig. 1.4), with a tendency to advance towards increased specialization. The present results shows that terminal taxa usually feed on fewer

Aristolochia species compared with basal taxa, and this pattern agrees with that found by Kelley and Farrell (1998). *Parides ascanius* could be suggested as an exception to this pattern, since it belongs to a basal clade of *Parides* and is a specialist in *Aristolochia macroura* [a fact confirmed through extensive field and laboratory data, including experiments with other available species of potential host-plants (Otero and Brown, 1986)]. These conclusions could be tested with the addition of more species of tailed *Parides* (see above) to confirm if they are part of this clade making *bunichus-ascanius* a terminal clade in group 1. In addition, the hypothesis that specialists are more sensitive than generalists to changes in abundance of their host plants (Futuyma and Moreno, 1988) could also be tested for *P. ascanius*. Otero and Brown (1986) argue that the main factor threatening this species is habitat destruction rather than host plant availability (*A. macroura* is common in most swampy coastal habitats in SE Brazil). The rarity of *P. ascanius* could be an example of habitat fidelity rather than host fidelity (Dobler *et al.*, 1996), since this species is specialized not only to its host plant, but also to the physical and biotic environment where this plant grows (Bernays, 1996).

The trend toward specialization among the troidine butterflies examined here could be a result of the geographic distribution of the species, reflecting the pattern proposed by Weintraub (1995): species with restricted geographical ranges tend to be specialists, while those with broad geographical ranges are usually generalists, and in this case, the diet breadth is mirroring the host-plant availability and abundance (Pasteels and Rowell-Rahier, 1991). This could explain monophagy of *Parides ascanius* and *Parides panthonus jaguarae*, two species with very restricted distributions (the first in swampy areas of the coastal plain in Rio de Janeiro and the second in narrow galley forests in central Minas Gerais). It is also interesting to note that, even if we are considering a probable subspecies here (*P. panthonus jaguarae*), this is not a factor of bias in our results, since another subspecies of this taxa, *P. panthonus aglaope*, is known to feed on up to five species of *Aristolochia* through its geographic range (Moss, 1920; Tyler *et al.*, 1994). Moreover,

additional data on host plant use in *Parides* could bear upon of the hypothesis of Fox and Morrow (1981), who argued that a species that uses many hosts over its geographical range could in fact be using only one or a few host-plants in each site. Gomez-Zurita *et al.* (2000) found that specialization in *Timarcha* beetles is dependent on geographical distribution and thus on the availability of host-plants. Again, *Parides panthonus*, with seven known subspecies, could be a good taxon for testing this hypothesis. Within this species, five subspecies have "broad" geographic distributions (*P. panthonus panthonus, P. p. aglaope, P. p. ecaudatus, P. p. callicles* and *P. p. lysimachus*) and two are very restricted (*P. panthonus jaguarae* and *P. panthonus castilhoi*). Knowledge about host plant use within these seven geographic entities could help to understand the relationships between distribution and host associations.

Hindwing tails – The significance of particular characters of butterfly wings, including tails, has been widely discussed, but no solid conclusions have been attained (Wootton, 1992). Our objective in the present paper is to map the presence of long tails in species of Troidini, especially *Parides*, to check an idea proposed in Tyler *et al*. (1994: 179), that the tailed *Parides* are the basal species of the genus. Even though the species of tailed *Parides* appear to belong mainly to basal clades (with the exception of *P. tros*, which is tailed and not basal), the absence of phylogenetic signal in this character makes it difficult to draw additional inferences. Again, additional species of tailed *Parides* could help to clarify the evolution of this morphological feature. Among Papilionidae as a whole, tails appear to have evolved and disappeared on multiple occasions, with the most basal taxa (e. g., *Baronia*, Parnassiinae) lacking them, and all of the more derived tribes containing both tailed and tailless species.

Habitat – There are no *a priori* hypotheses about ancestral habitats of Troidini. Based on our results, the Troidini is a group that originated from forest ancestors; colonization of open habitats and scrub forest was secondary in the group. This pattern is the same for all analysed genera except

Euryades, whose two known species are specialised on open habitats. The Australian genus *Cressida*, a proposed sister group of *Euryades* (see above) occurs in a variety of habitats including grassland and open forests. If this sister group relationship is confirmed, it could be reasonable to infer that the *Cressida+Euryades* clade had its origins from open forest/grassland ancestors with Austral origin.

Gregariousness of immatures – According to our results, "immatures solitary or in loose groups" is the plesiomorphic state for this character, and since all known Troidini immatures are considered aposematic [and probably all are chemically defended (Tyler *et al.*, 1994; Klitzke and Brown, 2000)], it is likely that, at least in Troidini, evolution of aposematism has occurred prior to the evolution of gregariousness (as proposed by Sillen-Tullberg, 1988). Gregariousness thus appeared in the ancestor of the genus *Battus*, as this character is shared by all known species in this genus (Tyler *et al.*, 1994). Also, the results show that gregariousness has arisen only once in Troidini, a conclusion different from that stated by Sillen-Tullberg (1988), who proposed a minimum of two (or three) events of evolution of gregariousness in this tribe. Additionally, host plant features cannot be neglected when discussing the evolution of this trait, strongly related with advantages of group feeding behaviour in some species of *Battus* (Fordyce and Agrawal, 2001; Fordyce and Nice, 2004).

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Capítulo 2

Chemical and phylogenetic relationships among *Aristolochia* L. (Aristolochiaceae) from Southeastern Brazil

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Abstract

A molecular phylogeny based both on the plastid gene *mat*K and on the non-coding region between the genes *trn*L-*trn*F, and a chemical relationship based on their sesquiterpenes pattern, were proposed for plants in the genus *Aristolochia* from SE Brazil. *Aristolochia* is a monophyletic genus, whose ancestral state is the presence of aristolochic acids (AAs) in the leaves. Species considered derived show only labdanoic acids (LAs) in leaves. The phenetic relationship recovered with sesquiterpenes does not agree with the phylogenetic relationships for *Aristolochia*, and three main clusters can be recognized, germacrene-D, germacrene-C and *Z*-caryophyllene groups. The distribution of AAs and LAs over the phylogeny of *Aristolochia* can be viewed as a result of the evolution of defenses against herbivory of phytophagous insects through the evolutionary history of these plants. On the other hand, the differentiation of sesquiterpene structures in species phylogenetically close can be suggested to be adaptations related to attraction of pollinators.

Key words: *Aristolochia*, molecular phylogeny, chemical similarity, aristolochic acids, labdanoic acids, character optimization.

Resumo

Uma filogenia baseada no gene cloroplástico *mat*K e na região não-codificadora entre os genes *trn*L-*trn*F, e uma relação química baseada no seu padrão de sesquiterpenos, foram propostas para as plantas do gênero *Aristolochia* do SE do Brasil. *Aristolochia* é um gênero monofilético, cujo estado ancestral é a presença de ácidos aristolóquicos (AAs) nas suas folhas. Espécies consideradas derivadas mostram apenas ácidos labdanóicos (LAs) nas folhas. A relação fenética recuperada com os sesquiterpenos não concorda com a relação filogenética para as *Aristolochia*, e três grupos principais podem ser reconhecidos: germacreno-D, germacreno-C e *Z*-cariofileno. A distribuição de AAs e LAs sobre a filogenia de *Aristolochia* pode ser vista como um resultado da evolução de defesas contra herbivoria de insetos fitófagos através da história evolutiva destas plantas. Por outro lado, a diferenciação das estruturas dos sesquiterpenos em espécies filogeneticamente próximas pode ser hipotetizada como resultado de adaptações relacionadas à atração de polinizadores.

Palavras chaves: *Aristolochia*, filogenia molecular, similaridade química, ácidos aristolóquicos, ácidos labdanóicos, otimização de caráter.

Introduction

The genus *Aristolochia* L. "sensu lato" (Aristolochiaceae, Aristolochioideae) consists of approximately 400 species centered in tropical regions, with some species inhabiting subtropical and temperate habitats (Kelly and González, 2003); *ca*. 90 species occur in Brazil (Leitão and Kaplan, 1992). The plants are subshrubs to lianas, and are cultivated as ornamentals because of their uncommon flowers (Judd *et al.*, 2002), which are zygomorphic and usually pollinated by flies (Brantjes, 1980; Costa and Hime, 1981, 1983; Wolda and Sabrosky, 1986; Hall and Brown, 1993; Sakai, 2002; Burgess *et al.*, 2004). These plants contain as main secondary compounds aristolochic acids [AAs – bitter, yellow, nitrogenous compounds Judd *et al.* (2002)], aristolactam benzylisoquinoline alkaloids, and mono-, sesqui-, di- and triterpenes, including derived diterpene labdanoic acids (LAs) (Teresa *et al.*, 1983; Lopes *et al.*, 1987; Lopes and Bolzani, 1988; Lopes *et al.*, 1990; Luiz *et al.*, 1990; Leitão and Kaplan, 1992; Leitão *et al.*, 1992; Vila *et al.*, 1997; Bomm *et al.*, 1999; Palmeira *et al.*, 2001; Wu *et al.*, 2001; Priestap *et al.*, 2002; Tsuruta *et al.*, 2002; Priestap *et al.*, 2003; Francisco *et al.*, 2004; Shi *et al.*, 2004; Wu *et al.*, 2004). Sesqui- and diterpenes are the most abundant chemical compounds in this genus, and 24 types of sesquiterpenes have been described so far from *Aristolochia* species, the major types including cadinanes, aristolanes, germacrenes and bicyclogermacrenes (Wu *et al.*, 2004). Plants in this genus have been widely studied mainly due to their pharmacological activities, as well as anfeedant, insecticidal, antibacterial and antifungal roles [see Garavito (1990) and Wu *et al.* (2004) for reviews].

All of the above cited compounds are known to mediate ecological interactions among plants and herbivores. Mono- and sesquiterpenes, e. g., can act as agents of interspecific communication, playing an important role in attracting pollinator insects to plants and as deterrent for herbivores, including insects, together with diterpenes (Harborne, 2001). LAs were first isolated from the

leaves of *A. esperanzae*, *A. cymbifera* and *A. galeata* by Lopes and Bolzani (1988), and are considered to deter the herbivory of some specialist Troidini butterflies (Papilionidae, Papilioninae) in derived *Aristolochia* species (Klitzke, 1992). AAs are suggested to be responsible for the chemical defense of Troidini (Brower and Brower, 1964; Rothschild *et al.*, 1970), since their larvae feed on *Aristolochia* plants, sequestering some of the secondary compounds found in the plants, which make the butterflies unpalatable to potential predators and parasitoids (Nishida and Fukami, 1989a; Sime, 2002). The same compounds can act as feeding stimulants, enhancing the growth of Troidini larvae (Chew and Robbins, 1984; Miller and Feeny, 1989; Nishida and Fukami, 1989b; Klitzke, 1992), but are deleterious to non-AA specialists Papilionini and Graphiini (Miller and Feeny, 1989). There are few studies on ecological activities of benzylisoquinoline alkaloid involving *Aristolochia*, but it is known that berberine is toxic to the Troidini *Parides bunichus* and slows larval growth of *Battus polydamas*; both AAs and benzylisoquinoline alkaloids have a strong deterrent effect on non-*Aristolochia* feeding herbivores (Miller and Feeny, 1983, 1989).

Klitzke (1992) separated the species of *Aristolochia* from Southeastern Brazil in three groups, based on their chemical features: 1. with AAs and without LAs in leaves (*A. sessilifolia*, *A. melastoma*, *A. rumicifolia*, *A. arcuata*, *A. macroura* and *A. triangularis*); 2. without AAs or LAs in leaves (*A. odora* and *A. elegans*) and 3. with LAs and without AAs in leaves (*A. esperanzae*, *A. cymbifera* and *A. galeata*). He suggested that the presence or absence of these compounds were due to Troidini herbivory pressure on *Aristolochia* species: proposed basal species of these plants have AAs but not LAs, and the derived ones acquired LAs, which are deterrent for most Troidini, and lost AAs, which are phagostimulants.

A hypothetical hierarchical relationship among *Aristolochia* species based on morphology, vegetative and flower characters was proposed by Tyler *et al.* (1994) and Brown *et al.* (1995). Recently, Garavito (1999) and Kelly and González (2003) proposed a phylogeny for

Aristolochioideae and Aristolochiaceae, respectively, based on morphological characters, and Murata *et al.* (2001) constructed a molecular phylogeny to 50 species of *Aristolochia* using the gene *mat*K.

Our goal in this study was to infer the interspecific relationships of *Aristolochia* species occurring in SE Brazil and used as host-plants by Troidini butterflies, using sequences of plastid regions, and to correlate it with chemical characters, such as AAs and LAs, which were also studied by Klitzke (1992), and sesquiterpenes. Although terpenes have been widely studied in *Aristolochia* (see references above), they have not yet been used to infer relationships among species of this genus. In conifers, for example, sesqui-, di- and triterpenes have been found to be valuable for chemosystematic investigations (Otto and Wilde, 2001). Likewise, they were shown to be relevant to the chemical relationships within *Cannabis* (Hillig, 2004) and the *Achillea millefolium* group (Kubelka *et al.*, 1999). In addition, volatile oil composition was used to discriminate species in *Bidens pilosa* complex (Grombone-Guaratini *et al.*, 2005), and *Teucrium polium* varieties (Kamel and Sandra, 1994). Using our inferred interspecific phylogeny of *Aristolochia* as a template for these chemical compounds, we discussed possible scenarios for the evolution of such substances by selective pressures of specialist herbivorous and pollinator insects.

Materials and methods

Molecular phylogeny of Aristolochia

Intact leaves of 12 species of *Aristolochia* occurring in SE Brazil were collected in the field (Table 2.1). Vouchers of all samples have been deposited in the Herbarium of UNICAMP (UEC). Upon collection the leaves were stored in liquid N_2. Previously published sequences of six species of *Aristolochia* were obtained from GenBank (Murata *et al.*, 2001). The final matrix has 14 taxa, including 12 *Aristolochia* and two representatives of other genera of Aristolochiaceae, *Asarum caudatum* and *Saruma henryi*, used as outgroups (Table 2.1). These closely related genera belong to the basal Asaroideae subfamily (Neinhuis *et al.*, 2000; Kelly and González, 2003).

Total genomic DNA was extracted using hexadecyltrimethylammonium bromide (CTAB) as described by Stewart and Via (1993) from intact frozen leaves of *Aristolochia* species. The DNA was stored in TE buffer at -20°C. For each of the specimens we sequenced two regions. The plastid gene *mat*K, located within the intron of the gene *trn*K (Hilu and Liang, 1997), was sequenced for those species not previously sequenced by Murata *et al.* (2001) in their study on the phylogeny of *Aristolochia*. To sequence this gene we used the same primers used in Murata's study (Table 2.2), with the following thermal cycling protocol: 96°C for 3 min, 35 cycles of 96°C for 1.5 min, 51°C for 1 min, 72°C for 2 min, and a final extension period of 72°C for 10 min. The other region was located between *trn*L (UAA) and *trn*F (GAA) cpDNA genes. We used the primers proposed by Taberlet *et al.* (1991) to sequence this region (primers *c* and *f* – Table 2.2), following the same cycling profile used for the *mat*K gene.

PCR products were cleaned up by using a GFXTM PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech), and then submitted to a sequence reaction using the protocol of ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit. PCR fragments were then

sequenced in an ABI 377 automated sequencer. All fragments were sequenced in both directions, using the same primers used for the PCR reactions (Table 2.2). Sequences were analyzed with SeqEd™ program version 1.0.3 (Applied Biosystems, Inc.), and aligned manually by the Se.Al program (Rambaut, 1996). We used the translated amino acid sequences and the sequences of other *Aristolochia* species available in GenBank (Murata *et al.*, 2001) to align the sequences of the *mat*K gene, and to align the non-coding region sequences we used the rules proposed by Borsch *et al.* (2003). All sequences were deposited in GenBank (accession numbers in Table 2.1).

Table 2.1. Species of *Aristolochia* sampled, with localities and GenBank accession numbers.

Species	Locality	Voucher Number (UEC)	GenBank Accession Numbers	
			***mat*K**	***trn*L-*trn*F**
Outgroup				
Asarum caudatum Lindley	Corvallis, OR, USA	———	AY781532	AY781539
Saruma henryi Oliver	China (cult.)*	———	AB060736*	AY145340#
	Bonn Bot. Garden#			
Aristolochia				
Aristolochia arcuata Masters 1885	Atibaia, SP, Brazil	132947	AB060786*	AY781540
	Campinas, SP, Brazil	132946+		
Aristolochia chamissonis (Klotzch) Duchartre 1864	Belo Horizonte, MG, Brazil	135626	AY781533	AY781541
Aristolochia cymbifera Mart. & Zucc. 1824	Mogi-das-Cruzes, SP, Brazil	135627	AB060789*	AY781542
Aristolochia cynanchifolia Mart. & Zucc. 1824	Rio de Janeiro, RJ, Brazil	135625	AY781534	AY781543
Aristolochia elegans Masters 1885	Piracicaba, SP, Brazil	132944	AB060790*	AY781544
Aristolochia esperanzae O. Kuntze 1898	Mogi-Guaçú, SP, Brazil	135628	AY781535	AY781545
Aristolochia galeata Mart. & Zucc. 1824	Atibaia, SP, Brazil	132942	AY781536	AY781546
Aristolochia gigantea Mart. & Zucc. 1824	Campinas, SP, Brazil	132941	AB060793*	AY781547
	Mogi-das-Cruzes, SP, Brazil	135633+		
Aristolochia macroura Gomez 1812	Campinas, SP, Brazil (cult.)	132948	AB060795*	AY781548
Aristolochia melastoma Manso 1864	Campinas, SP, Brazil	135624	AY781537	AY781549
Aristolochia paulistana Hoehne 1927	Piracicaba, SP, Brazil	132943	AY781538	AY781550
Aristolochia triangularis Cham. 1832	Atibaia, SP, Brazil	132945	AB060803*	AY781551

* Sequences obtained from GenBank (Murata *et al.*, 2001)

Sequence obtained from GenBank (Borsch *et al.*, 2003)

+ Samples used only for chemical analyses

Table 2.2. Primers used in this study.

Gene	Name	F/R	Sequence (5'→3')
*mat*K	*mat*K-1412F*	F	ATATAATTCTTATGTATGTG
	*mat*K-1470R*	R	AAGATGTTGAT(C/T)GTAAATGA
	*mat*K-AF[#]	F	CTATATCCACTTATCTTTCAGGAGT
	*mat*K-8R[#]	R	AAAGTTCTAGCACAAGAAAGTCGA
*trn*L-*trn*F	C[+]	F	CGAAATCGGTAGACGCTACG
	f[+]	R	ATTTGAACTGGTGACACGAG

* Johnson and Soltis (1994); [#] Ooi *et al.* (1995); [+] Taberlet *et al.* (1991)

Phylogenetic analyses

To explore the phylogenetic relationships among *Aristolochia* species, we initially used the Partition Homogeneity Test of PAUP* 4.0b10 (Swofford, 2002) to assess congruence among the two molecular data sets. We performed the test under parsimony, using the following parameters: heuristic search, TBR branch-swapping, with 100 random addition sequences, and 500 replicates to generate the null hypothesis. Partitions between *mat*K and the intergenic spacer *trn*L-*trn*F were homogeneous (P=0.564), and we carried out the phylogenetic analyses using the combined data set.

Maximum Parsimony (MP) analyses were performed with PAUP* 4.0b10 (Swofford, 2002) on the entire data set using heuristic search with 500 random taxon addition replicates, TBR branch-swapping, gaps scored as missing data, and all characters equally weighted. A strict consensus tree was computed whenever multiple equally parsimonious trees were obtained. The consistency index (CI) and the retention index (RI) were calculated in PAUP "tree scores" option. The robustness of each branch was determined using the non-parametric bootstrap test (Felsenstein, 1985), with 1000 replicates and 10 random taxon additions. Bremer support and partitioned Bremer support values (to obtain the contribution of each data set to the Bremer support values of the combined analysis) (Bremer, 1988; Bremer, 1994; Baker and DeSalle, 1997; Baker *et al.*, 1998) were calculated using

TreeRot (Sorensen, 1999), in conjunction with PAUP* 4.0b10 (Swofford, 2002). The analysis was conducted with 100 random taxon addition replicates, TBR branch-swapping and 100 trees held in each replicate.

Bayesian analyses were carried out in Mr. Bayes (Huelsenbeck and Ronquist, 2001; Huelsenbeck *et al.*, 2001; Huelsenbeck *et al.*, 2002) to investigate the effects on the results under the most restrictive assumptions of data analysis. The program MODELTEST 3.06 (Posada and Crandall, 1998) was used to determine the available substitution model with the best fit to each partitioned data set, thus, Bayesian analyses were performed under the model GTR+G+I [General Time-Reversible model (Rodrígues *et al.*, 1990), with gamma distribution (Γ) and with proportion of invariable sites (I)]. All substitution model parameters (gamma shape parameter, proportion of invariable sites, character state frequencies, substitution rates of GTR model) were allowed to vary across partitions (= plastid regions). We conducted six simultaneous chains for 1.0×10^6 generations, sampling trees every 100 cycles. Stability of the process was assessed by plotting the likelihood scores against generation time (Lin and Danforth, 2004). The first 1000 trees were discarded as "burn in". For all analyses, *Saruma henryi* was used as outgroup to root the tree.

Character optimization

The distribution of AAs and LAs over the MP molecular phylogeny proposed for *Aristolochia* was investigated using MacClade 3.08 program (Maddison and Maddison, 1999). Character states were treated as unordered and of equal weights. To test whether there is a phylogenetic signal in the character traced (that is, if the distribution of these traits among taxa can be explained by their phylogenetic relationships), we used the methodology proposed by Wahlberg (2001), modified from the PTP test described by Faith and Cranston (1991). The test consists in comparing the number of steps of the tree constructed with the actual data with the number of steps

obtained for each random reshuffling of the states of each separated character. We performed 300 random reshufflings of character states among the fixed terminal taxa, with the equally weighted data set, using the option "shuffle" in the MacClade program (Maddison and Maddison, 1999). The probability (*P*) that the observed pattern does not differ randomly is given by the number of replications as short as or shorter than the tree obtained with the actual data, plus one, divided by the number of replications. Following Faith and Cranston (1991), a significant phylogenetic signal is observed when *P* is less than 0.05, and here, the minimal value should be 0.003 (number of trees as short or shorter than the original tree + 1/300).

Chemical similarity among Aristolochia species

Undamaged and fully expanded leaves of the same samples of *Aristolochia* used in molecular phylogeny were sampled in the field as above to be analyzed for their sesquiterpene composition (Table 2.1). Upon sample, the leaves were kept in 30 mL of CH_2Cl_2 at -15°C until the extraction, and GC and GC-MS analyses.

The leaves of each sample were extracted with Polytron®, and the dichloromethane residue reduced by vacuum at room temperature. The dichloromethane extracts of *A. esperanzae*, *A. cymbifera* and *A. galeata* were methylated with diazomethane (standard conditions) before GC and GC-MS analyses, since they presented LAs. The resulting extracts were analyzed by GC-MS-EI on a Hewlett Packard-6890 gas chromatograph system with a fused capillary column (30m x 0.25mm x 0.25µm, HP-5MS, Crossbond 5%-phenyl-95%-dimethylpolysiloxane) directly coupled to a selective mass detector Hewlett Packard 5973. Conditions of injection were modified from Adams (1995): injector temperature = 240°C; oven temperature program = 60°C-300°C, 3°C/min; splitless injection during 1.50 min, carrier gas = He (1 mL/min), constant flow; sample volume 0.2 or 1 µL. An analysis in split mode with port injector at 150°C was also carried out to verify if Cope

rearrangement was occurring in germacrene-A and B yielding β- and δ-elemene, respectively (Teisseire, 1994; De Kraker *et al.*, 2001; Quintana *et al.*, 2003). Retention Indexes (RIs) of each compound were calculated according to van den Dool and Kratz (1963). The compounds eluted in the sesquiterpene region (Patitucci *et al.*, 1995) were tentatively identified comparing their RIs and mass fragmentation patterns with literature values (Adams, 1995). In order to obtain the relative abundance of each compound, the samples were analyzed on a Hewlett Packard-6890 GC system with a fused capillary column (30m x 0.25mm x 0.25μm, SA-5, Crossbond 5%-phenyl-95%-dimethylpolysiloxane, Sigma-Aldrich) directly coupled to a flame ionization detector. The conditions of injection were the same as above.

The relative abundance of each compound (arcsin transformed) was used for statistical analyses using Euclidean distances for similarity determination and Ward´s method as cluster procedure. In addition, a principal component analysis (PCA) was conducted (Legendre and Legendre, 1998).

Determination of presence or absence of aristolochic acids

We analyzed the leaves of four species of *Aristolochia* (*A. chamissonis*, *A. cynanchifolia*, *A. gigantea* and *A. paulistana*) in order to complement the data obtained by Klitzke (1992) on the presence and absence of AAs and LAs in *Aristolochia* leaves. We followed the methodology proposed by Urzúa and Priestap (1985) to carry out the extraction of AAs. Fresh leaves were extracted with MeOH (40 mL x 3); the extracts were combined, dried by vacuum at room temperature, and diluted in 10 mL of MeOH + 50 mL of 2% $NaHCO_3$, and then extracted with $CHCl_3$ (30 mL x 4). The aqueous basic layer was acidified with HCl to pH ≈ 3 and then extracted with $CHCl_3$ (30 mL x 6). The $CHCl_3$ was completely evaporated and the residue recovered in a known volume of $CHCl_3$. The organic residue was chromatographed together with commercial AAs

standard (Aldrich Chem. Co.) on thin-layer plates (TLC) of silica gel 60 F_{254} (Merck) in $CHCl_3$:MeOH (6:4). The AAs were detected in visible light (yellow spots) and UV at Rf ≈ 0.54.

Results and Discussion

Molecular phylogeny of Aristolochia

The full data set of *Aristolochia* contained 2242 nucleotides, 1245 from *mat*K and 997 from the region between *trn*L-*trn*F. The total number of parsimony-informative sites in the full combined data set was 249.

Parsimony searches over the equally weighted combined data set resulted in 24 equally parsimonious trees, with 477 steps (CI=0.901; RI=0.861). The strict consensus tree is shown in Fig. 2.1. The genus *Aristolochia* appeared as a monophyletic group, with a strong bootstrap and Bremer support, and with *Aristolochia melastoma* at the basal position in relation to all other *Aristolochia*. The absence of stipules and the presence of a limb subpeltilabiate in *Aristolochia melastoma* can be considered characteristic to put it at a basal position within its genus (Capellari Jr., 1991). The phylogenetic relationship among the other *Aristolochia* species was not completely resolved at the internal nodes with the fragments we used here; however, the plants came up divided into three major groups, besides *A. arcuata* and *A. triangularis*, which appeared as a polytomy. *Aristolochia chamissonis* and *A. paulistana* clustered in one clade with strong bootstrap and Bremer support. *Aristolochia cymbifera*, *A. esperanzae*, *A. galeata* and *A. macroura* compose a clade with strong bootstrap and Bremer support, with *A. macroura* occupying a basal position and the other species forming an unresolved clade with moderate support. The last clade was composed of *A. cynanchifolia*, *A. elegans* and *A. gigantea*, with strong bootstrap and Bremer support. The contribution to the tree of each gene, assessed by Partitioned Bremer Support, showed that both regions contributed equally to most branches, although *mat*K data was a source of conflict at two branches (Fig. 2.1).

The topology of the tree obtained by Bayesian analysis was quite similar to that obtained by MP, with the Bayesian tree differing only in the position of *Aristolochia triangularis* (Fig. 2.2). This species clustered with *A. gigantea*, *A. cynanchifolia* and *A. elegans* with a strong Posterior Probability (PP) value, occupying a basal position in relation to them (Fig. 2.2).

Our phylogenetic hypothesis for *Aristolochia* from SE Brazil found with chloroplast regions agrees with most of the results found by Murata *et al.* (2001), who used only *mat*K sequences, although the complete set of species in each study is quite different. The close relationship between *Aristolochia gigantea* and *A. elegans* was also found by Murata *et al.* (2001), as well the phylogenetic proximity between *A. macroura* and *A. cymbifera*. Besides the molecular phylogeny proposed by Murata *et al.* (2001) for *Aristolochia*, Garavito (1999) constructed a cladistic phylogenetic hypothesis for the subfamily Aristolochioideae based on morphological characters of flowers and pollen; however, only one species which took part in our study, *A. melastoma*, was present, making impossible any comparison. The only hierarchical relationship proposed for *Aristolochia* from SE Brazil was made by Tyler *et al.* (1994) and Brown *et al.* (1995), and some of our results strongly agree with this proposal. As affirmed above, *A. melastoma* occupies a basal position within *Aristolochia*, the same position suggested by Tyler *et al.* (1994) and Brown *et al.* (1995). Likewise, they proposed that *A. esperanzae*, *A. galeata* and *A. cymbifera* must have a derived position within *Aristolochia*, what is confirmed by our results (Figs. 2.1 and 2.2).

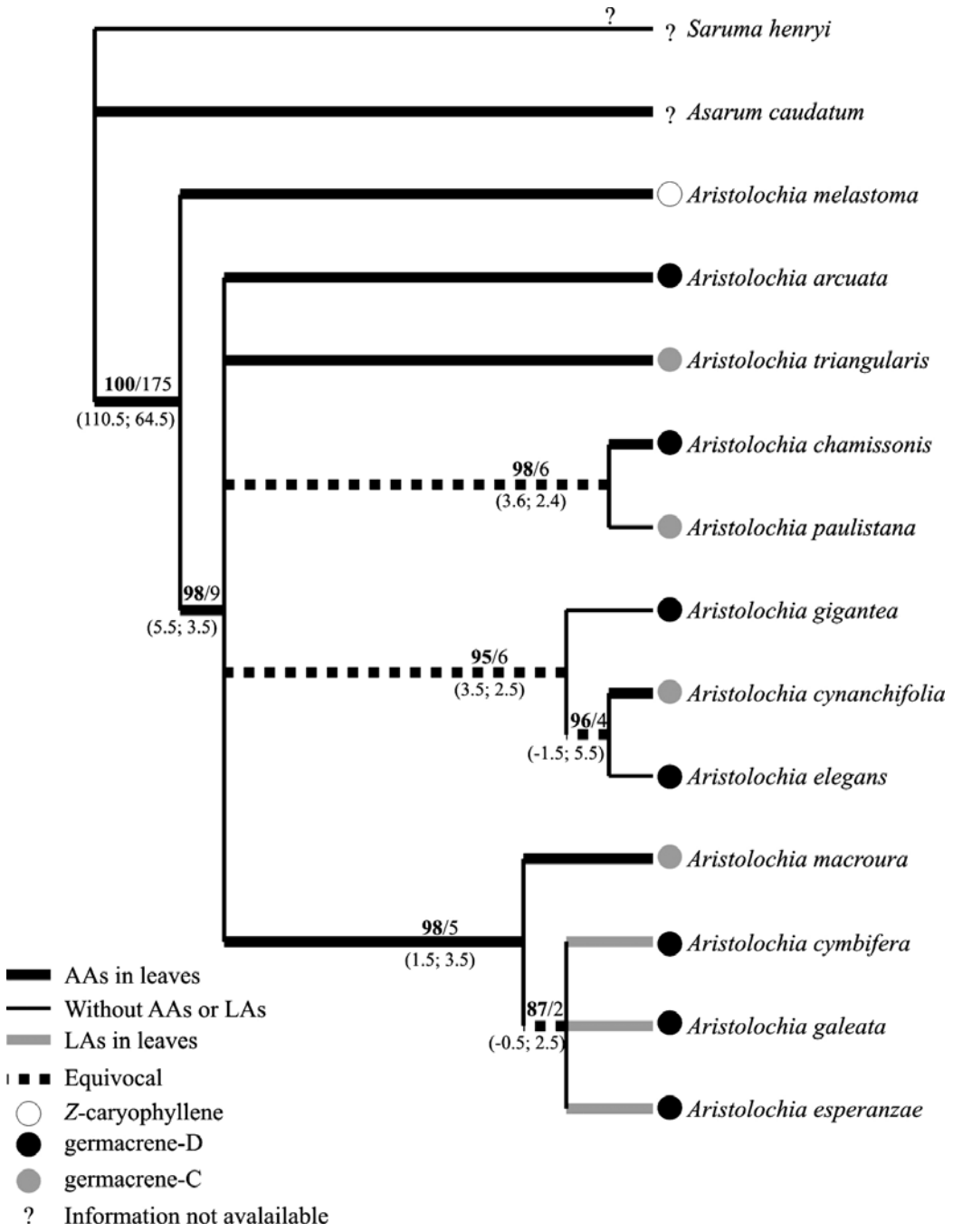

Figure 2.1. Phylogenetic relationship hypothesis among *Aristolochia* species. Strict consensus tree of 24 equally parsimonious trees based on combined data analysis (477 steps). Values above the branches indicate bootstrap values from 1000 replications and Bremer Support, respectively. Numbers below the branches are partitioned Bremer Support values, which indicate the contribution of *mat*K and non-coding region between *trn*L-*trn*F, respectively, to the Bremer support value of the combined analysis.

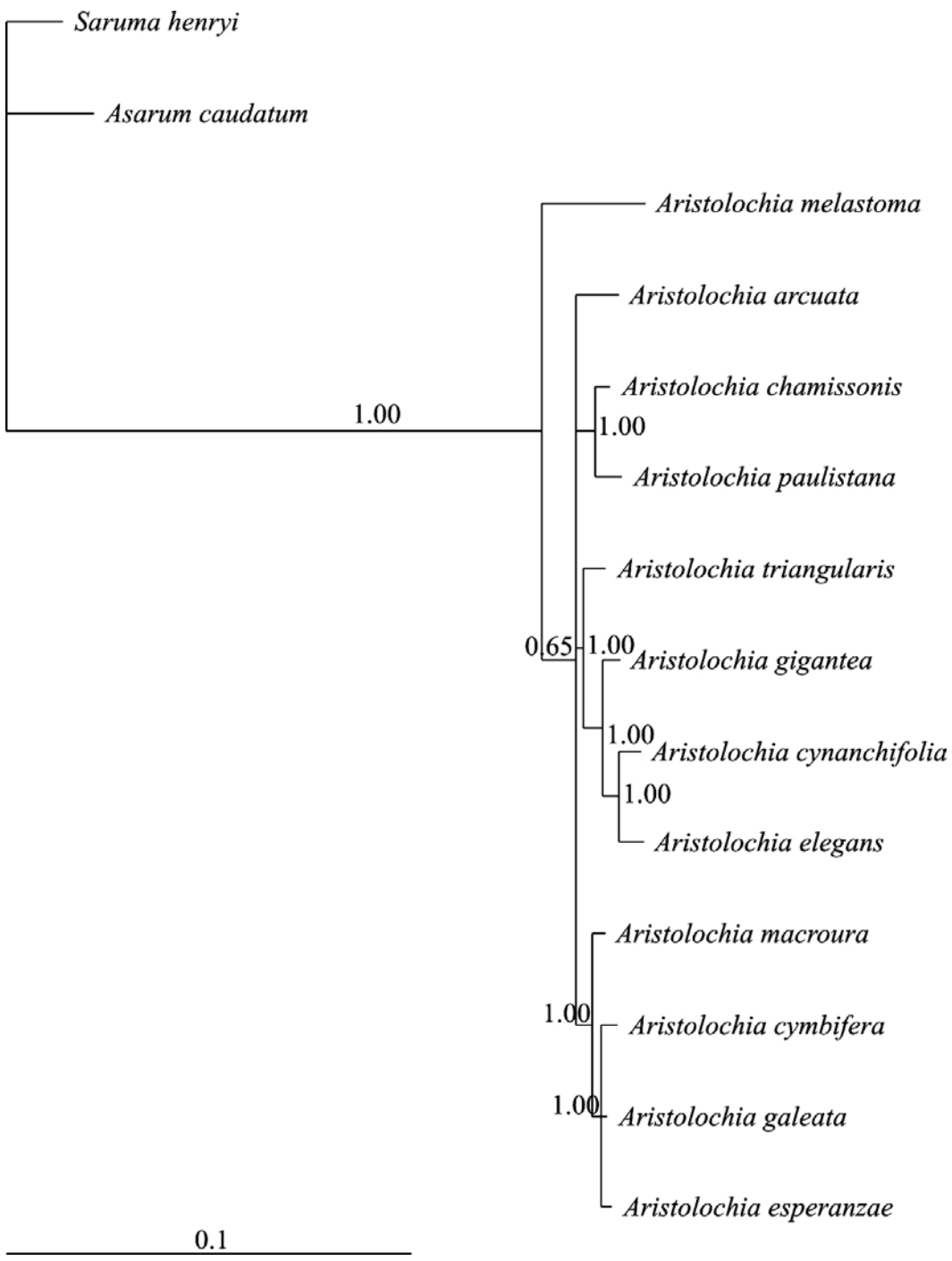

Figure 2.2. Phylogenetic tree inferred by Bayesian analysis from combined data set. Values above the branches indicate Bayesian Posterior Probability.

Chemistry of Aristolochia species

Fifty-one different compounds in the sesquiterpene retention time region were found in the 12 *Aristolochia* species. The relative abundance of each compound in each species is given in Table 2.3. Two sesquiterpenes tentatively identified as *E*-caryophyllene and bicyclogermacrene were found in all *Aristolochia* species studied, generally in high relative abundance (Table 2.3). Germacrene-D, found in high relative abundance in 10 of 12 *Aristolochia* species, was not found either in *A. cynanchifolia* or *A. paulistana*.

GC-MS analyses in split mode with port injector at 150°C showed that elemenes are Cope rearrangement products, derived from germacrenes. Sesquiterpenes with germacrene frameworks are often difficult to characterize by GC-MS, since germacrenes A, B and C, under some GC conditions, rearrange to the respective β-, γ- and δ-elemenes (Teisseire, 1994; De Kraker *et al.*, 2001; Quintana *et al.*, 2003) (Fig. 2.3).

Three main clusters can be recognized; the same groups as those recovered by cluster and PCA analyses (Figs. 2.4 and 2.5). *Group 1* is characterized by the high relative abundance of germacrene-D (RI 1480; ≈ 6-33%), and is composed of *Aristolochia arcuata, A. chamissonis*, *A. cymbifera*, *A. elegans*, *A. esperanzae*, *A. galeata* and *A. gigantea.* One individual of *Aristolochia triangularis* showed up in this cluster due to the high relative abundance of germacrene-D in the leaves. The sesquiterpene patterns of *A. gigantea* and *A. galeata* are not homogeneous among individuals, and their positions in relation to the other species within this group are unresolved; due to this, these species showed up more than once in the dendrogram (Fig. 2.4). *Group 2* is characterized by a high relative abundance of germacrene-C (inferred by their Cope rearrangement product δ-elemene - RI 1339; ≈ 16-49%), and is composed of *Aristolochia cynanchifolia*, *A. paulistana*, *A. macroura* and *A. triangularis*. Two individuals of *Aristolochia esperanzae* appear in this cluster because of their high relative abundance of germacrene-C. *Group 3* is composed only of *Aristolochia melastoma*, and is characterized by a high relative abundance of *Z*-caryophyllene (RI 1404; 38%).

Our data on sesquiterpenes prove to be useful in separating efficiently the *Aristolochia* species studied here. Indeed, the analyses using only the presence and absence of those compounds in each species also discriminate very well the *Aristolochia* species (data not shown). This is the first time that the sesquiterpenes are used to infer relationships among *Aristolochia*, despite the huge number of papers describing their terpenoid composition. Many diterpenes have been described for *Aristolochia*, but again no study was developed to infer interspecific relationships using this character.

We found three main groups of *Aristolochia* from SE Brazil in our cluster analyses (Fig. 2.4), which can be named germacrene-D, germacrene-C and *Z*-caryophyllene groups, and some of our results agree with the data available in the sesquiterpenes composition for *Aristolochia* species. *Aristolochia elegans* was placed in the germacrene-D group; this compound was the main sesquiterpene in the oil from its leaves (Vila *et al.*, 1997), although the sesquiterpene bicyclogermacrene was the most abundant. Here, this compound corresponds to 15% of the relative abundance of sesquiterpenes in leaves of *A. elegans*, and the most abundant sesquiterpene was tentatively identified as *E*-caryophyllene (35% of relative abundance, Table 2.3). Francisco *et al.* (2004) found that the major sesquiterpenes of *Aristolochia esperanzae* and *A. chamissonis* are caryophyllene, germacrene-B and spathulenol, the same results showed here. The main sesquiterpene we found in *A. chamissonis* was tentatively identified as *E*-caryophyllene, also found at a high relative abundance in leaves of *A. esperanzae* (Table 2.3).

We identified the presence of aristolochic acids in leaves of *Aristolochia chamissonis* and *A. cynanchifolia* in addition to the AAs found by Klitzke (1992) in *A. melastoma*, *A. arcuata*, *A. macroura* and *A. triangularis*. These compounds were not found here in leaves of *A. gigantea* and *A. paulistana*, nor in leaves of *A. elegans*, *A. esperanzae*, *A. cymbifera* and *A. galeata* (Klitzke, 1992).

Table 2.3. Relative abundance (mean ± SE) of sesquiterpenes in *Aristolochia* species. Numbers in parenthesis represent sampled individuals in each species.

Compounds	KI	KI Adams (1995)	*A. arcuata* (17)	*A. chamissonis* (2)	*A. cymbifera* (2)	*A. cynanchifolia* (3)	*A. elegans* (1)	*A. esperanzae* (9)	*A. galeata* (5)	*A. gigantea* (5)	*A. macroura* (16)	*A. melastoma* (10)	*A. paulistana* (1)	*A. triangularis* (13)
δ-elemene[1]	1334	1339	5.4±1.2	-	10.9±0.2	49.2±0.6	17.7	3.9±2.6	11.8±5.1	13.1±3.4	16.4±0.9	-	44.1	25.9±1.8
α-cubebene	1347	1351	<0.1	-	0.4±0.4	-	-	1.0±0.3	0.3±0.3	0.8±0.5	-	-	-	0.2±0.1
Citronellyl acetate	1355	1354	-	-	-	-	-	-	-	-	-	-	-	0.3±0.2
Cyclosativene	1362	1368	-	-	-	-	-	0.1±0.1	-	-	-	-	-	-
α-ylangene	1372	1372	-	-	-	-	-	3.6±0.9	-	-	-	-	-	-
α-copaene	1372	1376	5.8±0.5	-	7.2±1.1	-	2.2	-	3.5±1.1	3.6±0.5	1.2±0.1	2.8±0.2	-	3.8±0.5
β-bourbonene	1380	1384	-	3.7±0.1	0.6±0.6	-	-	2.7±0.5	3.0±0.9	0.3±0.3	0.8±0.2	1.0±0.4	-	2.4±0.3
β-elemene[2]	1383	1391	6.5±1.1	5.6±0.2	9.3±7. 3	-	1.5	0.7±0.7	1.7±1.0	3.4±0.3	2.4±0.2	0.6±0.4	1.2	14.1±2.3
β-elemene – isomer[2]	1389	1391	1.6±1.0	-	-	-	-	-	-	-	-	-	-	-
Cyperene	1400	1398	-	-	-	-	-	0.2±0.1	-	-	-	-	-	-
Unknown	1402		-	-	-	-	-	-	-	-	-	-	-	<0.1
Z-caryophyllene	1402	1404	-	-	-	-	11.2	-	-	-	0.1±0.1	37.6±4.1	-	<0.1
α-gurjunene	1425	1409	-	0.5±0.5	-	-	-	-	-	0.7±0.4	-	-	-	-
E-caryophyllene	1413	1418	19.9±0.8	39.5±2.0	15.9±1.9	10.0±2.0	34.6	18.8±1.7	30.2±4.8	33.7±2.5	40.7±1.1	12.6±3.4	10.2	12.4±0.9
β-gurjunene	1424	1432	-	1.5±1.5	1.2±1.2	-	-	0.9±0.5	1.9±1.2	0.8±0.5	-	-	-	1.5±0.6
γ-elemene[3]	1434	1433	0.5±0.3	-	11.8±4.3	-	-	0.1±0.1	-	0.9±0.4	-	-	-	4.6±0.6
Aromadendrene	1440	1439	2.0±0.6	-	-	-	-	-	-	0.3±0.3	-	-	-	<0.1
α-guaiene	1439	1439	-	-	-	-	-	0.2±0.1	-	-	-	3.5±1.0	-	0.7±0.2
α-himachalene	1444	1447	-	-	-	-	-	0.3±0.2	-	0.9±0.7	-	-	-	0.6±0.2
α-humulene	1448	1454	2.2±0.4	1.7±1.7	3.0±0.9	1.2±0.7	0.4	1.4±0.6	0.6±0.6	3.9±0.4	4.5±0.3	6.5±0.5	-	2.0±0.2
E-β-farnesene	1457	1458	-	-	-	-	-	-	-	-	0.3±0.1	0.1±0.1	0.5	-
Cis-muurola-4(14),5-diene	1461	1460	-	-	-	-	-	0.3±0.2	-	-	-	-	-	-
Seychellene	1458	1461	-	-	-	0.1±0.1	-	-	-	-	-	-	-	1.3±0.8
β-acoradine	1463	1466	-	-	-	-	-	0.1±0.1	-	-	-	-	-	0.2±0.1
β-chamigrene	1472	1475	-	-	-	-	4.2	-	-	2.0±0.9	0.7±0.2	4.6±1.0	-	0.7±0.2
Germacrene-D	1479	1480	33.1±1.6	19.8±5.2	22.9±9.4	-	6.3	22.2±3.8	25.8±2.5	8.6±1.6	3.1±0.2	6.7±2.8	-	8.4±2.1
β-selinene	1480	1485	-	-	-	-	-	0.2±0.1	-	-	-	-	-	0.4±0.2
Cis-β-guaiene	1482	1490	-	-	-	-	-	-	-	-	-	0.3±0.3	-	-
Bicyclogermacrene	1493	1494	10.0±0.8	24.0±1.7	8.5±1.9	38.8±2.3	15.2	22.7±2.5	11.9±3.6	18.9±3.8	15.3±0.8	9.2±1.1	40.3	10.7±0.8

Table 2.3. Extended.

Compounds	KI	KI Adams (1995)	*A. arcuata* (17)	*A. chamissonis* (2)	*A. cymbifera* (2)	*A. cynanchifolia* (3)	*A. elegans* (1)	*A. esperanzae* (9)	*A. galeata* (5)	*A. gigantea* (5)	*A. macroura* (16)	*A. melastoma* (10)	*A. paulistana* (1)	*A. triangularis* (13)
α-muurolene	1498	1499	-	-	-	-	-	-	-	-	-	0.4±0.3	-	-
Unknown	1503		1.8±1.1	0.2±0.2	-	-	-	0.7±0.1	-	-	0.2±0.1	-	-	1.0±0.3
α-farnesene	1506	1508	-	-	-	-	-	0.1±0.1	-	1.9±1.1	-	-	-	<0.1
β-bisabolene	1508	1509	-	-	-	-	-	-	-	0.2±0.1	-	-	-	-
γ-cadinene	1510	1513	0.6±0.3	-	1.8±0.4	-	-	1.3±0.7	3.9±0.8	1.2±0.9	0.2±0.2	8.2±1.2	-	-
7-epi-α-selinene	1510	1517	-	-	-	-	-	-	-	-	-	-	-	1.0±0.2
Unknown	1520		-	-	-	-	-	1.6±0.8	-	-	-	-	-	1.8±0.2
δ-cadinene	1520	1524	7.6±0.8	-	5.4±1.0	-	3.2	-	-	2.0±0.6	1.8±0.2	4.1±0.3	-	-
Cadina-1,4-diene	1526	1532	-	-	-	-	-	-	-	-	-	0.2±0.2	-	-
α-cadinene	1539	1538	-	-	-	-	-	0.2±0.04	-	-	-	-	-	-
Unknown	1545		-	-	-	-	-	-	-	-	-	-	-	0.3±0.1
Unknown	1546		-	-	-	-	-	<0.1	-	-	-	-	-	-
Elemol	1547	1549	-	0.4±<0.1	-	-	-	-	-	-	-	-	-	0.7±0.2
Unknown	1552		-	-	-	-	-	-	-	-	3.9±0.3	-	-	-
Unknown	1556		0.1±0.03	-	0.5±0.2	-	-	5.1±3.4	-	-	-	0.2±0.2	-	0.6±0.1
E-Nerolidol	1564	1564	-	-	-	-	1.1	0.4±0.3	4.0±2.5	-	-	-	-	0.4±0.1
Occidentalol	1545	1548	-	-	-	-	-	-	-	-	-	1.4±0.5	-	-
Ledol	1562	1565	-	-	-	-	-	-	-	-	-	0.1±0.1	-	-
Germacrene D-4-ol	1571	1574	1.2±0.3	2.5±1.4	0.5±0.5	-	2.2	-	1.3±0.5	2.8±1.1	3.1±0.6	-	3.6	3.2±0.5
Dendrolasin	1579	1574	-	-	-	0.7±0.1	-	-	-	-	2.8±0.4	-	-	-
Spathulenol	1573	1576	1.9±0.2	0.7±0.7	-	-	-	10.8±2.8	-	-	1.7±0.3	-	-	-
Caryoplyllene oxide	1580	1581	-	-	-	-	-	0.6±0.2	-	-	0.7±0.3	-	-	0.7±0.2

[1] Cope rearrangement from germacrene-C; [2] Cope rearrangement from germacrene-A; [3] Cope rearrangement from germacrene-B.

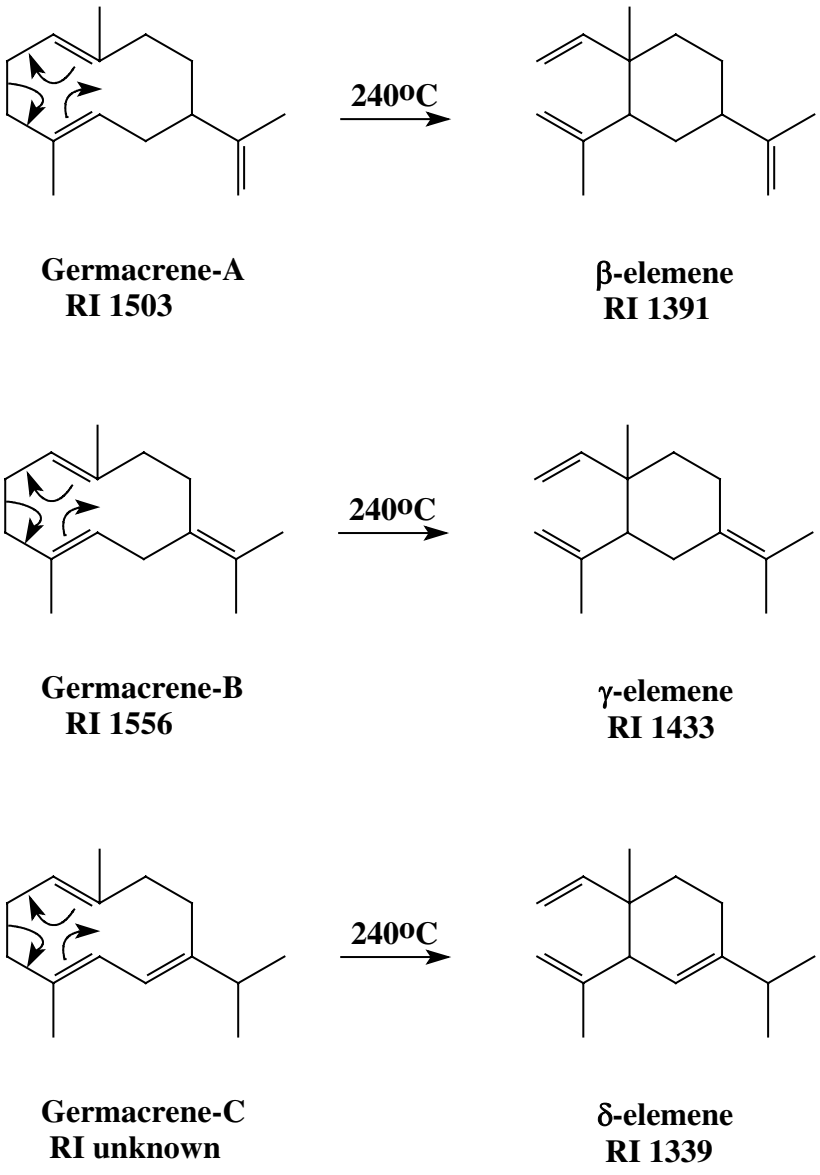

Figure 2.3. Cope rearrangement involving germacrene frameworks yielding elemenes [from Teisseire (1994)].

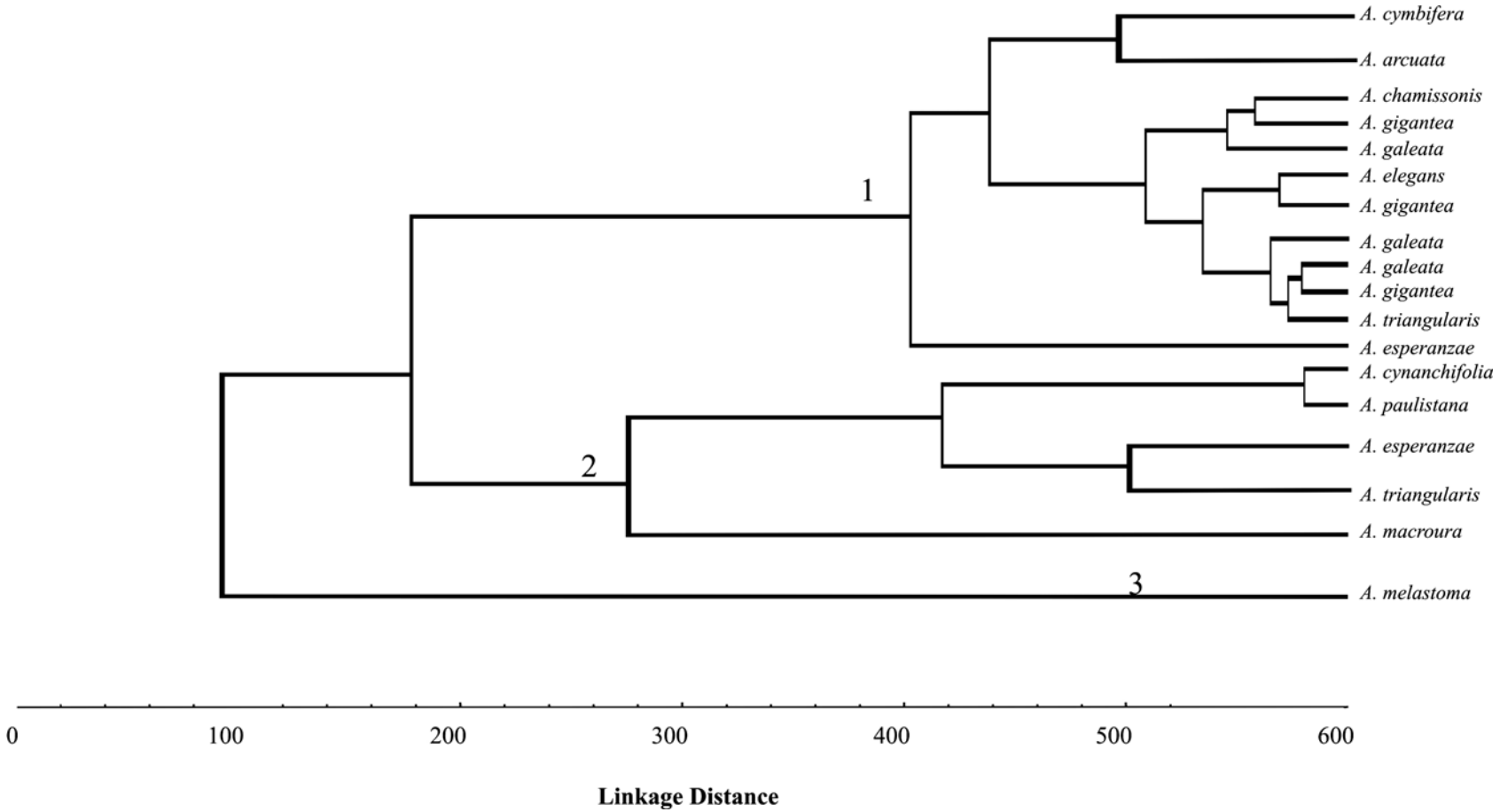

Figure 2.4. Dendrogram of *Aristolochia* based on chemical similarity. Numbers above internal branches indicate the three main groups according to Ward's clustering method of relative abundance of sesquiterpenes.

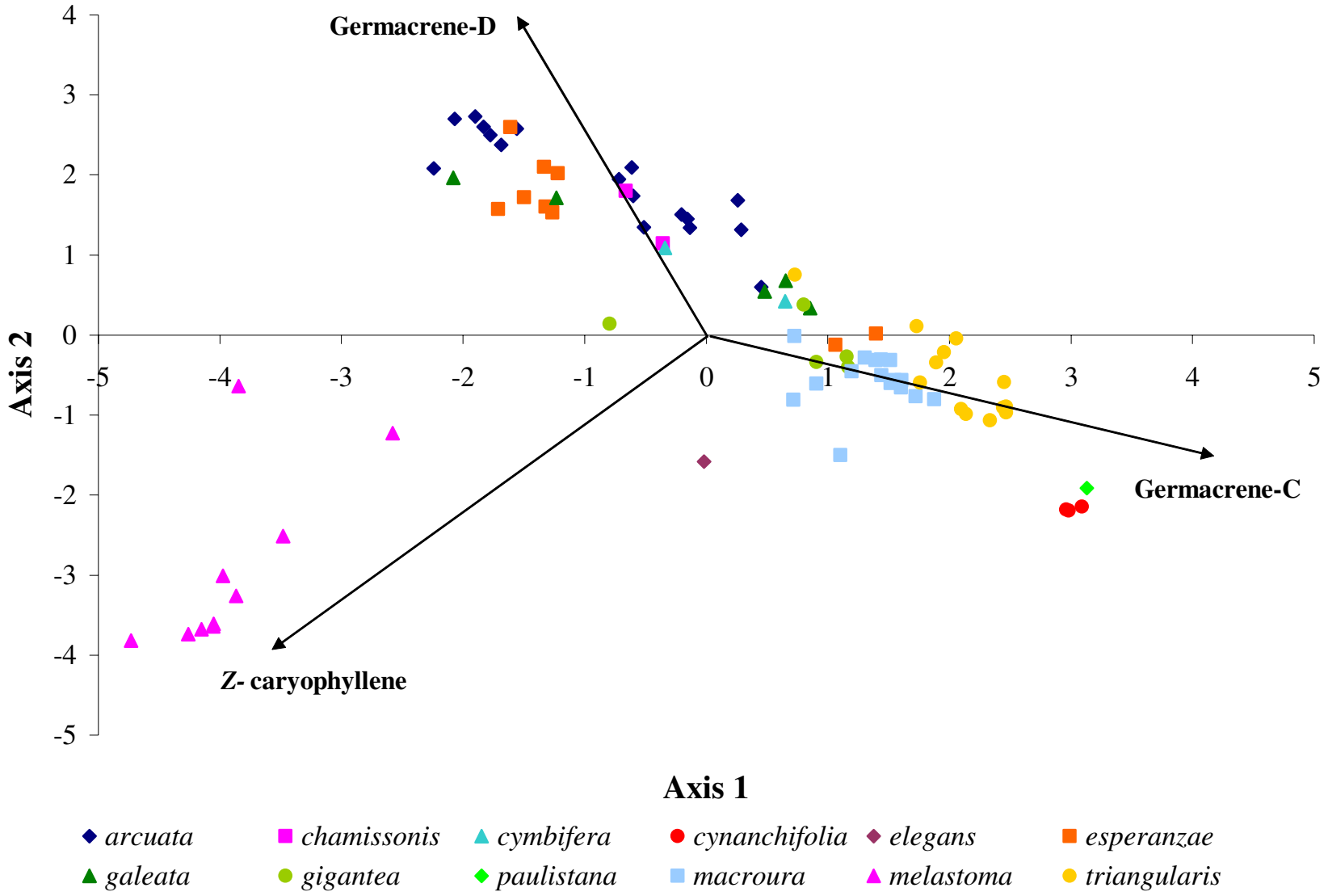

Figure 2.5. Ordination diagram of *Aristolochia* based on principal component analysis of relative abundance of sesquiterpenes. The axis 1 and 2 accounted for 28.89 and 20.46% of total variance, respectively.

Chemical and phylogenetic relationship

The ancestral state of the *Aristolochia* species studied here seems to be the presence of AAs in their leaves; however, the character tracing of AAs over the phylogeny of this genus did not show a significant phylogenetic signal ($P = 0.17$) (Fig. 2.1), although this can be due to the low resolution of the internal branches in the tree. Basal species, such as *Aristolochia melastoma*, show AAs in their leaves (Klitzke, 1992), and these compounds were also found in preliminary studies in leaves of *Euglypha* and *Holostylis* (Klitzke, 1992), *Thottea* spp (Nishida *et al.*, 1993) and *Asarum caudatum* (Kumar *et al.*, 2003). The last genus is considered basal within Aristolochiaceae (Garavito, 1999; Kelly and González, 2003), reinforcing the idea that the presence of AAs is a primitive trait in this family, as well as in the genus *Aristolochia*.

Likewise, the most derived node in the phylogeny of *Aristolochia*, joining *Aristolochia cymbifera*, *A. galeata* and *A. esperanzae* (despite of the lack of resolution at internal nodes in our phylogenetic hypothesis), is composed of species showing exclusively LAs in their leaves, instead of aristolochic acids (Lopes and Bolzani, 1988; Klitzke, 1992). Morphological evidence also joins these three species, since they all show stipules and a limb of calyx 2-labiate (Capellari Jr., 1991).

The phenetic relationships among *Aristolochia* found by sesquiterpene chemical similarity does not agree with our phylogenetic hypothesis (Figs. 2.1, 2.2, and 2.4). Species of *Aristolochia* highly similar in their sesquiterpene content, like *Aristolochia arcuata* and *A. cymbifera*, appear at different branches in our phylogenetic analyses.

Besides its basal phylogenetic position, *Aristolochia melastoma* was also the most dissimilar in its sesquiterpene pattern, and it is the only species to show the compound *Z*-caryophyllene at high relative abundance in the leaves. However, the more derived species (*Aristolochia cymbifera*, *A. galeata* and *A. esperanzae*), are very similar in sesquiterpene profile, and came up together in Group 1 in the dendrogram of sesquiterpene chemical similarity (Fig. 2.4). This similarity can facilitate

their use by herbivorous insects: the Troidini butterfly *Parides anchises*, e. g., although highly polyphagous, use preferentially *A. cymbifera*, *A. galeata* and *A. esperanzae* as host-plant (Brown *et al.*, 1995).

With the data of sesquiterpene pattern and the distribution of AAs and LAs over the phylogeny of *Aristolochia*, we are able to suggest hypothetical scenarios to explicate the evolution of these characters, based mainly on two of three scenarios proposed by Berenbaum and Seigler (1992) to explain the high phytochemical diversity in angiosperms. They cite, besides the pressure of abiotic environmental factors (e. g., high UV intensities), the pressure of herbivory by arthropods and the elaboration of attractants and rewards to pollinators as main causes of this diversity, affirming that these scenarios may not be mutually exclusive.

The distribution of AAs and LAs over the phylogeny of *Aristolochia* can be viewed under the light of the second scenario proposed by Berenbaum and Seigler (1992), that is, they may have arisen by pressures of herbivory of phytophagous insects. Ehrlich and Raven (1964) first suggested that herbivory pressures may have governed the secondary substances composition found in plants and (Brown *et al.*, 1991) applied this coevolutionary hypothesis to explain the interaction between Troidini butterflies and plants of *Aristolochia* species, considering the occurrence of changes in the classes of chemical compounds over the evolution of *Aristolochia* as defense against herbivory. Thus, derived species of *Aristolochia* may have lost AAs and acquired other defenses, such as LAs, to deter species of phytophagous insects specialized in Aristolochiaceae plants (Klitzke, 1992). In fact, all species of Troidini fed with leaves of *Aristolochia galeata* (with LAs) consumed less of these leaves and showed a smaller relative growth (Klitzke, 1992). In addition, the fraction of LAs of *A. galeata* and *A. esperanzae* (both species showing LAs), spread on the leaves of *Aristolochia* species usually accepted, inhibited their consumption, suggesting that LAs can work as an antifeedant to some species of Troidini (Klitzke, 1992). Only the most polyphagous species of

Troidini (*Parides neophilus* and *Battus polydamas*) were able to feed on *Aristolochia* species with LAs in their leaves (Klitzke, 1992). Additionally, the preferential use of species with LAs in their leaves by *Parides anchises* support the hypothesis of coevolution proposed by Brown *et al.* (1991), since this is a derived species within Troidini (Tyler *et al.*, 1994). However, these data can be interpreted as well by the theory of sequential evolution (Schoonhoven *et al.*, 1998) instead of by the coevolutionary hypothesis of Ehrlich and Raven (1964), that is, genetic changes in insect species may have enabled them to recognize plant species as hosts. By this theory, LAs may have arisen for other reasons rather than defense against herbivory, and some Troidini species may have been able to identify these plants and feed on them eventually, however, exerting little, if any, influence on *Aristolochia* chemical compounds evolution.

The third scenario of Berenbaum and Seigler (1992) (that is, pressure of pollinators) could be useful to enlighten the evolution of sesquiterpenes along the phylogeny of *Aristolochia*. In non-reproductive plant organs, chemical attractants of flowers may have secondarily (or simultaneously) defensive functions (Berenbaum and Seigler, 1992). Flowers of Magnoliacae, for example, emit many terpenoids (mainly hydrocarbon types), which are also emitted from damaged leaves (Azuma *et al.*, 1999); caryophyllene and bicyclogermacrene can be found both in flowers and leaves of Magnoliaceae (Azuma *et al.*, 1999). In some species of Nyctaginaceae, most of the total mono- and sesquiterpenes come from vegetation rather than from flowers, and both vegetative and floral volatiles are known to attract floral visitors (Levin *et al.*, 2001). If the volatile compounds of leaves of *Aristolochia* species are similar to those found in their flowers, we can infer that species closely related may have evolved different chemical compounds (sesquiterpenes) to attract different pollinators, as found in plants of the family Nyctaginaceae (Levin *et al.*, 2001). The pollination syndrome is quite specialized in *Aristolochia*, which have elaborate flowers with a highly modified calyx (Judd *et al.*, 2002). Flies are attracted to these flowers, primarily by odor (Sakai, 2002), and

are trapped within the flowerbase (utricle) to facilitate pollen deposition on the stigma (Burgess *et al.*, 2004). *Aristolochia* species show pollinator specificity (Brantjes, 1980; Siqueira, 1988), and species sharing the same type of pollinator have disjunct areas (Brantjes, 1980). Thorax height of the actual pollinating flies has an important role in this specificity; however, the selective action of the specific scents is suggested to be partly responsible for it (Hall and Brown, 1993). In our results, species of *Aristolochia* phylogenetically close show different sesquiterpenes (Fig. 2.1). The exceptions are *Aristolochia melastoma*, basal within *Aristolochia* and showing *Z*-caryophyllene as main compound, and the clade joining *A. cymbifera*, *A. esperanzae* and *A. galeata*, all them with germacrene-D at a high relative abundance. These species, however, show a different geographical range in the SE Brazil (Chapter 3). The differentiation of sesquiterpene profile in taxonomically close species, probably in order to attract different pollinators, may have low costs since the biosynthetic routes for production of *Z*-caryophyllene, germacrene-D and germacrene-C are very close, involving few biosynthetic steps from the same precursor, *trans-trans* farnesyl pyrophosphate [from Mann (1987) and Teisseire (1994)] (Fig. 2.6).

Improving our phylogenetic hypothesis [maybe using other genes, such as the ITS region (Liston *et al.*, 1999; Suh *et al.*, 2000; Bräuchler *et al.*, 2004; Kocyan *et al.*, 2004) or *rbcL* sequences (Rivadavia *et al.*, 2003)], carrying out experiments on the performance of Troidini butterflies on different host plants and their chemicals, and carrying out experiments with pollinator attraction of different *Aristolochia* species, will be necessary for the better understanding of the evolution of chemical compounds in *Aristolochia* species, and the role of herbivores and pollinators as selective pressure for it.

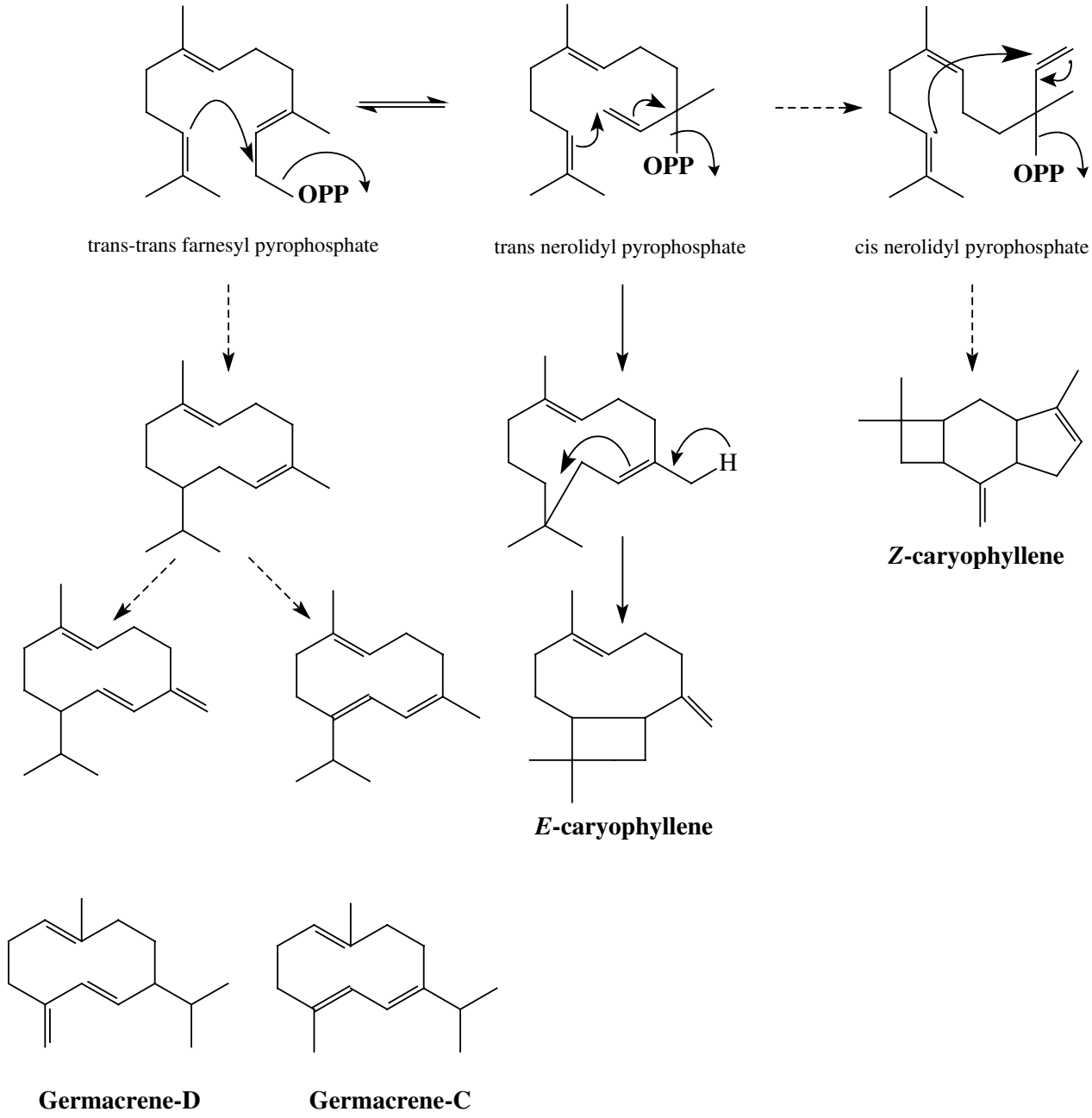

Figure 2.6. Biosynthetic pathway of the main sesquiterpenes present in *Aristolochia* [modified from Mann (1987) and Teisseire (1994)].

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Capítulo 3

Use of host-plants by Troidini butterflies: constraints to host shift

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Abstract

Molecular phylogenetic analyses were conducted to determine relationships and to investigate character evolution in the Troidini/*Aristolochia* interaction, trying to answer the following questions: 1) what is the present pattern of use of *Aristolochia* by these butterflies? 2) is the pattern we see today related to the phylogeny of plants or to their chemical composition? 3) can the geographical distribution of *Aristolochia* explain the host-plant use observed today? and 4) how did the interaction between Troidini and *Aristolochia* evolve? We found a significant congruence between the phylogenies of Troidini and *Aristolochia* and between the phylogeny of Troidini and the chemogram of *Aristolochia* when only the preferred host-plant associations were considered. However, the current pattern of host-plant use of these butterflies does not seem to be constrained by the phylogeny of their food plants, neither by the secondary chemicals in these plants nor by their geographical similarity. The current host-plant use in these butterflies seems to be simply opportunistic, with species with a wide geographical range using more species of host-plants than those with a more restricted distribution.

Key words: Troidini, *Aristolochia*, molecular phylogeny, pattern approach, character optimization, chemical similarity, geographical range

Resumo

Análises filogenéticas foram conduzidas para se determinar relações e para investigar a evolução de caracteres na evolução da interação entre Troidini e *Aristolochia*, tentando responder as seguintes questões: 1) qual o padrão de utilização de *Aristolochia* por estas borboletas? 2) o padrão visto atualmente está relacionado à filogenia das plantas ou à sua composição química? 3) a distribuição geográfica das *Aristolochia* pode explicar a utilização de plantas hospedeiras observada atualmente? e 4) como a interação entre Troidini e *Aristolochia* evoluiu? Foi encontrada uma congruência significativa entre as filogenias de Troidini e *Aristolochia* e entre a filogenia dos Troidini e o quimiograma de *Aristolochia* quando apenas as associações com as plantas hospedeiras preferenciais de Troidini foram consideradas. No entanto, o padrão atual do uso de plantas hospedeiras não parece ser limitado pela filogenia das mesmas, nem pelos químicos secundários encontrados nestas plantas nem pela sua similaridade geográfica. O uso atual de plantas hospedeiras nestas borboletas parece ser simplesmente oportunístico, com espécies com uma ampla distribuição geográfica usando mais espécies de plantas hospedeiras do que aquelas com distribuição mais restrita.

Palavras chave: Troidini, *Aristolochia*, filogenia molecular, enfoque de padrão, otimização de caráter, similaridade química, distribuição geográfica

Introduction

The interactions between herbivorous insects and their host-plants have been studied for a long time. Many studies have focused particularly in how these interactions evolved, considering key aspects the taxonomic conservatism in host utilization (Bernays, 1998; Janz *et al.*, 2001), and if there is a trend toward total specialism in this evolutionary history (an evolutionary "dead end") (Futuyma and Moreno, 1988). Taxonomically related species of insects frequently feed on taxonomically related host-plants, though precise concordance between insect and plant phylogenies is uncommon (Ehrlich and Raven, 1964; Holloway and Hebert, 1979; Mitter *et al.*, 1991; Janz and Nylin, 1998; Lopez-Vaamonde *et al.*, 2003). Such conservatism in host use is influenced basically by the secondary compounds found in the host-plants (Jaenike, 1990; Futuyma *et al.*, 1993; Bernays, 1998), and Fraenkel (1959) even proposed that the discriminatory use of certain plants by insects is the "raison d'être" of these substances in plants [see Jones and Firn (1991) for a different point of view]. Besides chemical compounds, other intrinsic and extrinsic factors also seem to constrain the use of host-plants by herbivores (Ronquist and Nylin, 1990), for example, female ovipositional preferences (Chew and Robbins, 1984) and the survival of the larvae on different plants, pressure of generalist natural enemies (Bernays and Graham, 1988), as well the geographical distribution of the species, because a herbivore cannot use a plant species it never encounters (Pasteels and Rowell-Rahier, 1991; Dobler *et al.*, 1996; Kelley and Farrell, 1998).

The concept that specialization can be an evolutionary dead end has been central in host-plant/herbivore evolutionary studies. However, it seems like that over evolutionary time, diet breath in insects (Colwell and Futuyma, 1971) has both increased and decreased (Bernays, 1998), sometimes leading to specialism (Moran, 1988; Ronquist and Nylin, 1990; Kelley and Farrell, 1998; Nosil, 2002), and others leading to generalism (Armbruster and Baldwin, 1998; Scheffer and

Wiegmann, 2000; Janz *et al.*, 2001). Specialization can be advantageous in several ways. Specialist herbivores could reduce the pathways of incoming chemical signals, leading to economy in neural machinery (Bernays, 1996). In the same way, specialist insects are able to invest less in detoxifying enzymes to struggle with few chemical compounds, instead of a set of different substances [see Futuyma and Moreno (1988) for a review]. In addition, secondary compounds should be poisonous to herbivorous insects that are not specially adapted to deal with them (Bernays and Graham, 1988). Specialization can yet enhances the use of sequestered toxin for defense against predators (Futuyma and Keese, 1992) and, in a competitive environment, specialists are supposed to exclude generalists if they use food more efficiently (Futuyma and Moreno, 1988).

Ehrlich and Raven (1964) treated the conservatism in host use by insects and proposed the hypothesis of coevolution to explain the step-by-step evolution of resistance in plants and adaptations to tolerance of their enemies, that Thompson (1989) called “escape-and-radiation” coevolution, characterized by periods of absent or reduced interaction between taxa. Despite the idea of coevolution between host-plants and herbivores being widespread, other explanations were proposed to explain the evolution of these associations and Ronquist and Nylin (1990) cite some other coevolutionary process models. In the *Arms Race type I* model (in a microevolutionary time) the effects of the defense (of plants) and counter defense (of herbivores) is less drastic than in the model proposed by Ehrlich and Raven (1964) – that Ronquist and Nylin (1990) call *Arms Race type II*; that is, defense in plants may not exclude totally insects from them. In the *Specialization* model, the general trend toward specialization in the insects occurs independently of evolutionary changes in the plants. In the *Late Colonization* model (Jermy, 1984, 1993; Percy *et al.*, 2004), the colonization occurred onto already existing plant groups, so insect groups may be much younger than the plants on which they live [although Ehrlich and Raven (1964) are always cited as defenders of the Arm Race model, in their paper they used the example of the Papilionidae/Aristolochiaceae

association to state that "is likely that the major diversification of Papilionidae took place after the evolution of Aristolochiaceae"]. Finally, the *Opportunistic* model points out that insects have switched so frequently to new host-plants that the historical pattern is lost (Ronquist and Nylin, 1990).

The close association between butterflies of the tribe Troidini (Papilionidae; Papilioninae) and their host-plants on the genus *Aristolochia* (Aristolochiaceae, Aristolochioideae) is a suitable model for studies on the evolution of host-plant/herbivore relationships. The tribe Troidini is predominantly tropical, with most species concentrated in the lowland forests of Central and South America and in the IndoAustralian region (Weintraub, 1995). The tribe includes 130 species divided into 12 genera, three of which occur in the Neotropics: *Battus* (11 species), *Euryades* (2 species), and *Parides* (34 species) (Tyler *et al.*, 1994). The family Aristolochiaceae (comprising 7 genera and approximately 450 species) is distributed mainly over the tropical and temperate regions (Judd *et al.*, 2002). The genus *Aristolochia* "sensu lato", the largest in the Aristolochiaceae, consists of approximately 400 species (Kelly and González, 2003), with *ca*. 90 species occurring in Brazil (Leitão and Kaplan, 1992). The plants are subshrubs to lianas, containing as main secondary compounds aristolochic acids, aristolactam benzylisoquinoline alkaloids, mono-, sesqui-, di- and triterpenes (Teresa *et al.*, 1983; Lopes *et al.*, 1987; Lopes and Bolzani, 1988; Lopes *et al.*, 1990; Luiz *et al.*, 1990; Leitão and Kaplan, 1992; Leitão *et al.*, 1992; Vila *et al.*, 1997; Bomm *et al.*, 1999; Palmeira *et al.*, 2001; Wu *et al.*, 2001; Priestap *et al.*, 2002; Tsuruta *et al.*, 2002; Priestap *et al.*, 2003; Francisco *et al.*, 2004; Shi *et al.*, 2004; Wu *et al.*, 2004).

The interaction between Troidini and *Aristolochia* has been used as classic example of coevolution (Ehrlich and Raven, 1964; Brown *et al.*, 1991), although Weintraub (1995) affirmed that the occurrence of host shifts at lower taxonomic levels in this group do not support the hypothesis of parallel cladogenesis. The traits of this association agree with most of the premises

under the coevolutionary hypothesis: Troidini use almost exclusively plants of *Aristolochia*, and their larvae are known for sequestering the secondary compounds present in their host-plants (Klitzke, 1992), which make them apparently unpalatable to many potential predators and parasitoids (Brower and Brower, 1964; Rothschild *et al.*, 1970). Nishida and Fukami (1989) and Sime (2002) suggested that aristolochic acids, which have pharmacological effects in vertebrates (Brown *et al.*, 1981), can be responsible for the chemical defense in Troidini. The adult butterflies advertise their unpalatability through aposematic coloration, making them notable in their roles as unpalatable models in mimicry rings (Tyler *et al.*, 1994).

Brown *et al.* (1995) demonstrated evidence that basal Troidini use preferentially basal *Aristolochia* species, while derived butterflies also use derived host species, suggesting a high coevolutionary linkage between these butterflies and their host-plants. Indeed, the Troidini can vary greatly in their diet breadth (Weintraub, 1995), and the most polyphagous taxa tend to be the butterflies with the widest geographical and ecological ranges. According to Weintraub (1995), monophagy appears to be relatively uncommon at the subgeneric and species level, occurring in species with restricted distributions.

Here, molecular phylogenetic analyses were conducted to determine relationships and to investigate character evolution in the Troidini/*Aristolochia* interaction, using what Ronquist and Nylin (1990) called “pattern approach”, that is the use of the phylogenies of associated groups to reconstruct the coevolutionary history of the interaction. The main questions concerning the evolution of that interaction are: 1) what is the present pattern of use of *Aristolochia* by these butterflies? 2) is the pattern we see today related to the phylogeny of plants or to their chemical composition? 3) can the geographical distribution of *Aristolochia* explain the host-plant use observed today? and 4) how did the interaction between Troidini and *Aristolochia* evolve?

Materials and methods

Relationships among Troidini butterflies

Molecular phylogeny of Troidini

The study of the evolutionary history of the host-plant use of Troidini butterflies was limited to those species occurring in Southeastern Brazil, for which the most complete data on host-plant use is avalaible, as well as data on host-plant secondary compounds (Brown *et al.*, 1981; Otero and Brown, 1986; Brown *et al.*, 1991; Morais and Brown, 1991; Klitzke, 1992; Tyler *et al.*, 1994; Brown *et al.*, 1995; Klitzke and Brown, 2000; Freitas and Ramos, 2001). Table 3.1 lists all host-plants used by the species of New World Troidini studied here, obtained from several literature and field sources.

The phylogenetic hypothesis for the Troidini used in this paper was obtained by a Maximum Parsimony (MP) analysis of the mitochondrial genes *cytochrome oxidase I* and *II* (COI and COII) and the nuclear gene *elongation factor-1*α (EF-1α) (Chapter 1). It was sequenced a total of 2169 bp from the mitochondrial DNA and 1161 bp from EF-1α, and the MP analysis was carried out with PAUP* 4.0b10 (Swofford, 2002). This tree was considered the best phylogenetic relationship hypothesis of New World Troidini (Fig. 3.1). Species for which there was no information on host-plant use have been pruned from Fig. 1.3 (Chapter 1). A great number of Troidini studied here use several *Aristolochia* species, and for these butterflies we constructed the tree with polytomies, as many as the number of host-plant species used.

Table 3.1. Species of Troidini butterflies used in this study, with general distribution and host-plants used in SE Brazil (host-plants marked with * were analyzed for secondary compounds pattern, geographical distribution and phylogenetic relationships).

Species	General Distribution	*A. arcuata**	*A. brasiliensis*	*A. chamissonis**	*A. cymbifera**	*A. cynanchifolia**	*A. elegans**	*A. esperanzae**	*A. galeata**	*A. gigantea**	*A. littoralis*	*A. macroura**	*A. melastoma**	*A. odora*	*A. paulistana**	*A. rumicifolia*	*A. triangularis**	References
Battus crassus (Cramer, [1777])	Costa Rica to W of Equador and S and SE Brazil				X		X	X				X						2, 3, 7
Battus polydamas (Linnaeus, 1758)	South and Central America, some places of North America	X			X		X	X	X	X	X	X	X		X		X	1, 2, 3, 4, 5, 6, 7, 8
Battus polystictus (Butler, 1874)	ES to SP in Brazil; SP to Paraguay and Buenos Aires	X							X	X			X				X	6, 7
Parides agavus (Drury, 1782)	SE Brazil to Paraguay and Argentina	X					X	X		X	X		X			X	X	1, 5, 7, 8
Parides anchises (Linnaeus, 1758)	Panama, through Colombia and W Venezuela, almost all regions in Brazil	X	X		X		X	X	X		X	X	X	X	X	X	X	1, 5, 7, 8, 9
Parides ascanius (Cramer, [1775])	Coastal RJ in SE Brazil											X						2
Parides bunichus (Hübner, 1821)	Piauí (Brazil) to Argentina, Coastal SC, Brazil	X					X	X			X		X				X	1, 5, 8
Parides neophilus (Geyer, 1837)	Amazon Basin and Central and SE Brazil. South America	X					X	X					X					5, 6, 7
Parides panthonus jaguarae (Foetterle, 1902)	Belo Horizonte, MG, Brazil			X														10
Parides proneus (Hübner, 1831)	SE Brazil highlands from MG and ES to SC and W into Paraguay	X					X	X					X					5, 8
Parides tros (Fabricius, 1793)	SE Brazil, wet Coastal mountains from ES to SC	X				X										X		7, 8
Parides zacynthus (Fabricius, 1793)	Coastal Brazil from 5° to 28° S											X		X	X		X	7, 8

References: (1) Brown *et al.* (1981); (2) Otero and Brown (1986); (3) DeVries (1987); (4) Spade *et al.* (1988); (5) Morais and Brown (1991); (6) Tyler *et al.* (1994); (7) Brown *et al.* (1995); (8) Klitzke and Brown (2000); (9) Freitas and Ramos (2001); (10) Correa, F.C.C. (pers. com.).

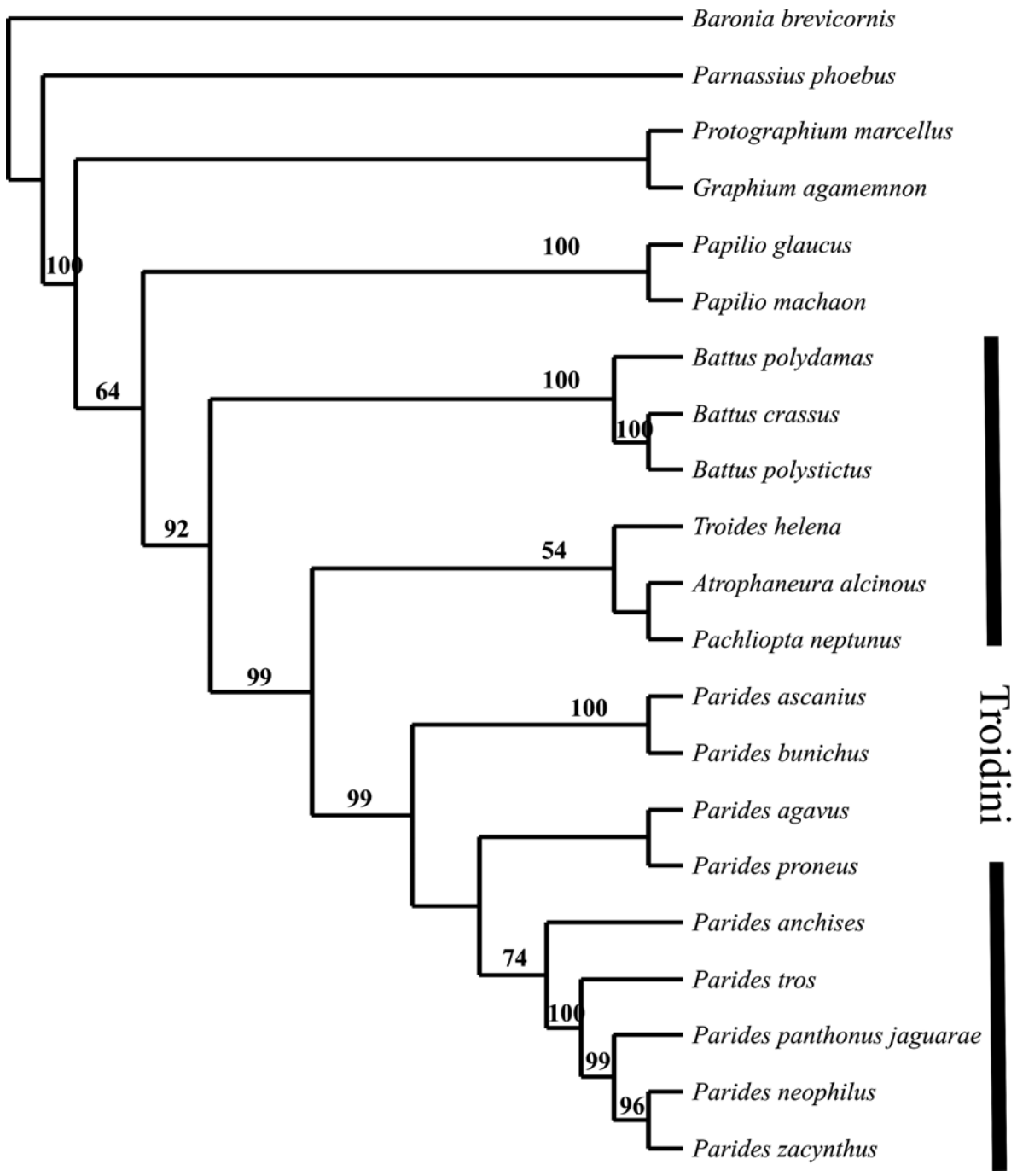


Figure 3.1. Phylogenetic relationship hypothesis of Troidini butterflies. Most parsimonious tree based on combined data analysis (3148 steps; CI=0.475; RI=0.472). Values above the branches indicate bootstrap values from 1000 replications (where it exceeds 50%).

Geographic relationship of Troidini

Maps of distribution of the 12 species of Troidini from SE Brazil studied through molecular phylogeny and host-plant use (Table 3.1) were constructed based on recent data obtained by the project BIOTA-FAPESP "Lepidoptera do Estado de São Paulo: diversidade, distribuição e recursos" and from Keith Brown (personal communication) (Appendix 1). The maps were drawn by using a baseline map of the SE region (including the states of São Paulo, Rio de Janeiro, Espírito Santo and Minas Gerais – 40°-54° W; 14°-26° S) on which were recorded the localities (cities) of occurrence of each species.

To estimate the geographic relationships of Troidini, a dendrogram based on the similarity of their geographical ranges (biogeogram) was constructed. Following Becerra and Venable (1999), the biogeogram was created using a transparent map of the SE region of Brazil of the same dimensions as the baseline map divided into squares of 0.5° longitude and latitude (Fig. 3.2), and counted the number of squares where each species is present. A matrix of Euclidian distances was constructed on the basis of the presence and absence of each species in each square, and the dendrogram was constructed with a cluster analysis using Ward's method (Legendre and Legendre, 1998).

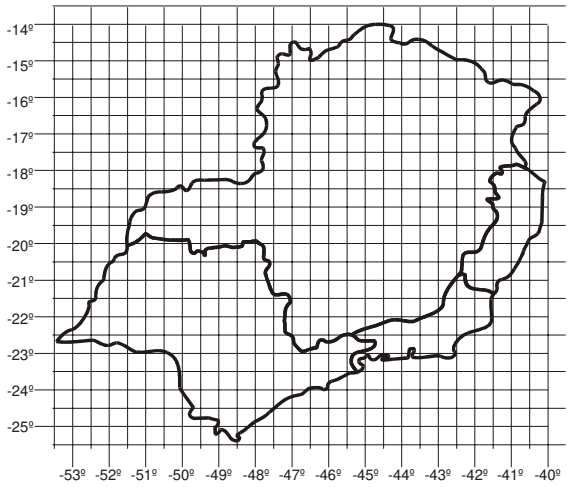


Figure 3.2. Baseline map of SE region of Brazil divided with grid of 0.5° latitude and longitude.

Relationships among Aristolochia species

Molecular phylogeny of Aristolochia

The phylogenetic hypothesis for the *Aristolochia* (Table 3.2) was obtained by a Maximum Parsimony (MP) analysis of two regions: the *mat*K gene, located within the intron of the chloroplast gene (cpDNA) *trn*K (Hilu and Liang, 1997), and the non-coding region of cpDNA located between *trn*L (UAA) and *trn*F (GAA) genes (Chapter 2). It was sequenced 2242 nucleotides, 1245 from *mat*K and 997 from the non-coding region between *trn*L-*trn*F. MP analyses were performed with PAUP* 4.0b10 (Swofford, 2002) on the entire data set, and a consensus tree was generated from the 24 most parsimonious trees (Fig. 3.3).

Table 3.2. Species of *Aristolochia* sampled, with localities of specimens.

Species	Locality
Aristolochia arcuata Masters 1885	Atibaia and Campinas, SP, Brazil
Aristolochia chamissonis (Klotzch) Duchartre 1864	Belo Horizonte, MG, Brazil
Aristolochia cymbifera Mart. & Zucc. 1824	Mogi-das-Cruzes, SP, Brazil
Aristolochia cynanchifolia Mart. & Zucc. 1824	Rio de Janeiro, RJ, Brazil
Aristolochia elegans Masters 1885	Piracicaba, SP, Brazil
Aristolochia esperanzae O. Kuntze 1898	Mogi-Guaçú, SP, Brazil
Aristolochia galeata Mart. & Zucc. 1824	Atibaia, SP, Brazil
Aristolochia gigantea Mart. & Zucc. 1824	Campinas, Mogi-das-Cruzes and Piracicaba, SP, Brazil
Aristolochia macroura Gomez 1812	Campinas, SP, Brazil (cult.)
Aristolochia melastoma Manso 1864	Campinas, SP, Brazil
Aristolochia paulistana Hoehne 1927	Piracicaba, SP, Brazil
Aristolochia triangularis Cham. 1832	Atibaia, SP, Brazil

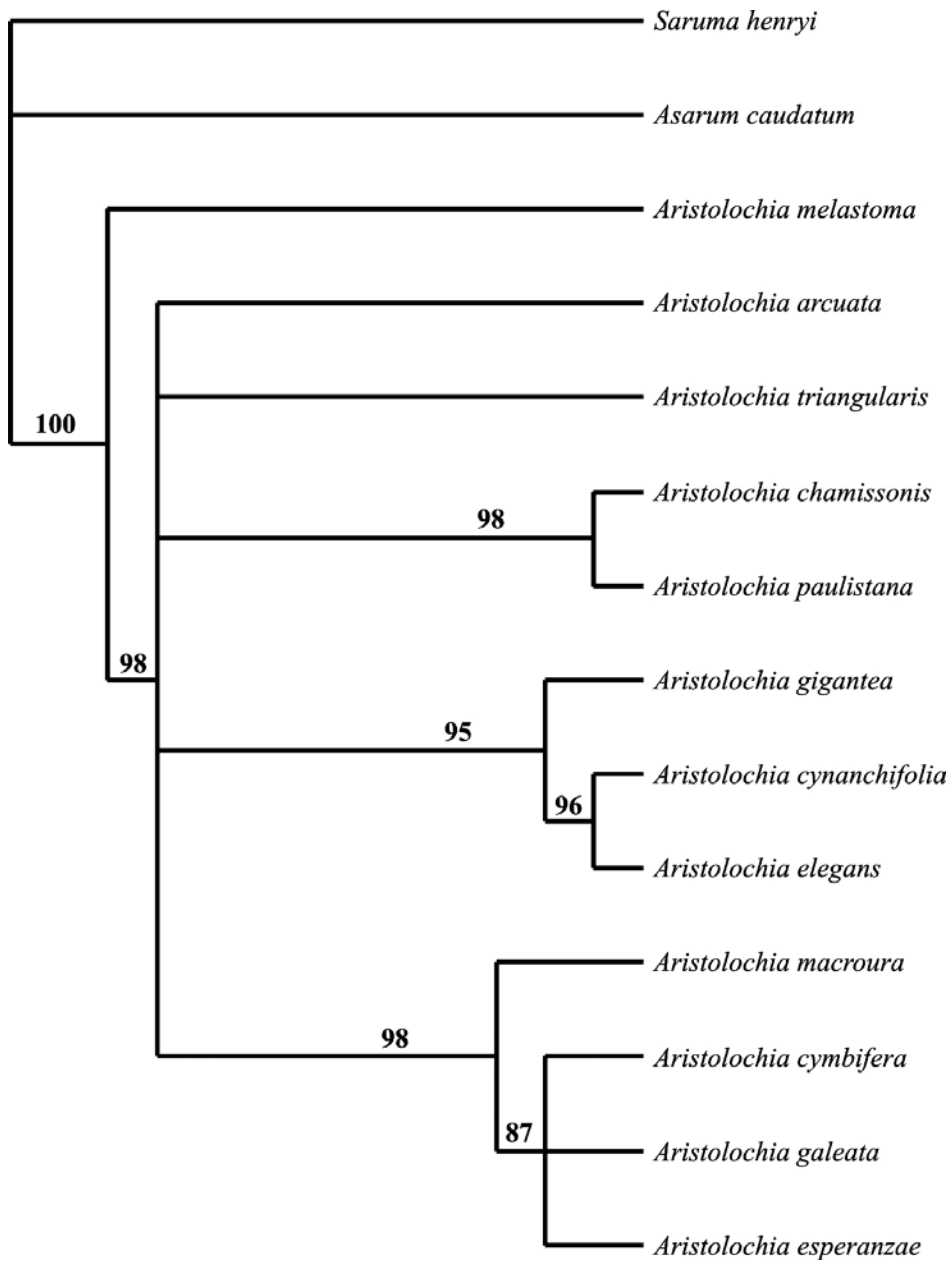


Figure 3.3. Phylogenetic relationship hypothesis among *Aristolochia* species. Strict consensus tree of the 24 equally parsimonious trees based on combined data analysis (477 steps). Values above the branches indicate bootstrap values from 1000 replications.

Chemical similarity among Aristolochia species

A dendrogram of chemical similarity (chemogram) of 12 *Aristolochia* species used as host-plants by Troidini butterflies in SE Brazil (Table 3.2) was constructed (Chapter 2) (Fig. 3.4). Euclidean distances were used for similarity determination and Ward´s method as cluster procedure of the relative abundance of fifty-one compounds eluted in the sesquiterpene region. Three main clusters can be recognized in Fig. 3.4. *Group 1* is characterized by the high relative abundance of germacrene-D; *Group 2* is characterized by a high relative abundance of germacrene-C and *Group 3* is characterized by a high relative abundance of *Z*-caryophyllene. One individual of *Aristolochia triangularis* and two individuals of *A. esperanzae* were pruned from the cluster analysis and from Fig. 2.1 because they were out of their main group (Chapter 2).

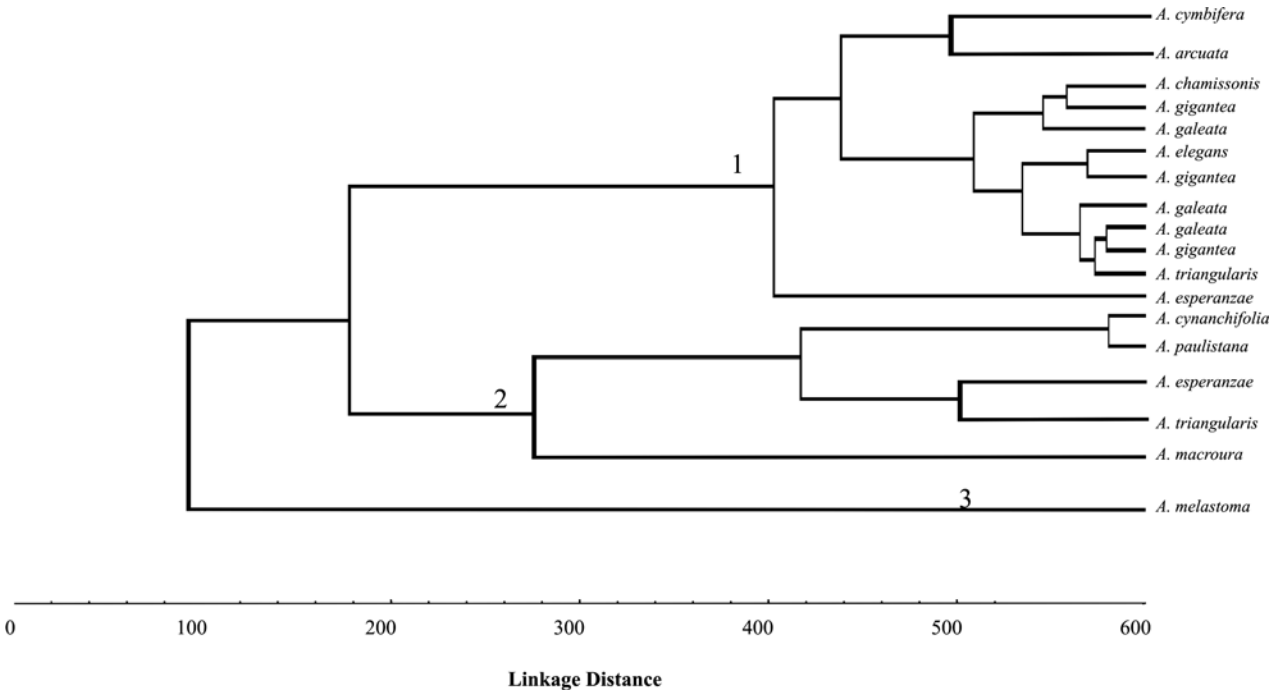


Figure 3.4. Dendrogram of *Aristolochia* based on chemical similarity (chemogram). Numbers above internal branches indicate the three main groups according to Ward's clustering method of relative abundance of sesquiterpenes.

Geographic relationship of Aristolochia species

Maps of distribution of 12 *Aristolochia* species (Table 3.2) were created using previously published distributions of *Aristolochia* species in SE Brazil (Hoehne, 1942; Capellari Jr., 1991) updated with more recent information from herbarium specimens (Appendix 2). The maps and the biogeogram of *Aristolochia* were constructed in the same way as to Troidini distributions (above).

Host-plant use pattern in Troidini

It was applied the approach of Brown *et al.* (1995) to link the species of butterflies to their host-plants to see if there is any phylogenetic pattern of host use and host shift, using the molecular phylogenies proposed for Troidini and for *Aristolochia*. We considered Brown *et al.* (1995) data on the host-plant use pattern in Troidini from SE Brazil, including the plant species used preferentially by each species.

Character optimization

To investigate the importance of the *Aristolochia* phylogeny in the Troidini evolutionary host shift were used two techniques: character tracing (Maddison and Maddison, 1999) and tree mapping (Page, 1993). The topology of the Troidini phylogeny (with polytomies) was compared with the topology of their host-plants, using MacClade 3.08 program (Maddison and Maddison, 1999) to perform analyses of character tracing (Becerra, 1997). Although the major objective was to map the host-plants onto the terminal branches, to understand the evolution of host-plant use it is necessary to know not only the character state of the living butterflies, but also of their ancestors, and using MacClade we can also infer the ancestral character state using the method of Maximum Parsimony (Maddison and Maddison, 1999). To test whether there is a phylogenetic signal in the characters

traced, it was used the methodology proposed by Wahlberg (2001), modified from PTP test described by Faith and Cranston (1991). It was performed 300 random reshufflings of character states among the fixed terminal taxa, with the equally weighted data set, using the option "shuffle" in MacClade program (Maddison and Maddison, 1999). The probability (*P*) that the observed pattern does not differ randomly is given by the number of replications as short as or shorter than the tree obtained with the actual data, plus one, divided by the number of replications. Following Faith and Cranston (1991), a significant phylogenetic signal is observed when *P* is less than 0.05, and here, the minimal value should be 0.003.

Tree mapping analyses were conducted in Component 2.0 program (Page, 1993) using all associations between Troidini and *Aristolochia* and only the preferred host-plant associations (Brown *et al.*, 1995). This is a statistical method which analyses historical associations between "hosts" and "associates" by creating a reconciled tree of both under the assumption that the relationship is due to "association by descent" (Page, 1993). This reconciled tree between host and associate cladograms or dendrograms can be tested statistically by two measures of fit, "leaves added", which is the difference between the number of nodes in the associate and reconciled trees, and "losses", which are the occasions in which an associate is absent where it should be present on the reconciled tree. The statistical test consists in comparing the "leaves added" and "losses" values of the reconciled tree with the distribution of these values in 1000 random trees of hosts generated by a Markovian model. The *P* value is obtained by the number of times that the "leaves added" and "losses" values are equal or shorter than these values in the reconciled tree (Page, 1990a, b). Tree mapping is a sensitive method even when the topologies compared are only loosely congruent, an expected picture for herbivorous insects which shift freely among hosts (Becerra and Venable, 1999).

In addition, MacClade 3.08 program (Maddison and Maddison, 1999) was used to see how the use of each host-plant is distributed along the Troidini phylogenetic tree, using the option "Trace

Character". We used the presence and absence of each *Aristolochia* in the diet of each butterfly as character states. Consistency Index was calculated in the ∑ menu in the tree window. DELTRAN (slow optimization - gains more likely to be homoplasious) and ACCTRAN (fast optimization - gains more likely to be homologies) tracing was applied to perform the analyses on character optimization whenever ambiguous changes were found (Maddison and Maddison, 1999).

Comparison of Troidini phylogeny with the chemogram and the biogeogram of Aristolochia

To investigate whether the plant chemical similarity and geographical distribution facilitated host shift by Troidini, the topology of the butterflies phylogeny (with polytomies) was compared with the topology of the *Aristolochia* chemogram and biogeogram, using MacClade 3.08 program (Maddison and Maddison, 1999) to perform analyses of character tracing (Becerra, 1997). Character states was random reshuffled 300 times among the fixed terminal taxa to test whether there is a phylogenetic signal in the characters traced (Wahlberg, 2001), with the equally weighted data set (Maddison and Maddison, 1999). Tree mapping was conducted in Component 2.0 program (Page, 1993), and performed using all associations between Troidini and *Aristolochia* and only the preferred host-plant associations, as above.

Comparison of Troidini and Aristolochia biogeograms

Tree mapping analyses were carried out in Component 2.0 program (Page, 1993) to compare the biogeograms of Troidini and *Aristolochia* in order to investigate if the use of hosts can be due only to the geographical ranges of the butterflies and their food plants. The analyses were performed using all associations between these butterflies and their host-plants and only the preferred host-plant associations.

Results

Geographic relationship of Troidini

The dendrogram obtained with the geographical similarity of Troidini consisted of three main clusters (Fig. 3.5). *Group 1* includes *Battus crassus*, *P. tros*, *Parides ascanius*, *P. panthonus jaguarae*, and *P. zacynthus*, butterflies with a relative narrow distribution, except *B. crassus* (Figs. 3.6 A, K, F, I, L). *Group 2* is composed of *Battus polydamas*, *Parides anchises*, *B. polystictus* and *P. agavus*, Troidini showing the broadest geographical range (Figs. 3.6 B, E, C, D). *Group 3* constains *Parides bunichus*, *P. neophilus* and *P. proneus*, butterflies as broad distributed as those from Group 2 but, except *P. proneus*, absent from the state of Espírito Santo (Figs. 3.6 G, H, J).

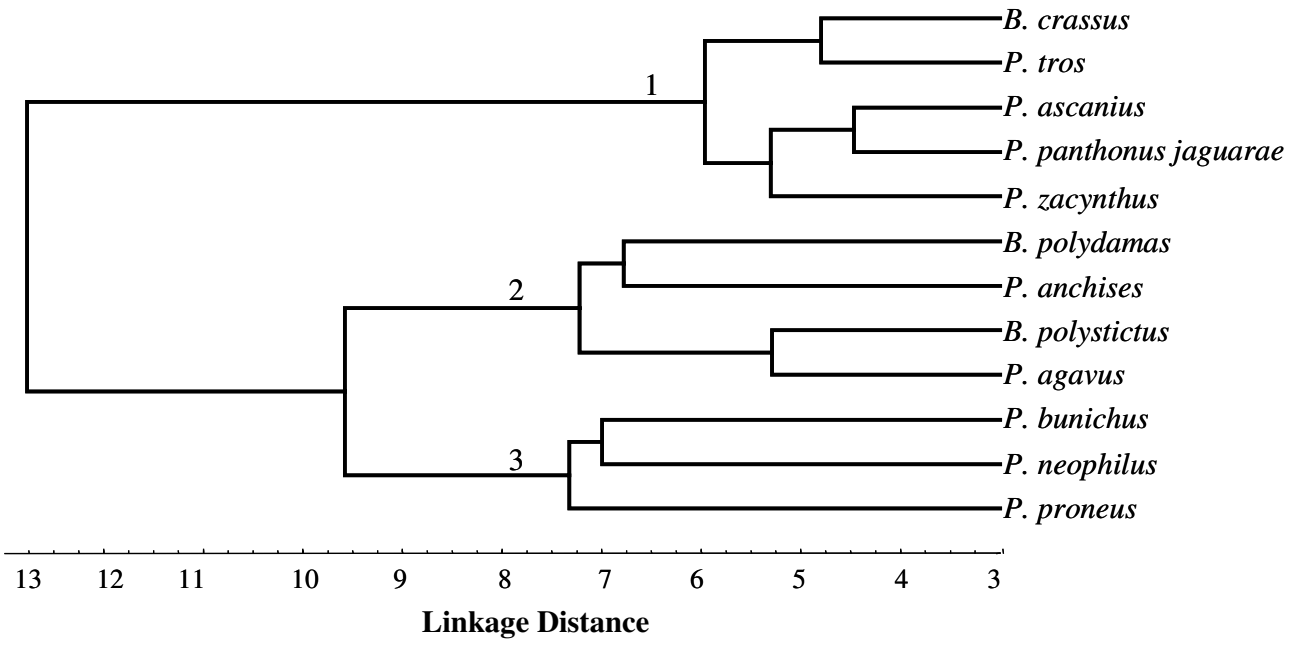
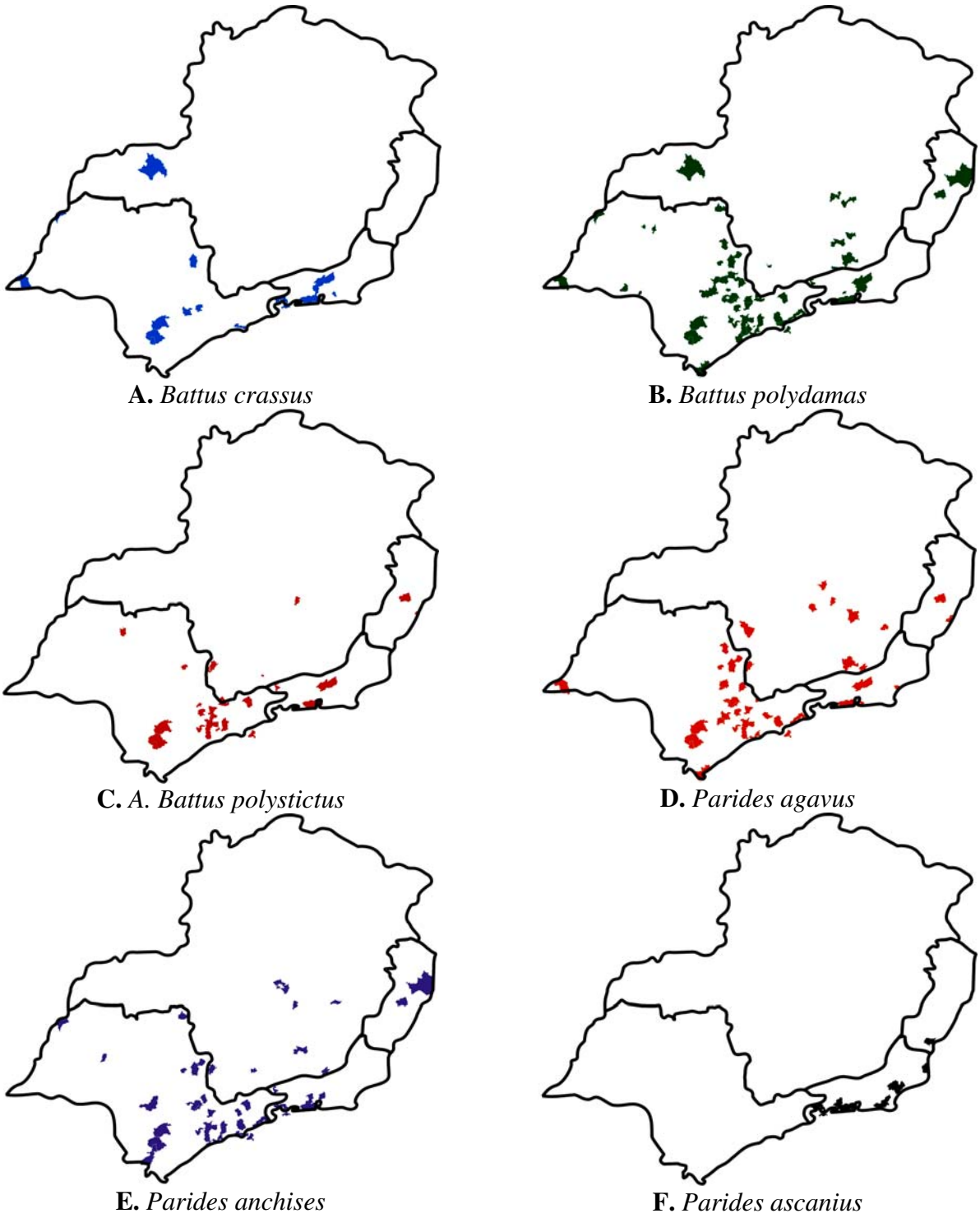


Figure 3.5. Dendrogram of Troidini based on their geographical similarity (biogeogram). Numbers above internal branches indicate the main biogeographic groups according to Ward's clustering method.



A. *Battus crassus*

B. *Battus polydamas*

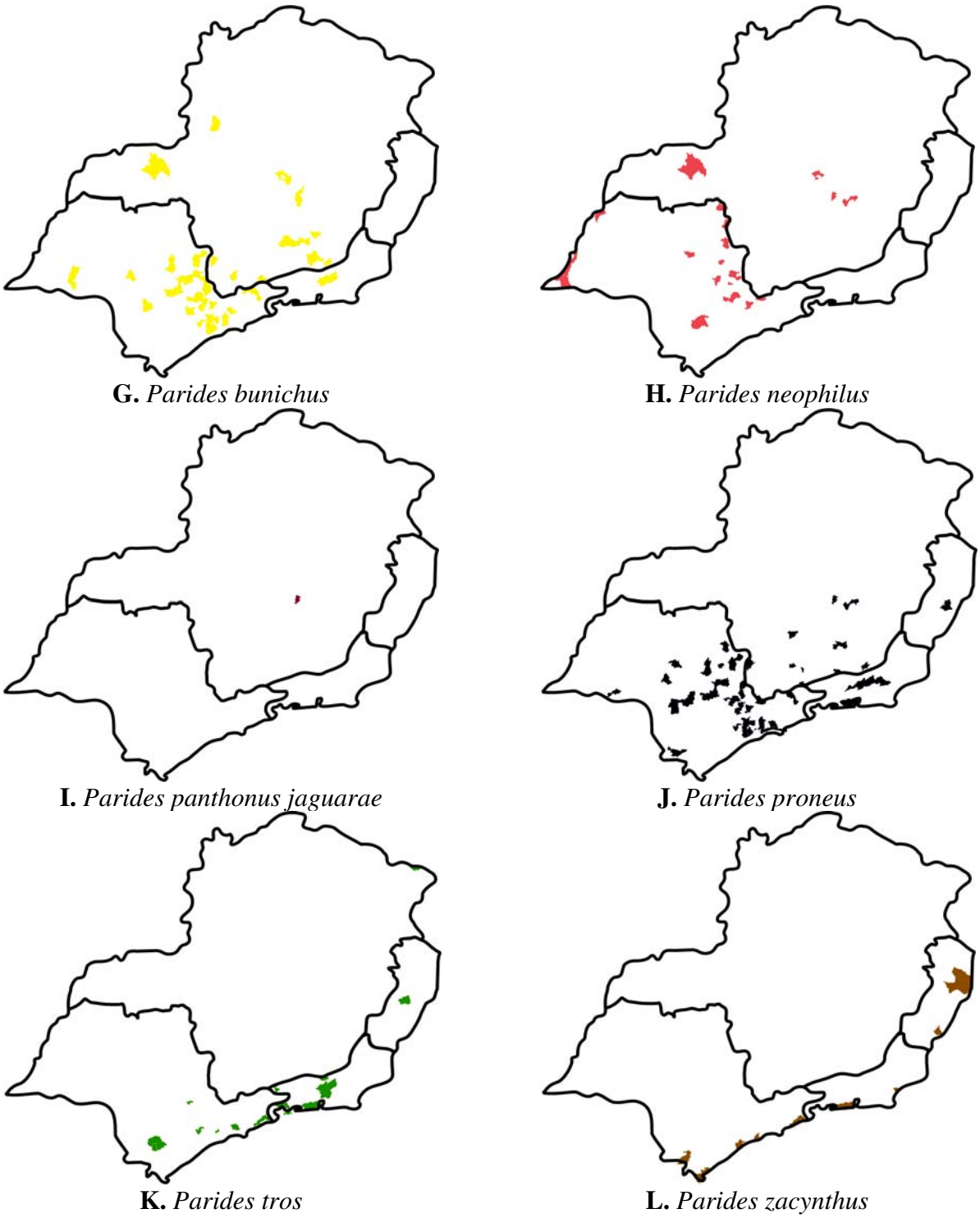
C. *A. Battus polystictus*

D. *Parides agavus*

E. *Parides anchises*

F. *Parides ascanius*

Figure 3.6. Geographical distribution of Troidini butterflies on the SE region of Brazil.



G. *Parides bunichus*

H. *Parides neophilus*

I. *Parides panthonus jaguarae*

J. *Parides proneus*

K. *Parides tros*

L. *Parides zacynthus*

Figure 3.6. Extended.

Geographic relationship of Aristolochia species

The cluster analysis with Ward's method identified four main groups of *Aristolochia* species according to the degree of similarity of their geographical ranges (Fig. 3.7). *Group 1* includes *A. arcuata*, *A. melastoma*, *A. gigantea, A. cymbifera* and *A. galeata*, all widely distributed mainly in the SE of São Paulo and Minas Gerais, and near the city of Rio de Janeiro (Figs. 3.8 A, J, H, C and G). *Group 2*, composed of *A. chamissonis*, *A. paulistana* and *A. triangularis*, includes mostly species whose geographic range includes the South of São Paulo (Figs. 3.8 B, K and L). *Group 3* contains *A. cynanchifolia*, *A. macroura* and *A. elegans*, and includes species occurring exclusively in Rio de Janeiro (*cynanchifolia*), in the North of Minas Gerais (*elegans*), and in coastal São Paulo, Espírito Santo and Rio de Janeiro (*macroura*) (Figs. 3.8 D, E and I). *Group 4* is composed exclusively of *A. esperanzae*, the only species inhabiting the NE of São Paulo and the W of Minas Gerais (Fig. 3.8 F).

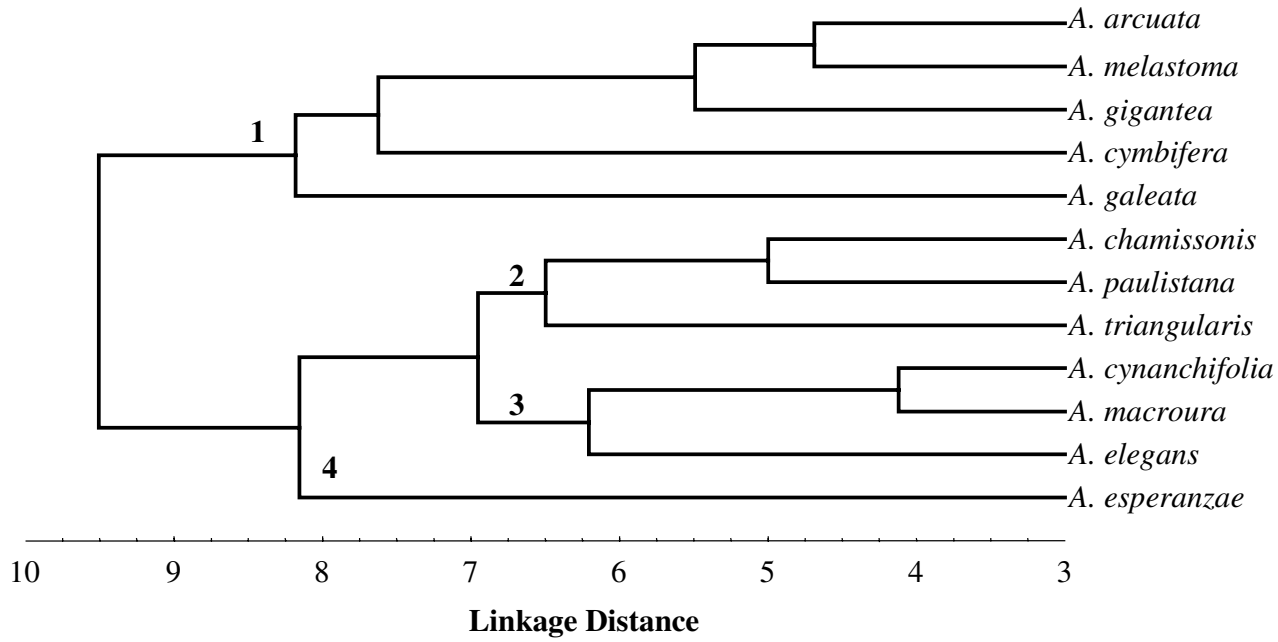


Figure 3.7. Dendrogram of *Aristolochia* based on their geographical similarity (biogeogram). Numbers above internal branches indicate the four main biogeographic groups according to Ward's clustering method.

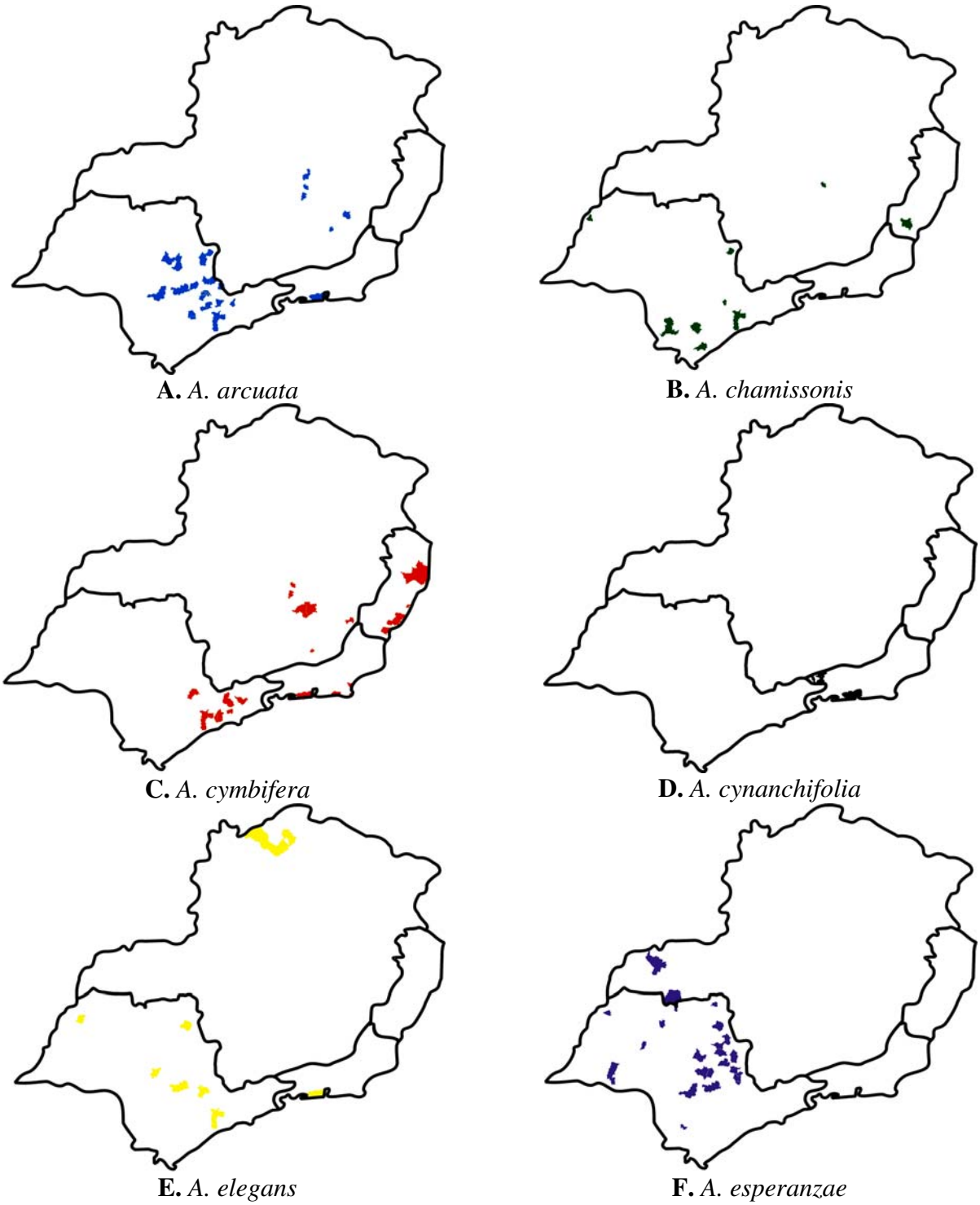
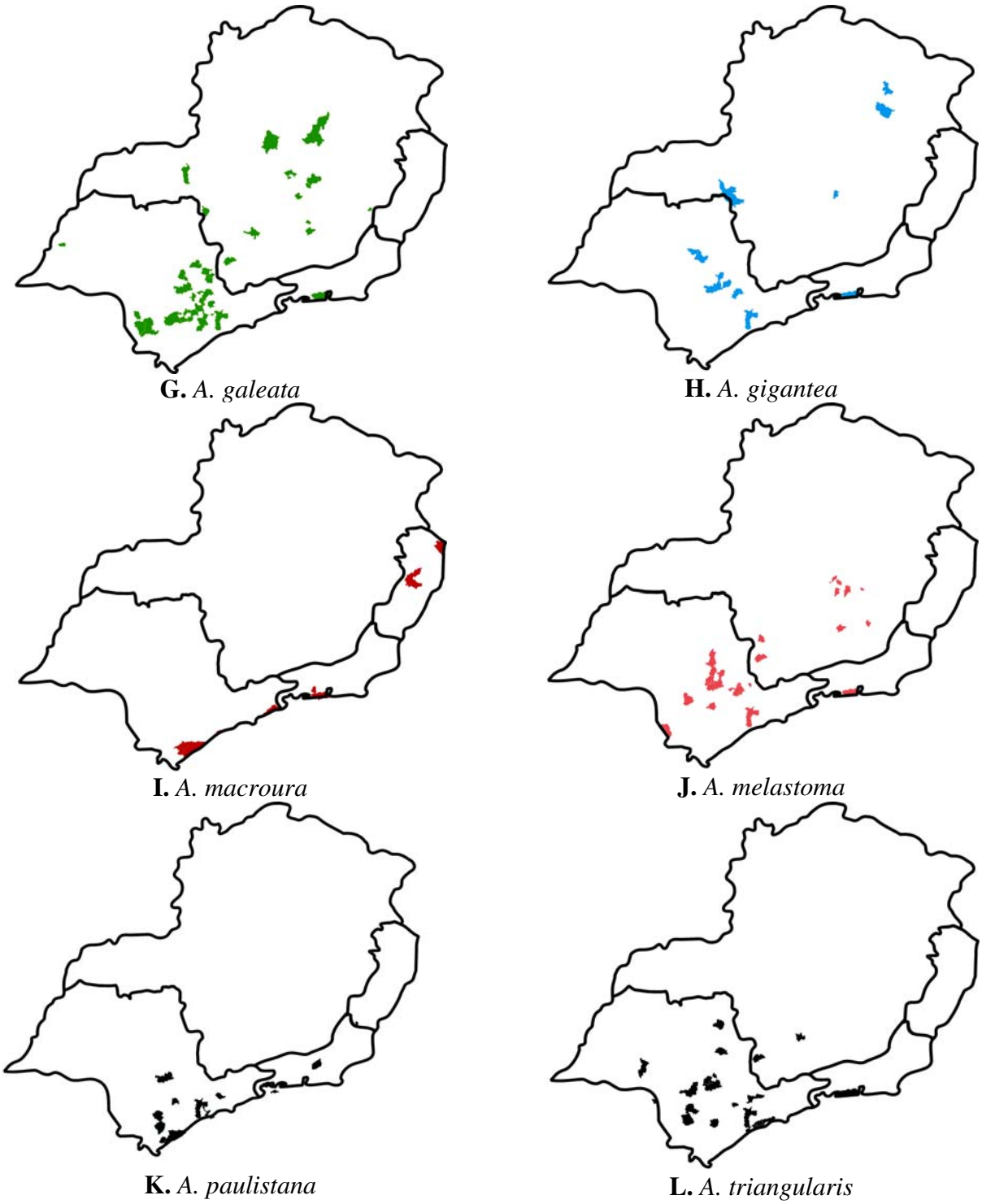


Figure 3.8. Geographical distribution of *Aristolochia* species on the SE region of Brazil.



G. *A. galeata*

H. *A. gigantea*

I. *A. macroura*

J. *A. melastoma*

K. *A. paulistana*

L. *A. triangularis*

Figure 3.8. Extended.

Host-plant use pattern in Troidini

Considering the phylogenies of Troidini and *Aristolochia* and linking the species of butterflies to their host-plants resulted in a chaotic picture (Fig. 3.9). Butterflies occupying different phylogenetic positions use indiscriminately the same host-plants. *Aristolochia melastoma*, for example, is used by *Battus polydamas* and *B. polystictus* (with basal position within Troidini) and also by *Parides neophilus* (occuping the most derived position). *A. esperanzae* is also used by butterflies occupying variable phylogenetic position (Fig. 3.9).

Battus polydamas and *Parides anchises*, the most polyphagous Troidini studied here, show different patterns of host-plant utilization. *Battus polydamas* use almost all *Aristolochia* species available, without any preference (Brown *et al.*, 1995), and belonging to all clades found in our phylogenetic hypothesis. *P. anchises* also feed on *Aristolochia* species of all clades, but uses preferentially three species which appeared together in the same clade (*A. cymbifera*, *A. galeata* and *A. esperanzae*) (Fig. 3.9).

Character optimization

Tracing the major clades of the *Aristolochia* phylogeny onto the Troidini phylogeny showed the widespread use of host-plants in all clades (Fig. 3.10). According to our results, the host-plant used by the ancestors of both *Battus* and *Parides* may have belonged to the clade joining *Aristolochia macroura*, *A. cymbifera*, *A. galeata* and *A. esperanzae*, and all *Parides* but *P. tros* and *P. panthonus jaguarae* use *Aristolochia* species belonging to that clade (Fig. 3.10). However, the distribution of this character does not seem to be determined by the the topology of Troidini ($P = 1.0$). The congruence between the phylogenies of Troidini and *Aristolochia* was not significant when used all associations

between them ("leaves added", $P = 1$; "losses", $P = 0.478$), but was significant when used only the preferred host-plant associations ("leaves added", $P = 0.018$; "losses", $P = 0.028$).

The distribution of each *Aristolochia* along the Troidini phylogeny showed different patterns (Fig. 3.11). *Aristolochia arcuata* is used by almost all Troidini studied here, except *Battus crassus*, *Parides ascanius*, *P. panthonus jaguarae* and *P. zacynthus* (Fig. 3.11 A). DELTRAN tracing indicated four gains and two losses of the use of this species, and ACCTRAN pointed out two gains and four losses, both counting six steps. *Aristolochia chamissonis* is used exclusively by *P. panthonus jaguarae*, and *A. cynanchifolia* is used exclusively by *P. tros* in Rio de Janeiro (Figs. 3.11 B and D). *Aristolochia cymbifera* is used by *Battus crassus* and *B. polydamas*, and by *P. anchises* (Fig. 3.11 C). DELTRAN tracing pointed out that its use may have arisen three times, and ACCTRAN that its use may have arisen twice and been lost once. *Aristolochia elegans*, *A. esperanzae* and *A. melastoma* present the same pattern of utilization by Troidini, and none are used by the most derived butterflies, except *P. neophilus* (Figs. 3.11 E, F and J). The use of the three species over the Troidini phylogeny showed six steps; DELTRAN tracing pointed out six gains, and ACCTRAN indicated two gains and four losses. *Aristolochia galeata* and *A. gigantea* present a quite similar pattern of use: both are used by *B. polystictus* and *B. polydamas*, and by only one species of *Parides*, *P. anchises* and *P. agavus*, respectively (Figs. 3.11 G and H). The optimized tree showed three steps: DELTRAN indicated three gains, and ACCTRAN two gains and one loss. *Aristolochia macroura* and *A. triangularis* are both used by *Battus* and *Parides* (Figs. 3.11 I and L). DELTRAN tracing of the use of *A. macroura* indicated that the use of this species may have arisen five times, and ACCTRAN pointed out that the gains may have taken place four times, and losses once. For *A. triangularis*, DELTRAN indicated six gains, and ACCTRAN five gains and one loss. *Aristolochia paulistana* is used by *B. polydamas* and *P. anchises* (the two most polyphagous species of Troidini) (Fig. 3.11 K). Both DELTRAN and ACCTRAN indicated three gains of its use.

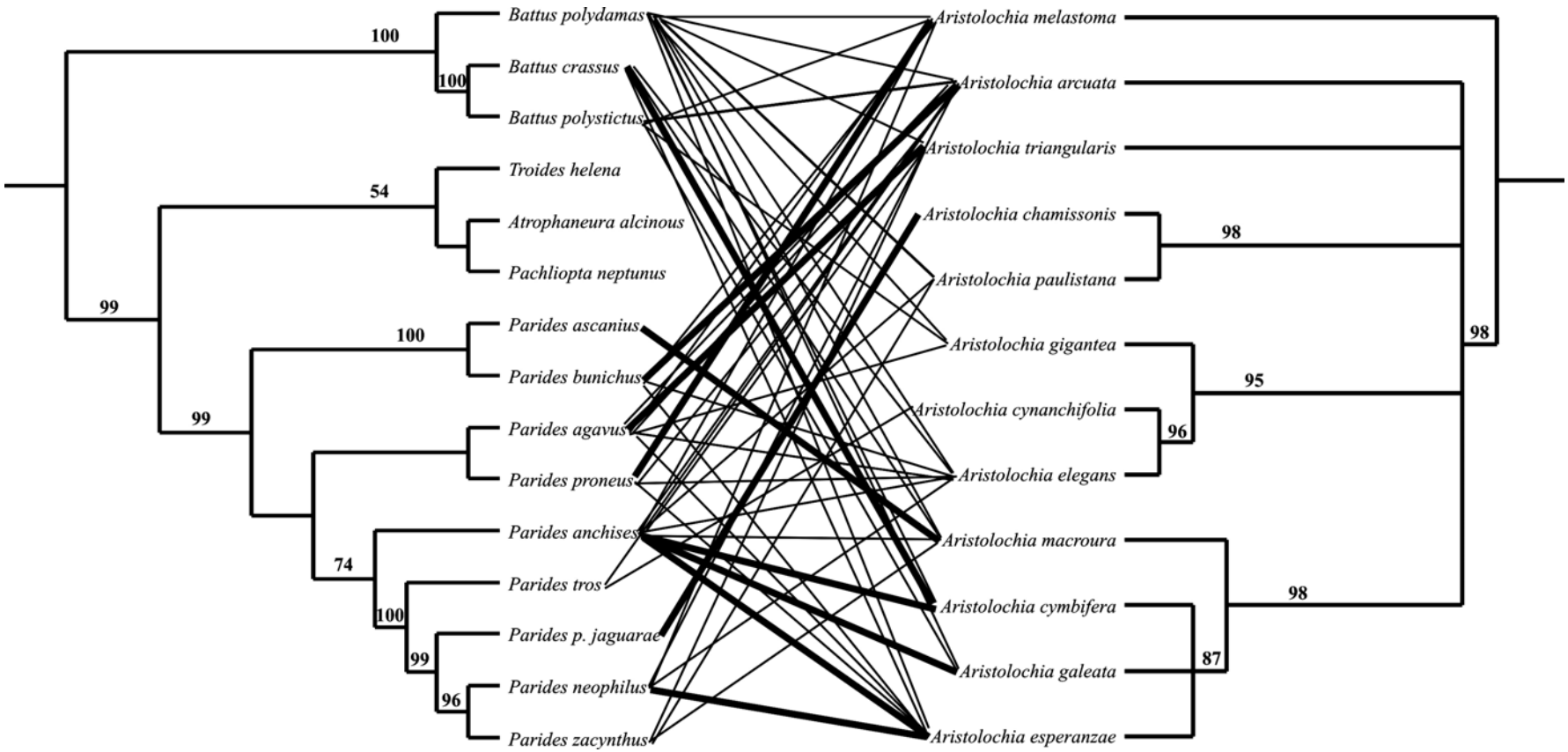


Figure 3.9. Interactions between New World Troidini butterflies and their larval host-plants on the genus *Aristolochia* in SE Brazil. Phylogenies are based on molecular data: COI, COII and EF-1α for butterflies, and *mat*K and non-coding region between *trn*F-*trn*L for plants. Strongest lines indicate preferred host-plant (Brown *et al*., 1995).

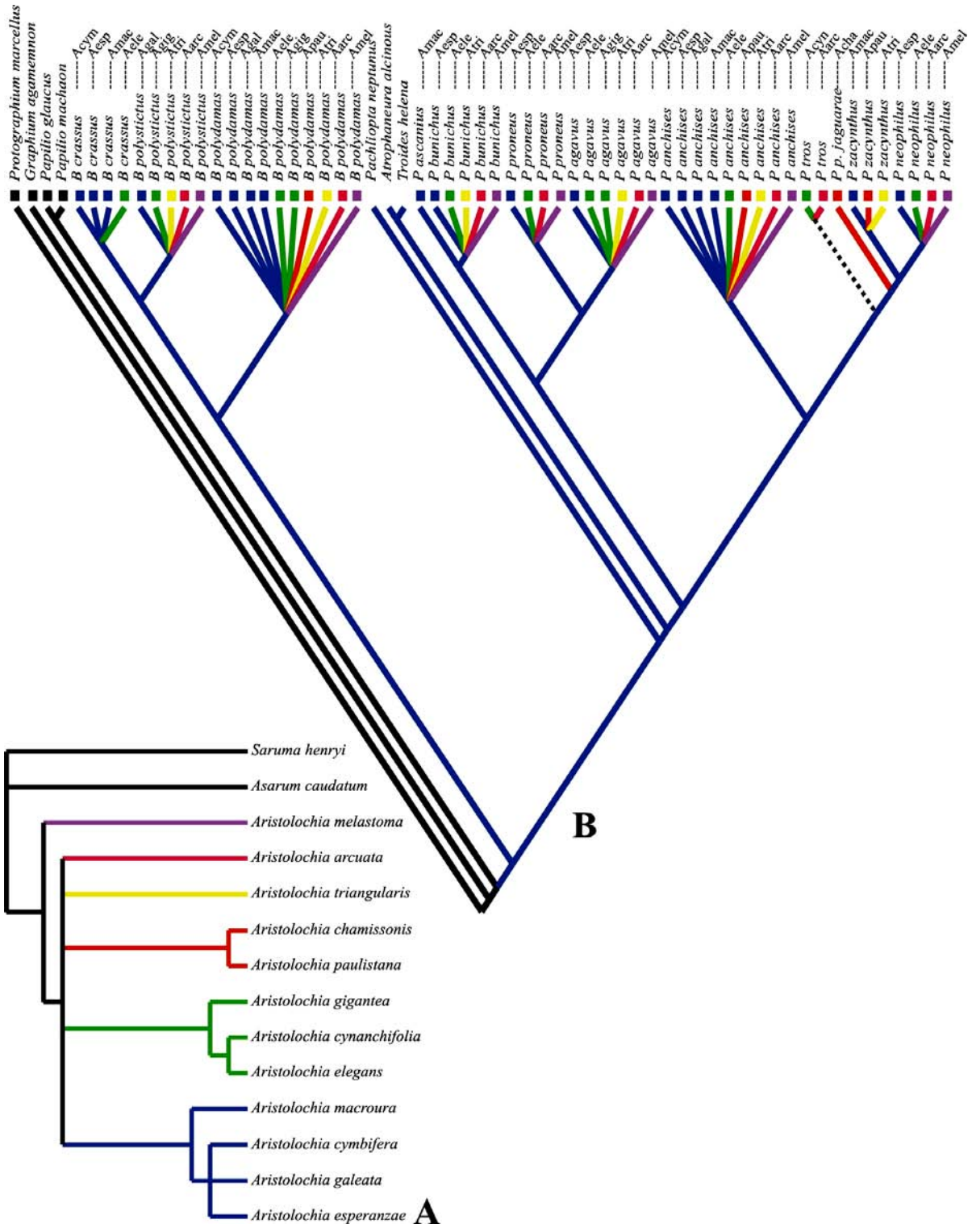
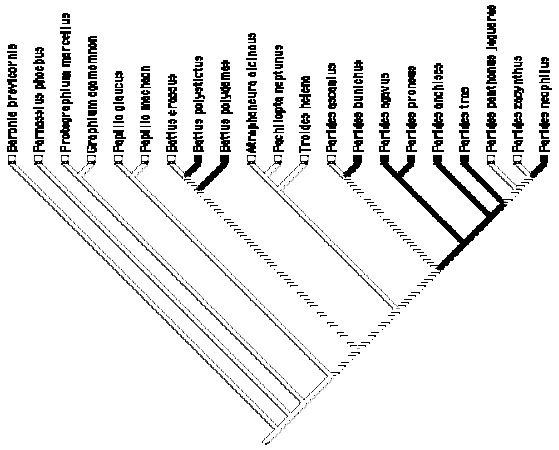
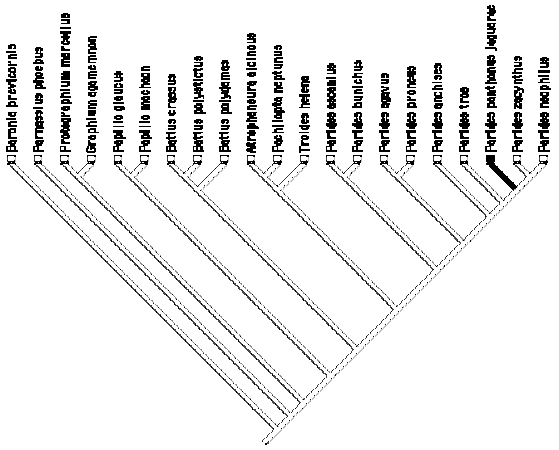


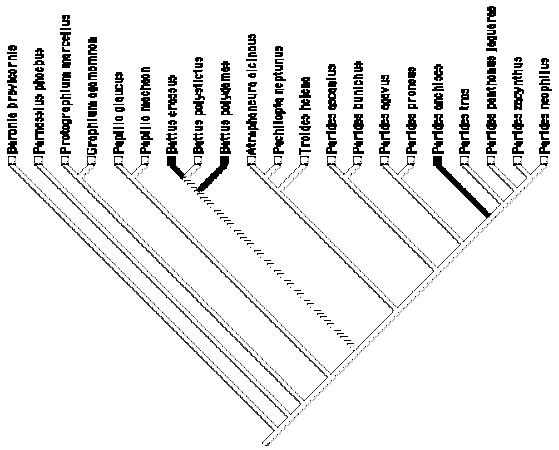
Figure 3.10. Comparison between Troidini and *Aristolochia* phylogenies. The six clades of *Aristolochia* (A) are traced onto the Troidini phylogenetic tree (B), with polytomies, as many as the number of host-plants used by each butterfly species.



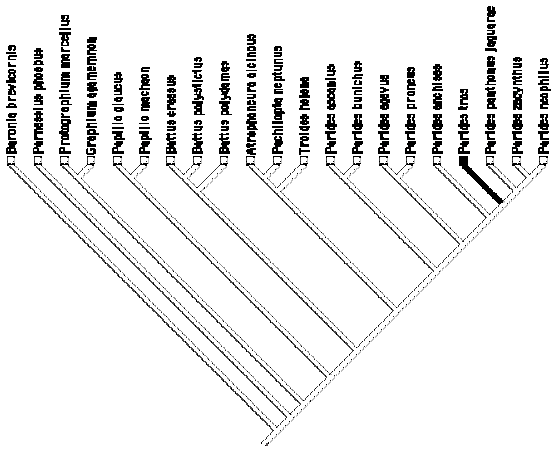
A. *A. arcuata*



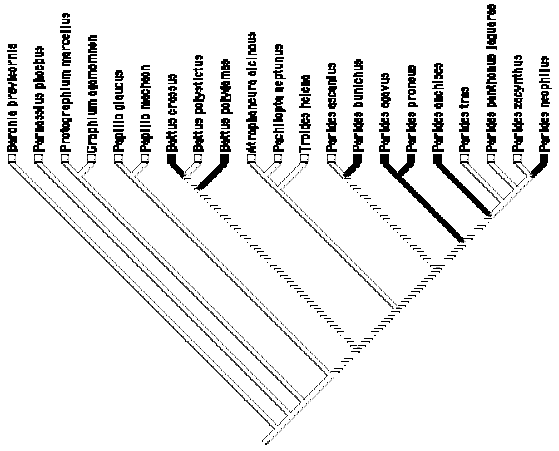
B. *A. chamissonis*



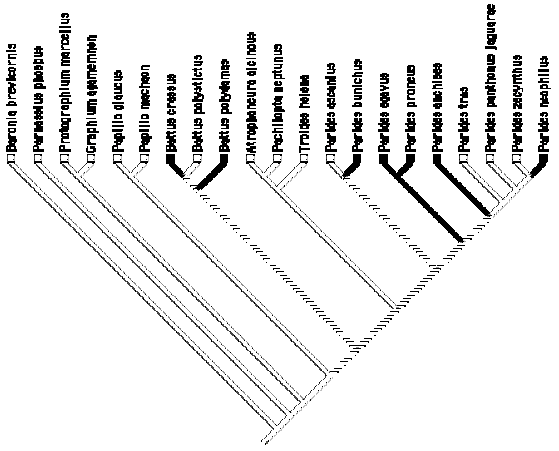
C. *A. cymbifera*



D. *A. cynanchifolia*

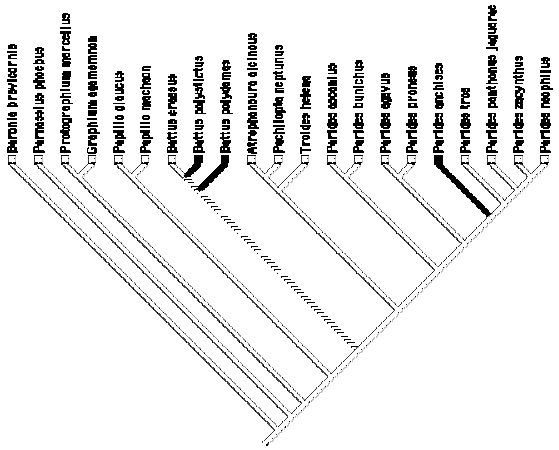


E. *A. elegans*

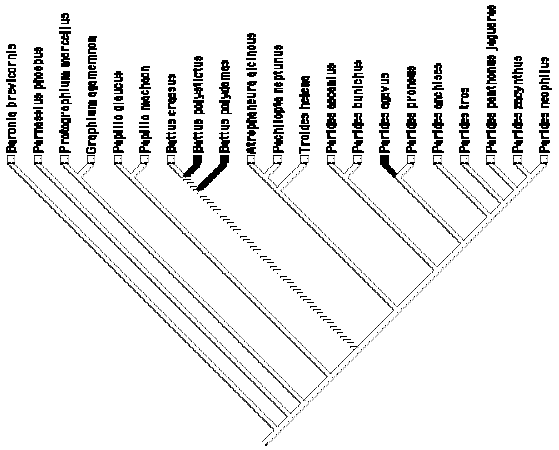


F. *A. esperanzae*

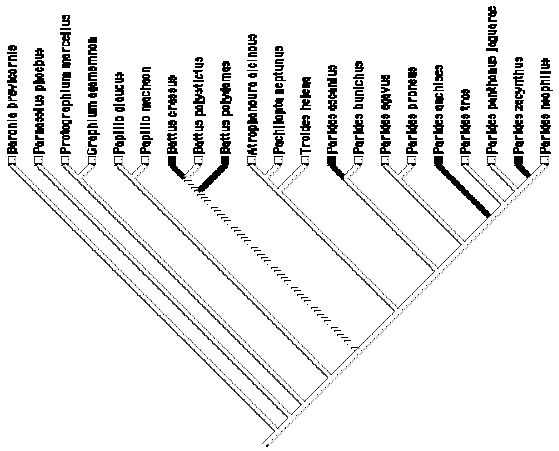
Figure 3.11. Host-plant utilization over Troidini phylogenetic tree. Black: presence of plant species in the butterfly diet; White: absence of plant species in the butterfly diet; Dashed line: equivocal node. (CI=0.25).



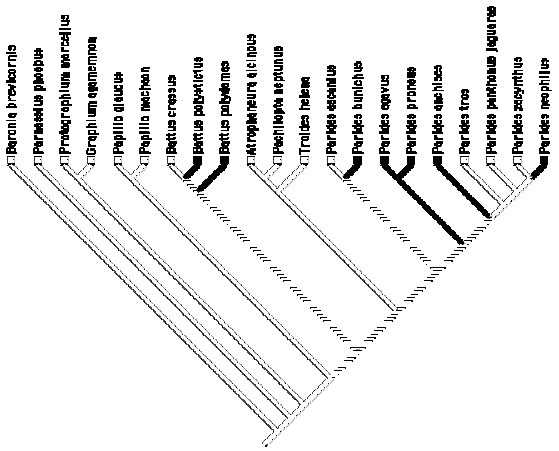
G. *A. galeata*



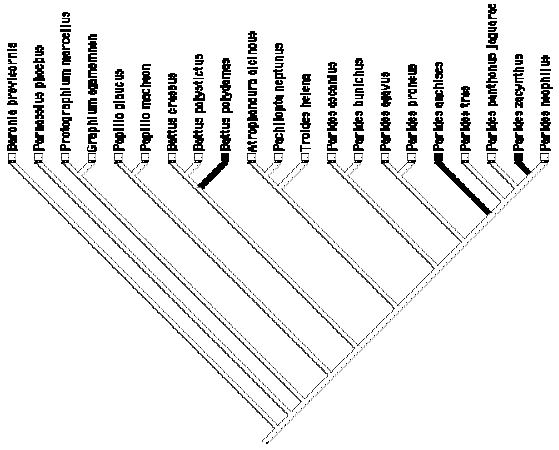
H. *A. gigantea*



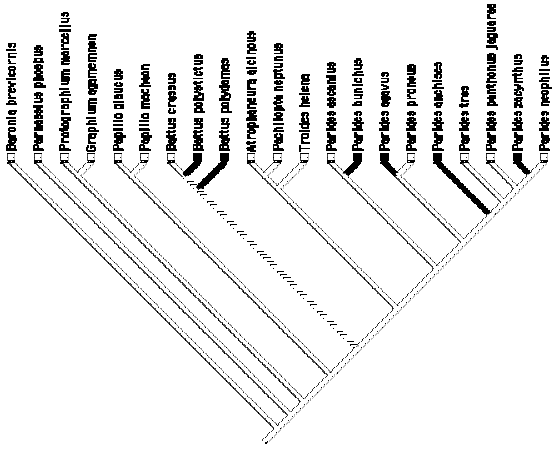
I. *A. macroura*



J. *A. melastoma*



K. *A. paulistana*



L. *A. triangularis*

Figure 3.11. Extended.

Comparison of Troidini phylogeny with the chemogram and the biogeogram of Aristolochia

The comparison between the phylogeny of Troidini and the chemogram of *Aristolochia* showed that the ancestors of *Battus* and *Parides* may have used to feed in host-plants belonging to the germacrene-D group, and the plants with a higher relative abundance of this compound are still used these butterflies (Fig. 3.12). On the other hand, both *Parides ascanius* and *P. zacynthus* feed exclusively in plants showing germacrene-C at high amounts (Fig. 3.12). The congruence between Troidini phylogeny and the *Aristolochia* chemogram was not significant when used all associations between them ("leaves added", $P = 1$; "losses", $P = 0.324$), but was significant when used only the preferred host-plants ("leaves added", $P = 0.019$; "losses", $P = 0.047$).

Character tracing of the geographic similarity of *Aristolochia* onto the Troidini phylogeny showed that the host-plant used by the ancestor of *Battus* and that of *Parides* may have came from different clusters (Fig. 3.13). The ancestor of *Battus* may have used to feed on host-plants from Group 1, that is, plants occurring in the SE of São Paulo and Minas Gerais, and near the city of Rio de Janeiro. On the other hand, the host-plant used by the ancestor of *Parides* may have belonged to Group 3, composed of plants with a less uniform distribution, and with two species showing a coastal range. At present, almost all Troidini feed on plants in Group 1, with species showing a broad distribution. Four species of Troidini from SE Brazil (*B. polydamas*, *P. bunichus*, *P. agavus* and *P. anchises*) use *Aristolochia* species from all geographic ranges. Otherwise, *B. polystictus* use host-plants almost exclusively from Group 1, with one representative of Group 2 (Fig. 3.13). The congruence between the Troidini phylogeny and the *Aristolochia* biogeogram was not significant when all species of host-plant used by each butterfly were considered ("leaves added", $P = 1$; "losses", $P = 0.687$). However, when only the preferred host-plants were considered, the congruence was significant for "leaves added" ($P = 0.029$) but not for "losses" ($P = 0.136$).

For both optimizations, the phylogenetic signal over the topology of Troidini was not significant ($P = 0.25$ for chemical similarity and $P = 0.46$ for geographical similarity).

Comparison of Troidini and Aristolochia biogeograms

The congruence between the biogeograms of Troidini and *Aristolochia* was not significant, according to tree mapping, using all host-plant associations ("leaves added", $P = 0.377$; "losses", $P = 0.486$). When only the preferred host-plants associations were considered, biogeograms were congruent for "leaves added" ($P = 0.02$) but not for "losses" ($P = 0.1$).

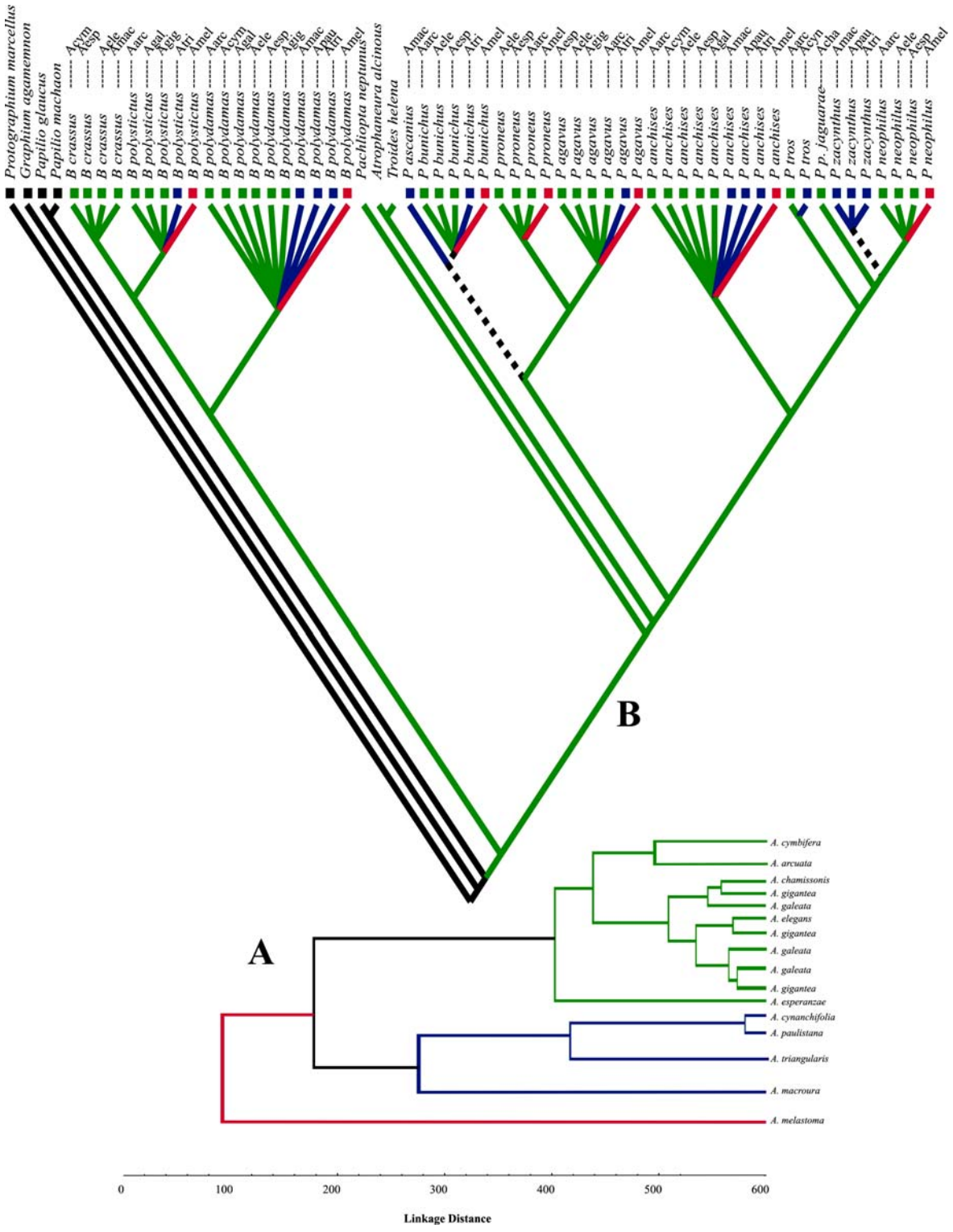


Figure 3.12. Comparison between Troidini phylogeny and *Aristolochia* chemogram. The three clusters of *Aristolochia* (A) are traced onto the Troidini phylogenetic tree (B), with polytomies, as many as the number of host-plants used by each butterfly species.

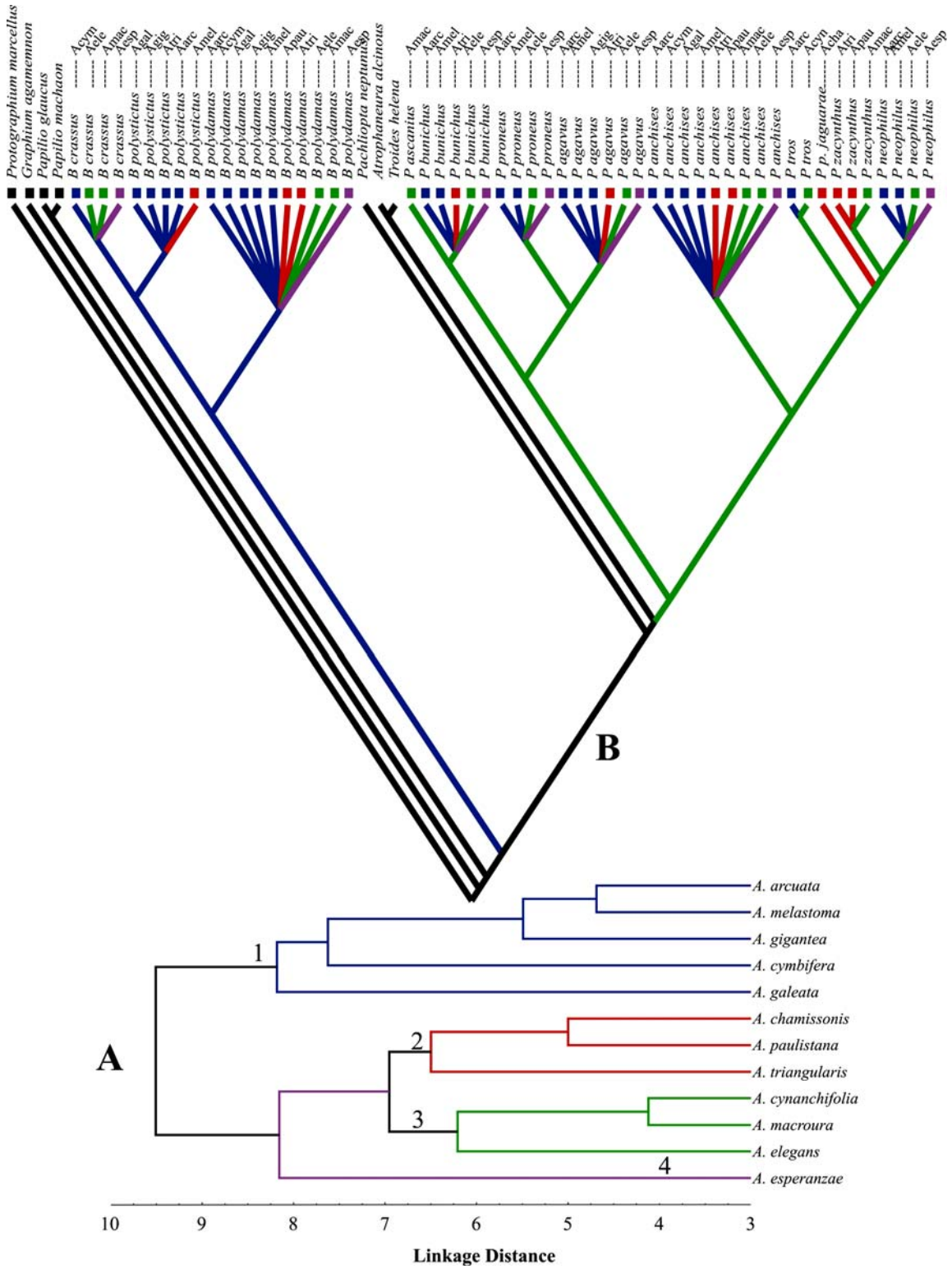


Figure 3.13. Comparison between Troidini phylogeny and *Aristolochia* biogeogram. The four clusters of *Aristolochia* (A) are traced onto the Troidini phylogenetic tree (B), with polytomies, as many as the number of host-plants used by each butterfly species.

Discussion

Host-plant phylogeny constraints

We did not find a strong indication in our analyses that basal Troidini use basal *Aristolochia*, or that derived species of these butterflies feed on derived species of their host-plants (Figs. 3.9 and 3.10). In fact, it seems that there is no current pattern of host-plant utilization based on the phylogenies of Troidini and *Aristolochia.* This does not agree with the proposal of Brown *et al.* (1995), who suggested a coevolutionary linkage between these butterflies and their host-plants. However, when we used only the preferred host-plant associations between Troidini and *Aristolochia*, we found a significant congruence between their phylogenies, but since these butterflies can feed many species of *Aristolochia*, it is difficult to say how their phylogenies could have constrained each others evolution. Total phylogenetic congruence is rarely found between herbivores and host-plant systems, and Thompson (1989) even says that parallel cladogenesis is just one result expected from reciprocal evolution and its absence is not a signal of lack of evolutionary interaction. Becerra (1997) found a weak influence of host-plant phylogeny in the evolution of host use in the *Blepharida*/*Bursera* interaction. On the other hand, Farrell and Mitter (1998) found an almost complete correspondence between the phylogenies of *Tetraopes* beetles and *Asclepias* used as host-plants.

It is intriguing that the comparison between the phylogenies of Troidini and *Aristolochia* have shown that the host-plant used by the ancestors of *Battus* and *Parides* may have came from the clade joining *Aristolochia macroura*, *A. cymbifera*, *A. galeata* and *A. esperanzae* (Fig. 3.10). Only *A. macroura* has aristolochic acids (AAs) in its leaves, while the other three species have only labdanoic acids (LAs) (Klitzke, 1992; Leitão and Kaplan, 1992). It was expected that the host-plant used by the ancestor of Troidini could show exclusively AAs in their leaves, but our results suggest that maybe the ancestral host-plant could show only LAs. According to the proposal of coevolution between Troidini

and *Aristolochia* (Brown *et al.*, 1991), these plants may have changed their classes of chemical compounds as a consequence of herbivore pressure. Hence, derived species of *Aristolochia* may have lost AAs and acquired other defenses, such as LAs, to deter species of herbivores specialized on Aristolochiaceae plants, which show AAs. The preferential use of species with LAs in their leaves by *Parides anchises* can support the hypothesis of coevolution proposed by Brown *et al.* (1991) since this is a derived species within Troidini (Chapter 1). However, we found here that the use of plants containing LAs may have arisen early in the evolutionary history of Troidini, at least for those species from SE Brazil. Although this may be true, a consistent body of data has shown evidence that the use of plants with AAs is more adavantageous for these butterflies. In fact, species of Troidini fed with leaves of *Aristolochia galeata* (with LAs) consumed less of these leaves and showed a smaller relative growth (Klitzke, 1992).

A possible explanation to the incogruences found between our results and these found by Klitzke (1992) could be the approach used in each study. Klitzke (1992) tested, among other things, the consume of different species of *Aristolochia* by Troidini, showing that some species are more consumed than others, and correlated these results with the chemical compounds found in *Aristolochia*. Here, we used an evolutionary approach based only on the presence/absence of each species of *Aristolochia* in the diet of each species of Troidini; however, we did not consider if one species is more used than others. Consequentely, maybe some records could be sub-optimal use of food plants, since polyphagous species may feed on less preferred host-plants due to the lack of their usual food plant at particular place and time (Fox and Morrow, 1981; Bernays and Graham, 1988).

Ancestor reconstructions are estimates based on data from extant terminals (Schluter *et al.*, 1997), and the parsimony method seeks to reconstruct ancestral states by choosing the states at each internal node that require the minimal number of steps on the tree (Maddison and Maddison, 1999). Thus, the widespread use of plants belonging to the clade of species showing LAs in their leaves must

have resulted in this state at the ancestral node. We can say with confidence that the presence of AAs and LAs does not seem to constrain the use of *Aristolochia* by Troidini currently, but how this use evolved is a matter of future studies.

Host-plant secondary chemicals constraints

We found that the phylogeny of Troidini and the chemogram of *Aristolochia* obtained with their sesquiterpene pattern are significantly congruent when we used only the preferred host-plant associations, as found when we compared the phylogenies of these butterflies and of their preferred host-plants; the significant values were quite similar in both comparisons ("leaves added", $P = 0.019$; "losses", $P = 0.047$ and "leaves added", $P = 0.018$; "losses", $P = 0.028$, respectively). However, once again it is difficult to interpret the role of the sesquiterpene similarity of *Aristolochia* in the host shift by Troidini, since these butterflies feed on a large number of host-plants. Janz and Nylin (1998) found that growth form is the more conservative aspect of host association between Papilinoidea and their host-plants, suggesting that other factors rather than plant chemistry may have an important role in shaping these associations. On the other hand, Becerra and Venable (1999) found that host-plant chemistry best explains the overall pattern of host shifts by *Blepharida* beetles, and Wahlberg (2001) also found that host-plant chemistry is a more conservative trait than host-plant taxonomy in the evolutionary history of host-plant use in the tribe Melitaeini. Berenbaum (2001) also suggested that chemical similarity in Apiaceae, rather than geographical proximity or phylogenetic relationship, is the most probable basis for the observed host-plant shifts.

The presence of a host-plant containing germacrene-D as main sesquiterpene in the ancestral node of Troidini can be explained by the preferred use of plants with this character by these butterflies. Only two species of *Parides*, *P. ascanius* and *P. zacynthus*, do not feed on host-plants containing germacrene-D at high relative abundance (Fig. 3.10). Pressure of pollinators was suggested to have

governed the evolution of different sesquiterpenes in *Aristolochia* (Chapter 2). Maybe, besides their differential role in attracting pollinator insects, these sesquiterpenes can attract differentially other floral visitors; that is, *P. ascanius* and *P. zacynthus* can be mostly attracted to plants containing a high relative abundance of germacrene-C. The same can be said about *Battus crassus* and *P. panthonus jaguarae*, which use *Aristolochia* plants belonging exclusively to germacrene-D group.

Although the sesquiterpene composition has worked well as a chemotaxonomic marker to separate *Aristolochia* species (Chapter 2), maybe there is not enough chemical difference among these species to constrain their use by Troidini. The main sesquiterpenes found on plants studied here are quite similar among species (Chapter 2). We can suggest that once the initial chemical barrier for using plants in the genus *Aristolochia* had been overcome, it was likely that all available species were used due to their chemical similarity. This assumption partly agrees with the coevolutionary hypothesis of Ehrlich and Raven (1964) when they say that some butterfly groups may have found a free adaptive zone to radiate on plants chemically defended against other herbivore insects, although the role of these insects in the radiation of their host-plants may have been insignificant (Thompson, 1989). In fact, Weintraub (1995) demonstrated that colonization events are relatively frequent in Troidini, and monophagy appears to be relatively uncommon at the subgeneric and species levels, occurring in species with restricted distributions.

To test that idea, the number of squares occupied by each Troidini in our baseline map (Fig. 3.2) was counted and plotted against the number of host-plants used by each one (Table 3.1). There was a significant positive correlation between the geographical range of butterflies and the number of host-plants used as food ($r^2 = 0.8286$, $P < 0.001$) (Fig. 3.14). This result agrees with Weintraub (1995) proposal that the most polyphagous Troidini are those with the broadest geographical range. Thus, butterflies with a larger distribution use a higher number of hosts; however, the widest distributed host-plants are not necessarily most used by these butterflies. Counting the number of squares occupied by

each species of *Aristolochia* and plotting this against the number of Troidini which feed on each one resulted in a non-significant correlation ($r^2 = 0.0854$, $P = 0.357$) (Fig. 3.15). This indicates that the butterflies may not occupe the entire range of their host-plants. In this way, host-plant availability cannot be considered a constraint to Troidini host shift, and other biotic or abiotic factors can provide different selective pressures at different times (Quinn *et al.*, 1997).

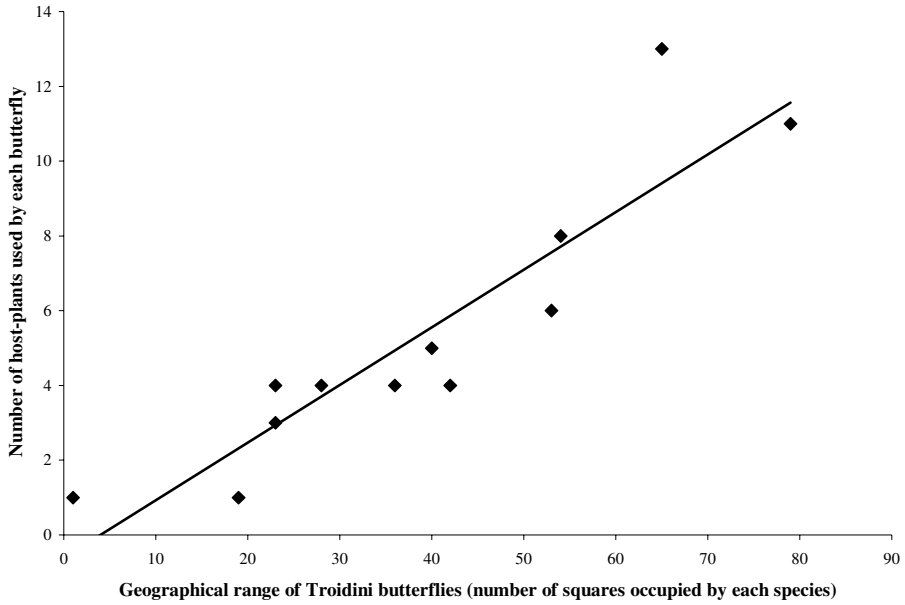


Figure 3.14. Relationship between the geographical distribution of Troidini butterflies and the number of host-plants in the genus *Aristolochia* used by each butterfly species.

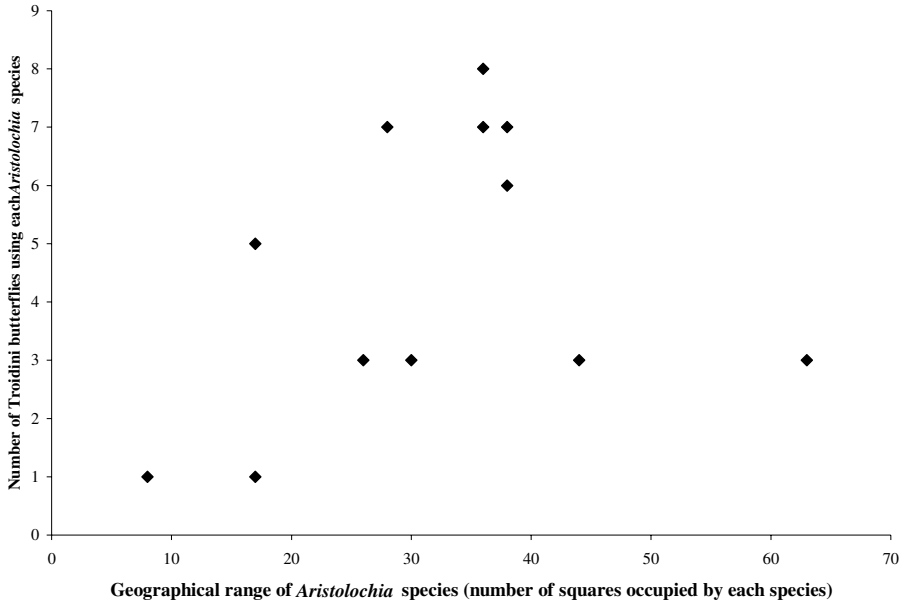


Figure 3.15. Relationship between the geographical distribution of *Aristolochia* and the number of Troidini butterflies which use each plant species as host-plant.

Host-plant geographic constraints

An alternative to host shifts among phylogenetic or chemically related host-plants is that insects may have shifted among hosts within a biogeographic area, since it can be easier to change host-plant if the geographical range of the new host is similar to that of the old host (Becerra and Venable, 1999). Here, the congruence between the phylogenetic topology of Troidini and the biogeogram of their host-plants was less significant than the correlation between the phylogeny of the butterflies and the phylogenetic tree and chemogram of their hosts. Becerra and Venable (1999) also found that geographical similarity is less important to host shifts in *Blepharida* beetles than the chemistry of their host-plants. Maybe, more important than the relation between insect phylogeny and host-plant

distribution could be insect and host-plant geographical similarity; however, we did not find a significant congruence between the biogeograms of Troidini and *Aristolochia* host-plants.

Conclusions

If we consider only the preferred host-plant associations between Troidini and *Aristolochia*, both phylogenetic relationships and sesquiterpene similarity among the host-plants may have constrained evolutionary host shifts by these butterflies. However, the present pattern of host-plant use by Troidini does not seem to be constrained by the phylogeny of their food plants, neither by the secondary chemicals in these plants nor by their geographical similarity. In general, the New World Troidini butterflies studied here feed on both basal and derived species of *Aristolochia*, species with or without AAs in their leaves, and even species showing only LAs. Besides, these butterflies accept equally well *Aristolochia* species showing distinct sesquiterpene patterns. And, except for strictly monophagous species, all Troidini use *Aristolochia* species with different geographical ranges. The current host-plant use in these butterflies seems to be simply opportunistic, with species with a wide geographical range using more species of host-plants than those with a more restrict distribution (Weintraub, 1995). This behavior might merely mirror the available choice and abundance of different host-plants in different regions within a butterfly's geographical range, even if these plants are not phylogenetically or chemically related (Fox and Morrow, 1981; Rowell, 1985; Pasteels and Rowell-Rahier, 1991; Percy *et al.*, 2004), showing the label pattern suggested by Bernays and Graham (1988) to host-plant use. In this way, the high host-plant fidelity at tribal level is lost at lower taxonomic levels.

Such "resource availability" pattern was found by Gomez-Zurita *et al.* (2000), who proposed that food specialization in *Timarcha* is dependent on geographical distribution. *Oreina* species provide an example of host-plant choice governed by available options rather than by plant relatedness and chemical similarity (Dobler *et al.*, 1996). In the same way, Pasteels and Rowell-Rahier (1991) affirmed that the frequent shift of host-plants by leaf beetles of the tribe Chrysomelini seems to be

opportunistic and not governed by chemical similarity among host-plants or by phylogenetic parallels between the beetles and their host-plants. The possible exception in Troidini for this availability model must be *Parides ascanius.* This species is a strict specialist on *Aristolochia macroura*, although other *Aristolochia* species are available in its habitats (Otero and Brown, 1986); however, the host-plant interactions in this species could be an example of habitat fidelity rather than host fidelity (Dobler *et al.*, 1996), since it is specialized not only to its host-plant, but also to the physical and biotic environment where this plant grows (Bernays, 1996).

The opportunisctic model can explain the present use of host-plants by Troidini butterflies, but do not clarify the starting point of the interaction. May be the best explanation is the colonization of available *Aristolochia* plants by these butterflies. To test this hypothesis, it were performed analyses to estimate divergence times to both butterflies and plants, using the program r8s v. 1.70 (Sanderson, 2004), with Penalized Likelihood method. r8s is a program which uses parametric, nonparametric and semiparametric methods to relax the assumption of constant rates of evolution among genes and lineages to obtain better estimates of rates and times (Sanderson, 2003). Branch lengths were estimated during Bayesian analyses under the model GTR+G+I. The minimum age to Papilionidae butterflies was fixed in 82.5 Mi years, according to Gaunt and Miles (2002). For Aristolochiaceae, the minimum age was fixed in 91.2 Mi years, according to Magallon and Sanderson (2001). The estimated age calculated for Troidini was 52.3 Mi years, and for *Aristolochia* it was 13.2 Mi years. This intriguing result may be due to the long branch separating *Aristolochia* from SE Brazil from the other two genera used here as outgroups to perform phylogenetic analyses. In addition, *Aristolochia* from South America are considered subclades of recent diversification (Fávio González, pers. com.), and to secure estimation of divergence time it was needed to include North and Center America *Aristolochia* species, believed to occupe basal positions in the genus, to perfume the phylogenetic analysis (Fávio González, pers. com.). A recent fossil of *Aristolochia* found in North of Colombia (Guajira) was estimated in 60

Mi years (Fávio González, pers. com.), and we can suppose that as the minimum age of the genus in South America. In this way, *Aristolochia* could be considered older than the Troidini butterflies which feed on them, what agrees wih the colonization hypothesis as a better explanation to the initial point of this interaction.

Our results indicate that change and potential for changing may be great in the Troidini/*Aristolochia* interaction. Plants and butterflies should be under constant selective pressure, and it is expected that interactions are being renewing at the same rhythm, in a dynamic ecological process. Thus, the data collected over time could turn the interactions picture still more chaotic.

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Discussão Geral

Para atingir o principal objetivo deste trabalho, que foi entender como a interação entre as borboletas da tribo Troidini e suas plantas hospedeiras no gênero *Aristolochia* evoluiu, foi usado o "enfoque de padrão" proposto por Ronquist e Nylin (1990), que sugere o uso das filogenias dos grupos associados para reconstruir a história coevolutiva da interação (sem com isto supor a obrigatoriedade de cladogênese paralela). Desta forma, o primeiro objetivo deste trabalho foi propor uma filogenia para os Troidini neotropicais baseada em dados moleculares, uma vez que não havia nenhuma proposta filogenética robusta usando este tipo de caráter para estas borboletas, principalmente para o gênero *Parides*. Os resultados encontrados para as relações entre os Troidini corroboraram aqueles obtidos anteriormente com dados morfológicos (Munroe e Ehrlich, 1960; Hancock, 1983; Miller, 1987) e com dados moleculares (Caterino *et al.*, 2001). De fato, a topologia geral foi mantida mesmo com a inclusão de taxa adicionais em alguns ramos, como a inclusão do gênero *Euryades* e espécies novas de *Battus* e *Parides*. A posição basal de *Battus* dentro dos Troidini, que havia sido questionada recentemente (Morinaka *et al.*, 1999), foi recuperada nesse estudo, e concorda com as propostas de Munroe e Ehrlich (1960), Hancock (1983) e Miller (1987), que colocaram este gênero na subtribo Battina, irmão da subtribo Troidina (que inclui todos os gêneros de Troidini, exceto *Battus*), correspondendo também à hipótese de Caterino *et al.* (2001). Estes resultados discordam daquele encontrado por Morinaka *et al.* (1999) que, usando unicamente o gene mitocondrial ND5, colocou *Battus* próximo aos Graphiini, e fora do grupo dos Troidini. No entanto, este resultado tem um fraco suporte, devido principalmente à amostragem taxonômica insuficiente e ao pequeno fragmento usado para as análises filogenéticas. Estes pontos foram considerados aqui, principalmente em relação ao número suficiente de bases analisadas, e na escolha de genes mitocondriais (COI e COII) e um gene nuclear (EF-1α). Em Papilionidae, estes genes foram usados com sucesso para elucidar relações entre

espécies e gêneros (Caterino e Sperling, 1999; Caterino *et al.*, 2001; Zakharov *et al.*, 2004), e Sperling (2003) recomenda seu uso combinado para estudos filogenéticos entre borboletas em geral.

A hipótese filogenética proposta aqui para os Troidini foi a primeira a enfocar detalhadamente o gênero *Parides*, uma vez que se amostrou 17 das 34 espécies reconhecidas para este gênero, com representantes de todos os grupos subgenéricos reconhecidos por Tyler *et al.* (1994). Estudos anteriores haviam examinado de uma a quatro espécies de *Parides* apenas (Tyler *et al.*, 1994; Morinaka *et al.*, 1999; Morinaka *et al.*, 2000; Caterino *et al.*, 2001). Esta amostragem mais intensa permitiu a identificação de linhagens diferentes dentro do gênero, com os *Parides* com cauda do SE do Brasil aparecendo numa posição basal em relação aos outros grupos. Algumas relações filogenéticas fortemente sustentadas, como a relação entre *Parides ascanius* e *P. bunichus*, apresentavam evidências anteriores, mas nunca haviam sido demonstradas com análises filogenéticas detalhadas. Estas duas espécies preferem habitats abertos ao invés de floresta (Tyler *et al.*, 1994), e já se demonstrou que elas podem formar híbridos, tanto no campo como no laboratório (Otero e Brown, 1986). Além disto, *Parides ascanius* é bastante similar morfologicamente aos adultos de *P. bunichus*, especialmente *P. bunichus chamissonia*, incluindo a genitália e elementos do padrão de coloração das asas (Otero e Brown, 1986; Tyler *et al.*, 1994). O forte suporte estatístico do ramo que junta estas duas espécies pode ser considerado um indicativo de que a hipótese filogenética proposta para todo o grupo pode estar contando de fato a história evolutiva dos *Parides*.

Mesmo com algumas questões permanecendo para serem estudadas, principalmente em relação à inclusão das demais espécies de *Parides* e à posição de *P. chabrias*, o presente estudo esclareceu as relações internas principais deste gênero, e ofereceu uma hipótese filogenética importante para se testar teorias ecológicas e biogeográficas na evolução deste grupo, como, por exemplo, a evolução no uso de plantas hospedeiras, que foi estudada aqui. Baseados nos dados disponíveis sobre o uso de plantas hospedeiras, o estado ancestral para Troidini e *Parides* parece ser o uso de muitas espécies de

Aristolochia, com uma tendência em direção a uma maior especialização. Os resultados de otimização de caráter demonstraram que os *taxa* terminais geralmente se alimentam em um menor número de espécies de *Aristolochia* quando comparados aos *taxa* basais, concordando com o dados encontrados por Kelley e Farrell (1998) para besouros do gênero *Dendroctonus*. Esta tendência em direção a uma maior especialização no uso de hospedeiros pode ser resultado da distribuição geográfica das espécies, refletindo o padrão proposto por Weintraub (1995), ou seja, espécies com distribuição geográfica restrita tendem a ser especialistas, enquanto aqueles com ampla distribuição são geralmente generalistas, de forma que a amplitude da dieta pode estar de fato refletindo a disponibilidade e abundância das plantas hospedeiras (Pasteels e Rowell-Rahier, 1991).

Após a obtenção desta hipótese filogenética para os Troidini, o segundo objetivo deste trabalho foi propor uma hipótese para a filogenia das suas plantas hospedeiras, todas pertencentes ao gênero *Aristolochia*. Esta foi a primeira proposta filogenética para as espécies de *Aristolochia* usadas como hospedeiros pelas espécies de Troidini do SE do Brasil, e as relações filogenéticas encontradas entre estas espécies concordam com muitas das relações sugeridas por uma análise hierárquica proposta para elas (Tyler *et al.*, 1994; Brown *et al.*, 1995). *Aristolochia melastoma*, por exemplo, ocupa uma posição basal em relação a todas as outras espécies de *Aristolochia* estudadas aqui, a mesma posição sugerida por Tyler *et al.* (1994) e Brown *et al.* (1995). Da mesma forma, encontrou-se nesse estudo que *Aristolochia esperanzae*, *A. galeata* e *A. cymbifera* ocupam a posição mais derivada neste gênero, o mesmo tendo sido proposto por aqueles autores.

Embora a árvore consenso considerada a melhor hipótese filogenética das *Aristolochia* do SE do Brasil tenha mantido alguns ramos internos sem uma resolução completa, esta hipótese permitiu o estudo da evolução de alguns caracteres químicos determinados ou conhecidos anteriormente para estas espécies. Como os compostos químicos de *Aristolochia* parecem determinar os padrões de exploração por Troidini, permitindo a identificação das plantas hospedeiras adequadas (Brown *et al.*, 1981),

entender como estes compostos evoluíram nas espécies de *Aristolochia* pode ajudar a entender como evoluiu a própria utilização destas plantas como hospedeiros por estas borboletas. Desta forma, pode-se sugerir que a distribuição de ácidos aristolóquicos (AAs) e labdanóicos (LAs) ao longo da filogenia das *Aristolochia* pode ter sido determinada por pressões seletivas de predadores herbívoros, uma hipótese defendida por Brown *et al.* (1991). Estes autores sugerem que teriam ocorrido mudanças nas classes de compostos químicos durante a evolução das *Aristolochia* em decorrência de pressões seletivas para aprimorar as defesas químicas contra herbivoria. Assim, espécies derivadas de *Aristolochia* teriam perdido AAs e adquirido outras defesas químicas, tais como LAs, para deter espécies de insetos fitófagos especializados em Aristolochiaceae (Klitzke, 1992). Um amplo conjunto de dados apoiando esta hipótese foi concatenado por Klitzke (1992), que demonstrou que borboletas Troidini comem e crescem menos em folhas de espécies de *Aristolochia* contendo LAs. Adicionalmente, apenas as espécies mais polífagas são capazes de comer em espécies de *Aristolochia* com estes ácidos (Klitzke, 1992).

A evolução da distribuição de sesquiterpenos ao longo da filogenia das *Aristolochia* pode ser interpretada à luz de pressões seletivas de polinizadores, uma vez que estes compostos podem desempenhar um importante papel em atrair insetos polinizadores para as plantas, além de outras funções de defesa contra herbivoria (Harborne, 2001). Em plantas de algumas famílias de angiospermas, os mesmos terpenóides encontrados nas flores podem estar presentes simultaneamente nas folhas, como em Magnoliaceae (Azuma *et al.*, 1999) e Nyctaginaceae (Levin *et al.*, 2001). Assim, se considerarmos que os compostos voláteis encontrados nas folhas de *Aristolochia* podem ser encontrados igualmente nas suas flores, pode-se inferir que espécies proximamente relacionadas podem ter evoluído diferentes sesquiterpenos para atrair diferentes polinizadores. Esta hipótese é apoiada pelas evidências de que espécies de *Aristolochia* apresentam especificidade no grupo de polinizadores,

geralmente espécies de moscas (Brantjes, 1980; Siqueira, 1988), e espécies que compartilham os mesmos polinizadores apresentam áreas de ocorrência disjuntas (Brantjes, 1980).

Com as hipóteses filogenéticas das borboletas Troidini e das suas plantas hospedeiras, foi então possível estudar de fato como esta interação evoluiu, e quais são os fatores que poderiam estar determinando o padrão atual de utilização de hospedeiros nestas borboletas. A comparação das filogenias de Troidini e *Aristolochia* não confirmou a proposta de Brown *et al.* (1995) de que espécies basais de Troidini se alimentam de espécies basais de *Aristolochia*, ou de que espécies derivadas destas borboletas se alimentam de espécies derivadas de plantas hospedeiras. De fato, não parece haver nenhum padrão na utilização de hospedeiros baseado nas filogenias de Troidini e *Aristolochia*. No entanto, quando se analisaram somente as associações de Troidini com suas plantas hospedeiras preferenciais, encontrou-se uma congruência significativa entre as duas filogenias, o que demonstra que a filogenia de *Aristolochia* pode ter tido um papel na determinação da escolha de hospedeiros por estas borboletas. Como atualmente cada espécie de borboleta pode se alimentar de várias espécies de *Aristolochia*, é difícil dizer como estas associações podem ter influenciado a evolução de cada grupo. Congruência filogenética total é raramente encontrada entre herbívoros e suas plantas hospedeiras. Becerra (1997), por exemplo, encontrou apenas uma fraca influência da filogenia das plantas na evolução no uso de hospedeiros na interação entre *Blepharida* e *Bursera*. Por outro lado, Farrell e Mitter (1998) encontraram uma correspondência quase completa entre as filogenias de besouros do gênero *Tetraopes* e plantas do gênero *Asclepias*.

Outros fatores além da filogenia das plantas hospedeiras poderiam estar definindo a mudança de hospedeiros na evolução dos Troidini e, desta forma, seria importante se conhecer não apenas as relações filogenéticas dos grupos de interesse, mas também as relações fenéticas obtidas com outros marcadores, neste caso, marcadores químicos e de distribuição geográfica. A comparação da filogenia dos Troidini com um dendrograma obtido com o padrão de sesquiterpenos das *Aristolochia*

(quimiograma) mostrou uma congruência significativa quando se consideraram apenas as associações entre as borboletas e suas plantas hospedeiras preferenciais, demonstrando um possível papel da composição química de *Aristolochia* na mudança de hospedeiros na evolução dos Troidini. No entanto, novamente é difícil afirmar como os compostos químicos de *Aristolochia* podem ter influenciado a evolução no uso de hospedeiros nestas borboletas, uma vez que elas se alimentam de várias espécies de plantas atualmente. Janz e Nylin (1998) encontraram que a forma de crescimento das plantas hospedeiras é um caráter mais conservado nas associações entre Papilinoidea e suas plantas hospedeiras, sugerindo que outros fatores além da química das plantas podem desempenhar papéis importantes na determinação destas associações. Por outro lado, outros autores têm demonstrado a importância da química das plantas na interação entre herbívoros e plantas hospedeiras. Becerra (1997) e Becerra e Venable (1999), por exemplo, encontraram que a química das plantas hospedeiras explica melhor o padrão geral de mudança de hospedeiros por besouros do gênero *Blepharida* e Wahlberg (2001) também encontrou que a química das plantas hospedeiras é um caráter mais conservado do que a sua taxonomia na história evolutiva do uso de hospedeiros na tribo Melitaeini. Berenbaum (2001) também sugere que a similaridade química em Apiaceae pode ter moldado as mudanças de hospedeiros observadas em vários *taxa*.

Dos três fatores testados, a similaridade geográfica entre as plantas hospedeiras foi o fator de menor relevância na mudança de hospedeiros em borboletas Troidini, como atesta a menor congruência encontrada entre a árvore filogenética dos Troidini e o biogeograma das *Aristolochia*.

O padrão atual de utilização de hospedeiros em Troidini não parece ser limitado pela filogenia das suas plantas hospedeiras, nem pelos químicos secundários encontrados nestas plantas ou pela sua similaridade geográfica. De fato, a utilização de hospedeiros nestas borboletas parece ser simplesmente oportunística, com espécies com uma ampla distribuição geográfica usando um maior número de espécies hospedeiras do que aqueles com uma distribuição geográfica mais restrita

(Weintraub, 1995). Este padrão pode estar meramente refletindo a escolha disponível e a abundância de diferentes plantas hospedeiras em diferentes regiões da distribuição geográfica de uma espécie de borboleta, mesmo que estas plantas não sejam relacionadas filogenética ou quimicamente (Fox e Morrow, 1981; Rowell, 1985; Pasteels e Rowell-Rahier, 1991; Percy *et al.*, 2004). Este padrão de disponibilidade de recursos na determinação no uso de plantas hospedeiras foi também encontrado em besouros *Oreina* (Dobler *et al.*, 1996) e Chrysomelini (Pasteels e Rowell-Rahier, 1991). Sendo assim, pode-se sugerir que uma vez ultrapassada a barreira inicial para utilização de plantas hospedeiras no gênero *Aristolochia* se tornou relativamente mais fácil para as borboletas Troidini se alimentar de todas as outras espécies de *Aristolochia* disponíveis. Espécies estritamente monófagas, como *Parides ascanius* e *P. panthonus jaguarae* podem apresentar este hábito unicamente devido à sua distribuição geográfica restrita, a primeira em áreas alagadas na planície costeira do Rio de Janeiro e a segunda em matas de galeria no centro de Minas Gerais.

Considerações finais

- Uma possível hipótese para o surgimento da interação Troidini/*Aristolochia* seria a colonização dessas espécies de plantas por estas borboletas. A partir daí, novas espécies de plantas podem ter sido adotadas como hospedeiros devido à sua disponibilidade, e estas novas interações podem ser mantidas principalmente por restrições evolutivas.

- Plantas e insetos herbívoros podem estar sofrendo pressões seletivas constantes, e é esperado que interações estejam se renovando no mesmo ritmo, em um processo dinâmico.

- Talvez o acúmulo de dados sobre a utilização de plantas hospedeiras por insetos herbívoros resulte em uma figura mais caótica para estas interações, pelo menos quando considerada cada espécie de inseto como um todo.

- Interações espécie-específica entre insetos e plantas hospedeiras podem estar ocorrendo em populações isoladas, levando a associações altamente especializadas regionalmente. Embora uma lista de plantas hospedeiras de uma espécie de inseto ao longo da sua distribuição geográfica possa ser longa, somente uma ou poucas espécies de plantas hospedeiras pode estar localmente presente. Desta forma, dentro de qualquer hábitat, um herbívoro pode ser mais especializado do que a lista de suas plantas hospedeiras pode indicar (Fox e Morrow, 1981).

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Apêndice

Appendix 1. Table of distribution of Troidini butterflies from SE Brazil.

Battus crassus			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Casa Branca			Angra dos Reis
Ilha Solteira			Petrópolis
Itapetininga			Rio de Janeiro
Itu			Teresópolis
Jundiaí			Tinguá
Ribeirão Bonito			Xerém
São Sebastião			
Teodoro Sampaio			
Uberlândia			
Battus polydamas			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Assis	Linhares	Alto Rio Doce	Guapimirim
Atibaia	Santa Teresa	Barbacena	Itatiaia
Araras		Belo Horizonte	Magé
Bragança Paulista		Extrema	Passa Três
Cajuru		Juiz de Fora	Petrópolis
Campinas		Rio Preto	Rio de Janeiro
Cananéia		Sabará	Teresópolis
Caraguatatuba		São João da Mata	
Casa Branca		Sta. Barbara	
Cosmópolis		Uberlândia	
Cotia			
Ilha Solteira			
Ilhabela			
Ithanhaém			
Itapetininga			
Itapira			
Joanópolis			
Jundiaí			
Mirassol			
Mogi das Cruzes			
Mogi Guaçú			
Monte Mor			
Paraibuna			
Paranapiacaba			
Pedregulho			
Pindamonhangaba			
Piracicaba			
Poloni			
Porto Ferreira			
Ribeirão Bonito			
Ribeirão do Sul			
Rio Claro			
Santos			
São Bernardo do Campo			
São José do Rio Pardo			
São Paulo			
São Roque			
São Vicente			
Souzas			
Sumaré			
Teodoro Sampaio			
Ubatuba			
Valinhos			
Battus polystictus			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Águas da Prata	Santa Teresa	Extrema	Itatiaia
Atibaia		Poços de Caldas	Petrópolis

Cotia		São Lourenço	
Ilhabela			
Itapetininga			
Joanópolis			
Jundiaí			
Mairiporã			
Mogi das Cruzes			
Paranapiacaba			
Pindamonhangaba			
Porto Ferreira			
Ribeirão Bonito			
Rio de Janeiro			
São José do Rio Preto			
São Paulo			
Souzas			
Teresópolis			
Valinhos			

Parides agavus

São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Atibaia	Vila Velha	Belo Horizonte	Barra de São João
Araras	Santa Teresa	Extrema	Niteró
Cajuru		Fervedouro	Petrópolis
Campinas		Itaúna	Rio de Janeiro
Cananéia		Juiz de Fora	Teresópolis
Casa Branca		Mar de Espanha	
Cordeirópolis		Mariana	
Cotia		Passos	
Ilhabela		Poços de Caldas	
Itapetininga		Sete Lagoas	
Itapira			
Itu			
Jacareí			
Jundiaí			
Monte Mor			
Paraibuna			
Porto Ferreira			
Ribeirão Bonito			
Santos			
São José do Rio Pardo			
São Paulo			
São Vicente			
Sorocaba			
Souzas			
Sumaré			
Teodoro Sampaio			
Ubatuba			
Valinhos			

Parides anchises

São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Amparo	Linhares	Barbacena	Angra dos Reis
Atibaia	Santa Teresa	Belo Horizonte	Imbariê
Avanhandava		Cambuquira	Itatiaia
Botucatu		Dionísio	Magé
Caçapava		Mar de Espanha	Nova Iguaçu
Campinas		Paraopeba	Passa Três
Casa Branca		Poços de Caldas	Rio de Janeiro
Cotia		Sete Lagoas	Rocha Leão
Ilha Solteira			Santa Cruz
Ilhabela			Xerém
Iporanga			
Itapetininga			
Mogi das Cruzes			
Mongaguá			
Paraibuna			
Pedregulho			
Pindamonhangaba			
Porto Ferreira			
Ribeirão Bonito			
Santos			
São José do Rio Pardo			
São Paulo			

São Sebastião
São Vicente
Souzas
Sumaré
Taubaté
Ubatuba
Valinhos

Parides ascanius

São Paulo	Espírito Santo	Minas Gerais	Rio de Janeiro
	Itapemirim		Angra dos Reis
			Araruama
			Barra de São João
			Barreira
			Cabiúnas
			Iguaba
			Itaguaí
			Rio das Ostras
			Rio de Janeiro
			Santa Cruz
			Saquarema
			Seropédica

Parides bunichus

São Paulo	Espírito Santo	Minas Gerais	Rio de Janeiro
Amparo		Alto Rio Doce	Petrópolis
Araras		Barbacena	Teresópolis
Atibaia		Belo Horizonte	
Bauru		Juiz de Fora	
Campinas		Lagoa Grande	
Campos do Jordão		Mar de Espanha	
Casa Branca		Nova Lima	
Cordeirópolis		Paraopeba	
Cotia		Passa Quatro	
Guaratinguetá		Poços de Caldas	
Itapira		Pouso Alegre	
Itatiba		Sabará	
Itatinga		São João Del Rei	
Itu		Sete Lagoas	
Jacareí		Uberlândia	
Jundiaí		Virgínia	
Mauá			
Mogi Guaçú			
Monte Alegre do Sul			
Monte Mor			
Piracicaba			
Rancharia			
Rio Claro			
Sabaúna			
São Bernardo do Campo			
São Carlos			
São Paulo			
São José do Rio Pardo			
Serra Negra			
Souzas			
Valinhos			

Parides neophilus

São Paulo	Espírito Santo	Minas Gerais	Rio de Janeiro
Atibaia		Belo Horizonte	
Cajuru		Paraopeba	
Campinas		Peti	
Eng. Coelho		Santa Bárbara	
Ilha Solteira		Uberlândia	
Itapetininga			
Joanópolis			
Matão			
Mogi Guaçú			
Monte Mor			
Patrocínio Paulista			
Pedregulho			
Porto Ferreira			
Presidente Epitácio			
Rio Claro			

Teodoro Sampaio

Parides panthonus jaguarae			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
		Belo Horizonte	

Parides proneus			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Amparo	Santa Leopoldina	Barbacena	Miguel Pereira
Apiaí		Belo Horizonte	Nova Friburgo
Araras		Cambuquira	Petrópolis
Atibaia		Campo Belo	Rio de Janeiro
Avaré		Carmo da Cachoeira	Teresópolis
Bauru		Caxambú	
Bertioga		Mar de Espanha	
Botucatu		Passa Quatro	
Cajuru		Peti	
Campinas		Poços de Caldas	
Casa Branca		Rio Preto	
Cotia			
Extrema			
Guaratingueta			
Ibitinga			
Itapira			
Itatiba			
Joanópolis			
Jundiaí			
Maracaí			
Mauá			
Mogi das Cruzes			
Piracicaba			
Porto Ferreira			
Rio Claro			
Salesópolis			
São Bernardo do Campo			
São Carlos			
São José do Rio Pardo			
São Paulo			
São Sebastião			
Souzas			
Sumaré			

Parides tros			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Cotia	Santa Tereza	Jordânia	Angra dos Reis
Paranapiacaba			Guapimirim
Picinguaba			Itatiaia
Ribeirão Bonito			Magé
Salesópolis			Petrópolis
Sumaré			Rio de Janeiro
Ubatuba			Teresópolis
			Três Rios
			Xerém

Parides zacynthus			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Cananéia	Anchieta		Manguaratiba
Iporanga	Sooretama		Rio das Ostras
Itanhaém	Linhares		Rio de Janeiro
Picinguaba			
Santos			
Ubatuba			

Appendix 2. Table of distribution of *Aristolochia* from SE Brazil.

Aristolochia arcuata			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Amparo	Caparaó	Baldin	Magdalena
Araraquara		Belo Horizonte	Rio de Janeiro
Atibaia		Caeté	
Botucatu		Jequeri	
Cabreúva		Lagoa Santa	
Campinas		Mauá	
Igaratá		Viçosa	
Itirapina			
Jequeri			
Jundiaí			
Limeira			
Mogi Mirim			
Piracicaba			
São Carlos			
São José da Boa Vista			
São José do Rio Pardo			
São Paulo			
Sabaúna			
Socorro			
Sorocaba			
Sumaré			
Votorantim			
Aristolochia chamissonis			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Registro	Cachoeira do Itapemirim	Lagoa Santa	
São Miguel Arcanjo			
Souzas			
São José do Rio Pardo			
São Paulo			
Itapeva			
Itapura			
Aristolochia cymbifera			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Campinas	Anchieta	Belo Horizonte	Bahia de Sepetiba
Mogi das Cruzes	Guaraparí	Carangola	Cabo Frio
Santa Branca	Iconha	Coronel Pacheco	Cachoeira
São José dos Campos	Itapemirim	Lagoa Santa	Freschal
Suzano	Linhares	Mariana	Magé
Taubaté		Ouro Preto	Rio de Janeiro
Aristolochia cynanchifolia			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
			Rio de Janeiro
			Taquari
Aristolochia elegans			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Batatais		Januária	Rio de Janeiro
Campinas			
Jaú			
Piracicaba			
São Paulo			
Sud Mennuci			
Sumaré			
Aristolochia esperanzae			
São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Araraquara		Frutal	
Avanhandava		Ituiutaba	
Batatais			
Botucatu			
Brotas			
Buritizal			
Cajuru			
Casa Branca			
Itapira			

Itirapina
Mogi Guaçú
Mogi Mirim
Piracicaba
Pirassununga
Rancharia
Santa Rita do Passa Quatro
Santo Antônio da Posse
São José do Rio Preto
Suzanópolis
Taquarivaí
Vitoriana

Aristolochia galeata

São Paulo	Espírito Santo	Minas Gerais	Rio de Janeiro
Águas de São Pedro	Guarapari	Betim	Rio de Janeiro
Araçoiaba da Serra		Carandaí	
Atibaia		Diamantina	
Barueri		Douradinho	
Campinas		Entre Rios	
Capivari		Furnas	
Ibiúna		Ibiraci	
Iracemápolis		Lagoa Santa	
Indaiatuba		Manhu-Mirim	
Ipeúna		Medanha	
Iporó		Nova Ponte	
Itaberá		Paraopeba	
Itapetininga		Poços de Caldas	
Itirapina		Três Marias	
Itu			
Jundiaí			
Mogi Guaçú			
Mogi Mirim			
Monte Mor			
Piracicaba			
Pirassununga			
Pinhal			
São Paulo			
Salto de Pirapora			
São Roque			
Sarapui			
Sorocaba			
Sumaré			
Taquarituba			
Tupi			

Aristolochia gigantea

São Paulo	Espírito Santo	Minas Gerais	Rio de Janeiro
Araraquara		Belo Horizonte	Rio de Janeiro
Campinas		Minas Novas	
Januária		Sacramento	
Limeira		Virgem da Lapa	
Matão			
Piracicaba			
Rio Claro			
São Paulo			

Aristolochia macroura

São Paulo	Espírito Santo	Minas Gerais	Rio de Janeiro
Cananéia	Colatina		Atafona
Guarujá	Conceição da Barra		Cabiúnas
Iguape			Rio de Janeiro
Monguaguá			Seropédica
Pariquera-Açú			
Pincinguaba			
Registro			

Aristolochia melastoma

São Paulo	Espírito Santo	Minas Gerais	Rio de Janeiro
Águas de São Pedro		Belo Horizonte	Rio de Janeiro
Campinas		Caeté	
Itararé		Carandaí	
Itatinga		João Monlevade	
Itirapina		Lagoa Santa	

Jaguariúna		Monte Belo	
Monte Alegre do Sul		Poços de Caldas	
Piracicaba		Pedro Leopoldo	
Rio Claro		Viçosa	
São Carlos			
São Paulo			
Sumaré			
Tatuí			
Valinhos			

Aristolochia paulistana

São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Cubatão	Norte Rio Doce		Angra dos Reis
Guarujá			Teresópolis
Iguape			
Jacareí			
Mamparra			
Pariquera-Açú			
Piracicaba			
Santo André			
São Miguel Arcanjo			
São Paulo			
Sete Barras			
Sorocaba			

Aristolochia triangularis

São Paulo	**Espírito Santo**	**Minas Gerais**	**Rio de Janeiro**
Angatuba		Lavras	Rio de Janeiro
Atibaia		Poços de Caldas	
Batatais			
Bertioga			
Botucatu			
Buri			
Cabreúva			
Cubatão			
Guarujá			
Ipeúna			
Marília			
Monguaguá			
Pilar do Sul			
Piracaia			
Piracicaba			
Santa Rita do Passa Quatro			
Santos			
São Paulo			
São Pedro			
São Vicente			
Tietê			
Timburi			