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FILOGENIA MOLECULAR, TAXAS EVOLUTIVAS, TEMPO DE DIVERGÊNCIA E HERANÇA ORGANELAR EM *Passiflora* L. (PASSIFLORACEAE)

Valéria Cunha Muschner

Orientador: Francisco Mauro Salzano
Co-Orientadora: Loreta Brandão de Freitas

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SUMÁRIO

RESUMO

Passiflora é um gênero neotropical que apresenta uma grande variabilidade floral e foliar, o que dificulta enormemente sua classificação taxonômica. A característica mais marcante do gênero é a corona de filamentos em suas flores. Para melhor entender a taxonomia de *Passiflora*, foram analisadas sete regiões de seu DNA, englobando os genomas plastidial, mitocondrial e nuclear de 104 espécies. Essas espécies incluem 19 dos 23 subgêneros da classificação morfológica antiga e todos os quatro subgêneros da classificação mais recente, também baseada em caracteres externos. Os resultados corroboraram a proposta mais recente, que divide o gênero em quatro subgêneros (*Astrophea*, *Decaloba*, *Deidamioides* e *Passiflora*), com a adição de mais um, *Tryphostemmatoides*. Os três subgêneros com o número de espécies mais representativo (*Astrophea*, *Decaloba* e *Passiflora*) formam grupos monofiléticos estatisticamente bem fundamentados. No entanto, *Deidamioides* não é monofilético em todas as análises e marcadores, enquanto que *Tryphostemmatoides* é representado por apenas uma espécie. Foram também estudadas as prováveis datas de surgimento de *Passiflora* e sua diversificação nos três principais subgêneros, através do estudo de quatro regiões do DNA, englobando também os três genomas, em 70 espécies. Verificou-se que o gênero *Passiflora* deve ter aparecido há cerca de 42 milhões de anos atrás (Ma); *Decaloba* parece ter sido o primeiro subgênero a se estabelecer (35 Ma), enquanto que os subgêneros *Astrophea* e *Passiflora* devem ter se diversificado há 24 Ma. Estes dois subgêneros parecem ter tido uma radiação rápida em comparação a *Decaloba*, que possui árvores filogenéticas com comprimentos dos ramos significativamente maiores que os outros dois. As adaptações morfológicas a diferentes polinizadores devem ter sido as principais responsáveis pela radiação rápida. A herança organelar de dois subgêneros, *Decaloba* e *Passiflora*, foi investigada através de um e quatro híbridos interespecíficos, respectivamente. A herança

foi estritamente materna para a mitocôndria e o cloroplasto em *Decaloba*, enquanto que no subgênero *Passiflora* ocorreu herança materna para o DNA mitocondrial e paterna para o DNA plastidial. Esses resultados reforçam os dados obtidos pela filogenia, no que se refere à diferenciação e diversificação dos subgêneros, pois há evidências de que a herança materna dos cloroplastos seja ancestral em plantas.

ABSTRACT

Passiflora is a neotropical genus which presents a high floral and foliar variability, making its taxonomic classification very difficult. The most marked characteristic of the genus is the corona of filaments in its flowers. To better understand *Passiflora*'s taxonomy seven regions of its DNA, including the plastid, mitochondrial, and nuclear genomes, were studied in 104 species. These species include 19 of the 23 subgenera of the old morphological classification and all the four subgenera of the most recent classification. The results confirmed the most recent proposal, which divides the genus in four subgenera (*Astrophea*, *Decaloba*, *Deidamioides* and *Passiflora*) with one more addition, *Tryphostemmatoides*. The three subgenera with the most representative number of species (*Astrophea*, *Decaloba* and *Passiflora*) form statistically well supported monophyletic groups. But *Deidamioides* is not monophyletic in all analyses and markers, while *Tryphostemmatoides* is represented by just one species. Estimations were also made of the date of *Passiflora* emergence and its diversification in the three main subgenera by the study of four DNA regions, also including the three genomes, in 70 species. The *Passiflora* genus should have appeared at about 42 million years ago (Ma); *Decaloba* was the first established subgenus (35 Ma) while the *Astrophea* and *Passiflora* subgenera should have diversified at 24 Ma. These two latter subgenera seem to have had a fast radiation in comparison to *Decaloba*, which presents significantly higher branch lengths in the phylogenetic trees as compared to the two others. Morphological adaptations to different pollinator agents could be the main responsible for this fast radiation. The organelle inheritance of two subgenera, *Decaloba* and *Passiflora*, was investigated through the study of respectively one and four interspecific hybrids. The inheritance was strictly maternal for the mitochondria and chloroplast in *Decaloba*, while in the *Passiflora* subgenus maternal inheritance for the mitochondrial DNA but paternal for chloroplast DNA occurred. These

results strengthen the phylogenetic data which differentiated the subgenera, as well as the subgenera divergence, since there is evidence that chloroplast maternal inheritance is ancestral in plants.

CAPÍTULO I

INTRODUÇÃO

I.1. A Família Passifloraceae

A família Passifloraceae ocorre, predominantemente, em áreas tropicais e subtropicais, possuindo cerca de 17 gêneros e 650 espécies, que se distribuem nas Tribos Paropsieae e Passifloreae. Essas espécies são trepadeiras ou lianas com gavinhas axilares (inflorescências modificadas), ocasionalmente podem ser arbustos ou até árvores e, nestes casos, as gavinhas estão ausentes (Judd *et al.* 1999). Essas plantas possuem uma grande variabilidade foliar e floral. A característica mais marcante da família é a presença de uma corona filamentosa em suas flores que, segundo Judd *et al.* (1999) apoiaria a monofilia de Passifloraceae. Para esses autores, a tribo Paropsieae, que contém árvores e arbustos que perderam as gavinhas, provavelmente representa um complexo basal parafilético dentro da família, enquanto que Passiflorieae é monofilética, como evidenciado pelo hábito escandente, gavinhas axilares e flores especializadas. No Novo Mundo a família é representada por cinco gêneros (*Passiflora*, *Mitostemma*, *Dilkea*, *Ancistrothyrsus* e *Tetrastylis*, embora esse último já tenha sido incluído ao gênero *Passiflora* por Feuillet & MacDougal, 2003 e Muschner *et al.*, 2003), todos da tribo Passifloreae. As famílias Malesherbiaceae e Turneraceae foram incluídas em Passifloraceae pela APG II (2003), por possuírem glicosídios cianogênicos. Além disso, Turneraceae e Passifloraceae têm glândulas foliares e transmissão biparental ou paterna dos cloroplastos (Shore *et al.* 1994; Ulmer & MacDougal 2004; V. C. Muschner *et al.* em preparação) e Malesherbiaceae e Passifloraceae apresentam a corona de filamentos.

I.2. O Gênero *Passiflora* L.

O nome do gênero, passiflora, origina-se do termo "flor da paixão ou *passionflower*", atribuído por Cieza de León em 1553, numa alusão à crucificação de Cristo. O nome do gênero foi adotado por Carl von Linné, em 1753, no *Species Plantarum* (Ulmer & MacDougal 2004).

O gênero *Passiflora* L. é o mais representativo da família Passifloraceae possuindo cerca de 525 espécies. Encontra-se distribuído em regiões tropicais do Novo Mundo, raramente na Ásia e Austrália. As características do gênero são: presença de corona de filamentos e cinco estames e órgão sexuais elevados em uma coluna conspícua, o androginóforo. Outra característica peculiar é a grande variabilidade foliar encontrada nesse gênero, que segundo MacDougal (1994) é a maior encontrada entre todas as angiospermas. Suas flores também são muito variáveis em tamanho e cor, com a corona e o perianto diversamente orientados e desenvolvidos, sendo que todas essas características devem ter surgido de um processo co-evolutivo com os agentes polinizadores (MacDougal 1994). A reprodução funciona predominantemente por fecundação cruzada. A autofecundação é rara e, quando ocorre, forma frutos menores e com poucas sementes (Semir & Brown 1975). Várias classes de animais atuam como polinizadores das diferentes espécies do gênero, mas em geral a melitofilia (abelhas) é a síndrome floral predominante; também ocorrem, em um número menor de espécies, as síndromes da ornitofilia (beija-flores), quiropterofilia (morcegos) e esfingofilia (mariposas) (Semir & Brown 1975; Koschnitzke 1993; Koschnitzke & Sazima 1997; Varassin & Silva 1999; Varassin *et al*. 2001). A dispersão das sementes é freqüentemente feita por aves e morcegos, que são atraídos pela coloração e pelo cheiro dos frutos maduros (Semir & Brown 1975), embora

pequenos mamíferos já tenham sido observados alimentando-se dos frutos de algumas passifloras (Williams *et al.* 2000; Koehler-Santos *et al.* in press).

Devido à grande variabilidade floral e foliar encontrada em *Passiflora*, a classificação taxonômica é bastante complexa.

I.3. A Classificação Taxonômica

A taxonomia de *Passiflora* está baseada em diversos caracteres florais e vegetativos, levando a uma complexa subdivisão taxonômica em subgêneros, seções e séries. De acordo com Killip (1938), o gênero poderia ser subdividido em 22 subgêneros e dez seções, mas Escobar (1989) sugeriu mudanças nessa classificação, descrevendo um novo subgênero. Ambas as classificações foram baseadas somente em características morfológicas, especialmente de estrutura floral.

Mais recentemente, Feuillet & MacDougal (2003) agruparam todas as espécies de *Passiflora* em quatro categorias principais ou subgêneros: *Astrophea*, *Decaloba*, *Deidamioides* e *Passiflora*. A classificação desses autores também é baseada, exclusivamente, em características morfológicas.

Nosso grupo já vem estudando este gênero há alguns anos quanto a aspectos moleculares, tanto no que se refere à delimitação taxonômica (Muschner *et al.* 2003) quanto aos processos de especiação (Lorenz-Lemke *et al.* 2005). Muschner *et al.* (2003), baseados na análise de duas seqüências de DNA não codificadoras (ITS do rDNA nuclear e espaçador intergênico *trn*L-*trn*F do cloroplasto) e uma codificadora (*rps*4 do cloroplasto), sugeriram a redistribuição dos 12 subgêneros estudados (segundo a classificação de Killip 1938) para apenas três, devido à formação de três clados com altos valores de suporte

estatístico (*bootstraps* > 99). Interessantemente, foram evidenciados diferentes tamanhos de ramos entre os três clados. O tempo de geração de cada espécie pode estar envolvido na diferença encontrada entre dois dos clados (Passiflora e Decaloba). Apesar da pouca informação sobre fatores ecológicos que possam estar envolvidos nesse processo, Benson *et al.* (1975) foram os primeiros a afirmar que espécies do subgênero *Decaloba* de Killip (pertencente ao clado Decaloba de Muschner *et al.* 2003) possuem tempo de geração mais curto que espécies do subgênero *Passiflora* (componente do clado Passiflora de Muschner *et al.* 2003). É possível que este fator possa estar acelerando a taxa evolutiva nas espécies do subgênero *Decaloba*. Os resultados de Muschner *et al.*. (2003) corroboram perfeitamente a nova classificação proposta por Feuillet & MacDougal (2003), podendo os clados observados serem nomeados como subgêneros.

O subgênero *Astrophea* é, indubitavelmente, o mais diferenciado dentro do gênero *Passiflora*, pois algumas dessas espécies não se parecem muito com as espécies do gênero à primeira vista. São 57 espécies de árvores, arbustos ou lianas arbustivas, com folhas não-lobadas, sendo a maioria nativa do norte da América do Sul. Os arbustos e, principalmente, as árvores, nos quais foram perdidas as gavinhas, diferem consideravelmente das outras espécies trepadeiras devido ao hábito e presença de crescimento secundário do lenho. A grande árvore *P. macrophylla*, por exemplo, produz as maiores folhas do gênero, podendo atingir até 95 cm de comprimento. Esse subgênero é dividido por Feuillet & MacDougal (2003) em duas superseções e cinco seções. As espécies da seção *Astrophea* são arbóreas ou arbustivas e possuem flores com tubo floral curto e coloração branca, o que forma um contraste com a corona amarelada. Presume-se que essas espécies sejam polinizadas por grandes abelhas, mas a literatura carece de estudos sobre este tema. Já a seção *Boryastrophea* tem espécies que são predominantemente lianas e apresentam flores

alaranjadas ou purpúreas, com um tubo floral conspícuo, mais longo que as sépalas, e uma corona de filamentos reduzida. A ecologia floral destas espécies sugere que elas sejam polinizadas por beija-flores.

O subgênero *Decaloba* inclui mais de 200 espécies, geralmente pequenas trepadeiras, com a maioria das espécies possuindo flores pequenas e folhas variegadas ou bi-lobadas. A grande maioria das espécies é polinizada por abelhas ou vespas, mas existe um número delas adaptadas à polinização por beija-flores (especialmente aquelas com flores avermelhadas e androginóforo longo como, por exemplo, *P. murucuja*), além de uma comprovadamente polinizada por morcegos noturnos (*P. penduliflora*). A maioria das espécies possui flores brancas ou esverdeadas; no entanto, a palheta de cores florais é quase completa neste subgênero. Atualmente as espécies estão distribuídas em oito superseções, sendo a superseção *Decaloba* a maior delas com cerca de 120 espécies. Nesta superseção foram incluídos os subgêneros *Murucuja*, *Pseudomurucuja*, *Psilanthus* e *Astephia* de Killip (1938), além das espécies de *Decaloba*. As espécies do subgênero *Adopogyne* de Killip (1938) foram incluídas na superseção *Multiflora*.

O subgênero *Passiflora* compreende cerca de 240 espécies caracterizadas por flores grandes, que geralmente têm uma corona com faixas de diversas cores. A corona é também a plataforma para as abelhas e outros insetos que são atraídos pelo odor destas flores. Esse subgênero contém também muitas espécies com a corona de filamentos reduzida e que são polinizadas por beija-flores. A maioria delas é classificada na superseção *Tacsonia*. As superseções *Distephana* e *Coccinea* também apresentam espécies polinizadas por beija-flores. É neste subgênero que podem ser encontradas plantas de importância econômica, tais como *P. edulis*, *P. ligularis* e *P. tarminiana*. Com exceção de *P. edulis* f. *edulis*, *P. tarminiana* e *P. foetida*, quase todas as espécies do subgênero *Passiflora* são auto-

incompatíveis e requerem polinização cruzada. Os subgêneros *Adenosepala*, *Calopathanthus*, *Distephana*, *Dysosmia*, *Dysosmioides*, *Granadilla*, *Granadillastrum*, *Manicata*, *Rathea*, *Tacsonia*, *Tacsonioides* e *Tacsoniopsis* de Killip (1938) e Escobar (1989) foram agregados ao subgênero *Passiflora*, que se encontra dividido em seis superseções.

O subgênero *Deidamioides* é o menor de todos, contendo apenas 13 espécies relativamente primitivas. Esse estado primitivo é confirmado pelo surgimento das flores diretamente a partir das gavinhas, um fenômeno raro no gênero *Passiflora*. *Deidamioides* é subdividido em cinco seções, duas das quais são monoespecíficas. O gênero *Tetrastylis* foi incluído neste subgênero dentro da seção *Tetrastylis* e as duas espécies que o compõem passaram a ser denominadas de *P. ovalis* e *P. contracta*. Neste subgênero foram incluídos os subgêneros *Deidamioides*, *Tryphostemmatoides* e *Polyanthea* de Killip (1938).

I.4. A Origem das Angiospermas

Segundo Charles Darwin a origem das angiospermas seria "um abominável mistério", e ainda hoje esta questão permanece como um problema altamente controverso. Acredita-se que a radiação das angiospermas tenha ocorrido há cerca de 115 milhões de anos (Ma) atrás, aproximadamente na metade do Cretáceo, tendo o grupo dominado a flora terrestre há cerca de 90 Ma, situação que continua até os dias de hoje (Lidgard & Crane, 1988).

As relações entre as linhagens de angiospermas têm sido uma questão de difícil resolução, pois o enraizamento do clado, usando dados morfológicos, é problemático e o registro fóssil é insuficiente (Crane *et al.* 1995). Durante as duas últimas décadas, têm sido

alcançados progressos significativos no sentido da resolução deste problema. Análises filogenéticas combinando caracteres morfológicos e seqüências de DNA têm procurado resolver as relações entre as linhagens (Donoghue & Doyle 1989; Chase *et al.* 1993, 2000; Doyle *et al.* 1994; Soltis 1997; Nandi *et al.* 1998; Qiu *et al.* 1999; Soltis *et al.* 1999a, 2000; Savolainen *et al.* 2000a, b; Wikström *et al.* 2001; APG II 2003; Hilu *et al.* 2003), e têm sido observados padrões altamente congruentes com relação ao enraizamento do clado (Mathews & Donoghue 1999; Qiu *et al.* 1999; Soltis *et al.* 1999a; Sanderson 2003a; Davis *et al.* 2004; Bell *et al.* 2005). Estes achados têm renovado o interesse e o foco para o registro fóssil, particularmente aqueles dos depósitos do Cretáceo.

Davies *et al.* (2004) usaram a abordagem metodológica de *supertrees* para reconstruir uma árvore datada das angiospermas a partir de dados moleculares. Eles concluíram que suas análises indicam um padrão ligeiramente lábil da taxa de diversificação, e corroboram a suspeita de Darwin de que não existe uma explicação simples para o mistério da diversificação desse grupo de plantas. A calibragem da diversificação utilizada para as maiores linhagens não mostrou uma radiação rápida recente das basais. Houve, porém, numerosas mudanças nas taxas de diversificação com aumentos grandes nas taxas evolutivas em períodos recentes. A diversificação não seria dirigida por poucas grandes inovações-chave, mas por um processo mais complexo no qual haveria instabilidade com "vencedores" e "perdedores" em todos os níveis e mudanças repetidas.

Os registros fósseis mais antigos das angiospermas são os de pólen do período Valanginiano-Hauterivano, 141-132 Ma antes do presente, AP (Brenner & Bickoff 1992; Huge 1994; Brenner 1996). O depósito do Aptiano-Albiano (125-97 Ma AP) de Portugal, que contém rápida expansão de diversidade morfológica em flores, sementes e pólen, também foi bastante estudado (Friis *et al.* 1999). Outros estudos, referentes a depósitos do

Cenomaniano-Campaniano, têm indicado idades mais recentes que variam de 97-74 Ma AP (Basinger & Dilcher 1984; Herendeen *et al.* 1999). A discussão sobre a idade das angiospermas é ampla e longa, e até que sejam encontrados registros fósseis mais completos, não será esgotada com facilidade. Uma alternativa que pode ajudar na estimativa do tempo de divergência deste grupo é a utilização de seqüências de DNA.

Wikström *et al.* (2001), baseando-se no seqüenciamento do DNA de três regiões codificadoras (genes *rbc*L e *atp*B do DNA plastidial e *18S* do DNA ribossomal nuclear), estimaram que as angiospermas teriam surgido entre o final e a metade do Jurássico (179-158 Ma AP) e que as eudicotiledôneas teriam surgido do início do Jurássico até a metade do Cretáceo (147-131 Ma AP). Estas estimativas são mais antigas que as obtidas com os registros fósseis, mas estão sendo bem aceitas por diversos pesquisadores em tentativas de datar a origem de diversos grupos de plantas (Sanderson 2003a; Davies *et al.* 2004; Bell *et al.* 2005). No presente estudo estaremos aceitando a proposição de que o ramo que deu origem à família Passifloraceae teria surgido há 36-32 Ma AP (Wikström *et al.* 2001).

I.5. Taxas de Substituição Nucleotídica em Plantas

I.5.1. Cloroplasto

O genoma plastidial (cpDNA) é uma molécula com aproximadamente 150 mil pares de bases (kbp), que codifica cerca de 100 funções genéticas (Clegg & Zurawski 1991) e sua organização molecular e evolução têm sido amplamente estudadas. Estudos de mapeamento gênico dessa organela em algas e plantas terrestres confirmaram a impressão de forte conservação de seu conteúdo gênico (Clegg *et al.* 1994; Martin *et al.* 2005). Entre

as plantas terrestres o conteúdo gênico é quase totalmente conservado, embora tenham sido demonstradas transferências de função do genoma plastidial para o nuclear (Downie & Palmer 1991; Huang *et al.* 2005).

O conteúdo gênico conservado e a taxa de substituição nucleotídica relativamente baixa em genes codificadores de proteínas, têm tornado o genoma plastidial ideal para estudos evolutivos em plantas (Clegg 1993; Martin *et al.* 2005). Durante os últimos dez a quinze anos, houve uma explosão de publicações sobre filogenias moleculares construídas a partir do cpDNA (p. ex. Chase *et al.* 1993; Soltis *et al.* 2000; Stefanović & Olmstead 2004; Salamin *et al.* 2005; Young & dePamphilis 2005).

O interesse por filogenias precisas supera o âmbito puramente descritivo. A origem das adaptações morfológicas pode ser colocada em um contexto filogenético para reconstruir a seqüência precisa das mudanças genéticas (e moleculares) que originaram novas estruturas (p. ex., Clegg *et al.* 1994) ou os genes de desenvolvimento são usados como marcadores filogenéticos para explicar a diversidade na estrutura floral, que ainda é o principal elemento de identificação taxonômica (Becker & Theiβen 2003; Kaufmann *et al.* 2005).

Estudos têm demonstrado, por outro lado, que alguns genes do cloroplasto violam a constância do relógio molecular, porque as taxas evolutivas variam entre as linhagens mais abrangentes de plantas (Bousquet *et al.* 1992; Gaut *et al.* 1992). No entanto, tem sido sugerido que a maioria dessas variações seria devida a diferenças no tempo de geração (Gaut *et al.* 1992; Doyle & Gaut 2000). Segundo esses últimos autores, tanto o relógio molecular calibrado pelo tempo, quanto o calibrado pelo tempo de geração são consistentes com a Teoria da Neutralidade.

A grande maioria das análises filogenéticas envolvendo grandes grupos taxonômicos em níveis acima de famílias envolvem o gene *rbcL*, codificador da subunidade maior da rubisco (Soltis *et al.* 2000; APG II 2003; Salamin *et al.* 2005). O gene *rps4*, codificador da proteína 4 da subunidade menor do ribossomo plastidial, tem sido pouco utilizado em estudos sobre a filogenia de plantas (Nadot *et al*. 1994; Souza-Chies *et al.* 1997; Soltis *et al.* 2002; Rydin *et al.* 2004). No entanto, o estudo realizado por Muschner *et al.* (2003), com 35 espécies do gênero *Passiflora*, demonstrou que essa região tem grande potencial para desvendar as relações filogenéticas dentro deste gênero.

Regiões não-codificadoras do cpDNA têm sido amplamente utilizadas nas análises filogenéticas em níveis taxonômicos intra e interespecíficos (Mes *et al.* 2000; Muschner *et al.* 2003), devido às taxas de substituição nucleotídica elevadas. Dentre as regiões plastidiais não-codificadoras melhor estudadas nas análises filogenéticas de angiospermas encontram-se o espaçador intergênico *trnL-trnF* e o intron do gene *trnL* (Mes *et al.* 2000; Holt *et al.* 2004; Lledó *et al.* 2005). Em 1991, Taberlet *et al.* publicaram *primers* universais para essas regiões, os quais vêm sendo utilizados numa extensa gama de espécies com excelentes resultados (Chen *et al.* 2005; Wang *et al.* 2005).

Por outro lado, as seqüências não-codificadoras, algumas vezes, contêm mais inserções/deleções (indels) que substituições (Golenberg *et al.* 1993), as quais podem dificultar o alinhamento das seqüências e a determinação das homologias (Kelchner 2000). Enquanto alguns autores argumentam que os indels não devem ser tratados como caracteres informativos (Golenberg *et al.* 1993), outros alegam que eles contêm informação filogenética importante e que, por esse motivo, devem ser incluídos nas análises (p.ex. Kelchner 2000).

I.5.2. Genes Nucleares

Um gene nuclear amplamente utilizado para a filogenia das angiospermas é o *18S* rDNA (p.ex. Chaw *et al.* 2000; Soltis *et al.* 2000). O DNA ribossomal (rDNA) é a região do genoma que codifica os componentes do RNA dos ribossomos. O rDNA eucariótico está organizado em *tandem*, com milhares de cópias no genoma. Cada unidade de repetição consiste de genes que codificam a subunidade menor (*18S*), a subunidade maior (*26S*) e o rDNA nuclear 5,8S, sendo que essas regiões codificadoras são separadas por espaçadores (Schlötterer 1998). Essas cópias são homogeneizadas por evolução em concerto, que pode ocorrer devido à permuta desigual e/ou conversão gênica (Hamby & Zimmer 1992; Koch *et al.*. 2003).

Nickrent & Soltis (1995) estudaram a taxa evolutiva e a resolução filogenética do gene *18S* rDNA inteiro bem como as do gene *rbcL* de 59 angiospermas. A comparação mostrou que o *rbcL* é cerca de três vezes mais variável que o *18S* rDNA. No entanto, por causa do maior comprimento deste último, a razão do número de sítios filogeneticamente informativos por molécula é somente 1,4 vezes maior para o *rbcL* que para o *18S* rDNA. As análises de parcimônia mostraram que diversos clados foram fortemente apoiados pelos dois genes, levando os autores a concluir que as seqüências do *18S* rDNA inteiras são suficientemente variáveis para o desenvolvimento de estudos filogenéticos no grupo das angiospermas.

Diversos autores têm publicado filogenias robustas das angiospermas a partir do gene *18S* rDNA, por exemplo, Soltis *et al.* (1997), que analisaram 223 espécies, e Soltis *et al.* (2000), que estudaram 560 espécies. Apesar disso, o *18S* rDNA sozinho forneceu poucos caracteres filogeneticamente informativos para resolver adequadamente as relações

entre e dentro dessas plantas (Soltis *et al.* 1999b; Kim *et al.* 2004). Kuzoff *et al.* (1998) demonstraram que a seqüência do gene *26S* rDNA tem um grande potencial para a reconstrução filogenética, em níveis taxonômicos comparáveis aos investigados com o *18S* rDNA. Eles concluíram ainda que o *26S* rDNA evolui de 1,6 a 2,2 vezes mais rápido e tem cerca de 3,3 vezes mais sítios informativos que o *18S* rDNA. Além disso, propuseram que os segmentos de expansão do *26S* rDNA evoluem de 1,2 a 3 vezes mais rápido que o gene *rbcL*, com 1,5 vezes mais sítios informativos. A utilidade filogenética dessa região em angiospermas tem sido demonstrada em diversos estudos (p. ex. Fishbein *et al.* 2001; Zanis *et al.* 2002; Korall & Kenrick 2004; Schönenberger *et al.* 2005).

I.5.3. A Mitocôndria

Wolfe *et al.* (1987) mostraram que as taxas de substituição sinônimas em genes mitocondriais de angiospermas são anormalmente baixas (cerca de 50 a 100 vezes menor) quando comparadas às dos genes mitocondriais de mamíferos. Além disso, demonstraram que as taxas de substituição do mtDNA são algumas vezes menores que às do cloroplasto e 10 a 20 vezes menores que as taxas de substituição dos genomas nucleares. Palmer & Herbon (1988) confirmaram que as taxas de substituição nucleotídica do genoma mitocondrial inteiro são baixas (inclusive em regiões não codificadoras) e mostraram separação entre as taxas de evolução de seqüências e estrutura. Estudos subseqüentes têm confirmado essas taxas de substituição muito baixas (Gaut 1998; Muse 2000). No entanto, variações moderadas nas taxas de substituição sinônimas (maiores que 7 vezes) têm sido encontradas na comparação entre diversos grupos de plantas (Eyre-Walker & Gaut 1997; Laroche & Bousquet 1999; Whittle & Johnston 2002). Na maioria dos casos, a correlação é

feita com genes plastidiais e / ou nucleares. Forças evolutivas que operam nos dois genomas organelares ou nos três genomas, como transmissão dos genomas organelares ou efeito do tempo de geração, respectivamente, são explicações para esses padrões. Estudos filogenéticos, embora desenvolvidos sem um enfoque quantitativo, sugerem que exista uma certa variação entre os diversos grupos de plantas (Beckert *et al.* 1999; Bowe *et al.* 2000; Chaw *et al.* 2000; Barkman *et al.* 2004; Davis *et al.* 2004). Cho *et al.* (2004) encontraram taxas de substituições sinônimas do mtDNA extraordinariamente elevadas e variáveis no gênero *Plantago* (Plantaginaceae). Estes autores tentaram explicar essa alta taxa de variação no gênero e argumentaram que a transferência de genes mitocondriais para o núcleo, os níveis elevados de edição de RNA, o tempo de geração, e mecanismos de mutação e reparo poderiam ser os responsáveis.

Mas o interesse em incluir um terceiro genoma nas análises filogenéticas tem aumentado. O mtDNA tem sido relativamente pouco utilizado na filogenia de plantas devido, também, ao alto grau de recombinação intra-molecular que apresenta na maioria das espécies vegetais estudadas, o que torna difícil o estudo de grandes regiões de seu DNA (Palmer & Herbon 1988). A análise de pequenas regiões do mtDNA pode vir a minimizar este problema. Por esse motivo, regiões intrônicas e espaçadoras têm-se mostrado uma promessa na sistemática molecular de níveis taxonômicos moderadamente mais baixos (Demesure *et al.* 1995; Freudenstein & Chase 2001; Duminil *et al.* 2002; Dombrovska & Qiu 2004). Um dos primeiros destes fragmentos do mtDNA a ser investigado é um intron do gene da NADH desidrogenase. A subunidade I desse gene (*nad1*) tem cinco exons (*a-e*) em *Oenothera*, alguns dos quais estão altamente dispersos e envolvidos no mecanismo de processamento em *trans* (Wissinger *et al.* 1991). O intron entre os exons *b* e *c* é relativamente pequeno (de 1422-1464 pb em *Triticum*, *Citrullus* e

Oenothera). Esse é um intron do grupo II, que está envolvido no mecanismo de processamento em *cis*, cuja estrutura secundária característica facilita seu auto-processamento. Na primeira vez que essa região foi usada em análises filogenéticas, foi identificada pouca variação inter e intra-específica (Demesure *et al.* 1995). No entanto, Chen & Sun (1998) encontraram variação comparável à de outros marcadores plastidiais e nucleares em espécies de *Spiranthes*, e Freudenstein & Chase (2001) observaram ampla variação, principalmente relacionada a eventos de inserção/deleção, dentro da família Orchidaceae. Embora essa não seja uma região codificadora, acredita-se que esteja sujeita a pressão seletiva devido à estrutura de sua alça principal, necessária para o auto-processamento. Outras regiões do mtDNA parecem conter informações filogenéticas muito úteis (Freudenstein & Chase 2001), principalmente relacionadas com eventos de inserção/deleção. A subunidade cinco do gene da NADH desidrogenase também possui cinco exons e introns evolvidos com o processamento em *trans* (Souza *et al.* 1991). Laroche *et al.* (1997) encontraram uma boa variação entre espécies de angiospermas para o intron entre os exons *d* e *e*, e embora até o momento essa região não tenha sido estudada em análises filogenéticas de angiospermas, ela parece promissora, pelo menos para algumas espécies.

I.6. O Relógio Molecular

Até pouco tempo o estudo da evolução dos organismos era baseado somente em registros fósseis e evidências genéticas a nível protéico. Com o advento da biologia molecular e o surgimento de ferramentas poderosas de bioinformática, a facilidade de acessar e analisar seqüências de DNA tem aumentado, tornando possível, portanto, a

comparação entre espécies através de seus genomas. Com essas novas tecnologias foram desenvolvidas filogenias moleculares detalhadas para diversos grupos de plantas, nos mais diferentes níveis taxonômicos.

Uma das pressuposições usadas para a produção de filogenias moleculares é a hipótese do relógio molecular. Essa hipótese propõe que a taxa de mudanças evolutivas em nível de seqüências de DNA é constante em diferentes linhagens. A hipótese ainda é bastante controversa, gerando discussões entre os biólogos evolutivos, especificamente o debate neutralista x selecionista. Os neutralistas tomam o relógio molecular como base para inferências sobre a evolução dos organismos supondo a constância das taxas de mutação neutras (Kimura 1968, 1969, 1983). Já os selecionistas consideram as variações encontradas no relógio molecular como evidência contra essa constância. As taxas de substituições nucleotídicas, ou fixação, em um sítio nucleotídico por ano (k) em uma população diplóide de tamanho 2N, são iguais ao número de novas mutações (neutras, deletérias ou vantajosas) que surgirem por ano (u) multiplicado por sua probabilidade de fixação (f). A expressão matemática para isso é $k = 2N.u.f$ (Li, 1997). Para uma mutação neutra, a probabilidade de fixação é simplesmente o inverso do tamanho populacional (1/2N); então a taxa de substituição de uma mutação deste tipo é $k = (2N).(1/2N).u = u$. Portanto, a sua taxa de substituição depende somente da taxa de mutação e não de outros fatores, como o tamanho populacional.

Kimura (1968) postulou que a maioria das mutações, em nível molecular, seria devida à fixação aleatória das mutações neutras ou quase neutras. Essa hipótese é hoje conhecida como a teoria neutra da evolução molecular (Kimura, 1983). No entanto, durante os anos 70 muitos dados comparativos, principalmente para seqüências protéicas de mamíferos, forneceram exemplos de heterogeneidade no ritmo evolutivo e o debate

sobre a constância do relógio molecular começou. Wu & Li (1985) demonstraram que as taxas evolutivas de roedores são maiores que as de humanos. As variações encontradas entre as taxas de substituições podem ser causadas pelas diferenças no tempo de geração entre as espécies, ou seja, espécies que têm um tempo de geração mais curto teriam taxas de substituição nucleotídica maiores que aquelas que têm um tempo longo de geração. Por exemplo, Gaut *et al.* (1992), através de análises realizadas com o gene *rbc*L do cloroplasto, verificaram que em gramíneas as taxas de substituições nucleotídicas eram cinco vezes maiores que em palmeiras. No entanto, Whittle & Johnston (2003), após a análise de 24 pares de espécies filogeneticamente independentes (cada par contendo uma espécie com história de vida anual e outra perene, ou espécies arbóreas com tempos de geração respectivamente curto e longo), não encontraram qualquer evidência de que o tempo de geração estivesse correlacionado com as taxas evolutivas das regiões nucleares estudadas.

A avaliação precisa do relógio molecular exige que o tamanho dos ramos de uma árvore filogenética sejam proporcionais; para satisfazer esta exigência há, muitas vezes, a remoção de algumas espécies da árvore, o que, em muito casos, impossibilita a datação das divergências, além de fazer uso ineficiente dos dados (Yang & Yoder 2003).

A definição do tamanho do ramo é um dos principais motivos da deficiência dos métodos que utilizam o pressuposto do relógio molecular. Este é interpretado como o produto da taxa de evolução (μ) pelo tempo (T), que representa a quantidade de evolução ocorrida desde o evento da cladogênese. Ao contrário do que é comumente pensado, o tamanho do ramo não representa a taxa evolutiva μ (Yang & Yoder 2003). Para calcular o tamanho de um ramo é necessário que se suponha a constância de um dos fatores acima, para que se calcule o outro. Em uma árvore de máxima verossimilhança ou de distância em que os ramos variam livremente, supõe-se que T é igual a 1, sendo as diferenças

observadas oriundas exclusivamente da variação na taxa μ. O grande problema dos métodos que testam o relógio molecular é que eles não calculam μ e *T* independentemente, ou seja, sem supor a constância das taxas ao longo dos ramos. A decomposição das duas quantidades que definem o tamanho de um determinado ramo não é simples, pois a ausência de informações *a priori* impossibilita tal cálculo. No entanto, é possível obter-se informações em relação aos valores de *T* devido ao registro fóssil, permitindo que se conheça algo com relação aos tempos de divergência entre as espécies.

I.7. O Relaxamento do Relógio Molecular

Sanderson (1997) foi o primeiro a explorar a questão da decomposição do tamanho dos ramos de uma árvore filogenética. Ele propôs um método que estima o tempo de divergência quando as taxas evolutivas variam entre as linhagens, supondo que as mudanças são autocorrelacionadas e que a taxa de mudança é herdada, a partir de uma linhagem ancestral, por seus descendentes imediatos. Através de técnicas de otimização, o método procura pela solução que minimiza as taxas inferidas de mudança.

O programa "r8s", criado a partir do método proposto e descrito acima (Sanderson 2003b), estima as taxas absolutas de evolução molecular e os tempos de divergência. A estimativa dos parâmetros é feita de diversas formas, a partir dos padrões de máxima verossimilhança, em um contexto global, ou postulando um relógio molecular local, para métodos experimentais semiparamétricos ou não paramétricos que relaxam a estringência de sua admissão usando procedimentos homogêneos. O ponto de partida é uma árvore filogenética e a estimativa do comprimento dos ramos (número de substituições ao longo dos ramos). Além disso, podem ser adicionados um ou mais pontos de calibragem para

permitir uma escala das taxas e tempos para unidades reais. Essas calibragens podem ser realizadas de duas maneiras: a escolha de uma idade fixa para um nó da árvore, ou a imposição de uma idade mínima ou máxima em um determinado nó, o que geralmente reflete o conteúdo de informações obtidas a partir das evidências fósseis. Os nós terminais podem ocorrer em qualquer ponto de calibragem, permitindo a investigação da taxa de variação nas filogenias. Finalmente, é possível determinar-se todos os tempos de divergência e avaliar a taxa de variação molecular a partir de diversos modelos de ajustamento (*smoothing*).

O algoritmo de Sanderson (2002) não utiliza distribuições probabilísticas para modelar a evolução das taxas e dos tempos e, portanto, é fundamentalmente não-paramétrico.

Thorne *et al.* (1998), Kishino *et al.* (2001) e Thorne & Kishino (2002) propuseram uma outra solução para o problema da dissociação de tempos e taxas. Esses autores admitiram explicitamente distribuições probabilísticas. O método usa uma abordagem baiesiana para inferir os tempos de divergência e as taxas evolutivas. A idéia básica é adotar distribuições *a priori* dos parâmetros μ e T e, posteriormente, verificar o impacto dos dados (alinhamento das seqüências e pontos de calibragem) nessas distribuições.

Este enfoque tem sido amplamente utilizado no cálculo de tempos de divergência, pois possibilita o uso de múltiplos genes e de modelos evolutivos complexos, ao contrário do método de Sanderson (1997, 2002). Além disso, incorpora a adoção de intervalos temporais para os pontos de calibragem. Simulações feitas pelos autores indicam que o método é robusto e valores *a priori* diferentes dos parâmetros tendem a convergir para uma mesma distribuição posterior (Kishino *et al.* 2001, Thorne & Kishino 2002).

I.8. Padrões de Herança Organelar em Plantas

As plantas possuem três genomas: nuclear, plastidial e mitocondrial. Cloroplastos e mitocôndrias são organelas eucarióticas de origem endossimbiôntica. A maioria das células eucarióticas contém dezenas a centenas de mitocôndrias que geram energia, enquanto as células das plantas contêm dezenas a centenas de cloroplastos que desempenham a fotossíntese como principal função. Mitocôndrias e cloroplastos são herdados de uma maneira não-Mendeliana em todos os organismos estudados (revisões em Birky 1995, 2001). A herança dos genomas citoplasmáticos é freqüentemente materna, mas existem numerosas exceções que resultam em diferentes graus de herança paterna ou biparental do mtDNA ou cpDNA (Koperlainen 2004). Essa variedade de padrões de herança sugere que têm sido adotadas diferentes estratégias entre os diferentes organismos considerados. A perda do padrão universal da herança materna também indica que o sistema não-Mendeliano não é provavelmente uma mera conseqüência da assimetria no tamanho dos gametas. Por causa dos variáveis graus de herança uniparental, segregação durante as divisões mitóticas e meióticas e múltiplas cópias desses genomas em cada célula, processos evolutivos que agem nos genomas do cloroplasto e da mitocôndria diferem daqueles que governam os de genomas nucleares.

A maioria das angiospermas exibe herança materna do cpDNA, mas cerca de um terço dos gêneros investigados mostra algum grau de herança biparental dos cloroplastos (Corriveau & Coleman 1988, Mogensen 1996, Zhang *et al.* 2003). Por outro lado, pouco se sabe sobre a herança do mtDNA em plantas, mas esse parece ser maternalmente herdado (Sodmergen *et al.* 2002; Mohanty *et al.* 2003). Dentre as angiospermas melhor investigadas estão o gênero *Actinidia*, que possui herança estritamente paterna do cpDNA

(Testolin & Cipriani 1997, Chat *et al.* 1999); as espécies *Medicago sativa* e *Turnera ulmifolia*, que exibem herança paterna, materna e biparental (Shore & Triassi 1998); e o gênero *Pelargonium*, que possui um padrão de herança biparental para ambos cpDNA e mtDNA (Guo & Hu 1995). A herança paterna do mtDNA já foi detectada em *Cyclobalanopsis glauca* (Lin *et al.* 2003). Casos de padrões de herança organelar não-usuais têm sido registrados na análise de híbridos interespecíficos ou intragenéricos produzidos artificialmente, como é o caso do gênero *Larrea*, onde a herança do genoma plastidial é paterna (Yang *et al.* 2000). Em cruzamentos entre *Citrus-Poncirus* (Moreira *et al.* 2002), a herança do mtDNA é parcialmente biparental.

Dentre as gimnospermas, as coníferas herdam o cpDNA, exclusiva ou predominantemente do genitor masculino, enquanto que outros grupos, como *Ephedra*, *Ginkgo* e *Zamia*, parecem ter herança materna dos cloroplastos (Mogensen 1996; Koperlainen 2004). Dependendo do grupo de gimnospermas, o mtDNA pode ter herança materna, paterna ou biparental.

Existe uma variedade de mecanismos pelos quais as organelas podem ser ou não transmitidas para a prole. Os mecanismos que resultam na supressão da herança citoplasmática paterna em angiospermas incluem a exclusão ou perda de organelas citoplasmáticas das células germinativas ou espermáticas, a exclusão do citoplasma masculino na fusão gamética e a degradação do DNA organelar dentro da célula germinativa e/ou células espermáticas (Morgensen 1996, Nagata *et al.* 1997). A eliminação materna pode ser ocasionada pela transformação das organelas citoplasmáticas ao longo do ovo/zigoto ou pela sua degeneração antes da fusão gamética (Morgensen 1996, Brums & Owens 2000). No entanto, nenhum desses mecanismos de eliminação é perfeitamente efetivo em todos os casos.

Liu *et al.* (2004) acreditam que o desenvolvimento do controle da herança do genoma dos cloroplastos ocorreu independentemente nas angiospermas, sendo possível que a herança materna tenha se tornado dominante antes do seu surgimento Estes autores pressupõem, ainda, que os mecanismos para a herança dos cloroplastos nessas plantas devam ter se desenvolvido independentemente e mais tardiamente que os de herança da mitocôndria, devido ao fato de que o modo de herança dos plastídios varia consideravelmente, estando associados aos processos de especiação.

O entendimento da diversidade genética é necessário, tanto para o desenvolvimento de estratégias eficientes de conservação, quanto para o delineamento de programas de exploração sustentável da biodiversidade. Análises moleculares são úteis para avaliar a diversidade e filogenia das espécies e para a compreensão dos processos de especiação. A determinação do modo de herança dos plastídios e mitocôndrias em plantas é um passo importante para o uso desses genomas no traçado da história evolutiva das espécies e na compreensão de suas implicações.

CAPÍTULO II

OBJETIVOS

II.1. Geral

O objetivo geral desse trabalho é contribuir para o esclarecimento da história evolutiva do gênero *Passiflora* a partir da utilização de seqüências de DNA e diversos métodos de análise.

II.2. Específicos

1. Analisar as relações filogenéticas dentro do gênero *Passiflora*, principalmente no que diz respeito ao seu agrupamento em subgêneros, e contribuir para o melhor entendimento da taxonomia clássica.
2. Correlacionar os resultados obtidos com características ecológicas e bioquímicas.
3. Datar o surgimento de *Passiflora* e a diversificação de seus principais subgêneros, associando essas datas com eventos biogeográficos.
4. Determinar os modos de herança organelar no gênero e tentar correlacioná-los com a filogenia e a datação de sua diversificação evolutiva.

CAPÍTULO III

1º ARTIGO

A ser submetido para a revista *Systematic Biology*

PHYLOGENETIC RELATIONSHIPS AMONG *Passiflora* (PASSIFLORACEAE) SPECIES: MOLECULAR DATA STRENGTHEN A NEW TAXONOMIC PROPOSAL FOR SUBGENERA

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Phylogenetic relationships among *Passiflora* (Passifloraceae) species: Molecular data strengthen a new taxonomic proposal for subgenera

VALÉRIA C. MUSCHNER[1], ALINE P. LORENZ–LEMKE[1], PAKISA D. TOGNI[1], ARMANDO C. CERVI[2], SANDRO L. BONATTO[3], FRANCISCO M. SALZANO[1], AND LORETA B. FREITAS[1]

[1]*Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Caixa Postal 15053, 91501–970 Porto Alegre, RS, Brazil; E–mail: muschner@ufrgs.br; loreta.freitas@ufrgs.br*

[2]*Departamento de Botânica, Universidade Federal do Paraná, Caixa Postal, 19031, 81531–970, Curitiba, PR, Brazil*

[3]*Centro de Biologia Genômica e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Ipiranga 6681, 90610–001 Porto Alegre, RS, Brazil*

Correspondence: Loreta B. Freitas, Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Caixa Postal 15053, 91501–970 Porto Alegre, RS, Brazil. Phone: 55 51 33166715. Fax: 55 51 33166727. E–mail: loreta.freitas@ufrgs.br

Abstract – *Passiflora* is a genus with more than 500 species, showing large flower complexity and diversity; its habit range from climbing herbaceous shrubs to trees. These characteristics condition taxonomic problems. The genus' first classification divided it into 22 subgenera, and afterwards an additional subgenus was added to them. However, recently a new classification system grouped the 23 subgenera in just four. To further understand and complement a first phylogeny of the genus published by us, 104 species, representing 19 of 23 subgenera of the first classifications, and all four of the most recent proposal were investigated. Seven molecular markers were used: (a) the *rbcL* and *rps4* genes, *trnL* intron, and *trnL-trnF* intergenic spacers of plastid DNA; (b) *nad1* b/c and *nad5* d/e of mitochondrial DNA; and (c) a partial sequence of the *26S* nuclear DNA, totaling about 6,300 base pairs. The monophyly of *Astrophea*, *Decaloba*, and *Passiflora* subgenera was highly supported independently of the phylogenetic analysis employed, but the fourth subgenus (*Deidamioides*), as originally proposed, proved to be polyphyletic. We hereby classified a restriction in the delimitation of *Deidamioides*, and the addition of E. P. Killip's *Tryphostemmatoides* to the subgenera classification, that therefore would be composed by five taxonomic units.

Key Words: *Passiflora*, phylogeny, taxonomic classification, cpDNA, mtDNA, nuclear DNA, combined genetic analysis.

The Passifloraceae family of the Malpighiales order is composed by 19 genera (APG II, 2003), one of its characteristics being the presence of cyclopentenoid cyanogenic glycosides and of a hypanthium-like structure that does not bear the stamens. *Passiflora* is the largest genus of the family with 525 species distributed especially in the tropical region (Vanderplank, 1996; Cervi, 1997; Ulmer and MacDougal, 2004). The majority of these species is herbaceous, but there also shrubs and trees among them. Killip (1938) and MacDougal (1994) asserted that among the Angiosperms no other group presented such a large foliar diversity. In addition, its flowers displayed ample variation in size and color, with the corona and perianth showing diverse orientation and development. Coevolution with insect pollinators has been suggested as an explanation for these features (MacDougal, 1994). Based on morphology only (especially flower structures) Killip (1938) and Escobar (1989) concluded that the genus could be divided into 23 subgenera, with diverse series and sections.

Feuillet and MacDougal (2003), on the other hand, proposed a drastic taxonomic reevaluation of the genus that according to them would consist of only four subgenera (*Astrophea*, *Decaloba*, *Deidamioides* and *Passiflora*). The *Astrophea* subgenus remained unchanged in relation to the previous classification, with 57 species divided in six sections; 214 species were attributed to *Decaloba*, the majority with x = 6 chromosomes, distributed by eight supersections and five sections; 13 species, grouped into five sections, were classified as *Deidamioides*; while *Passiflora* included more than 236 species mainly with x = 9 chromosomes, separated in six supersections, 13 sections, and 11 series. This classification, however, did not include Old World species. Krosnick and Freudenstein (2005) studied species of this region (subgenus *Decaloba*, supersection *Disemma*) confirming the supersection monophyly.

The first *Passiflora* molecular phylogeny, published by Muschner et al. (2003), included 11 of the previously suggested 23 subgenera, studied for two non-coding regions [the nuclear ribosomal internal transcribed spacers (nrITS) and the plastid *trnL-trnF* spacer regions], while the *rps4* plastid gene was also investigated for a more restrict, but representative sample. They found three clearly defined clades (involving the *Passiflora*, *Decaloba*, and *Astrophea* subgenera) while the *Deidamioides* subgenus remained undefined due to the small number of studied species classified in it. Other attempts to elucidate the genus' phylogeny did not yield good association with morphologic and/or ecological data, and did not agree with Feuillet and MacDougal (2003) proposition in relation to the number of subgenera and their composition (Yockteng and Nadot, 2004; Plotze et al., 2005).

Sequences of plastid, mitochondrial, and nuclear (especially ribosomal) DNA have been extensively utilized to study plant (especially Angiosperm) phylogenies (Soltis et al., 1998; Qiu et al, 1999; Kuzoff and Gasser, 2000). This strategy of combining multiple genes with different functions from the three plant genomes should reduce the homoplasies generated by gene-function and/or genomic specific phenomena, such as heterogeneity of rates of change, GC-content bias, RNA editing, and protein structural constraints (Qiu et al., 1999). Rokas et al. (2003) showed that as the number of genes increases in a phylogenetic analysis, the better the tree reflects the species' phylogeny. The same type of relationship was examined by Rokas and Carrol (2005), who concluded that for phylogenetic precision the number of the genes considered is a more important determinant than the number of taxa examined. However, branch representativiness should also be taken into consideration, and when a large number of taxa is being studied the ideal number of markers should be decided in cost-benefit terms.

The objectives of the present work were: (a) to examine *Passiflora*'s three genomes, assessing their phylogenetic utility; (b) test the genus monophyly; (c) compare the results obtained with Feuillet and MacDougal's (2003) infrageneric classification; (d) evaluate different methods of phylogenetic reconstruction; and (e)generally verify the relationships among species in a large number of such taxa in this genus.

MATERIALS AND METHODS

Taxon sampling

The 104 species representing nineteen of Killip's (1938) and Escobar's (1989) subgenera, as well as all four Feuillet and MacDougal's (2003) subgenera investigated are listed in Table 1, together with representatives from seven other genera of Passifloraceae (*Adenia* Forssk*., Ancistrothyrsus* Harms, *Barteria* Hook.f., *Deidamia* Noronha ex Thouars, *Dilkea* Mast., *Mitostemma* Mast., *Paropsia* Noronha ex Thouars) utilized as outgroups. *Tetrastylis* Barb.Rodr. (re-classified as *Passiflora* by Feuillet and MacDougal [2003]), was also included in our analyses. This sampling involved all species of Passifloraceae from which we could obtain suitable material to extract DNA, containing taxa from a wide distributional range in South and Central America. Among the outgroups, representatives of the two tribes of Passifloraceae were considered, as well as members of the Turneraceae and Malesherbiaceae, included in the Passifloraceae by APG II (2003).

DNA Extraction, Amplification, and Sequencing

Total DNA was extracted from fresh leaves dried in silica gel or obtained from herbarium material, using Roy et al.'s (1992) method with a few adaptations.

Seven DNA regions were sampled: the *rbcL* and *rps4* genes, *trnL* intron and *trnL–trnF* intergenic spacer from the plastid genome; *nad1* b/c and *nad5* d/e introns from the mitochondrial genome; and a partial portion of the *26S* gene from the nuclear ribosomal genome. These regions were amplified with primers 1F and 1460R (Savolainen et al., 2000), rps45' and rps43' (Souza-Chies et al., 1997), c, d, e and f (Taberlet et al., 1991), nad1/2 and nad1/3 (Duminil et al., 2002), mt3 and mt6 (Souza et al., 1991), N–nc26S1 and 1229r (Kuzoff et al., 1998). Sequencing primers were used as listed by these authors except for the *nad1* b/c intron, for which we constructed internal primers specific for *Passiflora*. PCR products were purified using the polyethylene glycol / NaCl precipitation method of Dunn and Blattner (1987). Sequencing was performed on a MegaBace 1000 (Amersham Biosciences) machine using the DYEnamicTM ET termination cycle sequencing premix kit (Amersham Biosciences) and following the manufacturer's protocol. The sequences were deposited with Genbank (Accession nos. given in Table 1). Sequence alignments were conducted using the ClustalX 1.81 program (Thompson et al., 1994, 2001) and manually refined. Regions of ambiguous alignment were excluded from the analyses.

Model Selection and Phylogenetic Analyses

Models for maximum likelihood (ML), neighbor-joining (NJ), and Bayesian (BA) analyses were selected based in two approaches, the Akaike Information Criterion (AIC) of the 56 models implemented in Modeltest (Posada and Crandall, 1998) and the evaluation

of the same 56 models in DT–ModSel (Minin et al., 2003). The latter uses a Bayesian Information Criterion (BIC) that combines branch–length estimates, model fit, and a penalty for overfitting in a statistically rigorous way. Since the models chosen by BIC were practically identical to those selected by AIC, we decided to use the AIC approach.

All analyses were performed for (1) each region separately; (2) the cpDNA data; (3) the mtDNA data; and (4) the combined seven regions data. The combined set for all loci includes 6,382 nucleotides with 75 *Passiflora* species representing all subgenera investigated and eight outgroups (*Adenia*, *Barteria*, *Deidamia*, *Dilkea*, *Mitostemma*, *Paropsia*, *Malesherbia*, and *Turnera*). In the combined results, 2.87% of the cells were coded as missing information.

Maximum parsimony – Equally weighted parsimony (maximum parsimony or MP) analyses were performed by heuristic search with TBR branch swapping, the MULPARS option, and 10 random–addition replicates. Gaps were treated as missing data. Since an excessive number of most parsimonious trees did not allow searches to be completed for the each region separately under the described search parameters, a heuristic search of 1,000 random addition replicates was conducted, with 100 trees saved per replicate. Bootstrap statistical support (Felsenstein, 1985) was carried out with 1,000 replications of heuristic search and simple taxon addition, with the ALL TREES SAVED option. The g1 statistic (Hillis, 1991) of skewed tree-length distribution was calculated from 10,000 random trees, to measure the phylogenetic information content of the seven DNA regions independently and for the combined data.

Maximum likelihood – In each ML analysis we used the model of sequence evolution as suggested by Modeltest. The ML tree estimation used heuristic searches with neighbor-joining starting trees, and TBR branch swapping was conducted with PAUP*

(Swofford, 1998). We performed 100 replicates of nonparametric bootstrap (Felsenstein, 1985) using the FASTSTEP option, to obtain the confidence of the ML topology. The ML analysis was also performed in Treefinder (Jobb et al., 2004) and PHYML (Guindon and Gascuel, 2003), with appropriate models of sequence evolution. The trees obtained by these two last programs had their likelihood scores recalculated in PAUP* and were then employed to evaluate the performance in each of the three programs. For this procedure Shimodaira and Hasegawa's (1999; SH) test, as well as consensus trees among phylogenies obtained from different programs were used.

Distances – For the distance analyses, trees were constructed in PAUP* using the neighbor-joining method (NJ; Saitou and Nei, 1987) with models selected by Modeltest, proportional (p), and logDet (Steel, 1994; Lockhart et al., 1994) distances. LogDet or paralinear distances were calculated to test the possible influence of nucleotide composition differences in the phylogeny (Nei and Kumar, 2000). Reliability of the trees was tested using 10,000 bootstrap replications (Hedges, 1992).

Bayesian analyses – Bayesian phylogeny estimation (BA) was performed in MrBayes v3.0b4 (Ronquist and Huelsenbeck, 2003). Each gene was assigned to its own model of sequence evolution. One cold and three heated Markov chain Monte Carlo (mcmc) chains run for $2x10^6$ generations were used, with trees sampled every 100^{th} generation, using a random tree as a starting point and a temperature parameter value of 0.2. The mcmc runs were repeated three times as a safeguard against spurious results. Burn–in, or the time for each parameter to reach a stationary state, was determined by visual inspection when the log-likelihood values achieved an asymptote over a large number of generations (the first 2,000 trees were discarded; 200,000 generations for each

analysis). To calculate the posterior probability of each bipartition, a 50% majority–rule consensus tree was constructed from the remaining trees using PAUP*.

Evolutionary rates – The molecular–clock hypothesis was tested with the LR test by calculating the log likelihood score of the chosen model with the molecular clock enforced and comparing it with the log likelihood score without the molecular clock enforced (Muse and Weir 1992; Baldwin and Sanderson 1998). The number of degrees of freedom is equivalent to the number of terminals minus two (Sorhannus and Van Bell, 1999). The two-cluster test of Takezaki et al. (1995) was performed using PHYLTEST (Kumar, 1996) to evaluate the relative rates between clades. Additionally, average nucleotide diversities and their standard errors within each subgenus were calculated by the Mega3 program (Kumar et al., 2004) with the p-distance option and 2,000 bootstrap replications.

RESULTS

Data Set Characteristics

The general characteristics for each data set are summarized in Table 2; alignments are available at the Systematic Biology website. The number of taxa analyzed for each region differs because in specific cases we were unable to adequately amplify the DNA of a given segment. Some points needing consideration are present below.

rbcL – A nine base pair (bp), or three amino acid (aa) deletion was observed in *Paropsia braunii* between positions 120 and 130. It is not know if this deletion may affect the protein's function, but the possibility that in this species a pseudogene occurs at this region should be contemplated. *Passiflora sanguinolenta*'s sequence presented several stop

codons, also suggesting the presence of a pseudogene, and therefore was excluded from the analyses. The DNA of *P. clathrata*, *P. palmeri* e *Paropsia guinensii*, were only partially sequenced, since it was not possible to amplify the whole region. This suggests a large deletion or mutation in the annealing portion of the reverse primer. The nucleotide diversity observed in this region is quite high (0.039 ± 0.004; Table 3), and if data from 32 Malpighiales genera (extracted from GenBank) are compared (data not shown) the diversity obtained is 0.051 ± 0.004.

rps4 – Several insertion/deletion events (indels) were observed in this gene. *Passiflora suberosa*, *P. coriacea* e *P. xiikzodz* share a 21 bp (or 7 aa) insertion between positions 27 and 49, while *P. coriacea* and *P. xiikzodz* have another 33 bp insertion between 441 and 475.

trnL intron – Here also indels were found, and some characterize monophyletic groups. For instance a six bp deletion occurs between nucleotides (nt) 157 and 154 in *Decaloba*.

trnL–trnF intergenic spacer – *P. coriacea*, *P. suberosa* e *P. xiikzodz* presented a 15 bp insertion between nt 105 and 121. Almost all species (exception *P. clathrata*) show a 11 bp insertion between positions 66 and 78 as compared to outgroups. *P. actinia*, *P. elegans* e *P. sidaefolia* have a five nt duplication between positions 316 and 322.

nad1 b/c intron – Several indels were observed here, but our previous study (Muschner et al., 2003), showed that the majority is not phylogenetically informative. Therefore, they were not considered in the final alignment. Large deletions occur between nt 686 and 1645. A deletion of 768 bp, starting at position 687, is found in *P. umbilicata*, while another of 684 bp, beginning at nt 747, is observed in *P. mixta*, *P. trifoliata* and *P. tripartita var. mollissima*. Additionally, starting at nt 810, there is a 250 bp deletion in *P.*

multiflora and other of 805 bp in *Barteria fistulosa*. A deletion of 450 bp, beginning at nt 1,125, is observed in *P. actinia*, *P. caerulea*, *P. edmundoi*, *P.racemosa*, *P. reflexiflora*, *P. sidaefolia*, *P. sprucei* e *P. tenuifila*. The evolutionary meaning of these large deletions will be discussed by V. C. Muschner et al. in a forthcoming paper. Finally, an 11 bp deletion between nt 161 and 173 is found in all species of the *Decaloba* subgenus, but is also present in *Deidamia sp.* and *Turnera subulata*.

nad5 d/e intron – Indels can occur, but are less frequent than those found in the other noncoding regions. *Paropsia braunii* has a large 173 bp insertion starting at nt 441 and another of 22 bp beginning at nt 610; species of the subgenus *Passiflora* share a five bp insertion between positions 316 e 322.

26S – This region practically does not show indels, that when present involve one nucleotide only.

Phylogenetic Analyses

The topologies of all trees, independently of the types of analysis employed, were very similar; therefore only selected examples of those obtained by Bayesian or maximum likelihood methods will be presented. The models selected by Modeltest are listed in Table 4.

The g1 statistics of the distribution of 10,000 random trees indicates that all seven combinations of data sets are significantly structured, suggesting that the DNA sequence variation is not random with respect to phylogeny.

Generally, all trees (Figures 1 – 5) showed three monophyletic groups, corresponding to the *Astrophea*, *Decaloba*, and *Passiflora* subgenera, plus a small set with *P. cirrhiflora* and *P. ovalis*. *P. tryphostemmatoides* is consistently positioned at the base of

the *Astrophea* subgenus. In what follows we will consider first the cpDNA, mtDNA and nrDNA separately, and afterwards the combined analyses.

Chloroplast DNA – *Decaloba*, *Astrophea*, and *Passiflora* clusters are highly supported by all methods used (MP, NJ, ML and BA; for the latter see Figure 1), bootstrap (BS) and posterior probabilities (PP) being higher than 80 in almost all isolated analyses. The exceptions were *rbcL* and the *trnL–trnF* intergenic spacer, in which *Astrophea* is not supported. However, when the four plastid regions are considered together the statistical support for *Decaloba* are equal to 100 in all analyses, vary between 99 and 100 for *Passiflora*, and between 88 (ML and BA), 74 (MP) and 60 (NJ) for *Astrophea.* The coding regions (*rbcL* and *rps4*) show a better resolution within each of the three groups when compared to the noncoding segments (*trnL* intron and *trnL–trnF* intergenic spacer). In the combined analysis (Figure 1) species having the same main pollinator generally group together.

Mitochondrial DNA – In the isolated analyses only the *Decaloba* monophyly is supported. But when the two regions are considered jointly *Astrophea* is also supported in the ML and BA (Figure 2) approaches. The interspecific relationships are also better supported when the two regions are considered together.

Nuclear DNA – If the 26S nrDNA gene is considered in isolation, again only the *Decaloba* and *Astrophea* clusters are supported (Figure 3).

Combined data – Tests using combined data from different systems showed limitations in the detection of incongruences (Reeves et al., 2001; Yoder et al., 2001); therefore we decided, as Wiens (1998), Reeves et al. (2001) and van den Berg et al. (2005), to closely examine especially groups supported in the individual analyses. But note that in

this case (Figures 4 and 5) *Decaloba*'s monophyly is supported by PP and BS values of 100, of 98 to 100 for *Passiflora*, and of 94 to 100 for *Astrophea*.

Evolutionary Rates

A difference that can be visually observed especially in Figures 4 and 5 is the larger branches of the *Decaloba* clade when compared to the others. A Z statistic ($p < 0.005$) calculated with the PHYLTEST package rejects a constant rate of evolution of the subgenus *Decaloba* in relation to the others for all DNA regions examined (except the *trnL–trnF* intergenic spacer). This test was made with an equal number of species for each subgenus, since it showed to be very conservative when all species were compared. The result of the LR test of the molecular–clock hypothesis for the combined data set is $–2 Ln = 508.96$, $df = 81$, $P < 0.001$. The nucleotide diversity was always higher in *Decaloba*, as compared to the other main subgenera (Table 3).

Comparison Among Phylogenetic Methods

The methods employed (Maximum Parsimony, Neighbor–Joining, Maximum Likelihood, Bayesian), in a general way generated very similar topologies, although a few within-cluster relationships changed (data not shown). The SH test among the ML trees generated by PAUP*, PHYML, and Treefinder showed that they do not differ significantly.

Taxonomic Implications

Decaloba relationships – This subgenus according to Feuillet and MacDougal (2003), groups Killips' (1938) *Adopogyne*, *Astephia*, *Decaloba*, *Murucuja*,

Pseudomurucuja and *Psilanthus* subgenera. Our results highly support this view (Figures 1 and 5). For instance, *P. penduliflora* and *P. tacsonioides*, as well *P. tulae* and *P. murucuja*, placed in different subgenera by Killip (1938), (but not by Feuillet and MacDougal, 2003), generally cluster together in the majority of our trees. *P. coriacea*, *P. suberosa*, and *P. xiikzodz* groups together in all trees. Morphologically they are peculiar by the absence of petals and Feuillet and MacDougal (2003) placed them in supersection *Cieca* . *P. sanguinolenta*, inserted in the *Decaloba* supersection by Feuillet and MacDougal (2003), was first classified in the *Psilanthus* subgenus by Killip (1938). This last author mentioned that due to its leaf shape, integument, and bract absence this species should show affinity with *P. rubra*. Since we could only study the *trnL-trnF* intergenic spacer of *P. rubra*, its relationship of *P. sanguinolenta* and *P. capsularis* is supported by a PP = 71 (data not shown). The relationships of *P. sanguinolenta* and *P. capsularis* is supported in all trees with PP or BS = 100, then our results support their position in section *Xerogona*. *P. misera*, *P. pohlii*, *P. organensis* and *P. tricuspis* generally group together also. According to Killip (1938) *P. misera* and *P. tricuspis* are related due to their relatively wide inner corona. *P. organensis* leaves can also be easily confounded with those of *P. pohlii* and *P. tricuspis*. Feuillet and MacDougal (2003) cluster these species together in section *Decaloba*. Our results confirm this classification this and relate them with other *Decaloba* species. Another interesting cluster is that observed in the cpDNA tree (Figure 1) between *P. helleri* and *P. talamancensis* which, according to Ulmer and MacDougal (2004) show almost identical leaves. *P. lancetillensis* and *P. microstipula* occur in a basal position in the *Decaloba* clade, with high support in all analyses except that of the *26S* gene (Figure 3). According to MacDougal and Hansen (2003) they are intimately related; Feuillet and MacDougal (2003) classified them in the *Pterosperma* section of the *Decaloba* subgenus,

but Killip (1938) and Feuillet and MacDougal (1999) placed them in the *Deidamioides* subgenus. Our results confirm their relationships with *Decaloba*, but in a divergent position from the remaining of this subgenus species (Figures 4 and 5).

Astrophea relationships – This subgenus includes species identified with the same label by Killip (1938) and Feuillet and MacDougal (2003) and the clade is highly supported. According to Feuillet and MacDougal (2003) the *Astrophea* subgenus can be divided into two supersections: *Astrophea* and *Pseudoastrophea*. Our analyses, in a general way, confirm the existence of these groups, but with some exceptions. A larger number of species of this subgenus should be studied to verify this question.

Passiflora relationships – This group is composed by Killip's (1938) *Calopathanthus*, *Distephana*, *Dysosmia*, *Dysosmioides*, *Granadillastrum*, *Passiflora*, *Tacsonia* and *Tacsonioides* subgenera, as well by Escobar's (1989) *Manicata*. This composition is the same proposed by Feuillet and MacDougal (2003). There are clusters with high statistical support within this subgenus, such as *P. actinia*, *P. elegans* and *P. sidaefolia* in the combined analyses and cpDNA tree (Figures 1, 4 and 5). Actually, Muschner et al. (2003) and Lorenz–Lemke et al. (2005) verified that they are evolutionary closely related. Another well supported cluster refers to *P. clathrata*, *P. foetida* and *P. palmeri* of Killip's (1938) *Dysosmia* subgenus, placed in the subgenus *Passiflora*, supersection *Stipulata* and section *Dysosmia* by Feuillet and MacDougal (2003). *P. campanulata*, *P. setulosa* and *P. villosa* classified in the *Dysosmioides* subgenus by Killip (1938) cluster with high values of statistical support. *P. speciosa* and *P. vitifolia* were classified in subgenus *Distephana* by Killip (1938). Feuillet and MacDougal (2003) placed them in supersection *Distephana* of *Passiflora* and we confirmed this classification. They are both pollinated by hummingbirds. A seven-species cluster with a high bootstrap value

formed by *P. antioquiensis*, *P. trisecta*, *P. manicata*, *P. mathewsii*, *P. tripartita var. mollissima*, *P. mixta* and *P. trifoliata* was observed. These species were variously classified in different groups by morphological analysis (Killip, 1938; Escobar, 1989), but were included in the supersection *Tacsonioides* by Feuillet and MacDougal (2003).

Undetermined relationships – *P. cirrhiflora*, classified in the subgenus *Polyanthea* by Killip (1938), and *Tetrastylis* (*Passiflora*) *ovalis*, included by Feuillet and MacDougal (2003) in the *Deidamioides* subgenus, generally clustered together. This grouping however shows different positions in the trees of the present study, with undetermined relationships to *Decaloba* and/or *Passiflora*. Besides, the statistical support of this relationship is high only in the Bayesian analyses of the combined 7 regions (Figure 4) or of the cpDNA tree (Figure 1).

P. tryphostemmatoides, classified in Killip's (1938) *Tryphostemmatoides*, subgenus, but included in the *Deidamioides* subgenus by Feuillet and MacDougal (2003), occurs closer to the *Astrophea* base in all but one of our trees, differing markedly from species of this subgenus in its mtDNA (Figure 2). We suggest that this species could be considered a separate subgenus.

Outgroups – In the phylogenies involving the seven DNA regions (Figures 4 and 5) *Passiflora* can be considered as a monophyletic group. *Dilkea johannesii* and *Mitostemma brevifilis* generally occur together at the base of the *Passiflora* genus cluster, with the exception of the *26S* nrDNA tree (Figure 3). According to Killip (1938), species of these genera are morphologically very similar, especially in their woody habit. *Deidamia* and *Adenia* always cluster with high statistical support. Curiously, *Adenia* has dioic flowers, while *Deidamia* (and the majority of the Passifloraceae) is hermaphroditic. Rooting of the trees was made with *Malesherbia* and *Turnera*, since despite being included

in the Passifloraceae they are phylogenetically more distant (Wikström et al., 2001). But in all trees the outgroup genera that cluster together are *Barteria* and *Malesherbia*. The first is classified in Old World's (Africa and Madagascar) Paropsieae, while *Malesherbia* occurs in the Andes and coastal deserts of Chile, Peru and Argentina (Gengler–Nowak, 2003). *Turnera*, distributed in subtropical and tropical America (Neffa and Fernández, 2000) and *Paropsia*, which occurs in the Old World together with *Barteria* and *Malesherbia*, are in a more basal position as compared to *Adenia* and *Deidamia*.

DISCUSSION

Taxonomic Implications

The three main clades found in this study had already been detected by Muschner et al. (2003) in a first analysis with a smaller number of taxa and molecular markers. Yockteng and Nadot (2004) also identified the existence of these clades, but proposed five other subgenera.

The *Decaloba* subgenus was highly supported in the present study, despite the fact that only a fraction (14%) of the 214 species classified in it had been studied. Six of the eight supersections proposed by Feuillet and MacDougal (2003) were studied and the majority was confirmed by us. For example, *P. coriacea*, *P. suberosa* e *P. xiikzodz*, classified by them in the *Cieca* supersection, clustered together in our trees. However, *P. capsularis* and *P. sanguinolenta* of the *Xerogona* section were inside the *Decaloba* section in the combined analyses. Species classified by Feuillet and MacDougal (2003) as the *Pterosperma* supersection were always observed in a basal position in relation to *Decaloba* in the present study. According to Ulmer and MacDougal (2004) there are two basal

supersections in this subgenus, *Pterosperma* and *Hanhniopathanthus*, but the latter was not represented in our studies. These authors also maintained that *Pterosperma* would be most primitive, since it possesses flowers borne off the tendrils.

Another well-supported subgenus is *Astrophea*, with 57 species according to Feuillet and MacDougal (2003), and represented here by 12 (21%) species. In a general way our results confirmed the two supersections proposed by these authors, but a cluster well supported in almost all of our trees, *P. candida* plus *P. citrifolia*, were classified in different supersections (*Pseudoastrophea* and *Boryastrophea*, respectively) by them. In the two phylogenies including all data (Figures 4 and 5) *Astrophea* is basal in relation to *Passiflora*, with high statistical support. These species, in contrast to other *Passiflora* that are herbaceous, lianas or small shrubs, consist of trees, shrubs or woody climbing plants. Besides, the genus characteristic tendrils are reduced to spines or aculei and the leaves can reach 95 cm of length, as in *P. macrophylla* (Ulmer and MacDougal, 2004). As asserted by Benson et al. (1975), this position suggests that the arborescent habit may be ancestral in the genus. This is confirmed by the *Dilkea* – *Mitostemma* group's position, basal to *Astrophea*, since according to Killip (1938) they could be wood vines, subscandent shrubs, or small trees, as the Old World genera. Kim et al (2004) maintained that the woody habit is the ancestral state in several major eudicotyledon clades. Besides *Dilkea* also does not posses tentrils.

The subgenus *Passiflora* is composed by 236 species, of which 24% are here represented. This subgenus is also highly supported in our analyses, confirming Feuillet and MacDougal's (2003) classification. However, although in some cases we could discern agreement with supersections, sections and/or series of these authors, discrepancies also occur. For instance, *P. mendoncaei*, *P. reflexiflora* and *P. luetzelburgii*, placed in the

Tacsonioides subgenus by Killip (1938) and classified in the *Stipulata* supersection, *Tacsonioides* section by Feuillet and MacDougal (2003), do not cluster in our phylogenies.

In our analyses the *Deidamioides* subgenus proposed by Feuillet and MacDougal (2003) is polyphyletic. Similar results were obtained by Krosnick and Freudenstein (2005). We suggest that of the three species studied here (*P. cirrhiflora*, *P. ovalis* e *P. tryphostemmatoides*), only two should be classified as *Deidamioides*, since *P. tryphostemmatoides* does not cluster with them and is placed in the trees near *Astrophea*, although with clear differences. A further suggestion, therefore, is that *P. tryphostemmatoides* be mantained in the *Tryphostemmatoides* subgenus of Killip (1938).

Our proposal is that the genus *Passiflora* should be classified in five subgenera: *Astrophea*, *Decaloba*, *Passiflora*, *Deidamioides* and *Tryphostemmatoides*.

Comparison Among the Three Main Clades

There are significant differences among the three main clades. To begin with, the species of subgenus *Decaloba* present significantly smaller flowers than the *Passiflora* or *Astrophea* subgenera ($P < 0.05$), as initially reported by Muschner et al. (2003). In addition, Souza et al. (2004) investigated the variation in genome size in eight *Passiflora* taxa, seven of the *Passiflora* and one of the *Decaloba* subgenera. *P. suberosa*, the only *Decaloba* representative, was the species with the lowest 2C DNA content.

V. C. Muschner et al. (unpublished) analyzed four *Passiflora* interspecific hybrids, three from the *Passiflora* and one from the *Decaloba* subgenera, in relation to the sequences of four DNA regions (*rps4* gene, *trnL* intron, *trnL–trnF* and *psbA–trnH* intergenic spacers) and observed that the first group presents exclusively paternal while the

second shows maternal cpDNA inheritance. On the other hand, the mtDNA is inherited exclusively from the maternal parent in all these hybrids.

Studies on the reproductive systems have been generally performed in *P. edulis*, of the *Passiflora* subgenus (Rêgo et al., 1999, 2000; Suassuna et al., 2003), no reports existing in *Decaloba* or *Astrophea*. The subgenus *Passiflora* presents autocompatible and autoincompatible species (Vasconcelos, 1991; Lindberg and Olesen, 2001; Rêgo et al., 2000; Suassuna et al., 2003). According to Lewis (1979), *Passiflora* species with large flowers are autoincompatible, while those with small flowers are autocompatible. The same is true in *Amsinckia* (Boraginaceae; Barret, 2002).

As reported by Snow and MacDougal (1993) and De Melo et al. (2001), the *Decaloba* subgenus presents x = 6, while the *Passiflora* subgenus shows x = 9 chromosomes [but *P. foetida*, of the latter subgenus and studied by De Melo et al. (2001) has x = 10]. The *Astrophea* subgenus would have x = 12 like *Adenia*, strengthening its basal position within the genus. But De Melo and Guerra (2003) suggested that x = 6 would be the ancestral genome for the genus, while groups with x = 9, x = 10 and x = 12 would have a tetraploid origin with descending disploidy and inactivation of some redundant gene sites, especially those of 5S rDNA.

Plotze et al. (2005) developed a morphometric method for the analysis of *Passiflora* leaves. They studied six species of the *Passiflora* and four of the *Decaloba* subgenera and detected two clusters, one composed by four of six *Passiflora* species and the other with species of the two subgenera. Therefore, no clear association between this characteristic and subgenus classification was found, leaf form not being a good indicator for this type of comparison.

As previously mentioned Passifloraceae species, including those previously classified as Turneraceae or Malesherbiaceae, recently referred as the Passifloraceous group (APG II, 2003) are known to produce, with some few exceptions, cyclopentanoid cyanohydrin glycosides 1-10. Flacourtiaceae and Achariaceae species also produce these compounds, but no other plants have this property (Clausen et al., 2002). Jaroszewski et al. (2002) verified the presence of these substances in *P. apetala*, *P. cuneata*, *P. indecora*, *P. kalbreyeri*, *P. murucuja*, *P. perfoliata*, *P. biflora*, *P. discophora* e *P. herbetiana*, of the *Decaloba* subgenus and in *P. x violacea*, a hybrid between *P. caerulea* e *P. racemosa*, of the *Passiflora* subgenus. No such compounds were found in *P. aurantia*, *P. gibertii*, *P. ligularis*, *P. manicata*, *P. platyloba* and *P. tripartita*, species of the *Passiflora* subgenus, nor in *P. lindeniana* of the *Astrophea* subgenus. It is therefore possible that the presence of these substances is restricted to *Decaloba*, their occurrence in the *Passiflora* hybrid being due to the exceptional opening of the respective metabolic route due to the hybridization process.

The association *Heliconius-Passiflora* is one of the better studied insect-plant associations. Heliconiine butterflies only feed, as larvae, Passifloraceae leaves. Benson et al. (1975) and Brown (1981) reviewed the Heliconiine-Passifloraceae interaction and concluded that there is specificity between determined groups of *Heliconius* and different *Passiflora* subgenera. Ehrlich and Raven (1964) suggested that these correlations can be explained by coevolutive processes responsible for the high present diversification of modern plants and herbivorous insects. Species of the *Heliconius numata-melpomene* group feed especially *Granadilla* (of the *Passiflora* subgenus) plants. According to these authors both taxa are considered primitive in their genera, possessing non-specialized morphology and behavior. On the other hand, members of the *Heliconius erato-charitonia*

species group feed plants of the *Plectostemma* (*Decaloba*) subgenus, while larvae of the *Heliconius sara-sapho* group feed plants both of the *Plectostemma* (*Decaloba*) and *Astrophea* subgenera. These other associations could be derived phenomena. Smiley (1985) confirmed these relationships and concluded that the *Decaloba* subgenus evolved without a chemical barrier against herbivory, while many species of the *Passiflora* subgenus are protected from a large number of *Heliconius* larvae. Besides, species of this subgenus developed morphologic structures which prevent or disfavor butterfly oviposition, such as those which resemble egg layers, or present high pilosity (Ulmer and MacDougal, 2004).

Maternal, Paternal, and Biparental Phylogenies

The mode of inheritance of a characteristic has obvious influence in its evolution (Harris and Ingram, 1991). In addition, traits inherited by parents of only one side can furnish precious details about sex-specific conditions important for the evolution of the organism as a whole, such as interpopulation gene flow (Collevatti et al., 2001, 2003; Liepelt et al., 2002). Ideally, therefore, evolutionary investigations should include, as was done in the present study, characteristics inherited just by the maternal or paternal line, as well as others with biparental inheritance (Shore and Triassi, 1998).

In plants and algae mitochondria and chloroplasts can be inherited through distinct sexual lineages. Generally, in hermaphrodite species both organelles are transmitted together by the gamete of the same sex, female in the majority of the Angiosperms (Dumolin-Lapègue et al., 1998, Moreira et al., 2002) and male in some Gymnosperms (Chesnoy, 1987; Hagemann, 1992). Exceptions, however, were observed; in Pinaceae (Neale and Sederoff, 1989) and *Actinidia* (Actinidiaceae) chloroplasts are paternally and

mitochondria maternally inherited (Chat et al., 1999; Burban and Petit, 2003); while the opposite was observed in *Musa acuminata* (Musaceae) and *Cucumis* (Cucurbitaceae) (Fauré et al., 1994; Havey et al., 1998).

Similarities and dissimilarities were obtained in our phylogenetic cpDNA, mtDNA, and nrDNA trees. All of them, however, point for *Decaloba* subgenus monophyly. On the other hand, when the placement of the *Passiflora* and *Astrophea* subgenera is considered, intergenomic differences occur. Mention was already made that the pattern of organelle inheritance differs among these subgenera; if some of the species considered had a hybrid origin, discrepancies could occur in relation to the others. Another question refers to different rates of nucleotide substitution; the differences observed between *Decaloba* and the other subgenera in this regard were already mentioned. But the intergenomic differences in such rates should also consider (Riesenberg, et al., 1996; Chat et al., 2004). In our case it should be pointed out that the four cpDNA regions presented five times more parsimoniously informative sites than the mtDNA and/or nrDNA markers.

Pollinization Agents

The high floral diversity found in the *Passiflora* genus is certainly intimately related to the different pollinization forms that exist in this genus. The corona of filaments, between the perianth and stamens, is highly variable, showing different colors, forms, smells, and filament disposition. According to Endress (1994), the series of most external filaments are involved in pollinator attraction, while the two inner ones furnish mechanical protection to the nectar chamber.

Passiflora ancestors were probably especially pollinated by bees. Hummingbird pollinization probably occurred independently more than once in the genus and in all

subgenera that were also pollinated by wasps, butterflies, and bats (MacDougal, 1994). Examples are seen in the phylogeny of Figure 1.

Pollination by small and large bees are found in *Passiflora* taxa with flattish to cup-shaped hypanthia, and with a range of erect, lateral to pendulous flowers. This flower form is generally found in the *Decaloba* subgenus, but also in several species of the *Passiflora* and *Astrophea* subgenera (MacDougal, 1994).

According to MacDougal (1994), hummingbird pollination is common in several *Passiflora* groups, and was documented in the *Granadillastrum*, *Tacsonia*, *Dysosmia* (presently *Passiflora*), *Decaloba*, *Murucuja* e *Pseudomurucuja* (presently *Decaloba*) subgenera. In our material widely separated species like *P. speciosa* and *P. vitifolia*, in the *Passiflora*, *P. sanguinolenta*, in the *Decaloba*, and *P. amoena*, in the *Astrophea* subgenera are all pollinated by hummingbirds, suggesting independent origins. The latter observation agrees with Ulmer and MacDougal's (2004) assertion that hummingbirds pollinate species of the *Astrophea Boryastrophea* section (to which *P. amoena* belongs).

Passiflora species typically pollinated by bats (*P. mucronata* and *P. galbana*, for instance) seem to attract the animals by their scent (Sazima and Sazima, 1978). This type of pollination seems also to have independently developed many times in *Passiflora*, as suggested by its presence in evolutionary distinct species (*P. penduliflora* in the *Decaloba*, *P. galbana*, *P. trisecta* and *P. trifoliata* in the *Passiflora*, and *P. ovalis* in the *Deidamioides* subgenera).

CONCLUSIONS

The present study illustrates the utility of molecular analyses for a series of comparisons of both evolutionary and taxonomic importance. The observed pattern of

genetic variation shows clear intergenomic differences, one especially curious finding being the large deletions found in the *nad1* b/c intron. Also of note is the higher rate of change observed in the *Decaloba* clade, which is reflected in higher nucleotide diversity indices. The three main subgenera identified through the molecular approach show distinctiveness in flower and genome size, cpDNA inheritance, chromosome numbers, secondary compounds content, and diverse degree of relationships with pollinators and predators. In addition to these three subgenera we are proposing the existence of two others. Within these entities clear associations between patterns of molecular phylogenetic relationships and morphological traits could be found. We maintain that global evaluations such as this one, involving different genomes, good phylogenetic markers, and careful taxonomic sampling, are essential for the unraveling of the complex evolutionary histories of the organic world.

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Table 1: List of the species studied, their taxonomic classification, source of collection, and GenBank accession numbers for the DNA sequences.

Subgenera or tribe (Killip, 1938)	This study's proposal	Species	Data of collection	GenBank numbers						
				rbcL	*rps4*	*trnL*	*trnL-trnF*	*nad1* b/c	*nad5* d/e	*26S*
Astrophea	*Astrophea*	*P. amoena* L. K. Escobar	Italy, Ripalta Cremasca, Colection (MV)	DQ123300	DQ123407	DQ123017	DQ123486	DQ123214	DQ123128	DQ122935
		P. arborea Spreng.	Panama (RY)	DQ123301	DQ123408	DQ123018	DQ123487	DQ123215	DQ123129	DQ122936
		P. candida (P. & E.) Mast.	Italy, Ripalta Cremasca, Colection (MV)	DQ123302	DQ123409	DQ123019	N/A	DQ123216	DQ123130	N/A
		P. ceratocarpa Silveira	Brazil, PA (LCB)	DQ123303	DQ123410	DQ123020	DQ123488	DQ123217	DQ123131	DQ122937
		P. citrifolia (Juss.) Mast.	French Guiana (MV)	DQ123304	AY212311	DQ123021	AY210958	DQ123218	DQ123132	DQ122938
		P. haematostigma Mart. ex Mast.	Guaratuba, PR (ACC)	DQ123305	AY212292	DQ123022	AY032773	DQ123219	DQ123133	DQ122939
		P. kawensis Feuillet	French Guiana (RY)	DQ123306	DQ123411	DQ123023	DQ123489	DQ123220	DQ123134	DQ122940
		P. lindeniana Tr. & Pl.	Italy, Ripalta Cremasca, Colection (MV)	DQ123307	DQ123412	DQ123024	DQ123490	DQ123221	DQ123135	DQ122941
		P. macrophylla Spruce ex Mast.	Brazil (MV)	DQ123308	AY212313	DQ123025	AY210965	DQ123222	DQ123136	DQ122942
		P. mansoi (Mart.) Mast.	Chapadão do Sul, MS (ACC)	DQ123309	AY212307	DQ123026	AY102401	DQ123223	DQ123137	DQ122943
		P. pittieri Mast.	Italy, Ripalta Cremasca, Colection (MV)	DQ123310	DQ123413	DQ123027	DQ123491	DQ123224	DQ123138	DQ122944
		P. rhamnifolia Mast.	Cabo Frio, RJ (TSN)	DQ123299	DQ123406	DQ123016	DQ123485	DQ123213	DQ123127	N/A
Adopogyne	*Decaloba*	*P. multiflora* L.	Dominica (MV)	DQ123297	DQ123404	DQ123014	AY210967	DQ123211	DQ123125	DQ122933
Astephia		*P. penduliflora* Bertero ex DC.	Blois-France-Greenhouse (RY)	DQ123298	DQ123405	DQ123015	DQ123484	DQ123212	DQ123126	DQ122934
Decaloba		*P. capsularis* L.	Quatro Barras, PR (ACC)	DQ123312	DQ123415	DQ123029	AY032775	DQ123226	DQ123140	DQ122946
		P. coriacea Juss.	Colombia (MV)	DQ123313	DQ123416	DQ123030	AY210959	DQ123227	DQ123141	DQ122947
		P. helleri Peyer	Mexico (MV)	DQ123314	DQ123417	DQ123031	AY210962	DQ123228	DQ123142	DQ122948
		P. lobbi subsp. ayaucuchoensis Skrabal & Weigend[2]	Peru (MC)	DQ123315	DQ123419	DQ123032	DQ123493	N/A	N/A	N/A
		P. lobbi subsp. obtusiloba (Mast.) Skrabal & Weigend[2]	Peru (MC)	DQ123316	DQ123418	DQ123033	DQ123494	DQ123229	DQ123143	N/A
		P. misera HBK.	Santa Maria, RS (PASS)	DQ123317	DQ123420	DQ123034	AY032777	DQ123230	DQ123144	DQ122949

Table 1 (Cont.)

Subgenera or tribe (Killip, 1938)	This study's proposal	Species	Data of collection	GenBank numbers						
				rbcL	*rps4*	*trnL*	*trnL-trnF*	*nad1* b/c	*nad5* d/e	*26S*
		P. morifolia Mast. in Mart.	Brazil, RS (PASS)	DQ123318	AY212314	DQ123035	AY032780	DQ123231	DQ123145	DQ122950
		P. organensis Gardn.	Brazil, PR (ACC)	DQ123319	DQ123421	DQ123036	AY032779	DQ123232	DQ123146	DQ122951
		P. ornithoura Mast.	Guatemala (MV)	DQ123320	DQ123422	DQ123037	AY210968	DQ123233	DQ123147	DQ122952
		P. pohlii Mast. in Mart.	Pirapora, MG (ACC)	DQ123321	DQ123423	DQ123038	AY032778	DQ123234	DQ123148	DQ122953
		P. podlechii Skrabal & Weigend	Peru (MC)	N/A	DQ123403	DQ123013	DQ123483	DQ123210	N/A	N/A
		P. punctata L.	Peru (MV)	DQ123322	DQ123424	DQ123039	AY210969	DQ123235	N/A	DQ122954
		P. rovirosae Killip	Italy, Ripalta Cremasca, Colection (MV)	N/A	DQ123425	DQ123040	N/A	N/A	N/A	N/A
		P. rubra L.	Brazil, PE (MG)	N/A	N/A	N/A	AY032776	N/A	N/A	N/A
		P. rufa Feuillet	French Guiana (MV)	DQ123323	AY212315	DQ123041	AY210971	DQ123236	DQ123149	DQ122955
		P. sexflora Juss.	Dominican Republic (MV)	DQ123324	DQ123426	DQ123042	AY210974	DQ123237	DQ123150	DQ122956
		P. suberosa L.	Brazil, RS (PASS)	DQ123325	DQ123427	DQ123043	AY032774	DQ123238	DQ123151	DQ122957
		P. talamancensis Killip	Costa Rica (MV)	DQ123326	DQ123428	DQ123044	AY210976	DQ123239	DQ123152	DQ122958
		P. tricuspis Mast. in Mart.	Brazil, SP (MCD)	DQ123327	DQ123429	DQ123045	AY102396	DQ123240	DQ123153	DQ122959
		P. trifasciata Lemaire	Pitangui, MG (NFM)	DQ123328	DQ123430	DQ123046	AY210980	N/A	N/A	N/A
		P. truncata Regel	Brazil, SC (ACC)	N/A	DQ123431	DQ123047	AY102390	N/A	N/A	N/A
		P. vespertilio L.	Brazil, PA (LCB)	DQ123329	DQ123432	DQ123048	DQ123495	N/A	N/A	N/A
		P. xiikzodz MacDougal	Italy, Ripalta Cremasca, Colection (MV)	DQ123330	DQ123433	DQ123049	AY210975	DQ123241	DQ123154	DQ122960
Deidamioides		*P. lancetillensis* MacDougal & Meerman	French Guiana (MV)	DQ123331	AY212312	DQ123050	AY210963	DQ123242	DQ123155	DQ122961
		P. microstipula Gilbert & MacDougal	Mexico (MV)	DQ123332	DQ123434	DQ123051	AY210966	DQ123243	DQ123156	DQ122962
Murucuja		*P. murucuja* L.	Blois-France-Greenhouse (RY)	DQ123345	DQ123442	DQ123064	DQ123501	DQ123255	DQ123168	DQ122974
		P. tulae Urban	Puerto Rico (MV)	DQ123346	DQ123443	DQ123065	AY102392	DQ123256	DQ123169	DQ122975
Pseudomurucuja		*P. cupraea* L.	Bahamas (MV)	DQ123378	DQ123459	DQ123102	DQ123513	DQ123274	DQ123186	DQ122993

Table 1 (Cont.)

Subgenera or tribe (Killip, 1938)	This study's proposal	Species	Data of collection	GenBank numbers						
				rbcL	*rps4*	*trnL*	*trnL-trnF*	*nad1* b/c	*nad5* d/e	*26S*
		P. tacsonioides Griseb.	Blois-France-Greenhouse (RY)	DQ123379	DQ123461	DQ123103	DQ123514	DQ123275	DQ123187	DQ122995
Psilanthus		*P. sanguinolenta* Mast.	Ecology & Evolutionary Biology Conservatory, Univ. Connecticut (RY)	N/A	DQ123462	DQ123104	DQ123515	DQ123276	DQ123188	DQ122996
Calopathanthus	*Passiflora*	*P. racemosa* Brot.	Brazil, RJ (FCN)	DQ123311	DQ123414	DQ123028	DQ123492	DQ123225	DQ123139	DQ122945
Distephana		*P. coccinea* Aubl.	Peru (MC)	DQ123333	DQ123435	N/A	N/A	N/A	N/A	N/A
		P. speciosa Gardn.	Brazil, MS (ACC)	DQ123334	AY212293	DQ123052	AY102402	DQ123244	DQ123157	DQ122963
		P. vitifolia HBK.	Colombia (MV)	DQ123335	DQ123436	DQ123053	AY210977	DQ123245	DQ123158	DQ122964
Dysosmia		*P. clathrata* Mast.	Brazil, MG (FCN)	DQ123336	DQ123437	DQ123054	DQ123496	DQ123246	DQ123159	DQ122965
		P. foetida L.	Brazil, PE (NFM)	DQ123337	AY212291	DQ123055	AY032763	DQ123247	DQ123160	DQ122966
		P. palmeri var. sublanceolata Killip	Italy, Ripalta Cremasca, Colection (MV)	DQ123338	DQ123438	DQ123056	DQ123497	DQ123248	DQ123161	DQ122967
Dysosmioides		*P. campanulata* Mast.	Brazil, PR (ACC)	DQ123339	AY212317	DQ123057	AY032760	DQ123249	DQ123162	DQ122968
		P. setulosa Killip	Brazil, PR (ACC)	DQ123340	AY212297	DQ123058	AY032761	DQ123250	DQ123163	DQ122969
		P. villosa Vell.	Brazil, MG (ACC)	DQ123341	AY212308	DQ123059	AY102403	DQ123251	DQ123164	DQ122970
Granalillastrum		*P. antioquiensis* Karst.	Italy, Ripalta Cremasca, Colection (MV)	DQ123342	DQ123439	DQ123060	DQ123498	DQ123252	DQ123165	DQ122971
		*P. trisecta*Mast.	Blois-France-Greenhouse (RY)	DQ123343	DQ123440	DQ123061	DQ123499	DQ123253	DQ123166	DQ122972
Manicata		*P. manicata* (Juss.) Pers.	Blois-France-Greenhouse (RY)	DQ123344	DQ123441	DQ123062	DQ123500	DQ123254	DQ123167	DQ122973
Passiflora		*P. actinia* Hook	Brazil, RS (PASS)	DQ123347	AY212301	DQ123065	AY032767	DQ123257	DQ123170	DQ122976
		P. acuminata DC.	Brazil, PA (AL)	N/A	AY212301	DQ123066	DQ123502	N/A	N/A	N/A
		P. alata Curtis	Brazil, RS (PASS)	DQ123348	AY212323	DQ123067	AY032765	DQ123258	DQ123171	DQ122977
		P. ambigua Hemsl.	Brazil, MT (LCB)	DQ123349	DQ123444	DQ123068	DQ123503	DQ123259	DQ123172	DQ122978
		P. amethystina Mikan	Brazil, MG (MCD)	N/A	AY212323	DQ123069	AY102397	N/A	N/A	N/A
		P. caerulea L.	Brazil, RS (PASS)	DQ123350	AY212316	DQ123070	AY032772	DQ123260	DQ123173	DQ122979
		P. cincinnata Mast.	Brazil, MS (ACC)	DQ123351	AY212294	DQ123071	AY102400	DQ123261	DQ123174	DQ122980

Table 1 (Cont.)

Subgenera or tribe (Killip, 1938)	This study's proposal	Species	Data of collection	GenBank numbers						
				rbcL	*rps4*	*trnL*	*trnL-trnF*	*nad1* b/c	*nad5* d/e	*26S*
		P. edmundoi Sacco	Brazil, BA (NFM)	DQ123352	AY212302	DQ123072	AY102399	DQ123262	DQ123175	DQ122981
		P. edulis Sims	Brazil, RS (PASS)	DQ123353	AY212303	DQ123073	AY032769	DQ123263	DQ123176	DQ122982
		P. eichleriana Mast.	Brazil, RS (PASS)	DQ123354	AY212304	DQ123074	AY102388	N/A	N/A	N/A
		P. elegans Mast.	Brazil, RS (PASS)	DQ123355	AY212295	DQ123075	AY032766	DQ123264	DQ123177	DQ122983
		P.exura	Italy, Ripalta Cremasca, Colection (MV)	DQ123356	DQ123445	DQ123076	DQ123504	N/A	N/A	N/A
		P. gabrielliana sp. new	French Guiana (MV)	DQ123357	AY212319	DQ123077	AY210960	N/A	N/A	N/A
.		*P. galbana* Mast.	Camocin S. Felix, PE (NFM)	DQ123358	DQ123446	DQ123078	AY032770	DQ123265	DQ123178	DQ122984
		P. garkey Mast.	French Guiana (MV)	DQ123359	AY212320	DQ123079	AY210961	N/A	N/A	N/A
		P. incarnata L.	Brazil, SP (BGJ)	DQ123360	AY212306	DQ123080	AY032768	DQ123266	DQ123179	DQ122985
		P. ischnoclada	Brazil, PA (LCB)	N/A	DQ123447	DQ123081	DQ123505	N/A	N/A	N/A
		P. jilekii Wawra	Brazil, SC (ACC)	DQ123361	AY212318	DQ123082	AY102387	DQ123267	DQ123180	DQ122986
		P. kermesina Link & Otto	Brazil, SP (BGJ)	N/A	DQ123448	DQ123083	AY032762	N/A	N/A	N/A
		P. maliformis L.	Dominica (MV)	DQ123362	AY212321	DQ123084	AY210964	DQ123268	DQ123181	DQ122987
		P. miersii Mast. in Mart.	Brazil, SP (PASS)	DQ123363	DQ123449	DQ123085	AY102395	DQ123269	DQ123182	DQ122988
		P. mucronata Lam.	Brazil, PE (MG)	N/A	DQ123450	DQ123086	DQ123506	N/A	N/A	N/A
		P. nitida Kunth	Brazil, MT (LCB)	DQ123364	DQ123451	DQ123087	N/A	N/A	N/A	N/A
		P. odontophylla Harms ex Glaz.	Brazil, MG (FCN)	DQ123365	DQ123452	DQ123088	DQ123507	N/A	N/A	N/A
		P. quadrangularis L.	Brazil, SP (BGJ)	DQ123366	AY212322	DQ123089	AY032764	N/A	N/A	N/A
		P. recurva Mast in Mart.	Brazil, MG (ACC)	DQ123367	AY212310	DQ123090	AY102391	N/A	N/A	N/A
		*P. riparia*Mart.	Brazil, PA (LCB)	DQ123368	DQ123453	DQ123091	DQ123508	N/A	N/A	N/A
		P. serratifolia L.	Surinam (MV)	DQ123369	DQ123454	DQ123092	AY210973	N/A	N/A	N/A
		P. serratodigitata L.	Martinique (MV)	DQ123370	DQ123455	DQ123093	AY210972	N/A	N/A	N/A
		P. setacea DC.	Brazil, SP (BGJ)	DQ123371	AY212296	DQ123094	AY102398	N/A	N/A	N/A
		P. sidaefolia M. Roemer	Brazil, MG (MCD)	DQ123372	AY212298	DQ123095	AY102394	DQ123270	DQ123183	DQ122989
		P. sprucei Mast.	Italy, Ripalta Cremasca, Colection (MV)	DQ123373	DQ123456	DQ123096	DQ123509	DQ123271	DQ123184	DQ122990
		P. tenuifila Killip	Brazil, RS (PASS)	DQ123374	AY212299	DQ123097	AY032771	DQ123272	N/A	DQ122991

Table 1 (Cont.)

Subgenera or tribe (Killip, 1938)	This study's proposal	Species	Data of collection	GenBank numbers						
				rbcL	*rps4*	*trnL*	*trnL-trnF*	*nad1* b/c	*nad5* d/e	*26S*
		P. trintae Sacco	Brazil, BA (TSN)	DQ123375	DQ123457	DQ123098	DQ123510	N/A	N/A	N/A
		P. urubicensis Cervi	Brazil, SC (ACC)	N/A	AY212300	DQ123099	AY102393	N/A	N/A	N/A
		P. watsoniana Mast.	Brazil, BA (AS)	DQ123376	DQ123458	DQ123100	DQ123511	N/A	N/A	N/A
Tacsonia		*P. mathewsii* (Mast.) Killip	Blois-France-Greenhouse (RY)	DQ123380	DQ123463	DQ123105	DQ123516	DQ123277	DQ123190	DQ122994
		P. mixta L. f.	(RY)	DQ123381	DQ123464	DQ123106	DQ123517	DQ123278	DQ123191	DQ122997
		P. tripartita var. mollissima (Juss.) Poir.	(RY)	DQ123382	DQ123465	DQ123107	DQ123518	DQ123279	DQ123192	DQ122998
		P. trifoliata Cav.	Peru (MC)	DQ123383	DQ123466	DQ123108	DQ123519	DQ123280	DQ123193	N/A
Tacsonioides		*P. luetzelburgii* Harms	Brazil, BA (TSN)	DQ123384	DQ123467	DQ123109	DQ123520	DQ123281	DQ123194	DQ122999
		P. mendoncaei Harms	Brazil, PR (ACC)	DQ123385	DQ123468	DQ123110	AY102389	DQ123282	N/A	DQ123000
		P. reflexiflora Cav.	Ecuador (MV)	DQ123386	DQ123469	DQ123111	AY210970	DQ123283	DQ123195	DQ123001
		P. umbilicata (Griseb.) Harms	Blois-France-Greenhouse (RY)	DQ123387	DQ123470	DQ123112	DQ123521	DQ123284	N/A	DQ123002
Polyanthea	*Deidamioides*	*P. cirrhiflora* Juss.	Italy, Ripalta Cremasca, Colection (MV)	DQ123377	DQ123459	DQ123101	DQ123512	DQ123273	DQ123185	DQ122992
Genus		*P. ovalis*	Brazil, BA (TSN)	DQ123401	AY216662	DQ123122	AY210978	DQ123295	DQ123207	DQ123010
Tryphostemmatoides	*Tryphostemmatoides*	*P. tryphostemmatoides* Harms	Blois-France-Greenhouse (RY)	DQ123388	DQ123471	DQ123113	DQ123522	DQ123285	DQ123196	DQ123003
Passifloreae tribe		*Adenia isoalensis*	(MC)	DQ123389	DQ123472	DQ123115	N/A	DQ123286	DQ123198	DQ123004
		Adenia keramanthus	(MC)	DQ123390	DQ123473	DQ123114	AY102405	DQ123287	DQ123197	DQ123005
		Ancistrothyrsus sp.	Peru (MC)	DQ123391	DQ123474	N/A	DQ123523	N/A	N/A	N/A
		Deidamia sp.	(MC)	DQ123394	DQ123477	DQ123117	DQ123526	DQ123289	DQ123201	DQ123007
		Dilkea cf johannesii Barb. Rodr.	Peru (MC)	DQ123399	DQ123478	DQ123118	DQ123527	DQ123290	DQ123202	DQ123008
		Mitostemma brevifilis	Brazil, MS (ACC)	DQ123400	AY212309	DQ123119	AY102386	DQ123291	DQ123203	DQ123009
Paropsieae tribe		*Barteria fitulosa*	Nigeria (MC)	DQ123392	DQ123475	N/A	DQ123524	DQ123288	DQ123199	DQ123006
		Barteria sp.	(MC)	DQ123393	DQ123476	DQ123116	DQ123525	N/A	DQ123200	N/A
		Paropsia braunii	Tanzania (MC)	DQ123395	DQ123479	N/A	N/A	N/A	DQ123204	N/A
		Paropsia brazzeana	Africa (MC)	DQ123396	DQ123480	DQ123120	DQ123528	DQ123292	DQ123205	N/A

Table 1 (Cont.)

Subgenera or tribe (Killip, 1938)	This study's proposal	Species	Data of collection	GenBank numbers						
				rbcL	*rps4*	*trnL*	*trnL-trnF*	*nad1* b/c	*nad5* d/e	*26S*
		Paropsia guneensis	Gold Coast (MC)	DQ123397	DQ123481	N/A	N/A	N/A	N/A	N/A
		Paropsia madagascariensis	(MC)	AF206802	AY216663	DQ123121	AY102404	DQ123293	DQ123206	N/A
Malesherbiaceae		*Malesherbia linearifolia*	Chile (KG, SK)	DQ123402	DQ123482	DQ123123	DQ123529	DQ123294	DQ123208	DQ123011
Turneraceae		*Turnera subulata*	Brazil, BA (CB)	DQ123398	N/A	DQ123124	DQ123530	DQ123296	DQ123209	DQ123012

Collectors: ACC = A. C. Cervi; AL = A. Lins; AS = A. Selbach; BGJ = Banco de Germoplasma de Jaboticabal; CB = C. van den Berg; FCN = F. Campos Neto; KG = K. Gengler; LCB = L. C. Bernacci; MC=Mark Chase; MCD = M. C. Dornelas; MG = M. Guerra; MV = M. Vecchia; NFM = N. F. Melo; PASS = our group; RY = R. Yockteng; SK = S. Krosnick; TSN = T. S. Nunes.

Brazilian states: BA = Bahia; MG = Minas Gerais; MS = Mato Grosso do Sul; MT = Mato Grosso; PA = Pará; PR = Paraná; PE = Pernambuco; RJ = Rio de Janeiro; RS = Rio Grande do Sul; SC = Santa Catarina; SP = São Paulo

TABLE 2: Number of taxa and outgroups considered, sequence and phylogenetic tree characteristics of the data.

Characteristics	DNA regions									
			intron					Combined data		
	rbcL	*rps4*	*trnL*	*trnL–trnF*	*nad1* b/c	*nad5* d/e	*26S*	cpDNA	mtDNA	All
Number of taxa	93	100	99	100	77	72	71	99	77	74
Number of outgroups	17	14	12	12	11	13	9	15	9	9
Sequence characteristics										
Length of sequenced region (range)	683 – 1345	479 – 600	314 – 546	239 – 324	898 – 1610	1103 – 1357	722 – 1125	1111 – 2773	771 – 2063	4 430 – 5 913
Aligned length	1345	666	664	393	1787	1545	1127	3068	2187	6382
Analyzed aligned length	1345	666	664	393	843	1344	1127	3068	2187	6382
Variable sites	376	247	178	155	180	228	227	965	342	1480
Parsimony informative sites	237	178	109	71	90	83	123	592	121	705
Pairwise uncorrected distances (range, %)[a]	0 – 9.1	0 – 10.0	0 – 6.0	0 – 13.3	0 – 4.5	0 – 3.3	0 – 4.2	0 – 16.2	0 – 4.5	0 – 5.4
Average AT content	55.3	63.4	69.6	64.5	46.0	52.1	39.4	60.8	49.6	53.0
Tree characteristics										
Parsimony										
Number of trees	57	419	2 069	620	1 205	5 382	132	4	2 427	576
Length	951	519	294	271	208	320	549	2 233	438	2 862
CI/RI[b]	0.51/0.81	0.65/0.89	0.74/0.92	0.72/0.89	0.92/0.96	0.80/0.90	0.52/0.78	0.56/0.82	0.84/0.93	0.62/0.82
Likelihood										
PAUP score	7 813.471	3 956.488	2 858.708	2 212.631	2 575.551	3 846.605	4 598.699	18 268.774	6 040.234	27 418.611
PhyML score	7 812.643	3 998.949	2 834.748	2 160.828	2 544.575	3 895.092	4 635.927	18 294.505	6 022.834	27 430.730
Treefinder score	7 992.700	3 932.725	2 751.871	2 168.797	2 577.286	3 802.093	4 722.206	18 494.149	6 004.187	27 429.117

[a] Without outgroups.

[b] Consistency Index / Retention Index.

TABLE 3: Average values for the nucleotide diversity found in the seven DNA regions. Values within parentheses refer to standard errors.

	rbcL	*rps4*	*trnL* intron	*trnL–trnF*	*nad1* b/c	*nad5* d/e	*26S*
Total	0.039 (0.004)	0.053 (0.005)	0.017 (0.004)	0.043 (0.009)	0.015 (0.003)	0.013 (0.002)	0.006 (0.002)
Passiflora genus	0.033 (0.004)	0.041 (0.004)	0.025 (0.004)	0.041 (0.009)	0.013 (0.003)	0.011 (0.002)	0.012 (0.002)
Passiflora subgenus	0.013 (0.002)	0.006 (0.001)	0.008 (0.002)	0.014 (0.004)	0.005 (0.001)	0.001 (0.000)	0.007 (0.001)
Decaloba subgenus	0.029 (0.003)	0.033 (0.004)	0.021 (0.003)	0.033 (0.005)	0.009 (0.001)	0.010 (0.002)	0.016 (0.002)
Astrophea subgenus	0.007 (0.001)	0.018 (0.003)	0.013 (0.003)	0.014 (0.004)	0.001 (0.000)	0.001 (0.000)	0.008 (0.001)
Outgroups	0.053 (0.005)	0.095 (0,008)	0.020 (0.005)	0.051 (0.007)	0.042 (0.003)	0.027 (0.002)	0.029 (0.001)

TABLE 4: Models selected by Modeltest and used in the Bayesian (BA) and Maximum Likelihood (ML) inferences.

DNA region	Model Selected by AIC
rbcL	GTR+I+G
	Shape = 0.5651 Pinvar = 0.8313
rps4	TVM+I+G
	Shape = 1.1134 Pinvar = 0.2475
intron *trnL*	GTR+I+G
	Shape = 1.1285 Pivar = 0.1483
trnL–trnF	GTR+G
	Shape = 0.7787 Pivar = 0
nad1 b/c	TVM+G
	Shape = 0.7621 Pivar = 0
nad5 d/e	GTR+I+G
	Shape = 0.9423 Pivar = 0.3599
26S	GTR+I+G
	Shape = 0.5061 Pivar = 0.6080
cp	TVM+I+G
	Shape = 0.8897 Pinvar = 0.4484
mt	TVM+I+G
	Shape = 0.9362 Pinvar = 0.4690
Combined	GTR+I+G
	Shape = 0.8313 Pinvar = 0.5651

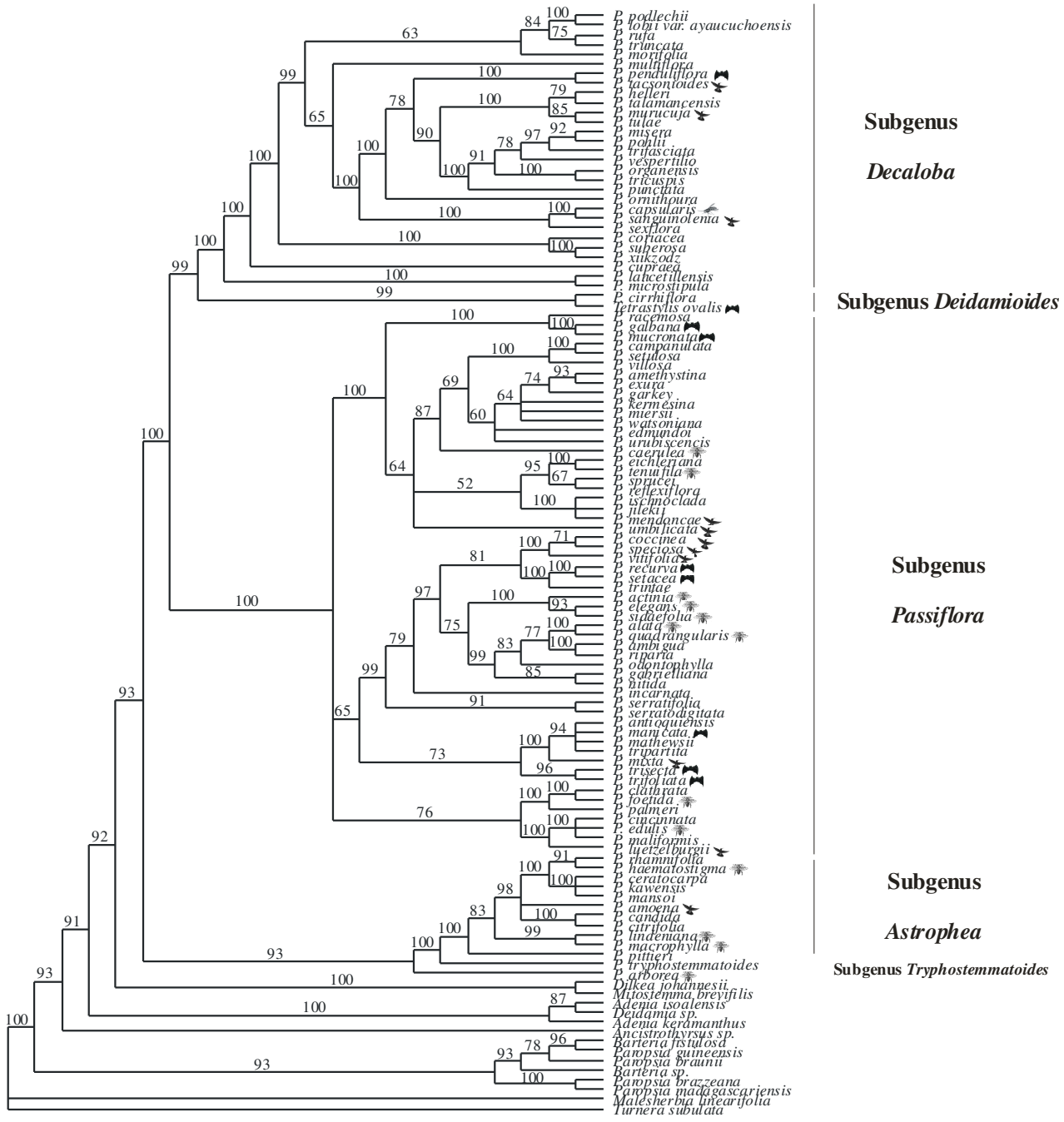
Figure 1: Phylogenetic tree based on the four cpDNA regions and obtained by the Bayesian approach. Numbers above the branches refer to posterior probabilities (PP) values. Pollinator agents are represented as follows:  = bat;  = hummingbird;  = wasp;  = bee.

Figure 2: Phylogenetic tree considering the two mtDNA regions, obtained by the Bayesian method. Numbers above the branches refer to posterior probabilities (PP) values.

Figure 3: Phylogenetic tree for the *26S* nrDNA gene obtained by the Bayesian approach. Numbers above the branches refer to posterior probabilities (PP) values.

Figure 4: Phylogenetic tree considering all seven DNA regions studied by the Bayesian analysis. Numbers above the branches refer to posterior probabilities (PP) values.

Figure 5: Maximum likelihood phylogenetic tree considering all the seven DNA regions. Numbers above the branches refer to bootstrap (BS) values.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P. villosa
P. amethystina
P. exura
P. garkeyi
P. kermesina
P. miersii
P. watsoniana
P. edmundoi
P. urubiscensis
P. caerulea
P. eichleriana
P. tenuifila
P. spruceii
P. reflexiflora
P. ischnoclada
P. jilekii
P. mendoncae
P. umbilicata
P. coccinea
P. speciosa
P. vitifolia
P. recurva
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P. riparia
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P. nitida
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P. macrophylla
P. pittieri
P. tryphostemmatoides
P. arborea
Dilkea johannesii
Mitostemma brevifilis
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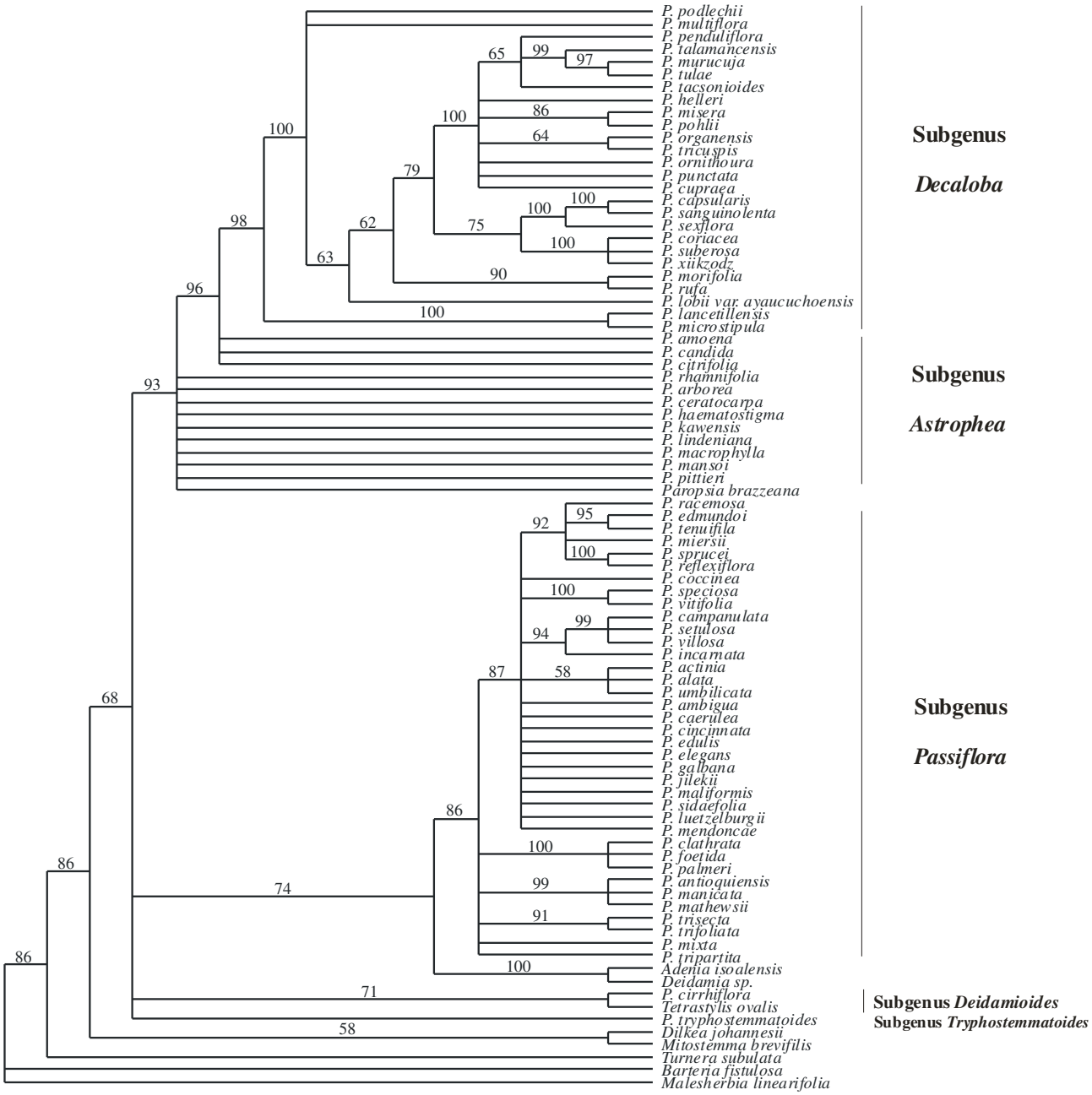
Deidamia sp.
Adenia keramanthus
Ancistrothyrsus sp.
Barteria fistulosa
Paropsia guineensis
Paropsia braunii
Barteria sp.
Paropsia brazzeana
Paropsia madagascariensis
Malesherbia linearifolia
Turnera subulata
Subgenus Decaloba
Subgenus Deidamioides
Subgenus Passiflora
Subgenus Astrophea
Subgenus Tryphostemmatoides

Figure 1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P. cirrhiflora
Tetrastylis ovalis
P. tryphostemmatoides
Dilkea johannesii
Mitostemma brevifilis
Turnera subulata
Barteria fistulosa
Malesherbia linearifolia
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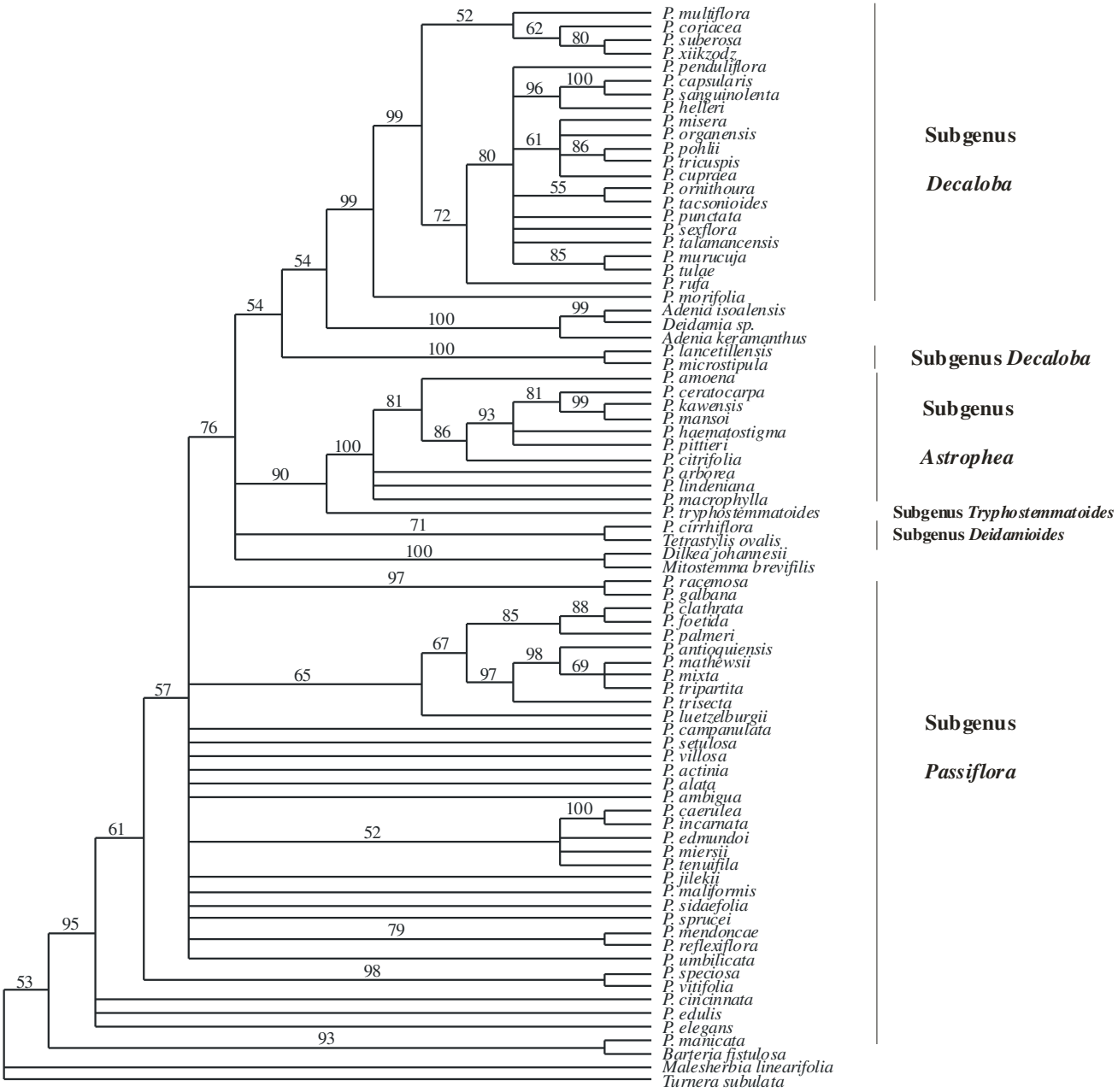
Subgenus Astrophea
Subgenus Passiflora
Subgenus Deidamioides
Subgenus Tryphostemmatoides

Figure 2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P. cincinnata
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P. elegans
P. manicata
Barteria fistulosa
Malesherbia linearifolia
Turnera subulata
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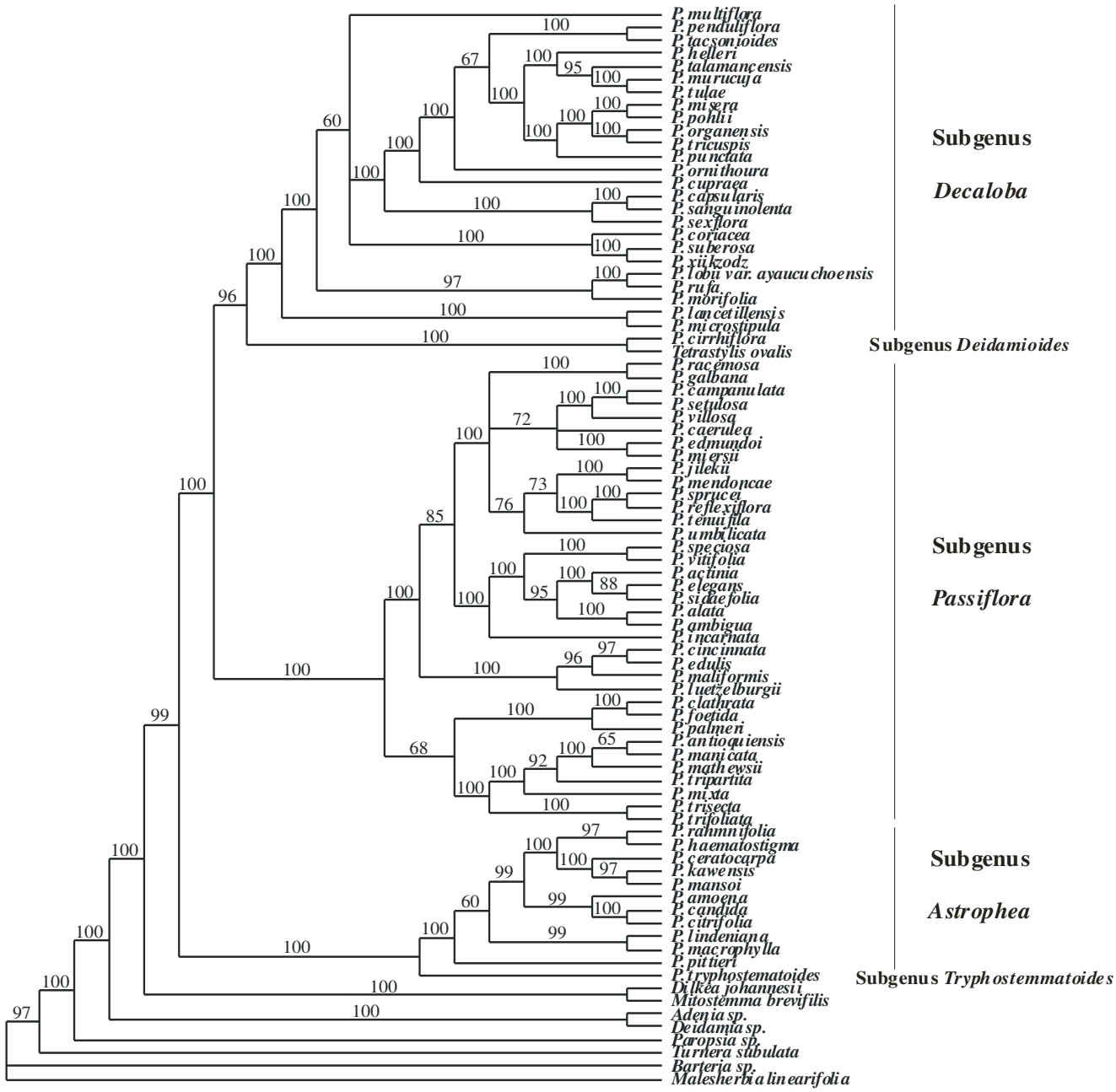
Subgenus Astrophea
Subgenus Tryphostemmatoides
Subgenus Deidamioides
Subgenus Passiflora

Figure 3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Subgenus Astrophea
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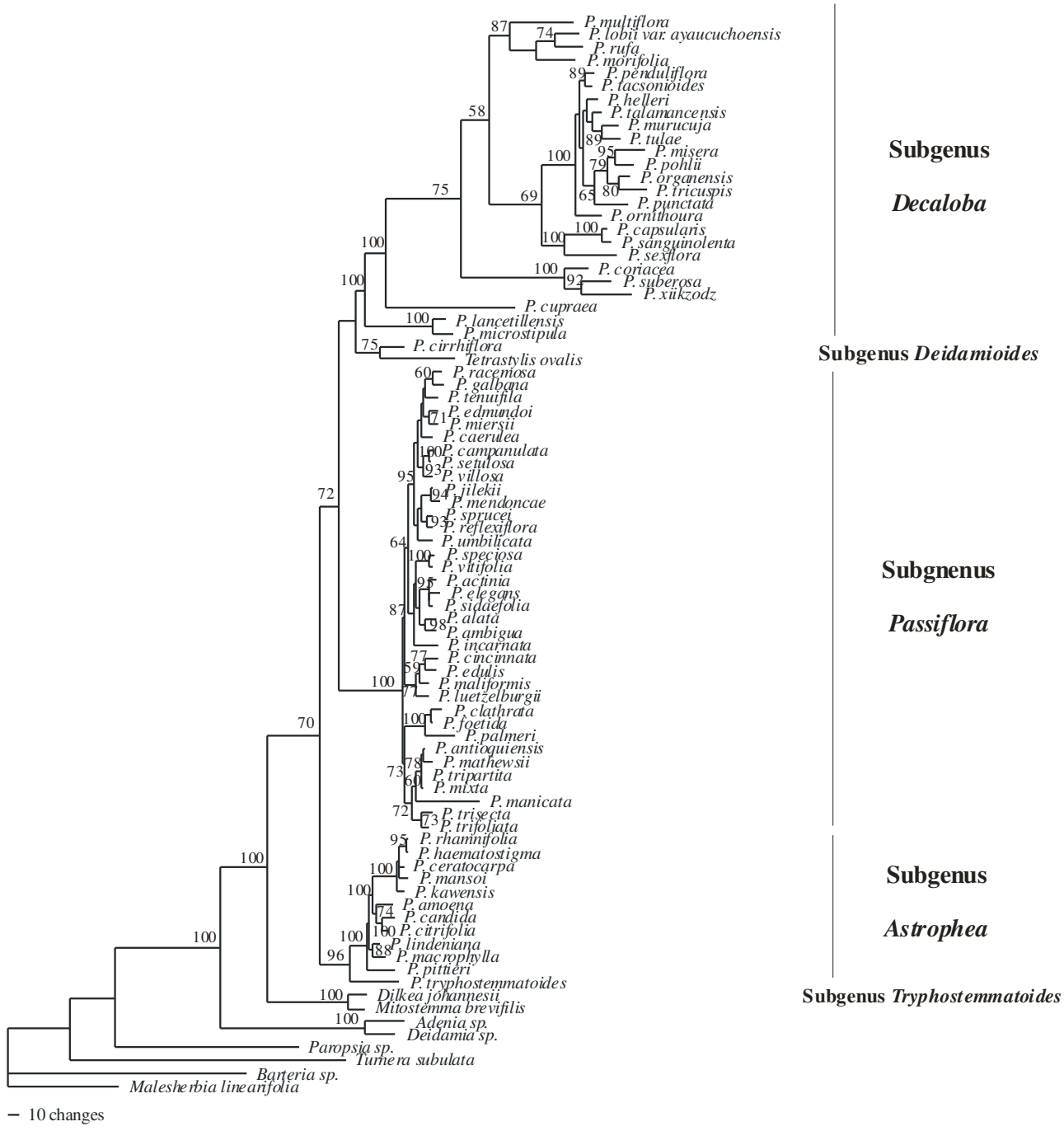
Figure 4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Paropsia sp.
Turnera subulata
Barteria sp.
Malesherbia linearifolia
10 changes
Subgenus Decaloba
Subgenus Deidamioides
Subgnenus Passiflora
Subgenus Astrophea
Subgenus Tryphostemmatoides

Figure 5

CAPÍTULO IV

2º ARTIGO

A ser submetido para a revista *Systematic Botany*

DIVERGENCE TIME AND EVOLUTIONARY RATES IN *Passiflora*

Running head: PASSIFLORA EVOLUTIONARY HISTORY

Divergence Time and Evolutionary Rates in *Passiflora*

VALÉRIA C. MUSCHNER[1], SANDRO L. BONATTO[2], FRANCISCO M. SALZANO[1] and LORETA B. FREITAS[1,3]

[1]Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Caixa Postal 15053, 91501–970 Porto Alegre, RS, Brazil

[2]Centro de Biologia Genômica e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Ipiranga 6681, 90610–001 Porto Alegre, RS, Brazil

[3]Author for Correspondence (loreta.freitas@ufrgs.br)

Address to which proofs should be sent
Loreta B. Freitas
Departamento de Genética, UFRGS
Caixa Postal 15053
91501–970 Porto Alegre, RS
Brazil
Phone: 55 51 33166715. Fax: 55 51 33166727.
E–mail: loreta.freitas@ufrgs.br

ABSTRACT A total of 70 species of *Passiflora* distributed over five subgenera, plus nine outgroups from related genera and representatives from eight other angiosperm families were examined in relation to two chloroplast (*rbcL*, *rps4*), one mitochondrial (b/c intron of the *nad1* gene), and one nuclear ribosomal (*26S*) DNA regions to establish evolutionary rates and divergence times. In a separate analysis the monophyly of Southeast Asian and Australian species of the *Disemma* section was confirmed. *Passiflora* shows heterogeneous substitution rates among subgenera, probably related to coevolution with pollinator agents. Its disjunct distribution (Americas / Southeast Asia–Australia) could probably be explained by Trans–Pacific dispersion which occurred about 42 million years ago (Ma). The first of the three main subgenera to separate was *Decaloba*, at about 35 Ma, the diversification of *Passiflora* and *Astrophea* occurring much later (24 Ma).

The advantages of DNA sequences for the inference of phylogenetic relationships among different organisms has been amply demonstrated (Savolainen et al. 2000; APG II, 2003; Muschner et al. 2003) and several studies had identified variation in the rates of evolution of genes and lineages (Avise 1994; Li 1997; Page and Holmes 1998; Hebert et al. 2002; Soltis et al. 2002). Those, in turn, led to the development of methods for the estimation of divergence time (Rambaut and Bromham 1998; Sanderson 2002; Thorne and Kishino 2002). The use of local strict molecular clock (Zuckerkandal and Pauling 1962, 1965), which follow the principles of the neutral theory of molecular evolution (Kimura 1983), calibrated by the fossils or tectonic moviments, is being increasingly used as a biogeographical tool for dating the divergence between clades (Renner 2004; Givinish and Renner 2004).

Renner et al. (2001) asserted that the difficulties encountered in the performance of biogeographic works dealing with tropical angiosperms, and specifically the identification of sister groups and divergence times, are due to their high variability and the scarcity of study material. Biogeographical analyses of the tropical flora attribute Transtropical disjunctions at high taxonomic levels to the Gondwana break–up (Raven and Axelrod 1974; Gentry 1982, 1993; Barlow 1990; Burnham and Graham 1999). This interpretation, however, imply divergence times of 100–90 millions of year ago (Ma) between the African neotropical clades, and higher values for taxa also found in Indochina and Southeast Asia. In addiction, fossils supporting the Gondwana break–up are available for only a few pantropical eudicot families. In the absence of an adequate fossil record for key areas like South America (Burnham and Graham 1999), the controversy between break–up Gondwana explanations and those which rely in more recent long–distance dispersion events for the interpretation of present distribution patterns remains unsettled.

Sanmartín e Ronquist (2004) found that in plants the hierarchical patterns observed by them were incongruent with the sequence of commonly accepted geological events, and that they seem to have been molded by more recent dispersion and extinction events that occurred in the southern hemisphere.

Passiflora L. is a large neotropical genus which has about 520 species. Its taxonomy was until recently rather complicated due to the high variability of its flowers and vegetative structures. Feuillet and MacDougal (2003), based in morphological characteristics, divided the genus in four subgenera: *Astrophea*, *Decaloba*, *Deidamioides*, and *Passiflora*. Muschner et al. (in mss.) studied seven DNA regions distributed over the chloroplast (cp), mitochondrial (mt) and nuclear (n) genomes, suggesting that the genus should be subdivided in the four above–indicated subgenera, plus *Tryphostemmatoides*. According to Ulmer and MacDougal (2004) *Astrophea* and *Deidamioides* occur in Central and South America, *Passiflora* in North and South America, while *Decaloba* has a disjunct distribution, with a group in North and South America and another in Southeast Asia and Australia (Fig. 1). *Tryphostemmatoides*, with only two species, was up to now only found in Colombia (South America).

Astrophea, *Decaloba*, and *Passiflora* exhibit monophyletic groups with high statistical support in the molecular analyses (Muschner et al. 2003; Krosnick and Freudenstein 2005). The latter authors based their analyses in six Southeast Asian and three Australian species of the *Disemma* section of the *Decaloba* subgenus.

The present study investigated four regions of the three genomes (cpDNA, mtDNA, nDNA) of 70 widely distributed species of *Passiflora*, to estimate the dates of the genus probable origin, of subgenera diversification, and their relationship with biogeographical and/or historical events.

MATERIALS AND METHODS

Taxon Sampling and Laboratory Methods. The *Passiflora* species studied, their subgenera classification, nine species from eight other genera used as outgroups, the source of the material, and the GenBank accession numbers of the sequences determined are displayed in Table 1. Since there is no fossil record for the Passifloraceae (maybe because these plants are pollinated by animals; see Proctor et al. 1996) sequences from representatives of seven other angiosperm genera for which fossil data information are available were obtained from GenBank for purposes of comparison and tree calibration. Information about them is given in Table 2.

Total DNA was extracted by Roy et al.'s (1992) method, and the regions studied were as follows: 1. cpDNA *rbcL* and *rps4* genes, amplified with primers 1F and 1460R (Savolainen et al. 2000) and rps45' and rps43' (Souza–Chies et al. 1997) respectively; 2. mtDNA intron b/c of the gene *nad1* gene, amplified by nad1/2 and nad1/3 (Duminil et al. 2002); and 3. partial nuclear ribosomal DNA *26S* gene, amplified by N–nc26S1 and 1229r (Kuzoff et al. 1998). Sequencing primers were the same indicated by the above–indicated authors, except for the *nad1* b/c intron, for which an internal primer was devised, specific for *Passiflora* (5' – ATTCACATAGAGACAGACT). PCR products were cleaned using the polyethylene glycol/NaCl precipitation method of Dunn and Blattner (1987). Sequencing was performed in a MegaBace 1000 machine (Amersham Biosciences) using a DYEnamicTM ET termination cycle sequencing premix kit (Amersham Biosciences), with the protocol provided by the manufacturer. Sequence alignments were manually conducted, with ambiguous regions being excluded from the analyses. The sequences were deposited in the Genbank (accession numbers provided in Table 1).

Molecular dating. Bayesian methods (Thorne et al. 1998. Kishino et al. 2001; Thorne and Kishino 2002) were employed using the MULTIDISTRIBUTE package (available at http://statgen.nscu.edu/thorne/multidivtime). This parametric approach relaxes the hypothesis of a strict molecular clock through a continuous autocorrelation in the substitution rates across the phylogeny and allows the use of several calibration time constraints.

MULTIDIVTIME divergence date estimates were performed in two stages: first the parameters of nucleotide substitution were estimated using the BASEML program of the PAML package (Yang 1997), using the F84 + G model (with five rate categories). Tree topology was estimated by maximum likelihood (ML) with the PAUP*4.0 program (Swofford, 1998) using the model selected by the MODELTEST (GTR + I + G) (Posada and Crandall 1998). The PAML2MODELINF program from the MULTIDISTRIBUTE package was employed to convert the BASEML output in an archive acceptable for ESTBRANCHES, also from the MULTIDISTRIBUTE package. The latter was used to calculate the variance–covariance matrix and the respective trees' branch lengths.

ESTBRANCHES outputs served as MULTIDIVTIME inputs and the divergence times were estimated using the appropriate calibration points. *A priori* and *a posteriori* branch ages, their standard errors, and the 95% confidence intervals were inferred via Markov chain Monte Carlo (MCMC) calculations. MCMC was run for 1,000,000 generations and sampled every 100 generations after an initial bur–in period of 10,000 cycles. To check for convergence analyses were run from two different starting points.

The following prior distributions were used in these analyses: 108 Ma (standard deviation, SD: 54 Ma) for the expected time between tip and root if there were no constraints; 0.001 (SD: 0.0005) substitutions per site per million year for the rate of the

root node; 0.1 (SD: 0.1) for the parameters which determine the magnitude of autocorrelation per million years; and 300 Ma for the larger value of the time unit between the root and the tips. The parameters were chosen following the MULTIDIVTIME program manual.

Old World *Passiflora*. Sequences of the cpDNA *trnL–trnF* and of the nrDNA ITS1 and ITS2 spacers previously published by Muschner et al. (2003) and Krosnick and Freudenstein (2005), in a total of 32 *Passiflora* species of the *Decaloba* subgenus, were used to confirm the *Disemma* section monophyly. For this purpose we utilized the maximum likelihood (using PAUP* 4.0) and Bayesian (MrBayes v3.0b4; Ronquist and Huelsenbeck 2003) programs.

RESULTS

Sequence and Tree Characteristics. The size of alignment of the four combined DNA regions was of 3,040 nucleotides, since due to distant relationships of the Passifloraceae with the other families used for tree calibration large regions had to be excluded from the analysis. The average rate of nucleotide substitution obtained by the MULTIDIVTIME program was of 0.0002 substitutions / site / million years. This rate is however higher in the *Decaloba* subgenus, as can be observed by the branch lenghts of the ML tree displayed in Fig. 2.

Since Krosnick and Freudenstein (2005) included a limited number of New World species in their analysis we decided to reanalyze their data including a larger number of *Decaloba* subgenus taxa. The results of the Bayesian analysis are presented in Fig. 3. *Disemma*'s section monophyly was confirmed with high statistical support (PP:

100; BS: 97), the Australian and Southeast Asian species separating as distinct clusters. Basically the same results were obtained using a maximum likelihood approach.

Divergence Date Estimates. Fig. 2 shows the divergence dates obtained. The separation between Old World (*Barteria* and *Paropsia* genera) and New World taxa appears to have occurred about 56 ± 18 million years ago (Ma). The next event (diversification of the Passifloraceae family in New World) should be have happened 44 ± 17 Ma, and shortly afterwards (42 ± 16 Ma) the branching of the *Passiflora* genus occurred. The first of the subgenera which separated from the others was *Decaloba* (35 ± 15 Ma), while the split between the two others should have occurred 24 ± 14 Ma.

DISCUSSION

This is the first study which considers *Passiflora*'s biogeographical history and its subgenera diversification. Wikström et al. (2001) in a work involving 560 plant families and three molecular markers (the *rbcL* e *atpB* cpDNA genes and *18S* nrDNA) estimated a diversification time for the Passifloraceae of 32–36 Ma. We are proposing an older (56 Ma) date, and note that Bremer et al. (2004), after studying six cpDNA markers in a larger number of Asterid species, also obtained older dates from those of the above–indicated authors for this group of plants. The application of molecular dating techniques to diverse sets of data are helping to identify changes in speciation rates and to address questions about the ecological, geographical, and temporal factors which may influence these rates (Penington and Dick 2004).

We tried to minimize the problems which may arise by use of calibration points just in the terminal nodes of a phylogeny (Sanderson 1997), by the inclusion of several

external groups, and the highest number of dates possible. Despite this fact, the ages derived from the present study should be considered with caution, as a first approximation of the main events which occurred in *Passiflora*'s evolution.

Even though the sampling at the molecular level of this study is lower than those of similar analyses (Renner 2004; Richardson et al. 2004; Bell and Donoghue 2005; Yuan et al. 2005) there are evidences for a Gondwanic origin of the Passifloraceae family, and according to Raven and Axelrod (1974) the migration between South America and Africa could have occurred even after the Gondwana break–up, 90–105 Ma. Morley (2003) reviewed the potential world migration routes for the megathermal angiosperms, suggesting, for example, that the South American and African connections should have existed up to the Oligocene (around 35 Ma). This should have happened via stepping stone dispersal across islands of the Rio Grande Rise and the Walvis Ridge (which according to Parrish 1993 was above water southwest of the coast of Africa up until that time), as well as through the Sierra Leone Rise.

Dispersion through Laurasia during the Eocene climatic optimum, when the conditions supported a tropical vegetation, could be the best explanation for many organisms that now have a disjunct distribution among the tropics of South America, Africa, and Southeast Asia (Richardson et al. 2004). Other studies (Renner et al. 2001; Davis et al. 2002) suggested a boreotropical migration into southern areas during the Oligocene and Miocene (35–23 Ma). Molecular phylogenetic studies have also demonstrated that the role of long–distance dispersals to explain modern distribution patterns may have been subestimated (Renner et al. 2001; Renner, 2004; Yuan et al. 2005).

The *Passiflora* disjunct distribution could be explained by a Trans–Pacific (Sanmartín and Ronquist 2004). The [(South America, New Zealand) Australia]

relationship is the most frequent found in the flora and fauna of the Southern Hemisphere, and is in conflict with the geologically predicted vicariance patterns (Renner et al. 2000; Winkworth et al. 2002). Sanmartín and Ronquist (2004) documented a highly asymmetrical plant directional dispersion, a westward long–distance dispersal from South América to New Zealand against the prevailing wind and oceanic currents (Winkworth et al. 2002). Instead of direct jumps, the dispersal could have occurred in a stepping–stone way along the Antarctic coastline (Renner et al. 2000). This hypothesis is supported by the presence of temperate florests in these areas until at least the Pliocene (Swenson and Bremer 1997; Sanmartín and Ronquist, 2004). This dispersal could have been mediated by the west–flowing East Wind Drift, which runs close to the Antarctic coast, or could have followed the West Wind Drift around Antarctica, involving dispersal first to the subantarctic islands (and/or Australia) and from there to New Zealand (Swenson and Bremer, 1997). This pattern would explain the clade monophyly that we observed in the Southeast Asian and Australian species of *Passiflora*.

Differences in evolutionary rates are widespread in plants (Muse 2000), and can be ascribed to intrinsic factors like genome type (cp, mt, nuclear) or specific regions, as well as to extrinsic ones like the speed of group speciaton or population size (Bousquet et al. 1992; Muse 2000; Andreasen and Baldwin 2001; Barraclough and Savolainen 2001). The heterogeneous and high rates of nucleotide substitution found in *Passiflora*, that do not seem to be gene- or genome-specific (data not shown), can be used as a comparison for those found in other taxa.

As was already mentioned, the *Decaloba* subgenus has larger branches than those of the *Passiflora* and *Astrophea* subgenera, which presented a pattern of accelerated radiation. The mechanisms that lead to high lineage diversification constitute a central

question in evolutionary biology (Malcomber 2002). The classical examples of rapid species radiation are all geographically restricted, with high sympatry levels. In these cases, low levels of interspecific competition in poor habitats have been suggested as important (Jensen 1990; Liem 1990). But key innovations could also increase the radiation of a given lineage (Hodges and Arnold 1994; Malcomber 2002). Although interspecific competition could be involved in the rapid diversification of the *Passiflora* and *Astrophea* subgenera, it is more likely that the key factors are related to plant morphology and pollination agents, as detailed below.

Species of the *Passiflora* subgenus are characterized by handsome flowers that are usually dominated by a corona that is usually zoned or banded in different colors. The corona is the seat of scent production, and this subgenus contains de majority of intensely fragrant species. The corona is also the landing platform for bees and other insects which are attracted by odor (Ulmer and MacDougal 2004). In the species pollinated by hummingbirds, however, the corona is usually reduced.

On the other hand, *Astrophea* flowers have a short floral tube and a white coloring that contrast with the often bright yellow corona. They are probably pollinated by large bees. In other section of this subgenus orange or reddish to purple flowers occur with a conspicuous floral tube that is longer than the sepals, and a reduced corona. In these cases the pollinators are probably hummingbirds (Ulmer and MacDougal 2004).

We are, therefore, observing a wonderful example of coevolution between flower morphology and pollination agents that are now being corroborated by molecular data.

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Table 1: Species studied, their subgenera classification, places of collection, and GenBank accession numbers of the sequences determined.

Subgenera	Species	Source of the material and collector names	GenBank numbers			
			rbcL	*rps4*	*nad1* b/c	*26S*
Astrophea	*P. amoena* L. K. Escobar	Italy, Ripalta Cremasca, Colection (MV)	DQ123300	DQ123407	DQ123214	DQ122935
	P. arborea Spreng.	Panama (RY)	DQ123301	DQ123408	DQ123215	DQ122936
	P. ceratocarpa Silveira	Brazil, PA (LCB)	DQ123303	DQ123410	DQ123217	DQ122937
	P. citrifolia (Juss.) Mast.	French Guiana (MV)	DQ123304	AY212311	DQ123218	DQ122938
	P. haematostigma Mart. ex Mast.	Guaratuba, PR (ACC)	DQ123305	AY212292	DQ123219	DQ122939
	P. kawensis Feuillet	French Guiana (RY)	DQ123306	DQ123411	DQ123220	DQ122940
	P. lindeniana Tr. & Pl.	Italy, Ripalta Cremasca, Colection (MV)	DQ123307	DQ123412	DQ123221	DQ122941
	P. macrophylla Spruce ex Mast.	Brazil (MV)	DQ123308	AY212313	DQ123222	DQ122942
	P. mansoi (Mart.) Mast.	Chapadão do Sul, MS (ACC)	DQ123309	AY212307	DQ123223	DQ122943
	*P. pittieri*Mast.	Italy, Ripalta Cremasca, Colection (MV)	DQ123310	DQ123413	DQ123224	DQ122944
Decaloba	*P. capsularis* L.	Quatro Barras, PR (ACC)	DQ123312	DQ123415	DQ123226	DQ122946
	P. coriacea Juss.	Colombia (MV)	DQ123313	DQ123416	DQ123227	DQ122947
	P. cupraea L.	Bahamas (MV)	DQ123378	DQ123459	DQ123274	DQ122993
	P. helleri Peyer	Mexico (MV)	DQ123314	DQ123417	DQ123228	DQ122948
	P. lancetillensis MacDougal & Meerman	French Guiana (MV)	DQ123331	AY212312	DQ123242	DQ122961
	P. microstipula Gilbert & MacDougal	Mexico (MV)	DQ123332	DQ123434	DQ123243	DQ122962
	P. misera HBK.	Santa Maria, RS (PASS)	DQ123317	DQ123420	DQ123230	DQ122949
	P. morifolia Mast. in Mart.	Brazil, RS (PASS)	DQ123318	AY212314	DQ123231	DQ122950
	P. multiflora L.	Dominica (MV)	DQ123297	DQ123404	DQ123211	DQ122933
	P. murucuja L.	Blois-France-Greenhouse (RY)	DQ123345	DQ123442	DQ123255	DQ122974
	P. organensis Gardn.	Brazil, PR (ACC)	DQ123319	DQ123421	DQ123232	DQ122951
	P. ornithoura Mast.	Guatemala (MV)	DQ123320	DQ123422	DQ123233	DQ122952
	P. penduliflora Bertero ex DC.	Blois-France-Greenhouse (RY)	DQ123298	DQ123405	DQ123212	DQ122934

Table 1 (Cont.)

Subgenera	Species	Source of the material and collector names	GenBank numbers			
			rbcL	*rps4*	*nad1* b/c	*26S*
	P. pohlii Mast. in Mart.	Pirapora, MG (ACC)	DQ123321	DQ123423	DQ123234	DQ122953
	P. punctata L.	Peru (MV)	DQ123322	DQ123424	DQ123235	DQ122954
	P. rufa Feuillet	French Guiana (MV)	DQ123323	AY212315	DQ123236	DQ122955
	P. sanguinolenta Mast.	Ecology & Evolutionary Biology Conservatory, Univ. Connecticut (RY)	N/A	DQ123462	DQ123276	DQ122996
	P. sexflora Juss.	Dominican Republic (MV)	DQ123324	DQ123426	DQ123237	DQ122956
	P. suberosa L.	Brazil, RS (PASS)	DQ123325	DQ123427	DQ123238	DQ122957
	P. tacsonioides Griseb.	Blois-France-Greenhouse (RY)	DQ123379	DQ123461	DQ123275	DQ122995
	P. talamancensis Killip	Costa Rica (MV)	DQ123326	DQ123428	DQ123239	DQ122958
	P. tricuspis Mast. in Mart.	Brazil, SP (MCD)	DQ123327	DQ123429	DQ123240	DQ122959
	P. tulae Urban	Puerto Rico (MV)	DQ123346	DQ123443	DQ123256	DQ122975
	P. xiikzodz MacDougal	Italy, Ripalta Cremasca, Colection (MV)	DQ123330	DQ123433	DQ123241	DQ122960
Passiflora	*P. actinia* Hook	Brazil, RS (PASS)	DQ123347	AY212301	DQ123257	DQ122976
	P. alata Curtis	Brazil, RS (PASS)	DQ123348	AY212323	DQ123258	DQ122977
	P. ambigua Hemsl.	Brazil, MT (LCB)	DQ123349	DQ123444	DQ123259	DQ122978
	P. antioquiensis Karst.	Italy, Ripalta Cremasca, Colection (MV)	DQ123342	DQ123439	DQ123252	DQ122971
	P. caerulea L.	Brazil, RS (PASS)	DQ123350	AY212316	DQ123260	DQ122979
	P. campanulata Mast.	Brazil, PR (ACC)	DQ123339	AY212317	DQ123249	DQ122968
	P. cincinnata Mast.	Brazil, MS (ACC)	DQ123351	AY212294	DQ123261	DQ122980
	P. clathrata Mast.	Brazil, MG (FCN)	DQ123336	DQ123437	DQ123246	DQ122965
	P. edmundoi Sacco	Brazil, BA (NFM)	DQ123352	AY212302	DQ123262	DQ122981
	P. edulis Sims	Brazil, RS (PASS)	DQ123353	AY212303	DQ123263	DQ122982
	P. elegans Mast.	Brazil, RS (PASS)	DQ123355	AY212295	DQ123264	DQ122983
	P. foetida L.	Brazil, PE (NFM)	DQ123337	AY212291	DQ123247	DQ122966
	P. galbana Mast.	Camocin S. Felix, PE (NFM)	DQ123358	DQ123446	DQ123265	DQ122984

Table 1 (Cont.)

Subgenera	Species	Source of the material and collector names	GenBank numbers			
			rbcL	*rps4*	*nad1* b/c	*26S*
	P. incarnata L.	Brazil, SP (BGJ)	DQ123360	AY212306	DQ123266	DQ122985
	P. jilekii Wawra	Brazil, SC (ACC)	DQ123361	AY212318	DQ123267	DQ122986
	P. luetzelburgii Harms	Brazil, BA (TSN)	DQ123384	DQ123467	DQ123281	DQ122999
	P. maliformis L.	Dominica (MV)	DQ123362	AY212321	DQ123268	DQ122987
	P. manicata (Juss.) Pers.	Blois-France-Greenhouse (RY)	DQ123344	DQ123441	DQ123254	DQ122973
	P. mathewsii (Mast.) Killip	Blois-France-Greenhouse (RY)	DQ123380	DQ123463	DQ123277	DQ122994
	P. miersii Mast. in Mart.	Brazil, SP (PASS)	DQ123363	DQ123449	DQ123269	DQ122988
	P. mixta L. f.	(RY)	DQ123381	DQ123464	DQ123278	DQ122997
	P. palmeri var. sublanceolata Killip	Italy, Ripalta Cremasca, Colection (MV)	DQ123338	DQ123438	DQ123248	DQ122967
	P. racemosa Brot.	Brazil, RJ (FCN)	DQ123311	DQ123414	DQ123225	DQ122945
	P. reflexiflora Cav.	Ecuador (MV)	DQ123386	DQ123469	DQ123283	DQ123001
	P. setulosa Killip	Brazil, PR (ACC)	DQ123340	AY212297	DQ123250	DQ122969
	P. sidaefolia M. Roemer	Brazil, MG (MCD)	DQ123372	AY212298	DQ123270	DQ122989
	P. speciosa Gardn.	Brazil, MS (ACC)	DQ123334	AY212293	DQ123244	DQ122963
	P. sprucei Mast.	Italy, Ripalta Cremasca, Colection (MV)	DQ123373	DQ123456	DQ123271	DQ122990
	P. tenuifila Killip	Brazil, RS (PASS)	DQ123374	AY212299	DQ123272	DQ122991
	P. tripartita var. mollissima (Juss.) Poir.	(RY)	DQ123382	DQ123465	DQ123279	DQ122998
	*P. trisecta*Mast.	Blois-France-Greenhouse (RY)	DQ123343	DQ123440	DQ123253	DQ122972
	P. umbilicata (Griseb.) Harms	Blois-France-Greenhouse (RY)	DQ123387	DQ123470	DQ123284	DQ123002
	P. villosa Vell.	Brazil, MG (ACC)	DQ123341	AY212308	DQ123251	DQ122970
	P. vitifolia HBK.	Colombia (MV)	DQ123335	DQ123436	DQ123245	DQ122964
Deidamioides	*P. cirrhiflora* Juss.	Italy, Ripalta Cremasca, Colection (MV)	DQ123377	DQ123459	DQ123273	DQ122992

Table 1 (Cont.)

Subgenera	Species	Source of the material and collector names	GenBank numbers			
			rbcL	*rps4*	*nad1* b/c	*26S*
Tryphostemmatoides	*P. tryphostemmatoides* Harms	Blois-France-Greenhouse (RY)	DQ123388	DQ123471	DQ123285	DQ123003
Other	*Adenia isoalensis*	(MC)	DQ123389	DQ123472	DQ123286	DQ123004
	Adenia keramanthus	(MC)	DQ123390	DQ123473	DQ123287	DQ123005
	Deidamia sp.	(MC)	DQ123394	DQ123477	DQ123289	DQ123007
	Dilkea cf johannesii Barb. Rodr.	Peru (MC)	DQ123399	DQ123478	DQ123290	DQ123008
	Mitostemma brevifilis	Brazil, MS (ACC)	DQ123400	AY212309	DQ123291	DQ123009
	Barteria fitulosa	Nigeria (MC)	DQ123392	DQ123475	DQ123288	DQ123006
	Paropsia madagascariensis	(MC)	AF206802	AY216663	DQ123293	N/A
	Malesherbia linearifolia	Chile (KG)	DQ123402	DQ123482	DQ123294	DQ123011
	Turnera subulata	Brazil, BA (CB)	DQ123398	N/A	DQ123296	DQ123012

Collectors: ACC = A. C. Cervi; BGJ = Banco de Germoplasma de Jaboticabal; CB = C. van den Berg; FCN = F. Campos Neto; KG = K. Gengler; LCB = L. C. Bernacci; MC=Mark Chase; MCD = M. C. Dornelas; MV = M. Vecchia; NFM = N. F. Melo; PASS = our group; RY = R. Yockteng; TSN = T. S. Nunes.

Brazilian states: BA = Bahia; MG = Minas Gerais; MS = Mato Grosso do Sul; MT = Mato Grosso; PA = Pará; PR = Paraná; PE = Pernambuco; RJ = Rio de Janeiro; RS = Rio Grande do Sul; SC = Santa Catarian; SP = São Paulo

Table 2. Information about the sequences obtained in the GenBank for representatives of eight angiosperm families for which fossil data are available, used for comparison and tree calibration. *Extracted from Wikström et al. (2001). Ma: million years ago; N/A: not available.

		GenBank numbers			
Family	Fossil age* (Ma)	*rbcL*	*rps4*	*nad1* b/c	26S
Euphorbiacae	58	AF530850	N/A	AY674695	AF479125
Elaeocarpaceae	58	AF206765	N/A	N/A	AF479128
Cucurbitaceae	58	AF206756	NC007144	AF453648	AF479108
Combretaceae	84	AF206826	N/A	N/A	AF479147
Buxaceae	104	AF203486	AY188234	N/A	AF389244
Buxaceae	104	AF543712	N/A	N/A	AF389243
Platanaceae	108	L01943	AY188229	AY832123	AF274662

FIG. 1. World map showing the Passifloraceae distribution. The genus *Passiflora*, however, is only found in the Americas, Southeast Asia and Australia.

FIG. 2. a) Maximum likelihood tree obtained using data from the four combined DNA regions. b) Chronogram from the four combined DNA regions. PASS = Subgenus *Passiflora*; DECA = Subgenus *Decaloba*; ASTR = Subgenus *Astrophea*.

FIG. 3. *trnL–trnF*, ITS1 and ITS2 interspacer regions. Bayesian tree, indicating as shaded areas the Australian and Southeast Asian species.

Figure 1

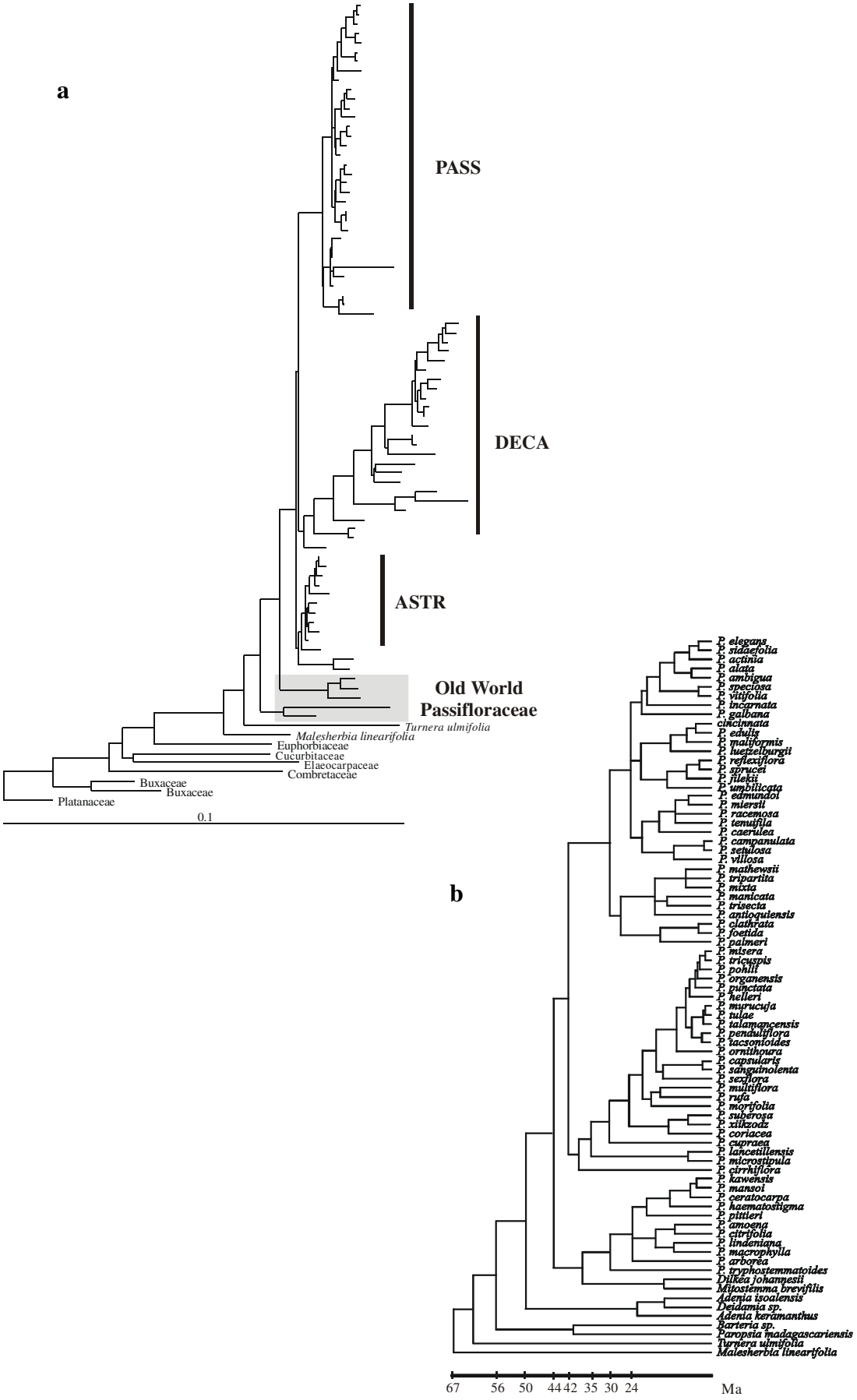
a
PASS
DECA
ASTR
Old World
Passifloraceae
Turnera ulmifolia
Malesherbia linearifolia
Euphorbiaceae
Cucurbitaceae
Elaeocarpaceae
Combretaceae
Buxaceae
Buxaceae
Platanaceae
0.1
b
P. elegans
P. nitidifolia
P. actinia
P. alata
P. ambigua
P. speciosa
P. vitifolia
P. incarnata
P. galbana
cincinnata
P. edulis
P. maliformis
P. luetzelburgii
P. reflexiflora
P. sprucei
P. filekii
P. umbilicata
P. edmundoi
P. miersii
P. racemosa
P. tenuifila
P. caerulea
P. campanulata
P. setulosa
P. villosa
P. mathewsii
P. tripartita
P. mixta
P. manicata
P. trisecta
P. antioquiensis
P. clathrata
P. foetida
P. palmeri
P. misera
P. tricuspis
P. pohlii
P. organensis
P. punctata
P. helleri
P. murucuja
P. tulae
P. talamancensis
P. penduliflora
P. tacsonioides
P. ornithopura
P. capsularis
P. sanguinolenta
P. sexflora
P. multiflora
P. rufa
P. morifolia
P. suberosa
P. xiikzodz
P. coriacea
P. cupraea
P. lancetillensis
P. microstipula
P. cirrhiflora
P. kawensis
P. mansoi
P. ceratocarpa
P. haematostigma
P. pittieri
P. amoena
P. citrifolia
P. lindeniana
P. macrophylla
P. arborea
P. trypanovitemmatoides
Dilkea johannesii
Mitostemma brevifilis
Adenia isoalensis
Deidamia sp.
Adenia kerrmanthus
Barteria sp.
Paropsia madagascariensis
Turnera ulmifolia
Malesherbia linearifolia
67 56 50 44 42 35 30 24
Ma

Figure 2

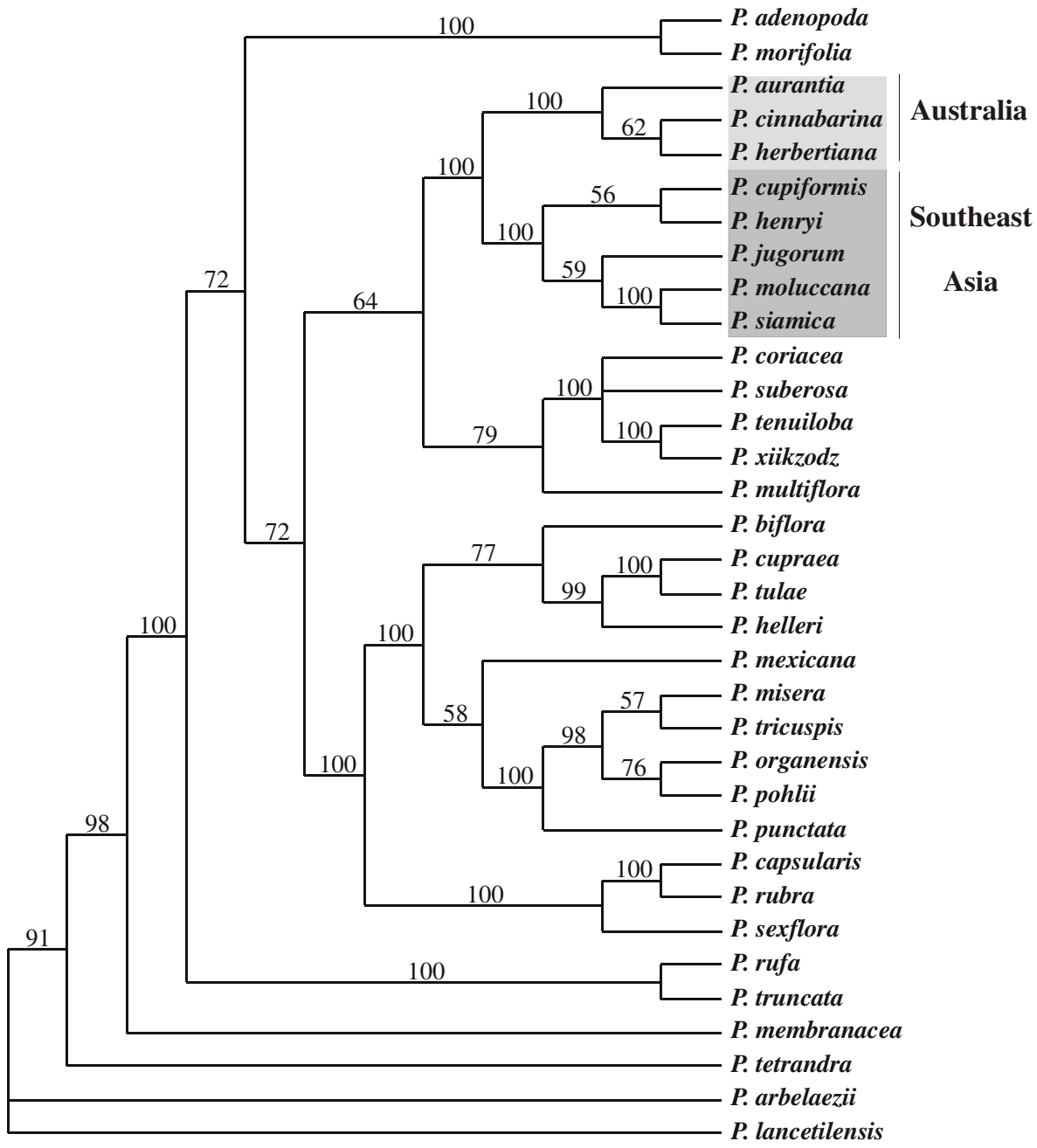
P. adenopoda
P. morifolia
P. aurantia
P. cinnabarina
P. herbertiana
Australia
P. cupiformis
P. henryi
P. jugorum
P. moluccana
P. siamica
Southeast Asia
P. coriacea
P. suberosa
P. tenuiloba
P. xiikzodz
P. multiflora
P. biflora
P. cupraea
P. tulae
P. helleri
P. mexicana
P. misera
P. tricuspis
P. organensis
P. pohlii
P. punctata
P. capsularis
P. rubra
P. sexflora
P. rufa
P. truncata
P. membranacea
P. tetrandra
P. arbelaezii
P. lancetilensis
100
100
62
100
56
100
59
100
72
64
100
100
79
72
77
100
99
100
100
58
57
98
100
76
98
100
100
91
100

Figure 3

CAPÍTULO V

3º ARTIGO

A ser submetido para a revista *American Journal of Botany*

ORGANELLAR INHERITANCE IN *Passiflora* (PASSIFLORACEAE)

ORGANELLAR INHERITANCE IN *PASSIFLORA* (PASSIFLORACEAE)[1]

VALÉRIA C. MUSCHNER,[2] ALINE P. LORENZ-LEMKE,[2] MAURIZIO VECCHIA,[3] SANDRO L. BONATTO,[4] FRANCISCO M. SALZANO,[2] AND LORETA B. FREITAS [2, 5]

[2]Programa de Pós-Graduação em Genética e Biologia Molecular, Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Caixa Postal 15053, 91501-970 Porto Alegre, RS, Brazil; [3]Via Roma 11/B, 26010 Ripalta Cremasca (CR) Italy; [4]Centro de Biologia Genômica e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Ipiranga 6681, 90610-001 Porto Alegre, RS, Brazil

[5]Correspondence: Loreta B. Freitas, Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Caixa Postal 15053, 91501-970 Porto Alegre, RS, Brazil. Phone: 55 51 33166715. Fax: 55 51 33166727. E-mail: loreta.freitas@ufrgs.br

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ABSTRACT

Analyses of four chloroplast (cp), one mitochondrial (mt), and one ribosomal nuclear (ITS) DNA regions studied in four artificial and one natural interspecific *Passiflora* hybrids indicated that while all mtDNAs were maternally inherited, the same was not true for cpDNA. The four hybrids (three induced and one natural) derived from species of the *Passiflora* subgenus showed paternal, but the one involving taxa of the *Decaloba* subgenus gave evidence of maternal transmission. These results are important for the ongoing studies which are being performed on the molecular evolution of this genus.

The two cytoplasmic plant genomes, chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) are generally inherited in an uniparental way (Birky 1995, 2001). Plastid inheritance has been more thoroughly studied in angiosperms (Corriveau and Coleman, 1988; Harris and Ingram, 1991; Mogensen, 1996; Zhang et al., 2003) than mtDNA inheritance (Sodmergen et al., 2002; Mohanty et al., 2003). Generally it was observed that in hermaphrodite species both organelles are transmitted together via a single–sex gamete which is female in the majority of the angiosperms (Dumolin-Lapègue et al., 1998, Moreira et al., 2002; Petit and Vendramin, 2005) but male in various gymnosperms (Chesnoy, 1987; Hagemann, 1992; Petit and Vendramin, 2005). Exceptions however have been observed in Pinaceae (Neale and Sederoff, 1989) and Actinidiaceae (*Actinidia*) (Chat et al., 1999; Burban and Petit, 2003), where the chloroplasts are paternally and the mitochondria maternally inherited. The opposite was observed in *Musa acuminata* (Musaceae) and in *Cucumis* (Cucurbitaceae), with the chloroplasts showing maternal and the mitochondria paternal inheritance (Fauré et al., 1994; Havey et al., 1998).

The *Passiflora* L genus belongs to the *Passifloraceae* family and is composed by 525 species mainly distributed in the Neotropical region; at present it is divided into four subgenera (Feuillet and MacDougal, 2003). The genus' species present considerable diversity, especially floral, the greater part of which is due to different expansions of the hypanthium. Another marked characteristic of the flowers is the corona of filaments, with a wide range of size, color and format which represent adaptations to different pollinators (MacDougal, 1994). *Passiflora* species have been cultivated for ornamental purposes and various interspecies hybrids were produced, so that up to 2003 over 300 hybrids and cultivated plants had been included in the *Passiflora Hybrids and Cultivars* (Ulmer and MacDougal, 2004a), thus increasing the morphological diversity of the group

Besides the morphological there is also considerable genetic variability. Muschner et al. (2003), studying 61 species, found high molecular variability for regions of the chloroplast and nuclear DNA. The results they obtained amply corroborated the new classification of the genus proposed by Feuillet and MacDougal (2003). Melo et al. (2001) described the basic chromosome number of various species, observing also variability in the genus (x=6, 9, 10 e 12). Within the *Passiflora* subgenus only x=6 was found, while in the *Decaloba* subgenus the other numbers were observed. Souza et al. (2004) measured the relative content of nuclear DNA and verified that the 2C nuclear content ranged from 3.16 to 5.36 for the diploid species and 1.83 for the tetraploid *P. suberosa* of the subgenus *Decaloba*.

Various analyses of the pollination mechanisms and of crossbreed compatibility have been developed, involving especially the species of higher economic interest, classified mainly in the subgenus *Passiflora.* In general, these species, which have much larger flowers than those of the subgenus *Decaloba*, present mechanisms of auto-incompatibility (Rêgo et al., 1999), reproducing by cross-fertilization, while *Decaloba* species are generally auto-compatible and reproduce by self-fertilization (Endress, 1994; Varassin and Silva, 1999).

Ulmer & MacDougal (2004b) mentioned Linda Escobar's unpublished findings on the paternal chloroplast inheritance in interspecific *Passiflora* hybrids. Previously, Corriveau and Coleman (1988), using cytological methods, suggested the possibility of biparental cpDNA inheritance in *P. edulis*. In both cases the hybrids which were analyzed were obtained by artificial crossbreeding. Lorenz-Lemke et al. (2005) reported a natural hybrid between *P. actinia* and *P. elegans*, with indirect evidence for paternal inheritance of the plastid genome. *Turnera ulmifolia,* a species of the Turneraceae family recently

inserted in Passifloraceae by APGII (2003), showed a tendency for paternal inheritance, although maternal and biparental inheritance were also observed (Shore and Triassi, 1998).

As part of an ongoing research program on *Passiflora* phylogenetics and population genetics (Muschner et al., 2003; Lorenz-Lemke et al., 2005), we studied the organelle inheritance in four artificial interspecies hybrids and one natural hybrid of *Passiflora*, evaluated through sequences of four cpDNA (*rps4*, *trnL* intron and the intergenic spacers *trnL-trnF* e *psbA-trnH*), one mtDNA (intron between exons *b* and *c* of the *nad1* gene) and one ribosomal nuclear DNA (ITS) regions.

MATERIALS AND METHODS

Plant material

The four hybrids analyzed were, 'P. Aurora' [*P. foe*tida (♀) x *P. palmeri* var. *sublanceolata* (♂)], 'P. Leida' [*P. incarnata* (♀) x *P. cincinnata* (♂)], 'P. Paola Gastaldo' [*P. incarnata* (♀) x *P. sprucei* (♂)], and 'P. Manta' [*P. xiikzodz* (♀) x *P. coriacea* (♂)]. The first three involved species of the *Passiflora*, while the latter included species of the *Decaloba* subgenera. To obtain them, the anthers covered with fresh pollen from the male parent were taken to the stigma of the female parent. The anthers of the mother plant had been cut off before its own pollen was mature. The pollination was performed in the early morning, and soon afterwards the flowers were covered with gauze to prevent contamination by unwanted pollen. The fruits obtained ripened in 2 or 3 months and the hybrid plants derived from them matured in two years. The interspecies natural hybrid (*P. actinia* x *P. elegans*) was described by Lorenz-Lemke et al. (2005).

DNA extraction, PCR amplification, sequencing, and analysis

Total DNA was extracted from young leaves, which were first dried in silica gel, using Roy et al.'s (1992) method. PCR amplification and sequencing were performed as follows: *rps*4, using the rps5 and trnS primers as described by Souza-Chies et al. (1997); *trnL-trnF* intergenic spacer and *trnL* intron, primers e, f and c, d, respectively, as described by Taberlet et al. (1991); for the *psbA-trnH* intergenic spacer, primers described by Sang et al. (1997). The *nad1/2* e *nad1/3* primers (Duminil et al., 2002) were used in the amplification of the b/c intron of the *nad1* gene, while the sequencing of the whole region was performed with the same PCR primer plus an internal primer specifically designed for *Passiflora* (5'-ATTCACATAGAGACAGACT). The internal transcribed spacers ITS1 and ITS2 of rDNA were amplified and sequenced with the primers described by Desfeux and Lejeune (1996).The PCR products were purified with PEG 20% (Dunn and Blattner, 1987) and the two strands were directly sequenced. Sequencing was performed on a MegaBace 1000 automatic sequencer (Amersham Biosciences) in accordance with the manufacturer's instructions. The sequences were deposited in the Genbank (Accession N^{os}). For the analysis of the natural hybrid between *P. actinia* (♂) and *P. elegans* (♀), the sequences of Lorenz-Lemke et al. (2005) and the analysis of the mitochondrial *nad1* b/c intron were utilized. Alignment of the sequences and identification of the variable sites were performed with the Mega 3.0 program (Kumar et al., 2004).

RESULTS AND DISCUSSION

The ITS nuclear regions were sequenced in the hybrids and their parents to confirm the hybrid condition of these individuals and eliminate the possibility of possible natural

contamination in our artificial fertilization process. By analyzing the heterozygote sites in the points where divergences in the parental sequences were detected, three of the four individuals were confirmed as interspecific hybrids. Due to the nature of the ITS region (scattered over all the genome and subjected to concerted evolution) the fact that no confirmation occurred for 'P. Paola Gastaldo' was not considered significant.

Characterization of the hybrids' sequences and those of their parents at the variable places is presented in Table 1. In relation to cpDNA, the parental species which originated the *'P. Aurora'* hybrid were identical for the 1685 nucleotides analyzed, except two *trnL* intron and two *psbA-trnH* bases. In these cases the hybrid presented a sequence identical to the *P. palmeri* (male parent) species. For the *'P. Leida'* and *'P. Paola Gastaldo'* hybrids the cpDNA inheritance (respectively 35 and 39 informative sites) was also strictly paternal. On the other hand the *'P. Manta'* hybrid (which was the only example of the subgenus *Decaloba* analyzed) presented a maternal pattern of inheritance (51 informative sites).

The mitochondrial DNA inheritance was strictly maternal in all the hybrids, in a total of 26 informative sites analyzed. Besides the point mutations, which were used to identify the maternal parent as the donor of the mitochondrial genome, we observed a large indel of 449 bp in the paternal *P. sprucei* and in *P. actinia*, as well as another of 5 bp in *P. coriacea*, which were not present in the hybrids.

The artificial hybrids results agree with Lorenz-Lemke et al.'s (2005) findings in a natural interspecies hybrid of *P. elegans* e *P. actinia*, both of the *Passiflora* subgenus. The sequences of the *trnL-trnF* and *psbA-trnH* chloroplast spacers of the hybrid were equal to those of *P. actinia*. The hybrid was observed in a *P. elegans* population, and the closest *P. actinia* population occurred 9 km apart. If the chloroplast inheritance of these species were maternal, two events involving long distances would have to have occurred. First, the *P.*

elegans pollen would have to have been transported to the *P. actinia* population and then, after the fruits had developed, their seeds would have to be dispersed far away from the plant and deposited in *P. elegans* territory. Given the rarity of the postulated events, the most economical hypothesis for the origin of the hybrid would be that a *P. elegans* plant had been impregnated by pollen from *P. actinia*, indicating a paternal origin for the plastids of this species. These observations reinforce the artificial hybrids findings and minimize the doubts about possible faulty manipulation in the artificial crosses as suggested by Birky (2001) as a source of error.

The mechanisms which result in different modes of inheritance of organelles between genera and within a given genus have been amply studied and discussed. They basically refer to the exclusion and/or degeneration of the paternal plastid during or after fertilization (Yang et al. 2000). However, a large number of alternatives have been compiled by different authors based mainly in cytological analyses after fecundation, to explain what happens to the maternal plastid (and its respective DNA) when the inheritance of the organelles is essentially paternal (Hagemann, 1992; Owens and Morris, 1990; Owens et al., 1995; Mogensen, 1996; Nagata et al., 1997). No such studies have been found for *Passiflora*, but the difference in mode of organellar inheritance confirms the distinction between the *Decaloba* and *Passiflora* subgenera observed in the molecular phylogeny (Muschner et al. 2003). Knowledge about the mode of inheritance of the cpDNA in *Passiflora* can provide important information for the interpretation of molecular data, especially in relation to cytoplasmic gene flow.

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TABLE 1: DNA regions showing informative variation about the interspecific hybrids and their parents.

trnL intron

	82	117	118	119	127	137	138	139	225	320	321	391	392	393	394	424	425
'P. Manta'	**C**	**T**	**-**	**-**	**-**	**A**	**A**	**T**	**-**	**C**	**G**	**T**	**-**	**C**	**A**	**A**	**A**
P. xiikzodz (♀)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. coriacea (♂)	A	G	T	T	T	-	-	G	a	T	-	A	A	T	T	C	T

trnL-trnF

	32	50	98	99	131	158
'P. Manta'	**T**	**T**	**G**	**C**	**C**	**A**
P. xiikzodz (♀)	.	.	.	.	.	.
P. coriacea (♂)	A	C	A	-	T	G

psbA-trnH

	41	50	57	67	75	77	78	83	115	147	168	182	183	184	185	186	191	192	196	197	198	199	200	247	269
'P. Manta'	**-**	**A**	**A**	**-**	**A**	**A**	**A**	**G**	**-**	**A**	**C**	**T**	**T**	**T**	**T**	**G**	**A**	**A**	**T**	**G**	**G**	**A**	**A**	**A**	**-**
P. xiikzodz (♀)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. coriacea (♂)	T	C	C	T	G	G	T	-	T	T	A	G	G	A	A	A	T	G	A	-	-	-	-	C	b

rps4 and *nad1*

	rps4 288	*rps4* 336	*rps4* 385	*nad1* 689	*nad1* 1251	*nad1* 1254
'P. Manta'	**C**	**C**	**A**	**A**	**G**	**c**
P. xiikzodz (♀)	.	.	.	.	.	.
P. coriacea (♂)	T	G	G	G	T	-

	trnL intron 388	*trnL* intron 389	*trnL-trnF*	*psbA-trnH* 117	*psbA-trnH* 236	*rps4*	*nad1*
'P. Aurora'	**T**	**T**	*	**T**	**A**	*	*
P. palmeri (♂)	.	.	*	.	.	*	*
P. foetida (♀)	-	-	*	-	-	*	*

trnL intron and *trnL-trnF*

	trnL 118	*trnL* 264	*trnL* 344	*trnL* 390	*trnL* 408	*trnL* 410	*trnL-trnF* 112	*trnL-trnF* 221	*trnL-trnF* 231
'P. Leida'	**G**	**T**	**A**	**-**	**A**	**T**	**d**	**G**	**C**
P. cincinnata (♂)	.	.	.	.	.	.	.	.	.
P. incarnata (♀)	-	-	G	T	G	G	-	C	T

psbA-trnH

	31	37	39	56	60	64	65	72	77	79	84	89	90	102	124	194	206	211	222	224	251	283
'P. Leida'	**A**	**G**	**A**	**A**	**G**	**A**	**T**	**T**	**T**	**A**	**G**	**-**	**T**	**-**	**e**	**G**	**T**	**A**	**T**	**G**	**G**	**T**
P. cincinnata (♂)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. incarnata (♀)	C	A	G	C	A	T	C	A	A	C	-	G	C	A	-	A	A	-	C	-	A	G

rps4 and *nad1*

	rps4 87	*rps4* 171	*rps4* 243	*rps4* 414	*nad1* 24	*nad1* 85	*nad1* 418	*nad1* 457	*nad1* 653	*nad1* 682	*nad1* 798	*nad1* 799	*nad1* 965	*nad1* 1163
'P. Leida'	**T**	**A**	**T**	**C**	**C**	**A**	**T**	**C**	**G**	**A**	**C**	**A**	**A**	**A**
P. cincinnata (♂)	.	.	.	.	A	C	G	T	A	G	T	G	G	C
P. incarnata (♀)	G	C	C	T	.	.	.	.	.	.	.	.	.	.

trnL intron and *trnL-trnF*

	trnL 118	*trnL* 165	*trnL* 243	*trnL* 346	*trnL* 349	*trnL* 390	*trnL* 410	*trnL-trnF* 61	*trnL-trnF* 94	*trnL-trnF* 213
'P. P. Gastaldo'	**G**	**-**	**G**	**T**	**T**	**T**	**T**	**G**	**G**	**G**
P. sprucei (♂)	.	.	.	.	.	.	.	.	.	.
P. incarnata (♀)	-	T	T	C	C	-	G	T	A	C

psbA-trnH

	31	56	62	79	101	103	117	118	124	162	166	184	185	186	187	188	189	194	198	211	213	217	221	239	248	271
'P. P. Gastaldo'	**A**	**A**	**A**	**A**	**-**	**C**	**T**	**C**	**f**	**A**	**G**	**T**	**T**	**T**	**T**	**C**	**T**	**A**	**A**	**T**	**G**	**G**	**-**	**G**	**T**	**T**
P. sprucei (♂)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
P. incarnata (♀)	C	C	C	C	A	A	-	A	-	C	T	A	A	G	A	A	A	T	G	C	A	A	A	A	G	G

rps4 and *nad1*

	rps4 87	*rps4* 243	*rps4* 414	*nad1* 20	*nad1* 24	*nad1* 50	*nad1* 139	*nad1* 418	*nad1* 457	*nad1* 682	*nad1* 798	*nad1* 799	*nad1* 823	*nad1* 988
'P. P. Gastaldo'	**T**	**T**	**C**	**T**	**C**	**C**	**T**	**T**	**C**	**A**	**C**	**A**	**T**	**g**
P. sprucei (♂)	.	.	.	C	A	T	G	G	T	G	T	G	G	-
P. incarnata (♀)	G	C	T	.	.	.	.	.	.	.	.	.	.	.

	trnL intron	*trnL-trnF* 30	*trnL-trnF* 99	*psbA-trnH* 47	*psbA-trnH* 159	*rps4*	*nad1* 19	*nad1* 988
'P. acti x P. eleg'	†	**h**	**G**	**T**	**-**	†	**T**	**i**
P. actinia (♂)	†	.	.	.	.	†	C	-
P. elegans (♀)	†	-	T	G	T	†	.	.

Note: Lower case a to i represent indels of variable size as follows: [a] 8 pb indel (st 225-232), [b] 22 pb indel (st 269-290), [c] 5 pb indel (st 1254-1258), [d] 8 pb indel (st 112-119), [e] 22 pb indel (st 124-145), [f] 13 pb indel (st 124-136), [g] 449 pb indel (st 988-1436), [h] 6 pb indel (st 30-35), [i] 449 pb indel (st 988-1436); st: sites; an asterisk indicates lack of variation, a dagger data not obtained, dots the same nucleotide (A, T, C, G) present in the hybrids.

CAPÍTULO VI

DISCUSSÃO

As classificações de Killip (1938) e Escobar (1989) dividiam o gênero *Passiflora* em 23 subgêneros apoiados somente em características morfológicas. Já Feuillet & MacDougal (2003) propuseram uma classificação que agrupa as espécies de *Passiflora* em apenas quatro subgêneros. Embora estes últimos autores também tenham baseado sua classificação predominantemente nos caracteres morfológicos, valeram-se da sinonimização de muitos *taxa* e da tentativa de introduzir algumas relações evolutivas no agrupamento. Muschner *et al.* (2003), numa análise filogenética a partir de seqüências de DNA nucleares e plastidiais, estudaram representantes de 12 subgêneros (de Killip 1938) e sugeriram que as espécies analisadas fossem redistribuídas em apenas três subgêneros. Os subgêneros, denominados clados, propostos por Muschner et al. (2003) foram concordantes com a classificação proposta por Feuillet & MacDougal (2003). Subseqüentemente, foram publicadas outras avaliações da classificação de Feuillet e MacDougal, usando uma abordagem filogenética molecular com outros marcadores, mas não apoiaram esta classificação (Yockteng & Nadot 2004).

No Capítulo III foram analisadas 104 espécies de *Passiflora* distribuídas em 19 dos 23 subgêneros de Killip (1938) e Escobar (1989), as quais compõem os quatro subgêneros de Feuillet & MacDougal (2003). Neste artigo, foram estudadas sete regiões do DNA, englobando os três genomas vegetais (plastidial, mitocondrial e nuclear). Os resultados obtidos corroboraram amplamente a classificação proposta por Feuillet & MacDougal (2003) com a adição de mais um subgênero (*Tryphostemmatoides*). Os subgêneros *Astrophea*, *Decaloba* e *Passiflora* formaram grupos monofiléticos com altos valores de suporte estatístico. No entanto, o subgênero *Deidamioides* proposto por Feuillet & MacDougal (2003) não se confirmou monofilético, tendo sido proposto sua divisão em dois, *Deidamioides* e *Tryphostemmatoides*. Krosnick & Freudenstein (2005), analisando

um número menor de espécies e outro conjunto de marcadores, também sugeriram a divisão deste subgênero da mesma forma. Analisar um número maior de espécies incluídas por Feuillet & MacDougal em *Deidamioides* poderá esclarecer as relações filogenéticas deste grupo. Como a maioria das espécies aqui inseridas pode ser encontrada no Brasil e somente poucos representantes ocorrem na Amazônia boliviana, um trabalho de revisão taxonômica do grupo está sendo proposto por pesquisadores de Feira de Santana (BA), envolvendo análises de marcadores moleculares (T.S. Nunes, comunicação pessoal).

Os três principais agrupamentos encontrados nas filogenias moleculares, subgêneros *Astrophea*, *Decaloba* e *Passiflora*, apresentam padrões ecológicos e bioquímicos bem diferenciados. Tais diferenças incluem: 1) tamanho das flores, significativamente menores em *Decaloba*; 2) conteúdo 2C de DNA, significativamente menor em *Decaloba*; 3) número cromossômico básico diferente para cada um dos três subgêneros; 4) *Decaloba* possui compostos cianogênicos, enquanto as espécies do *Passiflora* não os possuem; 5) especificidade na interação de grupos de *Heliconius* com diferentes subgêneros de *Passiflora*; 6) diferentes modos de herança do cloroplasto. Desta forma, pode-se usar com segurança e justificadamente as recomendações da APGII com relação à composição das classificações botânicas: filogenias moleculares, amplamente corroboradas e embasadas por características morfológicas e ecológicas.

A contribuição dos marcadores moleculares para esclarecer as relações infragenéricas quanto à divisão em subgêneros de *Passiflora* é evidente, ficando bastante claro que o número de regiões analisadas tem um papel crucial para que essas relações sejam estabelecidas. No entanto, a similaridade genética relativamente alta encontrada na maioria das espécies, exceto para as do subgênero *Decaloba*, e os resultados incongruentes obtidos para diferentes marcadores não permitiram a divisão dos subgêneros nas seções e

séries propostas por Feuillet & MacDougal (2003). Neste caso, resta a dúvida entre propor a não subdivisão dos subgêneros, porque estas outras classificações realmente não são monofiléticas, ou aceitar a atual classificação justificando a ausência dos agrupamentos pelos marcadores moleculares por sua inadequação para a inferência das relações interespecíficas do conjunto de *taxa* analisados. Aqui, também, o aumento no número de espécies de cada subgênero irá auxiliar na resolução das relações evolutivas. Sendo apenas uma questão metodológica, a análise de outros marcadores, com maior variabilidade genética, irá ajustar os agrupamentos. Se o número relativo de espécies de cada subgênero for considerado no Capítulo III e ponderada, ainda, a divergência genética dos marcadores estudados entre as espécies do subgênero *Decaloba*, pode-se propor que, pelo menos para este, as subdivisões em seções e séries não se confirmam com a filogenia molecular.

Um outro resultado interessante que se pode ainda observar no manuscrito do Capítulo III, que já havia sido destacado por Muschner *et al.* (2003), é a diferença nos comprimentos dos ramos da árvore filogenética entre os três subgêneros *Astrophea*, *Decaloba* e *Passiflora*. As espécies do subgênero *Decaloba* estão ligadas a ramos significativamente mais longos que as dos outros dois subgêneros. Interessantemente, essa característica não é gene ou genoma específico. O tempo de geração de cada espécie pode estar envolvido na diferença encontrada entre os subgêneros, embora se tenha pouca informação sobre fatores ecológicos que possam estar envolvidos nesse processo. Benson *et al.* (1975) foram os primeiros a afirmar que espécies do subgênero *Decaloba* possuem tempos de geração mais curtos que espécies do subgênero *Passiflora*. É possível que este fator possa estar acelerando a taxa evolutiva nas espécies do subgênero *Decaloba*, mas alguns autores questionam esta e outras possibilidades em plantas, sem no entanto

apresentar uma alternativa concreta (Whittle & Johnston 2003). Em todo o caso, deve-se destacar que somente entre estas espécies foram encontrados casos de autofecundação.

Análises de datação molecular têm sido realizadas em diversos grupos de plantas, tais como, *Notophagus* (Knapp *et al.* 2005), *Begonia* (Plana *et al. 2004*), Melastomataceae (Morley & Dick 2003) e Crypterionaceae (Conti *et al.* 2002). No Capítulo IV foram analisadas apenas quatro das sete regiões do DNA amostradas no manuscrito do Capítulo anterior (*rbcL* e *rps4* do cpDNA, intron b/c do gene *nad1* do mtDNA e região parcial do gene *26S* do nrDNA) devido a dificuldades no alinhamento do espaçador intergênico *trnL-trnF* e intron do gene *trnL* com os grupos com registro fóssil utilizados para a calibragem da árvore e carência de seqüências desses grupos para o intron d/e do gene *nad5*. A dificuldade no alinhamento foi decorrente da alta divergência das seqüências quando os diferentes gêneros são analisados. Através de uma abordagem que relaxa o relógio molecular estrito, procurou-se estimar as prováveis datas de surgimento do gênero *Passiflora* e a diversificação de seus subgêneros mais representativos. Os resultados indicam que o gênero *Passiflora* diversificou-se no Novo Mundo há aproximadamente 42 milhões de anos atrás (Ma), sendo *Decaloba* o primeiro subgênero a se diversificar (35 Ma), enquanto *Passiflora* e *Astrophea* parecem ter se diversificado há 24 Ma. A estimativa para o surgimento de Passifloraceae (56 Ma) é maior que a única estimativa para a família feita por Wikström *et al.* (2001), em um estudo envolvendo 560 famílias de angiospermas, que estimaram datas de 32-36 Ma para este grupo. Bremer *et al.* (2004) e Bell & Donoghue (2005) também estimaram datas mais antigas que as de Wikström *et al.* (2001) e assim, como aconteceu para as Passifloraceae, as discrepâncias podem ser decorrentes do aumento no número de seqüências nos trabalhos que focalizaram uma única família ou

gênero. A família Passifloraceae possivelmente teve uma origem Gondwânica, devido à distribuição disjunta apresentada pelos gêneros da família (vide Figura 1 do Capítulo IV).

Já com relação à distribuição disjunta do gênero *Passiflora*, pode-se sugerir que esta seja devida a eventos de dispersão à longa distância pelo Oceano Pacífico. Os principais agentes dispersores no Sul do Oceano Pacífico, segundo Winkworth *et al.* (2002), são provavelmente pássaros (albatrozes e *petrels*) que regularmente atravessam o Pacífico, além das dispersões pelo vento e correntes marinhas. Estas hipóteses já foram testadas e comprovadas para outras espécies vegetais que apresentam características semelhantes às observadas nas espécies de *Passiflora* (Renner *et al.* 2001; Knapp *et al.* 2005).

No manuscrito do Capítulo IV, mais uma vez, aborda-se a característica inerente às árvores filogenéticas em *Passiflora*: diferença no comprimento dos ramos entre os subgêneros *Astrophea*, *Decaloba* e *Passiflora*. Observa-se que os subgêneros *Astrophea* e *Passiflora* tiveram uma radiação rápida, provavelmente associada a adaptações florais a diferentes polinizadores. Segundo Malcomber (2002), inovações-chave devem ser as principais responsáveis pela radiação rápida em uma linhagem, sugerindo ainda que em *Gaertenera* esse padrão deva ter surgido devido a adaptações florais para polinizadores mais especializados. Na Figura 1 do Capítulo III tem-se uma pequena idéia sobre a interação das espécies do grupo com seus agentes polinizadores. As lacunas observadas na árvore devem-se ao total desconhecimento dos polinizadores das outras espécies.

Os resultados apresentados no Capítulo V dizem respeito ao padrão de herança organelar no gênero. Os híbridos interespecíficos e seus parentais em *Passiflora* apresentaram dois padrões de herança do cpDNA. Nas espécies do subgênero *Decaloba* os plastídios e seu DNA foram herdados do progenitor materno, enquanto que entre as

espécies do subgênero *Passiflora* foi o gameta masculino o doador destas seqüências. A herança das seqüências do mtDNA foi estritamente materna, independentemente do subgênero considerado. Diferentes padrões de herança do cloroplasto em um mesmo gênero já foram documentados em outros grupos (por exemplo, *Turnera* analisada por Shore & Triassi 1998 e *Syringa*, descrita por Liu *et al.* 2004). Estudos sobre o modo de herança organelar são muito importantes para ajudar a desvendar a história evolutiva das espécies de plantas e outros organismos e podem auxiliar no entendimento dos processos de especiação. Os resultados do Capítulo V reforçam ainda mais o proposto no Capítulo III com relação à monofilia dos subgêneros *Passiflora* e *Decaloba*. O estudo de Liu *et al.* (2004) demonstrou que a herança do cloroplasto está intimamente associada à filogenia de *Syringa*, gênero que é dividido em dois subgêneros, cada um deles com um modo de herança plastidial diferente. Em *Passiflora* isto também é verdadeiro, sugerindo que ocorreu um desenvolvimento independente do controle da herança dos plastídios nesses grupos de plantas. Liu *et al.* (2004) propuseram que é possível que a herança materna deva ter se tornado dominante antes do aparecimento das angiospermas, o que pode ser evidenciado pelo modo de herança plastidial materna na alga verde *Chlamydomonas*. Nossos achados também corroboram que a herança materna deva ser ancestral, como evidenciado pela idade de diversificação do subgênero *Decaloba* (35 Ma), que tem herança materna do cpDNA, em relação ao subgênero *Passiflora* (24 Ma), que possui herança paterna do cpDNA.

Muitos resultados foram aqui abordados e contribuíram para o esclarecimento de algumas questões relevantes no estudo deste complexo gênero de plantas. Alguns passos importantes foram dados no sentido de esclarecer aspectos da história evolutiva do gênero *Passiflora*: 1) a taxonomia do gênero, até então bastante complicada, pôde ser melhor

esclarecida e corrobora a classificação morfológica mais recente; 2) três subgêneros (os maiores em número de espécies) apresentaram altos valores de suporte estatístico sustentando sua monofilia e possuem características morfológicas e ecológicas diferentes que a corroboram; 3) a datação da diversificação de *Passiflora* e de seus três subgêneros maiores pôde ser correlacionada a eventos biogeográficos; 4) as diferentes taxas de substituição nucleotídica observadas na comparação dos subgêneros parecem estar correlacionadas com o tempo de geração e o modo de reprodução das espécies, não sendo características de alguns dos genes ou genomas estudados; 5) o padrão de herança organelar diferenciado é mais um aspecto a corroborar a filogenia molecular e a divisão do gênero em subgêneros.

No entanto, existem mais de 500 espécies conhecidas de *Passiflora* e muitas áreas ricas em diversidade biológica ainda não foram estudadas. O maior número de espécies diferentes tem sido encontrado em países como a Bolívia e a Colômbia, mas no Brasil não se sabe quantas ou quais são as espécies que ocorrem na Floresta Amazônica. Se forem consideradas todas as espécies já descritas, menos de 20% delas têm seu número cromossômico básico ou nível de ploidia conhecido; de menos de 10% sabe-se alguma coisa sobre a biologia floral ou forma de reprodução; apenas 1% é utilizada como fonte de compostos para a indústria farmacêutica ou para fins alimentícios. Somando todos os trabalhos já publicados sobre marcadores moleculares, de qualquer natureza, menos de 50% das espécies têm seu relacionamento evolutivo com outras espécies do gênero determinado. Nada é sabido sobre os aspectos de seu desenvolvimento floral e foliar, embora existam diversas sugestões sobre a importância das alterações morfológicas nos processos evolutivos do grupo, como aquisição de novos polinizadores, alterações no modo de reprodução, e aspectos neotênicos na forma das folhas. A corona de filamentos,

assim como a presença do androginóforo, é uma das características mais marcantes destas espécies, tendo sido diversas vezes proposta como o caráter diagnóstico que garante a monofilia do gênero, mas nada se sabe sobre sua origem ontogenética. Há, portanto, muito trabalho ainda por realizar.

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