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Programa de Pós-Graduação em Ecologia e Conservação de Recursos Naturais

Aspectos da evolução e da quebra da distilia em espécies de *Psychotria* L. e *Palicourea* (Aubl) (Rubiaceae)

Tese apresentada ao PPG em Ecologia e Conservação de Recursos Naturais da Universidade Federal de Uberlândia como requisito para obtenção do título de doutor em ecologia e conservação de recursos naturais.

Ebenézer Barbosa Rodrigues

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Uberlândia

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Ata da defesa de TESE DE DOUTORADO junto ao Programa de Pós-Graduação em Ecologia e Conservação de Recursos Naturais - Instituto de Biologia da Universidade Federal de Uberlândia.

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Às **14 horas** do dia **28** de **fevereiro** do ano de **2018, no auditório do Bloco 8C** – Campus Umuarama, da Universidade Federal de Uberlândia reuniu-se a Banca Examinadora, designada pelo Colegiado do Programa de Pós-Graduação em Ecologia e Conservação de Recursos Naturais, composta pelos Doutores: **Hélder Nagai Consolaro (UFG), Nicolay Lemes da Cunha (UFMS), Dênis Coelho Oliveira (UFU), Gudryan Jackson Barônio (UFU) e Paulo Eugênio de Oliveira (UFU)** orientador(a) do(a) candidato(a).

Iniciando os trabalhos, o(a) Presidente da mesa, Dr(a). Paulo Eugênio de Oliveira apresentou a Comissão Examinadora e o(a) candidato(a), agradecendo a presença do público e concedendo ao(à) Discente a palavra para a exposição do seu trabalho. A duração da apresentação do(a) Discente, o tempo de arguição e resposta foram estabelecidos conforme as normas do Programa.

A seguir, o(a) Senhor(a) Presidente concedeu a palavra aos examinadores, que passaram a arguir o(a) candidato(a). Finalizada a arguição, que ocorreu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais.

Em face do resultado obtido, a Banca Examinadora considerou o candidato **Aprovado**, sugerindo novo título para o trabalho (quando couber):

Esta defesa de Tese de Doutorado Acadêmico é parte dos requisitos necessários à obtenção do título de Doutor. O competente diploma será expedido após o cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU.

Nada mais havendo a tratar, foram encerrados os trabalhos às 18 horas e 10 minutos. Foi lavrada a presente ata que, após lida e aprovada, foi assinada pela Banca Examinadora.

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Índice

Resumo

Aspectos da evolução e da quebra da distilia em espécies de *Psychotria* L. e *Palicourea* (Aubl) (Rubiaceae)

As estratégias sexuais de plantas com flores são diversas. A distilia é uma dessas estratégias de reprodução, em que plantas apresentam dois morfos florais que ocorrem separadamente em cada indivíduo da população em uma proporção balanceada. Estames e estigmas das flores desses morfos florais apresentam alturas recíprocas entre eles, além disso, há também um sistema de incompatibilidade heteromórfico em populações distílicas que interrompe o crescimento de tubos polínicos oriundos de autopolinizações e polinizações intramorfo. Esta estratégia sexual é considerada como um mecanismo especializado na promoção de polinização cruzada em plantas. Entretanto, há transições evolutivas nesse sistema reprodutivo, envolvendo quebra do sistema de incompatibilidade, perda de um dos morfos florais e ausência de separação espacial dos órgãos sexuais nas flores, chamada homostilia. Em muitos grupos de plantas houveram estudos a respeito dos caminhos evolutivos dessas transições e seus efeitos na reprodução após a quebra da distilia. Nesta tese, foi estudada a distilia e sua quebra nos gêneros que contém o maior número de plantas com distilia, *Psychotria* L. e *Palicourea* Aubl. O estudo realizou uma reconstrução filogenética da distilia e suas variações em espécies desses dois gêneros e também investigou a seleção fenotípica em caracteres florais, a imprecisão em polinização e a integração fenotípica em espécies e populações com distilia e sua transição para outros sistemas reprodutivos. Além disso, foi verificada se a integração floral apresenta efeito na imprecisão em polinização de espécies e populações com distilia e sua quebra para outras estratégias reprodutivas. O estudo revelou que nas espécies de *Psychotria* e *Palicourea* a distilia é ancestral a diversificação dos gêneros. Houve evolução independente de transiçõe derivadas da distilia como monomorfismo, homostilia, monoicia e dioecia. Devido a distilia ser ancestral a esse grupo de espécies não foi possível inferir alguns dos modelos sugeridos para a evolução da distilia em plantas. As transições evolutivas foram registradas em espécies com ocorrência em ilhas ou em populações de habitats isolados indicando a importância de efeitos fundadores na evolução e no estabelecimento de espécies e populações com quebra da distilia. A morfologia da corola foi um melhor preditor do que a hercogamia (separação estigma-antera) para um maior fitness feminino, tanto na espécie com distilia quanto na espécie com monomorfismo. Ao nível interespecífico o estudo revelou que a precisão em polinização da espécie monomórfica estudada, *Psychotria prunifolia,* foi semelhante a da espécie com distilia, *Psychotria trichophoroides*. Porém, a integração fenotípica da morfologia floral da espécie distílica foi maior do que nas flores da espécie monomórfica. Já ao nível intraespecífico, populações com distilia apresentaram maior precisão em polinização do que as populações monomórficas de *Psychotria carthagenensis* e semelhante proporção de visitas de polinizares de probóscide curta e longa. A integração fenotípica também foi maior nas flores das populações distílicas do que nas populações com monomorfismo e há um forte efeito da integração do fenótipo floral na precisão em polinização em *P. carthagenensis*. As transições evolutivas do sistema reprodutivo de espécies de *Psychotria* e *Palicourea* parecem estar mais ligadas as pressões ecológicas locais do que com diferenças na morfologia dos polinizadores e representam a flexibilidade de adaptação das espécies desses gêneros a ambientes que favorecem a auto reprodução. Entretanto, entre populações a distilia é uma estratégia de reprodução que

proporciona alta precisão em polinização, mas espécies que apresentam monomorfismo podem ser tão eficientes em polinização cruzada quanto espécies com distilia.

Abstract

Evolutionary aspects in the evolution and breakdown of distyly in species of *Psychotria* L. and *Palicourea* (Aubl) (Rubiaceae)

Flowering plants have several mating strategies. Distyly is one these breeding strategies, in which, plants present two floral morphs that occurs separately in a balanced ratio in each plant population. Stamens and stigmas of these floral morphs have reciprocal heights among them, beyond that, that is also a heteromorphic system of incompatibility that interrupts the growth of pollen tubes coming from self-pollinations or intramorph pollinations. This breeding system strategy is considered as a specialized mechanism for cross-pollination in plants. But, there are evolutionary transitions in this breeding system, like the loss of one of the floral morphs and absence of spatial separation of the sexual organs in the flower, called homostyly. In many plant groups there were studies about the evolutionary pathways of these transitions and what the effects in reproduction after distyly breakdown. In this thesis, we studied distyly and its breakdown in the plant genera with the greatest number of species presenting distyly, *Psychotria* L. e *Palicourea* Aubl. The study used phylogenetic reconstruction of distyly and its variations in species of both genera and either investigated the phenotypic selection in floral traits, the pollination imprecision in species and populations with distyly and transitions to other breeding systems. We further test if floral integration present effect in pollination imprecision in populations with distyly and its breakdown towards other breeding strategies. The studied showed that distyly is ancestral to the diversification of *Psychotria* and *Palicourea* genera. There was independent evolution of the transitions derived from distyly as monomorphism, homostyly, monoecy and dioecy. Due distyly ancestrally in these species groups it was not possible to infer any of the models proposed for the evolution of distyly in plants. The evolutionary transitions were reported in species with occurrence in island or in populations in isolated habitats, indicating the importance of founder effects in the evolution and establishment of species and populations with distyly breakdown. The corolla morphology was a better predictor than herkogamy (spatial separation od sexual organs) for a high female fitness in either distylous *Psychotria trichophoroides* and in the monomorphic species, *Psychotria prunifolia*. At the interspecific level, our study showed that precision in pollination is similar in both the species. Furthermore, the phenotypic integration of the species with distyly was higher than the monomorphic one. But, at the intraspecific level, populations with distyly presented higher pollination precision than the monomorphic populations of *Psychotria carthagenensis* and similar proportion of short and long-tongued pollinators. The phenotypic integration also was higher in flowers of the distylous populations than in the populations with monomorphism and there was a strong effect of the integration of the floral phenotype in the pollination precision in *P. carthagenensis*. The evolutionary breeding system transitions in *Psychotria* and *Palicourea* species seems to be more linked to local ecological pressures rather to differences in morphology of pollinators, and, represent the flexibility of adaptation of species of these genera in habitats that favours selfing. However, at the population level, distyly is a strategy that promotes high pollination precision, but, species with monomorphism can be as efficient in cross-pollination as species presenting distyly.

Introdução geral

A heterostilia, um polimorfismo em que uma mesma espécie apresenta duas ou três morfologias florais, que, distinguem se pela altura dos órgãos sexuais masculinos e femininos nas flores, porém, há um posicionamento recíproco da altura de estigmas e anteras entre os morfos florais (hercogamia recíproca), além disso, em espécies heterostílicas há um sistema de incompatibilidade heteromórfico, em que apenas polinizações entre os distintos morfos florais produzem frutos (Ganders, 1979, Barrett, 1998). A distilia, é um dimorfismo floral amplamente distribuído nas Angiospermas em que há a presença do morfo longistilo (com estigmas posicionados acima da altura das anteras) e do morfo brevistilo (estigmas posicionados abaixo do nível das anteras), esses morfos ocorrem em igual proporção em populações distílicas, devido a seleção dependente da frequência e a incompatibilidade o eficiência dos polinizadores na transferência de pólen entre os morfos florais (Richards, 1986; Ganders, 1979; Barrett, 1992; Eckert *et al*., 1996; Barrett & Arroyo, 2012).

Há séculos plantas com polimorfismo floral são foco de estudo de historiadores naturais. Darwin (1877) e Dulberger (1992) relatam em seus manuscritos observações de outros pesquisadores indicando a existência de duas morfologias florais em plantas da mesma espécie em meados do século XVI, e a descrição de dois morfos florais em *Primula* no século XVIII. Entretanto, foi no século XIX que Darwin, com ajuda de outros historiadores naturais, compilou dados sobre as espécies que até então era conhecida a ocorrência de polimorfismo floral. Em seu livro "*The diferente forms of flowers on plants of the same species*" (1877), Charles Darwin registra em seus manuscritos populações em que os morfos ocorriam em proporções iguais e a esterilidade ocorrida após polinizações ilegítimas em flores de espécies distílicas e tristílicas. Darwin relata também ocorrência de monomorfismo e espécies com homostilia (ausência de hercogamia intrafloral). Darwin (1877) propôs então a funcionalidade desse sistema de reprodução: facilitar a transferência de pólen entre órgãos reprodutivos de distintas morfologias florais que apresentam deposição de pólen em partes diferentes do corpo de seus polinizadores. Darwin ainda propõe em seus estudos que a condição ancestral do sistema distílico seriam flores com hercogamia de aproximação com algum nível de autoesterilidade, e que flores com estigma curto e estames altos seriam originados pelo princípio da compensação e depois fixadas por seleção de morfologias flores com alturas recíprocas.

Já no século XX, alguns estudos buscaram entender quais os passos evolutivos desse sistema reprodutivo que envolve a ocorrência de hercogamia recíproca e um sistema de incompatibilidade. Diversos modelos evolutivos foram propostos (Anderson, 1973; Charlesworth & Charlesworth, 1979, Lloyd & Webb, 1992), a principal divergência entre os modelos evolutivos para a evolução da distilia é quanto qual seria a morfologia ancestral, se ela seria uma flor com homostilia ou uma flor com hercogamia de aproximação. Há também controversas sobre qual seria a ordem da evolução da hercogamia recíproca e do sistema de incompatibilidade, qual teria ocorrido primeiro? E também se haveria um controle genético (supergene model) comum para a morfologia floral e o sistema de incompatibilidade (Dowrick, 1956; Ganders, 1979; Muenchow, 1981) ou se o controle de ambos os fatores seria independente (Vuilleumier, 1967). Outros estudos, revelaram que para a manutenção da distilia altas taxas de transferência de pólen entre os morfos (reprodução disassortativa) são necessárias (Barrett, 1990; Baker *et al*., 2000; Arroyo *et al*., 2002; Thompson *et al*., 2003), e que o comportamento dos polinizadores também pode influenciar a manutenção da taxa equilibrada dos morfos em populações distílicas (Pérez-Barrales *et al*., 2007).

Com os recentes avanços da genética e da biologia molecular, novas descobertas a respeito dos mecanismos desse sistema de reprodução surgiram. Diferentes sítios de interrupção de crescimento de tubo polínico (reação de incompatibilidade) entre os morfos longistilo e brevistilo, indicariam que a incompatibilidade pode ter evoluído de forma separada nos morfos em Rubiaceae (Bawa & Beach, 1983; Gibbs, 1986; Ornduff, 1988). Cada morfo floral também pode apresentar proteínas distintas e específicas em seus grãos de pólen e estiletes, como em espécies de *Turnera* (Turneraceae) (Athanasiou & Shore, 1997). Foram identificados genes ortólogos associados a distilia em *Nimphoides peltata* (Menyanthaceae) (Li *et al*., 2017). Também foi identificada uma sequência de DNA associada apenas ao morfo brevistilo em *Primula* (Primulaceae) (Manfield *et al*., 2005). Recentemente, novos mecanismos da distilia foram publicados, foi encontrado a presença ou ausência de um gene que regula o dimorfismo em *Primula* (Huu *et al*., 2016; McClure, 2016). O morfo brevistilo possui esse gene e no morfo longistilo ele é ausente, e a presença desse gene causa inibição do alongamento de células do estilete, produzindo o estilete curto das flores brevistilas, enquanto a ausência do gene CYP^T permite o alongamento de células do estilete alto de flores longistilas e um outro gene GLO^T controlaria a altura dos estames (Huu *et al*., 2016; McClure, 2016). Outros estudos

apontam outros genes ligados ao controle da distilia em Linaceae, Polygonaceae e em Turneraceae. Assim, essas novas descobertas permitem uma visão mais ampla dos mecanismos de evolução e manutenção da distilia em espécies vegetais.

Entretanto, desde Darwin (1887), transições para outras estratégias de reprodução são relatadas em espécies com distilia, resultando principalmente em monomorfismo (ocorrência de apenas um dos morfos distílicos) e homostilia (ausência de hercogamia intrafloral) (Ganders, 1979; Hamilton, 1990). Há também ocorrência de outras modificações evolutivas a partir da distilia, como apomixia, ginodiocia (Baker, 1966) e dioicia (Ornduff, 1966, Beach & Bawa, 1980). O monomorfismo é atribuído como consequência de processos mediados por seleção do polinizador, distúrbios ambientais e de efeitos fundadores (Ganders, 1979, Pérez-Barrales *et al.*, 2007, Zhou *et al.* 2017). Diferenças no fluxo de pólen compatível entre os morfos também é considerado como um dos fatores que afetam a proporção igual dos morfos florais em populações distílicas, levando a uma maior quantidade de um morfo floral na população ou até mesmo na sua fixação (Baker *et al.*, 2000). Já a ocorrência de monomorfismo pode relacionado a distribuição geográfica da espécie como em populações centrais em *Luculia pinceana* (Zhou *et al.*, 2012) ou também um resquício da condição ancestral de monomorfismo na evolução da distilia como em *Narcissus* (Amaryllidaceae) (Graham & Barrett, 2004).

Por outro lado. a homostilia é relacionada a um fator genético, ocorrendo em uma taxa previsível devido a permutação nos genes que regulam a expressão da distilia em espécies heterostílicas (Richards, 1986; Shore & Barrett, 1990, Barrett, 1992). A hipótese de que a homostilia seria uma evidência de recombinação nos *loci* que controlam a distila parece ter origem nos estudos de Ernst (1936) em *Primula*, inferida em várias espécies de *Primula* com o uso de dados de ITS (Conti *et al.*, 2000) e reafirmada em análises filogeográficas (Zhou *et al.*, 2017). A homostilia ocasionada por recombinação no supergene também é apontada em transições evolutivas em *Turnera* (Barrett & Shore, 1987) e em *Villarsia albiflora* (Menyanthaceae) (Ornduff, 1988). Em *Exochaenium* (Gentianaceae) a homostilia teria relação com pressões seletivas exercidas por polinizadores (Kisling & Barrett, 2013). Entretanto, há também evidências de que a homostilia pode ocorrer sem ter origem na recombinação, como em *Primula vulgaris* (Li *et al.*, 2016). A homostilia também parece ter surgido por outras pressões seletivas além da recombinação gênica em *Linum* (Linaceae) (Richards, 1997). Já. em Pontederiaceae a evolução da homostilia a partir da tristilia teria ocorrência devido a uma perda de alelos

em espécies de *Eichornia* (Barrett, 1988). A ocorrência de homostilia também parece estar ligada com a distribuição geográfica, ocorrendo em populações marginais de *Amsinckia* (Boraginaceae) (Li & Jhonston, 2001). Desse modo, vários fatores genéticos e ecológicos são apontados como pressões seletivas para a quebra da distilia em plantas.

Essas transições evolutivas geralmente evoluem em ambientes com alta endogamia e condições ambientais que favoreçam a autopolinização (Ganders, 1979), e estão relacionadas a mudanças na integração fenotípica entre os caracteres da morfologia floral e também a diferenças na precisão em polinização cruzada em quebras nos aspectos morfológicos (hercogamia recíproca) e fisiológicos (sistema de incompatibidade) de plantas com polimorfismos florais (Sanchez *et al.*, 2008; Armbruster *et al.*, 2009; Sosenski *et al.*, 2010; Ferrero *et al.*, 2011). Estudos de reconstrução filogenética do sistema de reprodução com espécies distílicas e espécies com quebra da distilia apontam que o monomorfismo e a homostilia são derivados da distilia, e atribuem esses desvios a diferentes fatores como diferenças na ploidia entre as espécies, recombinação gênica, mudanças na morfologia dos polinizadores e também a fatores ligados a distribuição geográfica das espécies (Schoen *et al.*, 1997; Graham & Barrett, 2004; Truyens *et al.*, 2005; Mast *et al.*, 2006, Pérez Barrales & Arroyo, 2010).

Em Rubiaceae *Psychotria e Palicourea* são gêneros com bastante proximidade filogenética e com mais de 2000 espécies (Nepokroeff *et al.*, 1999), nos quais a distilia é considerada o sistema reprodutivo ancestral (Hamilton 1990; Taylor, 1997). Porém, algumas variações evolutivas da distilia também são registradas nesses gêneros, como diferentes níveis de autocompatibilidade em populações com ocorrência dos dois morfos florais (Hamilton 1990), monomorfismo (Sakai & Wright, 2001; Consolaro *et al.*, 2011; Rodrigues & Consolaro 2013; Sá *et al.*, 2016. A homostilia também ocorre nesses gêneros apesar de ser considerada rara (Hamilton, 1990), e há também registros de ocorrência de monoicia e dioicia nestas espécies (Watanabe *et al.*, 2015). Muitos estudos apontam o surgimento independente e de forma repetida da distilia e de suas transições evolutivas como em Boraginaceae (Schoen *et al.*, 1997, Ferrero *et al.*, 2011), em Primulaceae *(Mast et al., 2006)*, em Pontederiaceae (Khon *et al.*, 1996), em Linaceae (McDill *et al.*, 2009), Gentianaceae (Kissling & Barrett, 2013), Amaryllidaceae (Graham & Barrett, 2004) e também Polygonaceae (Wu *et al.*, 2017). Entretanto em Rubiaceae, sobretudo para *Psychotria* e *Palicourea*, gêneros com o maior número de espécies distílicas (Baker, 1958) e modelos para estudos evolutivos do sistema de reprodução em espécies vegetais

(Ganders, 1979; Neprokroeff *et al.*, 1999; Barrett, 1992, Barrett, 1988, Brennan, 2017), não se sabe a história evolutiva da distilia e das transições existentes em seu sistema reprodutivo.

Esta tese tem então o intuito de elucidar os caminhos evolutivos e ecológicos da distilia e de sua quebra em espécies de *Psychotria* e *Palicourea* (Rubiaceae). O primeiro capítulo tem por objetivo reconstruir a história filogenética da distilia e de suas variações reprodutivas em espécies desses gêneros. Já no segundo capítulo, são estudadas a inacurácia em polinização, a seleção fenotípica em caracteres florais e a integração floral em uma espécie distílica e uma espécie monomórfica de *Psychotria*. No terceiro e último capítulo são investigadas a variação intrapopulacional na ocorrência da distilia em *Psychotria carthagenensis* Jacq., a frequência de visita de polinizadores com probóscide longa e curta nas populações amostradas e se há diferenças na integração fenotípica e na precisão em polinização em populações distílicas e com quebra nos aspectos morfológicos e fisiológicos da distilia. Neste último capítulo também é analisado o efeito da integração floral na precisão em polinização das populações estudadas.

Referências Bibliográficas

Anderson WR. **1973**. A morphological hypothesis for the origin of heterostyly in the Rubiaceae. *Taxon* **22**: 537–542. https://doi.org/10.2307/1218628

Armbruster WS, Hansen TF, Pélabon C, Pérez-Barrales R, Maad J. **2009**. The adaptive accuracy of flowers: measurement and microevolutionary patterns. *Annals of botany* **103**: 1529–45. https://doi.org/10.1093/aob/mcp095

Athanasiou A, Shore JS. **1997**. Morph-specific proteins in pollen and styles of distylous *Turnera* (Turneraceae). *Genetics* **146**: 669–679. https://doi.org/10.1093/genetics/146.2.669

Baker, H. G. 1958. Studies in the reproductive biology of West African Rubiaceae. *Journal of the West African Science Association*, **4:** 9-24.

Baker, HG. 1966. The evolution, functioning and breakdown of heteromorphic incompatibility systems. I. The Plumbaginaceae. *Evolution*, **20:** 349-368. https://doi.org/10.1111/j.1558-5646.1966.tb03371.x

Baker AM, Thompson JD, Barrett SCH. **2000**. Evolution and maintenance of stigma-height dimorphism in *Narcissus*. I. Floral variation and style-morph ratios. *Heredity* **84**: 502–513. https://doi.org/10.1046/j.1365-2540.2000.00651.x

Barrett, SCH. 1988. Evolution of breeding systems in *Eichhornia* , a review. *Annals of the Missouri Botanical Garden* **75**: 741–760. https://doi.org/10.2307/2399363

Barrett, SCH. 1988. The evolution, maintenance and loss of self-incompatibility systems. In: *Reproductive Strategies of Plants: Patterns & Strategies* (Eds. J. & L. Lovett Doust), pp. 98–124. Oxford University Press, New York.

Barrett SCH. **1990**. The evolution and adaptive significance of heterostyly. *Trends in Ecology and Evolution* **5**: 144–148. https://doi.org/10.1016/0169-5347(90)90220-8

Barrett, SCH. 1992. Heterostylous genetic polymorphisms: model systems for evolutionary analysis**.** In: *Evolution and function of heterostyly* (S.C.H. Barret, ed.) Monographs on theoretical and applied genetics. Springer-Verlag, Berlin. https://doi.org/10.1007/978-3-642-86656-2_1

Barrett SCH. **1998**. The evolution of mating strategies in flowering plants. *Trends in Plant Science* **3**: 335–341. https://doi.org/10.1016/S1360-1385(98)01299-0

Barrett SCH, Arroyo MTK. **2012**. Variation in floral morph ratios in tristylous *Oxalis squamata* (Oxalidaceae): an Andean alpine endemic. *Botany* **90**: 1180–1185. https://doi.org/10.1139/b2012-075

Barrett SCH, Shore JS. **1987**. Variation and Evolution of Breeding Systems in the *Turnera ulmifolia* L . Complex. *Evolution* **41**: 340–354. https://doi.org/10.1111/j.1558-5646.1987.tb05802.x

Bawa KS, Beach JH. **1983**. Self-Incompatibility in the Rubiaceae of a Tropical Lowland Wet Forest. *American Journal of Botany* **70**: 1281–1288. https://doi.org/10.1002/j.1537-2197.1983.tb07917.x

Beach JH, Bawa KS. **1980**. Role of pollinators in the evolution of dioecy from distyly. *Evolution* **34**: 1138–1142. https://doi.org/10.1111/j.1558-5646.1980.tb04055.x

Brennan, AC. 2017. Distyly supergenes as a model to understand the evolution of genetic architecture. *American journal of botany*, **104:** 5-7. https://doi.org/10.3732/ajb.1600363

Charlesworth B, Charlesworth D. **1979**. The Maintenance and Breakdown of Distyly. *The American Naturalist* **114**: 499. https://doi.org/10.1086/283497

Consolaro H, Silva SCS, Oliveira PE. **2011**. Breakdown of distyly and pin-monomorphism in *Psychotria carthagenensis* Jacq. (Rubiaceae). *Plant Species Biology* **26**: 24–32. https://doi.org/10.1111/j.1442-1984.2010.00300.x

Conti E, Suring E, Boyd D, Jorgensen J, Grant J, Kelso S. **2000**. Phylogenetic relationships and character evolution in *Primula* L.: The usefulness of ITS sequence data. *Plant Biosystems* **134**: 385–392. https://doi.org/10.1080/11263500012331350535

Darwin C. 1877 *The different forms of flowers on plants of the same species*. London, UK: John Murray. https://doi.org/10.5962/bhl.title.46988

Dowrick J. **1956**. Heterostyly and homostyly in *Primula obconica*. *Heredity* **10**: 219–236. https://doi.org/10.1038/hdy.1956.19

Dulberger R. **1992**. Floral polymorphisms and their functional significance in the heterostylous syndrome. In: *Evolution and function of heterostyly*. Monographs on theoretical and applied genetics. 41–84. https://doi.org/10.1007/978-3-642-86656-2_3

Eckert CG, Manicacci D, Barrett SCH. **1996**. Genetic drift and founder effect in native versus introduced populations of an invading plant, *Lythrum salicaria* (Lythraceae). *Evolution* **50**: 1512–1519. https://doi.org/10.1111/j.1558-5646.1996.tb03924.x

Ernst, A. 1936. Heterostylie-forschung. *Zeitschrift für Induktive Abstammungs-und Vererbungslehre*, **71:** 156-230. https://doi.org/10.1007/BF01848862

Ferrero V, Chapela I, Arroyo J, Navarro L. **2011**. Reciprocal style polymorphisms are not easily categorised: the case of heterostyly in *Lithodora* and *Glandora* (Boraginaceae). *Plant biology (Stuttgart, Germany)* **13 Suppl 1**: 7–18. https://doi.org/10.1111/j.1438-8677.2009.00307.x

Ganders FR. **1979**. The biology of heterostyly. *New Zealand Journal of Botany* **17**: 607–635. https://doi.org/10.1080/0028825X.1979.10432574

Gibbs PE. **1986**. Do homomorphic and heteromorphic self-incompatibility systems have the same sporophytic mechanism? *Plant Systematics and Evolution* **154**: 285–323. https://doi.org/10.1007/BF00990130

Graham SW, Barrett SCH. **2004**. Phylogenetic reconstruction of the evolution of stylar polymorphisms in *Narcissus* (Amaryllidaceae). *American Journal of Botany* **91**: 1007–1021. https://doi.org/10.3732/ajb.91.7.1007

Hamilton CW. **1990**. Variations on a Distylous theme in mesoamericam *Psychotria* subgenus *Psychotria* (Rubiaceae). *Memoirs of the New York Botanical Garden* **55**: 62–75.

Huu CN, Kappel C, Keller B, Sicard A, Takebayashi Y, Breuninger H, Nowak MD, Bäurle I, Himmelbach A, Burkart M, *et al.* **2016**. Presence versus absence of CYP734A50 underlies the style-length dimorphism in primroses. *eLife* **5**. https://doi.org/10.7554/eLife.17956

Kissling J, Barrett SCH. **2013**. Variation and evolution of herkogamy in *Exochaenium* (Gentianaceae): Implications for the evolution of distyly. *Annals of Botany* **112**: 95–102. https://doi.org/10.1093/aob/mct097

Kohn JR, Graham SW, Morton B, Doyle JJ, Barrett SCH. **1996**. Reconstruction of the evolution of reproductive characters in Pontederiaceae using phylogenetic evidence from chloroplast DNA restrictio-site variation. *Evolution* **50**: 1454–1469. https://doi.org/10.1111/j.1558-5646.1996.tb03919.x

Li P, Johnston MO. **2001**. Comparative floral morphometrics of distyly and homostyly in three evolutionary lineages of *Amsinckia* (Boraginaceae). *Canadian Journal of Botany* **79**: 1332–1348. https://doi.org/10.1139/b01-107

Li J, Cocker JM, Wright J, Webster MA, McMullan M, Dyer S, Swarbreck D, Caccamo M, Van Oosterhout C, Gilmartin PM. **2016**. Genetic architecture and evolution of the S locus supergene in *Primula vulgaris*. *Nature Plants* **2**. https://doi.org/10.1038/nplants.2016.188

Li ZZ., Sun SS, Wang QF, Chen, J. M. 2017. RNA-Seq analysis of the distylous plant *Nymphoides peltata* identified ortholog genes between long-and short-styled flowers. *Frontiers in Ecology and Evolution*, **5:** 59. https://doi.org/10.3389/fevo.2017.00059

Lloyd DG, Webb CJ. 1992. "The evolution of heterostyly.In: *Evolution and function of heterostyly*. Monographs on theoretical and applied genetics. 151-178. https://doi.org/10.1007/978-3-642-86656-2_6

Mast AR, Kelso S, Conti E. **2006**. Are any primroses (*Primula*) primitively monomorphic? *The New phytologist* **171**: 605–16. https://doi.org/10.1111/j.1469-8137.2006.01700.x

Manfield IW, Pavlov VK, Li J, Cook HE, Hummel F, Gilmartin PM. **2005**. Molecular characterization of DNA sequences from the *Primula vulgaris* S-locus. *Journal of Experimental Botany* **56**: 1177–1188. https://doi.org/10.1093/jxb/eri110

McClure B. **2016**. Reproduction: The genetic basis of heterostyly. *Nature Plants*. **2.** https://doi.org/10.1038/nplants.2016.184

McDill J, Repplinger M, Simpson BB, Kadereit JW. **2009**. The Phylogeny of *Linum* and Linaceae Subfamily Linoideae, with Implications for Their Systematics, Biogeography, and Evolution of Heterostyly. *Systematic Botany* **34**: 386–405. https://doi.org/10.1600/036364409788606244

Muenchow, G. 1981. An S-locus model for the distyly supergene. *The American Naturalist*, **118:** 756-760. https://doi.org/10.1086/283866

Nepokroeff M, Bremer B, Sytsma KJ. **1999**. Reorganization of the genus *Psychotria* and Tribe Psychotrieae (Rubiaceae) inferred from ITS and rbcL sequence data. *Systematic Botany* **24**: 22. https://doi.org/10.2307/2419383

Ornduff, R. 1988. Distyly and incompatibility in *Villarsia congestiflora* (Menyanthaceae), with comparative remarks on *V. capitata*. *Plant systematics and evolution*, **159:** 81-83. https://doi.org/10.1007/BF00937426

Pérez-Barrales R, Arroyo J, Scott Armbruster W. **2007**. Differences in pollinator faunas may generate geographic differences in floral morphology and integration in *Narcissus papyraceus* (Amaryllidaceae). *Oikos* **116**: 1904–1918. https://doi.org/10.1111/j.0030-1299.2007.15994.x

Pérez-Barrales R, Arroyo J. **2010**. Pollinator shifts and the loss of style polymorphism in *Narcissus papyraceus* (Amaryllidaceae). *Journal of evolutionary biology* **23**: 1117–28. https://doi.org/10.1111/j.1420-9101.2010.01988.x

Richards AJ. **1986**. Plant Breeding Systems. *EdAllen Unwin*. London.

Richards, AJ. 1998. Lethal linkage and its role in the evolution of plant breeding systems. *Reproductive biology*, 71-83.

Rodrigues EB, Consolaro H. **2013**. Atypical distyly in *Psychotria goyazensis* Mull. Arg. (Rubiaceae), an intramorph self-compatible species. *Acta Botanica Brasilica* **27**: 155–161. https://doi.org/10.1590/S0102-33062013000100016

Sá T, Furtado MT, Ferrero V, Pérez-Barrales R, Rodrigues EB, dos Santos IG, Consolaro H. **2016**. Floral biology, reciprocal herkogamy and breeding system in four *Psychotria* species (Rubiaceae) in Brazil. *Botanical Journal of the Linnean Society* **182**: 689–707. https://doi.org/10.1111/boj.12476

Sakai S, Wright SJ. **2008**. Reproductive ecology of 21 coexisting *Psychotria* species (Rubiaceae): when is heterostyly lost? *Biological Journal of the Linnean Society* **93**: 125–134. https://doi.org/10.1111/j.1095-8312.2007.00890.x

Sanchez JM, Ferrero V, Navarro L. **2008**. A new approach to the quantification of degree of reciprocity in distylous (sensu lato) plant populations. *Annals of Botany* **102**: 463–472. https://doi.org/10.1093/aob/mcn111

Schoen DJ, Johnston MO, Lheureux a M, Marsolais J V. **1997**. Evolutionary history of the mating system in *Amsinckia* (Boraginaceae). *Evolution* **51**: 1090–1099. https://doi.org/10.1111/j.1558-5646.1997.tb03956.x

Shore JS, Barrett SCH. **1990**. Quantitative genetics of floral characters in homostylous *Turnera ulmifolia* var. *Angustifolia* Willd. (Turneraceae). *Heredity* **64**: 105–112. https://doi.org/10.1038/hdy.1990.13

Sosenski P, Fornoni J, Molina-Freaner FE, Weller SG, Domínguez C a. **2010**. Changes in sexual organ reciprocity and phenotypic floral integration during the tristyly-distyly transition in *Oxalis alpina*. *The New phytologist* **185**: 829–40. https://doi.org/10.1111/j.1469-8137.2009.03105.x

Taylor CM. **1997**. Conspectus of the Genus *Palicourea* (Rubiaceae: Psychotrieae) with the description of some new species from Ecuador and Colombia. *Annals of the Missouri Botanical Garden* **84**: 224–262. https://doi.org/10.2307/2400003

Thompson JD, Barrett SC, Baker AM. 2003. Frequency–dependent variation in reproductive success in Narcissus: implications for the maintenance of stigma–height

dimorphism. Proceedings of the Royal Society of London. Series B: Biological Sciences, 270: 949-953. https://doi.org/10.1098/rspb.2002.2318

Truyens S, Arbo MM, Shore JS. **2005**. Phylogenetic relationships, chromosome and breeding system evolution in *Turnera* (Turneraceae): Inferences from its sequence data. *American Journal of Botany* **92**: 1749–1758. https://doi.org/10.3732/ajb.92.10.1749

Vuilleumier, BS. 1967. The origin and evolutionary development of heterostyly in the angiosperms. *Evolution*, **21:** 210-226. https://doi.org/10.1111/j.1558-5646.1967.tb00150.x

Watanabe K, Aleck Yang TY, Nishihara C, Huang TL, Nakamura K, Peng CI, Sugawara T. **2015**. Distyly and floral morphology of *Psychotria cephalophora* (Rubiaceae) on the oceanic lanyu (orchid) Island, Taiwan. *Botanical Studies* **56**: 1–9. https://doi.org/10.1186/s40529-015-0091-9

Wu LY, Wang B, Schoen DJ, Huang SQ. **2017**. Transitions from distyly to homostyly are associated with floral evolution in the buckwheat genus (*Fagopyrum*). *American Journal of Botany* **104**: 1232–1240. https://doi.org/10.3732/ajb.1700189

Zhou W, Barrett SCH, Wang H, Li D-Z. **2012**. Loss of floral polymorphism in heterostylous *Luculia pinceana* (Rubiaceae): a molecular phylogeographic perspective. *Molecular ecology* **21**: 4631–45. https://doi.org/10.1111/j.1365-294X.2012.05707.x

Zhou W, Barrett SCH, Li HD, Wu ZK, Wang XJ, Wang H, Li DZ. 2017. Phylogeographic insights on the evolutionary breakdown of heterostyly. *New Phytologist*, **214:** 1368-1380. https://doi.org/10.1111/nph.14453

Capítulo I

Phylogenetic reconstruction of distyly and its breakdown in species of *Psychotria* L. **and** *Palicourea* Aublet **(Rubiaceae)**

Introduction

Heterostyly is genetic controlled floral polymorphism, in which individual plants express the phenotype of different floral morphs in the populations (Ganders, 1979; Barrett, 1992). Plants with distyly (dimorphic heterostyly) present two floral morphs: the short-styled flowers (thrum) with stamens under the pistil height, and the long-styled flowers (pin) with pistil above the stamens level. The morphs occur in a 1:1 ratio, a balanced proportion mediated by frequent-dependent-selection (Richards, 1986). The heights of female (pistil) and male (stamens) sexual organs are reciprocal among the floral morphs (reciprocal herkogamy), and heterostylous plants usually have diallelic self-incompatibility, so that only cross-pollinations between floral morphs result in ovules fertilization and seed set (Ganders, 1979; Barrett, 1992). However, other breeding system strategies often occur in plant species groups in which distyly seems to be widespread (Barrett & Shore, 2008). The most common breeding system transition is homostyly, the loss of intrafloral herkogamy with male and female reproductive whorls placed at the same height within the flowers (Ganders, 1979). The monomorphism, occurrence of populations with only one morph that resemble the pin (long-styled) or thrum (short-styled) morphs is also another transition reported in distylous plants (Ganders, 1979, Barrett, 1992). These atypical morphologies in distylous plants may happen in distinct levels, in flowers of a same plant, across individuals (Sakai & Wright, 2008), can be fixed in isolated populations (Consolaro *et al*., 2011) or also be fixed in the whole geographic distribution of the species (Rodrigues & Consolaro, 2013), Coelho *et al*., unpublished).

Several hypotheses have proposed pathways in which distyly may have evolved (Ernest 1936, Mather and Winton 1941, Baker 1966, Anderson 1973, Charlesworth and Charlesworth 1979, Lloyd & Webb 1992). Particularly for the family Rubiaceae, Anderson (1973) proposed a "Morphological hypothesis" for the origin of distyly: in this model, the ancestral breeding system prior to distyly was a flower with protandry and self-compatibility presenting delayed maturation and elongation of the style. Then, a

mutation makes the stigma matures below the anthers, giving origin to the short-styled morph, what enhanced self-pollination and seed output in these mutants, which survived and stablished. In Anderson's (1973) scenario, the first distylous Rubiaceae plants the short-styled flowers were self-pollinated and the long-styled outcrossing. So, by this hypothesis the floral polymorphism evolved prior to the establishment of a system of incompatibility. However, Charlesworth and Charlesworth (1979) published a distinct hypothesis for distyly evolution. Their model predicts the evolution of the incompatibility system before the morphological polymorphism. A homostylous ancestral flower presented self-incompatibility, but in this scenario, mutant plants with reciprocal placement of anthers and stigma (long-styled and short-styled morphs) would avoid self-interference and were selected, leading to distyly fixation. Another model for the evolution of distyly was proposed later by Webb and Lloyd (1992), their hypothesis included an ecological and taxonomic view of the plants that present dimorphic heterostyly. They predicted the evolution of distyly from an ancestral flower with approach herkogamy that was partially outcrossing. Then, a dominant mutation in style length, a morph with reversal herkogamy (short-styled morph) spread and fixed in the population, after that, the ancillary traits (system of incompatibility and ancillary floral polymorphisms) evolved in this reciprocal herkogamous population and distyly with self-incompatibility was finally stablished. Lloyd and Webb (1992) considered the morphology of homostylous flowers as derived from distyly caused by recombination in the supergene that controls heterostyly expression.

The phylogenetic reconstructions of distylous taxa in plant families revealed that monomorphism and homostyly (*sensu* Ganders 1979) are derived from distyly and their breeding system ancestral states results are more likely to support the model for distyly evolution proposed by Lloyd and Webb (1992) than the other models. Aside from the wide occurrence of heterostyly across plant families, studies approaching ancestral state reconstruction have not been well documented outside the Amaryllidaceae (Graham & Barrett, 2004), Boraginaceae (Schoen *et al.*, 1997), Passifloraceae (Truyens *et al.*, 2005) and Primulaceae (Mast *et al.*, 2006). These studies allow to comprehend if heterostyly evolved more than once in the species groups, the ancestral and intermediate states of this breeding system evolution, if deviations from distyly are derived or ancestral states of distyly, and what theoretical models for evolution can be inferred for distyly in these taxa (Barrett & Shore, 2008). The breeding system transitions were attributed to ploidy level

and recombination for the Passifloraceae (Truyens *et al.*, 2005) and the Primulaceae (Mast *et al.*, 2006), to pollinators pressures for the Amaryllidaceae (Pérez Barrales & Arroyo, 2010), and to ploidy level and occurrence of species with deviations from distyly in marginal habitats for the Boraginaceae (Schoen *et al*., 1997).

Apart from the families above, in Oxalidaceae and Rubiaceae, families with great number of heterostylous species, it has been predicted that heterostyly would have had multiple origins (Barrett & Shore, 2008). Monomorphic populations and homostyly (equal height of sexual organs) are hypothesized to be evolutionary stages derived from distyly in the Rubiaceae, (Ganders, 1979; Hamilton, 1990). Bir Bahadur (1968) estimated that the Rubiaceae have 416 distylous species distributed in 21 tribes. It is the family with the largest number of distylous species in the Angiosperms (Ganders, 1979). Its tribe Psychotriae is monophyletic, although both the genus *Psychotria* and *Palicourea* are paraphyletic with a complex phylogenetic relationship (Nepokroeff *et al*., 1999) and more than 2000 species (Baker, 1958; Naiki, 2012). The Rubiaceae species are also considered good model for testing breeding system evolution, by presenting distyly and several other reproductive strategies (Neprokoeff *et al*., 1999). But, despite the greatest number of distylous species, few studies were conducted approaching phylogenetic breeding system character build-up for the Rubiaceae. Sakai & Wright (2008) studied genetic relationships and breeding system transitions in 18 species of *Psychotria* L. in the Barro Colorado Island, in Panama. In this study they confirmed that breeding system transitions were derived from distyly and that they had independent evolution. Another phylogenetic study in the Rubiaceae breeding system variations approached genera of the subfamily Rubioideae (Ferrero *et al*., 2012), and pointed that distyly is ancestral to the Psychotrieae and Spermacoceae alliances, suggesting that more detailed studies at the species level are better to understand breeding system evolution in the Rubiaceae.

Ten years after Sakai & Wright (2008) approach about breeding system evolutionary aspects in the *Psychotria* genus, in our study we used phylogenetic reconstruction to trace the breeding system evolution in the genera *Psychotria* L. and *Palicourea* Aubl. species. We particularly aimed in the following issues: i) what breeding systems occur in the species and are them derived from distyly as proposed by Ganders (1979) and Hamilton (1990)?; ii) is distyly ancestral in *Psychotria* and *Palicourea*?; and how many times it has

evolved and been lost in the *taxa*?; iii) which of the theoretical models for evolution and breakdown of distyly can be inferred for these genera?

Material and methods

Molecular and breeding system data:

We downloaded DNA sequence data from species of the genera *Psychotria* and *Palicourea* available in GenBank (www.ncbi.nlm.nih.gov/genbank/) and then we analysed the data to find the highest number of species with similar DNA regions sequences. After that, we selected the most available DNA region: 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 26S ribosomal RNA. Finally, we select *Psychotria* and *Palicourea* species (n=47) with published studies on breeding system or for which breeding system information is known by field and/or herbarium observations (Supplementary table 1). We classified the breeding system of the species as proposed by Ganders (1979), considering homostyly when flowers presented no herkogamy and monomorphism as the sole occurrence of flowers which morphology looked like one of the distylous floral morphs. DNA sequences were edited and aligned using Geneious version 11.0 (http://www.geneious.com, Kearse *et al*., 2012) using the MAFFT algorithm. The nucleotides substitution model was estimated in Mega 7.0 (Kumar *et al*., 2015) and the best model was selected using the AIC values. The phylogenetic relationship of the species was estimated through Bayesian inference implemented in Beast 1.7 (Drummond *et al*., 2014). We used the software Tracer 1.5.0 (Rambaut & Drummond, 2009) to evaluate the effect sample size of the Bayesian phylogenetic sampled trees. Trees with noise in the estimated Bayesian posterior probabilities were excluded in a burn-in phase using TreeAnnotator (Drummond *et al*., 2014). Branch lengths and divergence time were estimated rooting and calibrating the tree with *Faramea multiflora* chosen as outgroup and the estimated age for fossil records of the genus *Faramea* Aubl. was the Oligocene (~34 m.y) (Graham, 2009).

We traced the ancestral states for breeding system in species of *Psychotria* and *Palicourea* based on the phylogeny of the data from the DNA region: 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 26S ribosomal RNA. Breeding system character state reconstruction was performed in

Mesquite 2.5 (Maddison, 2008). We used stochastic mapping character reconstruction using continuous Markov's chain model, that allows trait changes in all possible evolutionary pathways (Nielsen 2002). Independent evolution of the breeding systems of *Palicourea* and *Psychotria* was calculated using Pagel's Lambda considering free homoplasy of characters, which calculates likelihoods using a speciation/extinction model reduced from the BiSSE model, low likelihood values (e.g. closer to zero) indicate independent trait evolution (Maddison *et al*., 2007).

Results

Phylogenetic inference

The phylogenetic relationship of *Psychotria* and *Palicourea* species inferred by Bayesian Estimation of Sampled Trees was constructed using 716bp following the TRG + G nucleotides substitution model and it is shown in Fig. 1. The topology broadly agrees with phylogenetic studies in the Rubiaceae, either with ITS and rbcl data (Nepokroeff *et al*., 1999) or with ITS data (Razafimandimbison *et al*., 2008). We found the same uncertain resolution in the phylogenetic relationship among species of the *Psychotria* subg. *Heteropsychotria* and the *Palicourea* reported in other phylogenetic studies (Nepokroeff *et al*., 1999; Razafimandimbison *et al*., 2008).

Ancestral state inference

We found distyly and other four evolutionary breeding system transitions in species of the genera *Psychotria* and *Palicourea*: homostyly (absence of herkogamy), monomorphism (occurrence of one flower morphology that resemble the pin the thrum distylous flowers), dioecy and monoecy (Fig. 2). The breeding system transitions were all derived from distyly, they occurred in 14 of the 47 species of the study. Among the derivations we recorded 9 species with monomorphism (64.28%), 3 species with homostyly (21.42%), 1 species with dioecy (7.15%) and 1 species with monoecy (7.15%). The derived breeding systems were present across the species phylogeny: in *Psychotria* subg. *Psychotria*, *Psychotria* subg. *Heteropsychotria*, and *Palicourea*. Except for the Hawaiian species, the breeding system transitions occurred in species of all the other biogeographic regions (Fig. 2). Dioecy and monoecy were reported only in the Oriental

and Sino-Japanese regions and these species were ancestral (10- 13 m.y.) to all the other species with breeding system transition, while the earliest occurrence of homostyly and monomorphism respectively appeared in *Psychotria racemosa* (subg. *Heteropsychotria*) and in *Psychotria mapouriodes* (subg. *Psychotria*) with estimated age of 10 m.y. for these species. However, more recent species with estimated age of about 5 m.y. as *Palicourea guianensis*, *Palicourea montivaga*, *Psychotria tenuifolia* and *Psychotria carthagenensis* also presented breeding system derivations. Distyly is the ancestral breeding system in the genera *Psychotria* and *Palicourea* and distyly and its evolutionary breeding system transitions evolved independently in *Palicourea* and *Psychotria*, Pagel's lambda = 0.12, as Ganders (1979) and Hamilton (1990) predicted. Although we found no evidences that the breeding had evolved more than once in the taxa, there were no reversions from the derivate breeding systems to distyly, rejecting this part of their predictions.

Inferences in evolution and breakdown of distyly in Palicourea and Psychotria

Our phylogenetic reconstruction indicated that distyly have evolved in an ancestral taxon, prior to the speciation and diversification of *Palicourea* and *Psychotria*. All the breeding systems (monomorphism with approach herkogamy, homostyly, monoecy and dioecy) were directly derived from distyly. Our results corroborate the ancestrally of distyly in the Psychotriae alliance (Hamilton, 1990; Ferrero *et al*., 2012). There was no reversion from the derived breeding systems to distyly, excluding the possibility of inferences about the ancestral morphology of distyly in *Palicourea* and *Psychotria*.
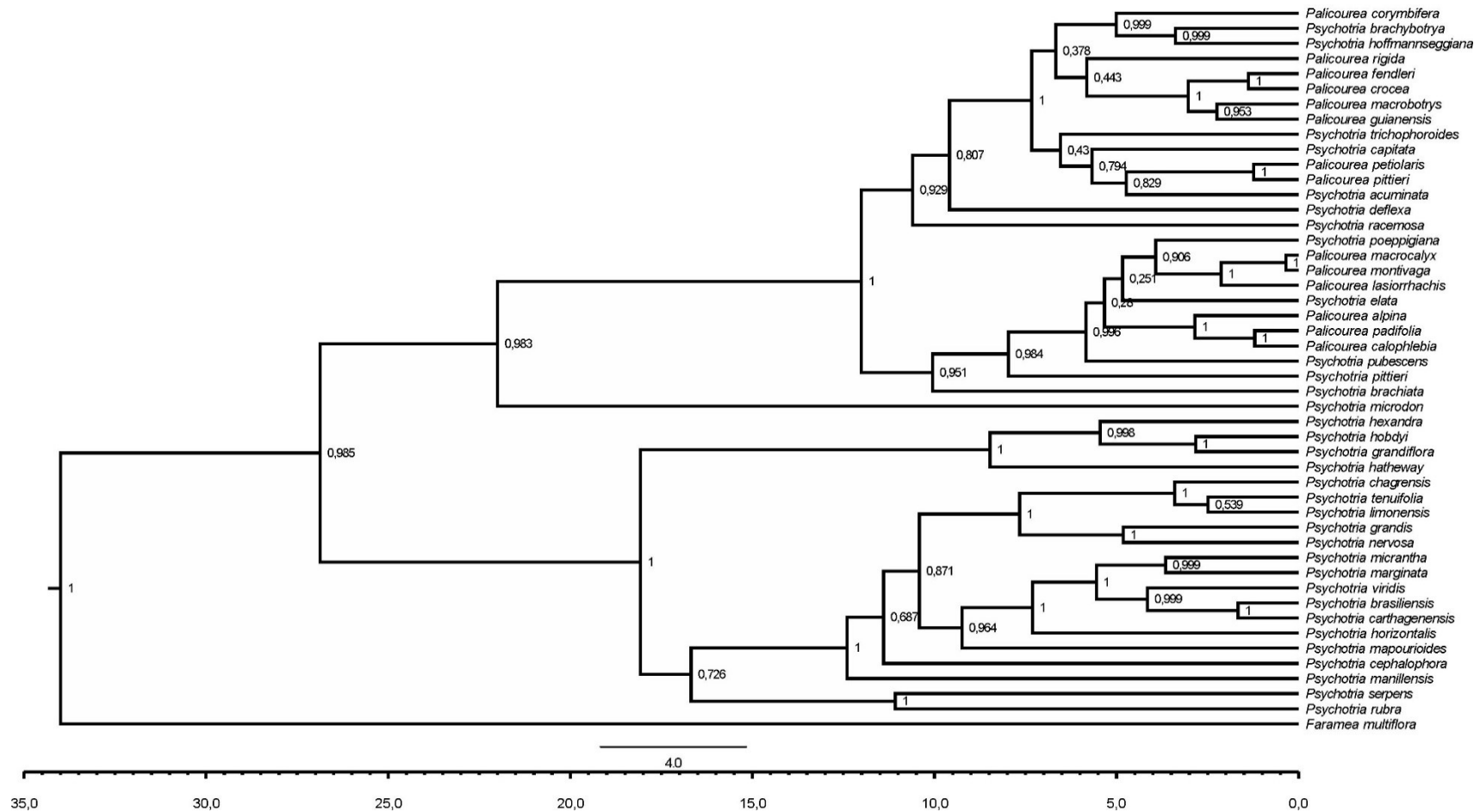
Figure 1: Species tree of *Psychotria* and *Palicourea* (Rubiaceae) inferred by BEAST (Bayesian Evolutionary Analysis by Sampling Trees) from sequences of 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 26S ribosomal RNA. Numbers at branches indicate Bayesian posterior probabilities. Scale bar is represented in million years (m.y)
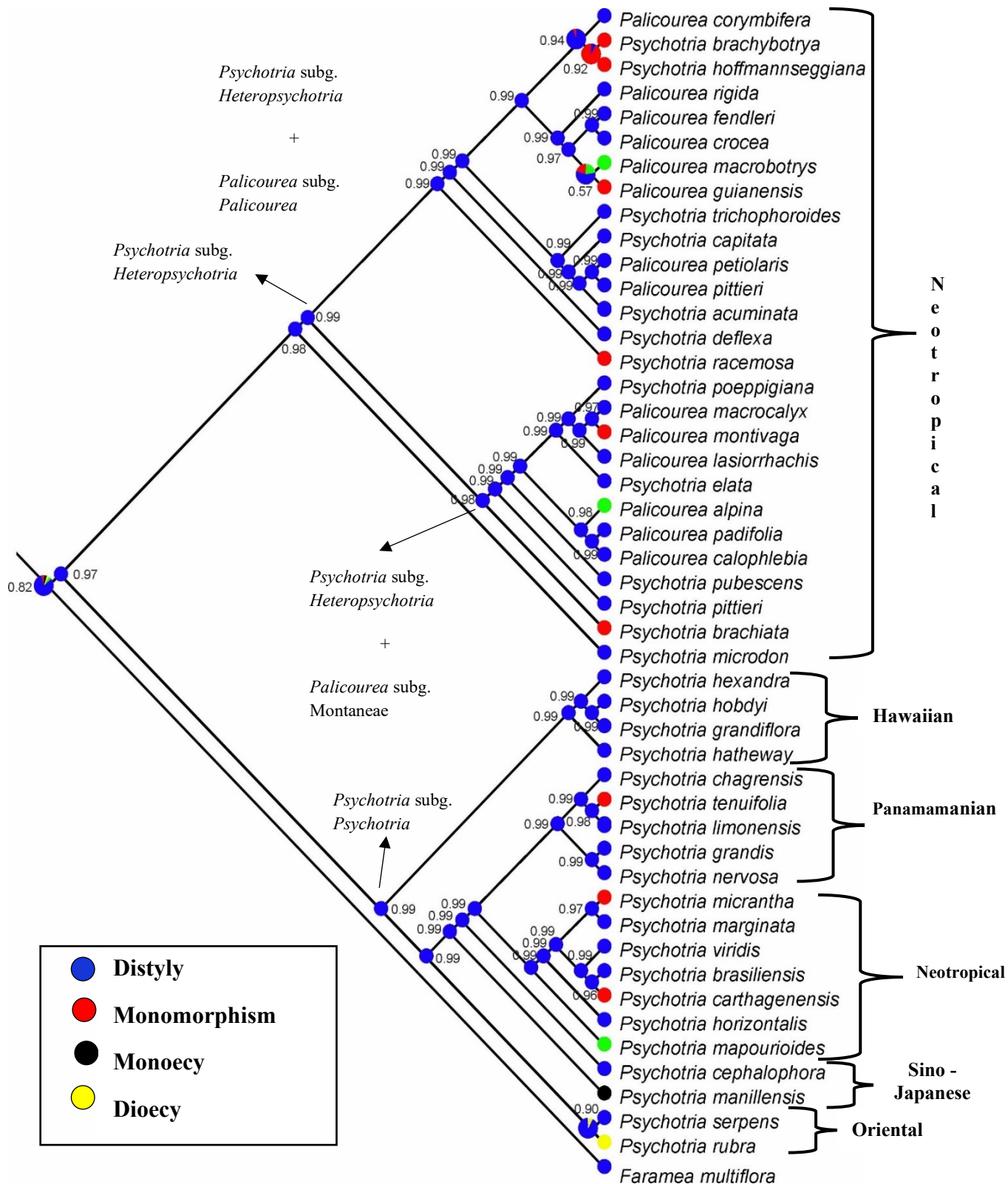
Fig. 2: Phylogenetic breeding system reconstruction of species of *Psychotria* and *Palicourea* (Rubiaceae) inferred by 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 26S ribosomal RNA data.

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Numbers at branches indicate proportional likelihoods of the most probably breeding system of each node. Taxonomic classifications following Taylor (1989) and Nepokroeff (1999). Biogeographical regions classifications following Holt *et al*. (2012)**.**

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Discussion

Our results indicate that the evolutionary breeding system transitions in the genera *Psychotria* and *Palicourea* all derived from distyly and relatively recent. They include variations in herkogamy (from reciprocal distylous herkogamy to monomorphism with approach herkogamy, and loss of herkogamy/homostyly) mostly in Neotropical groups, and variations toward gender specialization (dioecy and monoecy) in Paleotropical ones. These variations seem to be related to adaptions of the species to islands or isolated habitats favouring selfing and to differences in colonization and reproductive performance of the floral morphs, leading to the establishment of populations or even to species with breeding system transitions derived from distyly.

The breeding system deviations. monomorphism with approach herkogamy and homostyly have an ambiguous interpretation in breeding system reconstruction of heterostylous groups, by representing either a breeding system deviation or an ancestral condition in the evolution of distyly (Barrett & Shore, 2008). Nevertheless, evidences from evolutionary breeding system studies have showed that populations with monomorphic flowers and approach herkogamy can be derived from distyly in *Luculia pinceana* (Rubiaceae) (Zhou *et al*., 2012) and in *Primula chungensis* (Primulaceae) (Zhou *et al*., 2017). This same flower morphology can represent an ancestral state of distyly evolution in different *Narcissus* species (Amaryllidaceae) (Graham & Barrett, 2004). However, our results corroborate the ancestrally of distyly in the Psychotriae alliance proposed by Ferrero *et al*., (2012). In the genera *Palicourea* and *Psychotria* monomorphism with approach herkogamy, homostyly, dioecy and monoecy were traced only as derivations from distyly with no possible reversions.

The monomorphism of *Psychotria hoffmansegianna*, *Psychotria racemosa*, *Psychotria brachiata*, *Psychotria tenuifolia*, *Psychotria micrantha* (Sakai & Wright, 2008), *Palicourea guianensis* (Taylor, 1997) and *Palicourea montivaga* (Taylor, 1989) were reported in island populations studies in Central America. Otherwise, the monomorphism in *Psychotria carthagenensis* was reported in continental populations (Consolaro *et al*., 2011, Rodrigues *et al*., chapter 3). Distinct ecological aspects are discussed as factors for the origin of monomorphism with approach herkogamy in heterostylous plant groups. Breeding system variation across populations (distyly and monomorphism with approach

herkogamy) were associated to differences in pollinators morphology (short and long-tongued) in *Narcissus papyraceus* (Amaryllidaceae) (Pérez-Barrales & Arroyo, 2010). But, the monomorphism in populations of *Luculia pinceana* (Rubiaceae) seems to be linked to founder effects and differences in the self-incompatibility of the floral morphs (Zhou *et al.*, 2012). Founder effects may also be responsible for the origin of monomorphism in species of *Palicourea* and *Psychotria*. Among the species of our phylogenetic breeding system reconstruction, there are records of distylous populations for *Psychotria hoffmansegianna*, *Psychotria racemosa* and *Palicourea guianensis* in Brazilian forests (Sá *et al.*, 2013, pers observ). In *Psychotria carthagenensis* the monomorphism was also associated with populations with marginal distribution in the species potential distribution, in isolated forests fragments (Rodrigues *et al*. chapter 3). Thereby, the biogeographic history seems to influence the breeding system of *Psychotria* and *Palicourea* species and probably led to the colonization or the loss of one of the distylous morphs on these island and isolated populations.

Homostyly was reported in *Palicourea macrobotrys*, *Palicourea alpina* and *Psychotria mapourioides*. In *Palicourea macrobotrys* homostyly occurred in marginal habitat distribution of the species (Coelho & Barbosa, 2003) and in *Palicourea alpina* in island populations in Jamaica (Tanner, 1982), in these species homostyly seems to be fixed in the species level, since that is no record of distyly in either the species elsewhere (Taylor, 1997). Furthermore, in *Psychotria mapouriodes* homostyly occurred in one population in the Brazilian Northeastern region (Parque Estadual do Pau-Ferro, pers. obs.) in a rain forest fragment isolated amid the Caatinga vegetation (Veloso *et al*., 1991), but, in forests in the Cerrado, the species presents distyly (Tangará da Serra, Mato Grosso; Parque Nacional de Brasília, Distrito Federal Brazil, pers. obs). In many taxa the origin of homostyly is considered as a result of recombination in the distyly supergene, as the occurrence of homostyly in *Primula* (Conti *et al.*, 2000; Li *et al.*, 2016), in *Turnera* (Barrett & Shore, 1987) and in *Villarsia albiflora* (Menyanthaceae) (Ornduff, 1988). However, the origin of homostyly can be related to other ecologic pressures rather than recombination in the distylous supergene (Richards, 1997). In *Exochaenium* (Gentianaceae) the origin of homostyly have been driven by pollinator-mediated selection process (Kissling & Barrett, 2013). And, in *Amsinckia* (Boraginaceae) homostyly was attributed to populations that occurred in marginal ecological habitats of the species

(Schoen *et al.* 1997). There are no molecular studies with the distylous supergene for *Psychotria* and *Palicourea* species, actually for no Rubiaceae either, but probably homostyly follows the same genetic model proposed for other distylous plants groups, in which homostyly is the most common breeding system derivation from distyly (Barret & Shore, 2008). Nevertheless, in *Psychotria* and *Palicourea*, monomorphism was the most frequent breeding system transition and homostyly was the second one. Similarly, to the monomorphism in other species of *Palicourea* and *Psychotria*, homostyly was reported in island and isolated populations. Thus, colonization process evolving a founder homostylous or monomorphic populations may be the pathway for the origin of the fixed and the random occurrence of these breeding system transitions in the species of our study.

The high occurrence of monomorphism in species of *Psychotria* and *Palicourea* may occurs due to Rubiaceae present great variation in presence or lack of self-incompatibility (Bawa & Beach, 1983). The weakening or breakdown of physiological incompatibility in distylous plants population combined with inefficiency of pollinators in promoting cross-pollination is the most likely mechanism of deviations in the balanced morph ratio and even in the establishment of monomorphic populations (Barrett *et al*., 1989). Population size is another factor that can affect morph ratio or lead to fixation of a single morph in plant population and most of the times the long-styled morph is the one to be fixed (Barrett, 1993; Arroyo *et al*., 2002; Balogh & Barrett, 2016). Among the species of our study, only *Palicourea montivaga* presented short-styled monomorphism (Taylor, 1989), all the other monomorphic *Psychotria* and *Palicourea* represent establishment of long-styled monomorphic populations. It is hypothesized that the long-styled morph present better performance in founding populations than the short-styled morph, for its external positional of stigmas in the flowers, thus being more likely to receive pollen grains than the stigma of short-styled flowers (Baker *et al*. 2000). The rare occurrence of short-styled monomorphism may happen due reverse herkogamy being rare in plants (Lloyd & Webb, 1986). *Psychotria* and *Palicourea* species with monomorphism may represent plants with different responses to pressures linked to self-incompatibility and founder events in these insular and isolated habitats where the species are distributed.

Dioecy and monoecy were the breeding system in *Psychotria rubra* and *Psychotria manillensis*, respectively. The breeding system transition of both the species occurred in

the Japanese archipelago (Watanabe *et al*., 2013). Beach & Bawa (1980) presented a hypothesis for the evolution of dioecy from distyly. In their model, the origin of dioecy was caused by a gradual process, triggered by a disruption in the disassortative pollen flow among the distylous morphs and a shift in pollinator fauna (long-tongued to short-tongued), followed by unidirectional pollen flow and the later selection of the unisexual flower. Otherwise, Thomson & Barrett (1981) pointed out the importance of self-incompatibility ancestor in the evolution and selection of dioecy. However, for *Psychotria rubra* it is not known if the species present self-incompatibility or if there are distylous populations of the species outside the Japanese island and what the pathways for the directly evolution of dioecy from distyly in the species. The monoecy in *Psychotria manillensis* also occurred in a Japanese island habitat. Watanabe & Sugawara (2015) cited a possible association of monoecy with polyploidy in the species and suggested that chromosome doubling can be responsible for the origin of male and female flowers in a same plant, probably derived from its close related species, the dioecious *Psychotria rubra*. However, our results do not support this inference due to the independence in the evolution of both breeding system transitions. Monoecy it is not well studied, but most models for its evolution assumed its origin from a hermaphrodite ancestor (Charlesworth & Charlesworth, 1978; Beach &Bawa, 1980) with disruptive selection in male and female sex allocation (Barrett, 2002), usually associated with some degree of male sterility and finally, the selection of unisexual flowers (monoecy and dioecy). Thus, the evolution of unisexual flowers from distyly shall involve harmful effects of sexual interference in the ancestral distylous floral morphology (Casper & Charnov, 1982; Charlesworth, 1989; Charlesworth & Morgan, 1991). The evolution of gender specialization in *Psychotria rubra* and *Psychotria manillensis* represents breeding strategies derived from distyly. In insular habitats, like the Japanese Islands, these evolutionary transitions may ensure cross-pollination and eliminate the risks of sexual self-interference in these plants.

The breeding system transitions in *Psychotria* and *Palicourea* were recorded both in recent and older *taxa*, and most of the evolutionary transitions occurred in insular habitats plants. Corroborating the idea that heterostyly is rare or mostly absent in islands (Pailler *et al*., 1998), the breakdown of distyly have been observed commonly during species colonization in oceanic islands (Barrett *et al*., 1989; Sakai & Wright, 2007; Barrett &

Shore, 2008; Watanabe & Sugawara, 2015). However, this factor seems to be species specific, since in islands there are also species with distyly as *Psychotria cephalophora* and *Psychotria boninensis* and *Psychotria serpens* in the Japanese archipelago (Sugawara *et al.*, 2014; Watanabe *et al.*, 2015; Watanabe *et al.*, 2013) and *Psychotria deflexa*, *Psychotria chagrensis*, *Psychotria marginata* and other *Psychotria* species in Barro Colorado Island, Panama (Sakai & Wright, 2008). Probably, differences in dispersal performance and colonization of the morphs may give rise to distyly-derived breeding system in islands and isolated populations. In the continental populations, breeding system transitions may arise due to retraction and isolation of forests remnants and reduced populations, favouring the establishment of populations with breakdown of distyly.

Our results of the breeding system ancestral state reconstruction for *Psychotria* and *Palicourea* species do not contribute to the model of evolution of distyly proposed by Anderson (1973), there were no protandry as ancestral state or as breeding system deviation in the species of our study. The results of the phylogenetic reconstruction also do not agree with Charlesworth & Charlesworth (1979) predictions. Homostyly was always derived from distyly and there were no evidences of homostyly as ancestral breeding system of the species or reversion to distyly from homostyly (fig. 2). Our results corroborate Hamilton (1990) and Lloyd and Webb (1992) predictions about the derived floral morphology from distyly. As they proposed, homostyly and monomorphism were derived from distyly. However, the breeding system phylogenetic reconstruction of our study does not allow inferences using the Lloyd and Webb (1992) model for the evolution of distyly. We found no evidences of monomorphic flowers with approach herkogamy as ancestral breeding system state of distyly or reversions to distyly from flowers with approach herkogamy in *Psychotria* and *Palicourea*. Probably because distyly did arise much earlier than the origin and diversification of both genera.

The breeding system derivations from distyly evolved independently across the species of this study. They also were not associated with the phylogenetic divisions of the species, evolving in the subgenera of *Psychotria* (*Psychotria* and *Heteropsychotria*) and in both subgenera of *Palicourea* (*Palicourea* and *Montanae*). The shift from distyly to monomorphism with approach herkogamy and to homostyly does not seem to be linked with shifts in pollinator fauna either. The evolutionary breeding system transitions were

reported in species pollinated by insects (subgenus *Psychotria* and subgenus *Palicourea*) and by hummingbirds (some species of the subgenus *Heteropsychotria* and some species of the subgenus *Montanae*¸ respectively). Furthermore, the evolutionary breeding system transitions in *Psychotria* and *Palicourea* were all reported in populations and species that occurred in oceanic islands or isolated forests that are located amid Cerrado or Caatinga vegetations. Our results, indicate that distyly is indeed widespread in *Psychotria* and *Palicourea* across their Pantropical distribution, these species can present versatile strategies in their breeding system evolution to ensure reproduction in oceanic and continental insular habitats.

References

Anderson WR. **1973**. A Morphological Hypothesis for the Origin of Heterostyly in the Rubiaceae. *Taxon* **22**: 537–542. https://doi.org/10.2307/1218628

Arroyo J, Barrett SCH, Hidalgo R, Cole WW. 2002. Evolutionary maintenance of stigma-height dimorphism in *Narcissus papyraceus*(Amaryllidaceae). *American Journal of Botany* **89**: 1242–1249. https://doi.org/10.3732/ajb.89.8.1242

Baker, H. G. 1958. Studies in the reproductive biology of West African Rubiaceae. *Journal of the West African Science Association*, **4:** 9-24.

Baker, HG. 1966. The evolution, functioning and breakdown of heteromorphic incompatibility systems. I. The Plumbaginaceae. *Evolution*, **20:** 349-368. https://doi.org/10.1111/j.1558-5646.1966.tb03371.x

Baker AM, Thompson JD, Barrett SCH. **2000**. Evolution and maintenance of stigma-height dimorphism in *Narcissus*. I. Floral variation and style-morph ratios. *Heredity* **84**: 502–513. https://doi.org/10.1046/j.1365-2540.2000.00651.x

Balogh CM, Barrett SCH. 2016. Stochastic processes during invasion: the influence of population size on style-morph frequency variation in *Lythrum salicaria* (purple loosestrife). *International Journal of Plant Sciences* **177**: 409-418. https://doi.org/10.1086/686023

Barrett SCH. 1992. Heterostylous genetic polymorphisms: model systems for evolutionary analysis**.** In: *Evolution and function of heterostyly* (S.C.H. Barret, ed.) Monographs on theoretical and applied genetics. Springer-Verlag, Berlin. https://doi.org/10.1007/978-3-642-86656-2_1

Barrett SCH. 1993. The evolutionary biology of tristyly. In: *Oxford Surveys in Evolutionary Biology* (Eds. D. Futuyma & J. Antonovics), Vol. **9**: pp. 283–326. Oxford University Press, Oxford, U.K.

Barrett SCH 2002. The evolution of plant sexual diversity. *Nature Reviews Genetics* **3**: 274–284. https://doi.org/10.1038/nrg776

Barrett SCH., Morgan M, Husband BC. 1989. The dissolution of a complex polymorphism: the evolution of self-fertilization in tristylous *Eichhornia paniculata* (Pontederiaceae). *Evolution* **43**: 1398–1416. https://doi.org/10.1111/j.1558-5646.1989.tb02591.x

Barrett SCH, Shore JS. **1987**. Variation and Evolution of Breeding Systems in the *Turnera ulmifolia* L . Complex. *Evolution* **41**: 340–354. https://doi.org/10.1111/j.1558-5646.1987.tb05802.x

Barrett SCH,Shore JS. 2008. New insights on heterostyly: Comparative biology, ecology and genetics. In: *Self-Incompatibility in Flowering Plants: Evolution, Diversity and Mechanisms*. (Ed. V. Franklin-Tong). Springer-Verlag, Berlin. pp. 3–32. https://doi.org/10.1007/978-3-540-68486-2_1

Bawa KS, Beach JH. **1983**. Self-Incompatibility in the Rubiaceae of a Tropical Lowland Wet Forest. *American Journal of Botany* **70**: 1281–1288. https://doi.org/10.1002/j.1537-2197.1983.tb07917.x

Beach JH, Bawa KS. **1980**. Role of pollinators in the evolution of dioecy from distyly. *Evolution* **34**: 1138–1142. https://doi.org/10.1111/j.1558-5646.1980.tb04055.x

Casper BB, Charnov EL. 1982. Sex allocation in heterostylous plants. *Journal of Theoretical Biology*, **96:** 143-149. https://doi.org/10.1016/0022-5193(82)90217-X

Charlesworth D. 1989. Allocation to male and female function in hermaphrodites, in sexually polymorphic populations. *Journal of Theoretical Biology*, **139:** 327-342. https://doi.org/10.1016/S0022-5193(89)80212-7

Charlesworth B, Charlesworth D. **1979**. The Maintenance and Breakdown of Distyly. *The American Naturalist* **114**: 499. https://doi.org/10.1086/283497

Charlesworth B, Morgan MT, Charlesworth D. 1991. Multilocus models of inbreeding depression with synergistic selection and partial self-fertilization. *Genetics Research*, **57:** 177-194. https://doi.org/10.1017/S0016672300029256

Coelho CP, Barbosa AA a. 2003. Biologia reprodutiva de *Palicourea macrobotrys* Ruiz & Pavon (Rubiaceae): um possível caso de homostilia no gênero *Palicourea* Aubl.

Revista Brasileira de Botânica **26**: 403–413. https://doi.org/10.1590/S0100-84042003000300013

Consolaro H, Silva SCS, Oliveira PE. **2011**. Breakdown of distyly and pin-monomorphism in *Psychotria carthagenensis* Jacq. (Rubiaceae). *Plant Species Biology* **26**: 24–32. https://doi.org/10.1111/j.1442-1984.2010.00300.x

Conti E, Suring E, Boyd D, Jorgensen J, Grant J, Kelso S. **2000**. Phylogenetic relationships and character evolution in *Primula* L.: The usefulness of ITS sequence data. *Plant Biosystems* **134**: 385–392. https://doi.org/10.1080/11263500012331350535

Drummond AJ, Suchard MA, Xie D, Rambaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular biology and evolution*, **29:** 1969-1973. https://doi.org/10.1093/molbev/mss075

Ernst, A. 1936. Heterostylie-forschung. *Zeitschrift für Induktive Abstammungs-und Vererbungslehre*, **71:** 156-230. https://doi.org/10.1007/BF01848862

Ferrero V, Rojas D, Vale A, Navarro L. **2012**. Delving into the loss of heterostyly in Rubiaceae: Is there a similar trend in tropical and non-tropical climate zones? *Perspectives in Plant Ecology, Evolution and Systematics* **14**: 161–167. https://doi.org/10.1016/j.ppees.2011.11.005

Ganders FR. **1979**. The biology of heterostyly. *New Zealand Journal of Botany* **17**: 607–635. https://doi.org/10.1080/0028825X.1979.10432574

Graham SW, Barrett SCH. **2004**. Phylogenetic reconstruction of the evolution of stylar polymorphisms in *Narcissus* (Amaryllidaceae). *American Journal of Botany* **91**: 1007–1021. https://doi.org/10.3732/ajb.91.7.1007

Graham A. 2009. Fossil record of the Rubiaceae. *Annals of the Missouri Botanical Garden*, *96*(1), 90-108. https://doi.org/10.3417/2006165

Hamilton CW. **1990**. Variations on a Distylous theme in mesoamericam *Psychotria* subgenus *Psychotria* (Rubiaceae). *Memoirs of the New York Botanical Garden* **55**: 62–75.

Holt BG, Lessard JP, Borregaard MK, Fritz SA, Araújo MB, Dimitrov D, Nogués-Bravo D. 2012. An update of Wallace's zoogeographic regions of the world. *Science*, 1228282. https:// doi.org/10.1126/science/1228282

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Thierer T. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, **28:** 1647-1649. https://doi.org/10.1093/bioinformatics/bts199

Kissling J, Barrett SCH. **2013**. Variation and evolution of herkogamy in *Exochaenium* (Gentianaceae): Implications for the evolution of distyly. *Annals of Botany* **112**: 95–102. https://doi.org/10.1093/aob/mct097

Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular biology and evolution*, **33:** 1870-1874. https://doi.org/10.1093/molbev/msw054

Li ZZ., Sun SS, Wang QF, Chen, J. M. 2017. RNA-Seq analysis of the distylous plant *Nymphoides peltata* identified ortholog genes between long-and short-styled flowers. *Frontiers in Ecology and Evolution*, **5:** 59. https://doi.org/10.3389/fevo.2017.00059

Lloyd DG, Webb CJ. 1986. The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. *New Zealand journal of botany*, **24:** 135-162. https://doi.org/10.1080/0028825X.1986.10409725

Lloyd DG, Webb CJ. 1992. "The evolution of heterostyly.In: *Evolution and function of heterostyly*. Monographs on theoretical and applied genetics. 151-178. https://doi.org/10.1007/978-3-642-86656-2_6

Maddison WP, Midford PE, Otto SP. 2007. Estimating a binary character's effect on speciation and extinction. *Systematic biology*, **56:** 701-710. https://doi.org/10.1080/10635150701607033

Maddison WP. 2008. Mesquite: a modular system for evolutionary analysis. *Evolution*, **62:** 1103-1118. https://doi.org/10.1111/j.1558-5646.2008.00349.x

Mast AR, Kelso S, Conti E. **2006**. Are any primroses (*Primula*) primitively monomorphic? *The New phytologist* **171**: 605–16. https://doi.org/10.1111/j.1469-8137.2006.01700.x

Mather K, De Winton D. 1941. Adaptation and counter-adaptation of the breeding system in Primula. *Annals of Botany*, **5:** 297-311. https://doi.org/10.1093/oxfordjournals.aob.a087394

Naiki A. **2012**. Heterostyly and the possibility of its breakdown by polyploidization. *Plant Species Biology* **27**: 3–29. https://doi.org/10.1111/j.1442-1984.2011.00363.x

Nepokroeff M, Bremer B, Sytsma KJ. **1999**. Reorganization of the genus *Psychotria* and Tribe Psychotrieae (Rubiaceae) inferred from ITS and rbcL sequence data. *Systematic Botany* **24**: 22. https://doi.org/10.2307/2419383

Nielsen R. 2002. Mapping mutations on phylogenies. *Systematic biology*, **51:** 729-739. https://doi.org/10.1080/10635150290102393

Ornduff, R. 1988. Distyly and incompatibility in *Villarsia congestiflora* (Menyanthaceae), with comparative remarks on *V. capitata*. *Plant systematics and evolution*, **159:** 81-83. https://doi.org/10.1007/BF00937426

Pailler T, Humeau L, Figier J, Thompson JD. 1998. Reproductive trait variation in the functionally dioecious and morphologically heterostylous island endemic *Chassalia corallioides* (Rubiaceae). *Biological Journal of the Linnean Society*, **64:** 297-313. https://doi.org/10.1111/j.1095-8312.1998.tb00335.x

Pérez-Barrales R, Arroyo J. **2010**. Pollinator shifts and the loss of style polymorphism in *Narcissus papyraceus* (Amaryllidaceae). *Journal of evolutionary biology* **23**: 1117–28. https://doi.org/10.1111/j.1420-9101.2010.01988.x

Rambaut A., Drummond AJ. 2014. Tracer: MCMC trace analysis tool, version 1.5. *Available at website http://tree. bio. ed. ac. uk/software/tracer*.

Razafimandimbison SG, Rydin C, Bremer B. **2008**. Evolution and trends in the Psychotrieae alliance (Rubiaceae) - a rarely reported evolutionary change of many-seeded

carpels from one-seeded carpels. *Molecular phylogenetics and evolution* **48**: 207–23. https://doi.org/10.1016/j.ympev.2008.03.034

Richards AJ. **1986**. Plant Breeding Systems. *EdAllen Unwin*. London.

Richards, AJ. 1998. Lethal linkage and its role in the evolution of plant breeding systems. *Reproductive biology*, 71-83.

Rodrigues EB, Consolaro H. **2013**. Atypical distyly in *Psychotria goyazensis* Mull. Arg. (Rubiaceae), an intramorph self-compatible species. *Acta Botanica Brasilica* **27**: 155–161. https://doi.org/10.1590/S0102-33062013000100016

Sá T, Furtado MT, Ferrero V, Pérez-Barrales R, Rodrigues EB, dos Santos IG, Consolaro H. **2016**. Floral biology, reciprocal herkogamy and breeding system in four *Psychotria* species (Rubiaceae) in Brazil. *Botanical Journal of the Linnean Society* **182**: 689–707. https://doi.org/10.1111/boj.12476

Sakai S, Wright SJ. **2008**. Reproductive ecology of 21 coexisting *Psychotria* species (Rubiaceae): when is heterostyly lost? *Biological Journal of the Linnean Society* **93**: 125–134. https://doi.org/10.1111/j.1095-8312.2007.00890.x

Schoen DJ, Johnston MO, Lheureux a M, Marsolais J V. **1997**. Evolutionary history of the mating system in *Amsinckia* (Boraginaceae). *Evolution* **51**: 1090–1099. https://doi.org/10.1111/j.1558-5646.1997.tb03956.x

Sugawara T, Yumoto M, Tsuneki S, Watanabe K. **2014**. Incompatibility and reproductive output in distylous *Psychotria boninensis* (Rubiaceae), endemic to the Bonin (Ogasawara) Islands, Japan. *Journal of Japanese Botany* **89**: 22–26.

Tanner E. 1982. Breeding systems in a tropical forest in Jamaica. *Biological Journal of the Linnean Society* **18:**263-278. https://doi.org/10.1111/j.1095-8312.1982.tb02039.x

Taylor CM. 1989. Revision of *Palicourea* (Rubiaceae) in Mexico and Central America. *Systematic Botany Monographs*, 1-102. https://doi.org/10.2307/25027720

Taylor CM. **1997**. Conspectus of the Genus *Palicourea* (Rubiaceae: Psychotrieae) with the description of some new species from Ecuador and Colombia. *Annals of the Missouri Botanical Garden* **84**: 224–262. https://doi.org/10.2307/2400003

Thomson, JD, Barrett SCH. 1981. Selection for outcrossing, sexual selection and the evolution of dioecy in plants. *American Naturalist* **118**: 443–449. https://doi.org/10.1086/283837

Truyens S, Arbo MM, Shore JS. **2005**. Phylogenetic relationships, chromosome and breeding system evolution in *Turnera* (Turneraceae): Inferences from its sequence data. *American Journal of Botany* **92**: 1749–1758. https://doi.org/10.3732/ajb.92.10.1749

Veloso HP, Rangel Filho ALRR, Lima JCA. 1991. Classificação da vegetação brasileira, adaptada a um sistema universal. IBGE, Rio de Janeiro.

Watanabe K, Shimizu A, Sugawara T. **2013**. Dioecy derived from distyly and pollination in *Psychotria rubra* (Rubiaceae) occurring in the Ryukyu Islands, Japan. *Plant Species Biology*. https://doi.org/10.1111/1442-1984.12013

Watanabe K, Sugawara, T. 2015. Is heterostyly rare on oceanic islands?. *AoB Plants*, *7*. https://doi.org/10.1093/aobpla/plv087

Watanabe K, Aleck Yang TY, Nishihara C, Huang TL, Nakamura K, Peng CI, Sugawara T. **2015**. Distyly and floral morphology of *Psychotria cephalophora* (Rubiaceae) on the oceanic Lanyu (orchid) Island, Taiwan. *Botanical Studies* **56**: 1–9. https://doi.org/10.1186/s40529-015-0091-9

Zhou W, Barrett SCH, Wang H, Li D-Z. **2012**. Loss of floral polymorphism in heterostylous *Luculia pinceana* (Rubiaceae): a molecular phylogeographic perspective. *Molecular ecology* **21**: 4631–45. https://doi.org/10.1111/j.1365-294X.2012.05707.x

Zhou W, Barrett SCH, Li HD, Wu ZK, Wang XJ, Wang H, Li DZ. 2017. Phylogeographic insights on the evolutionary breakdown of heterostyly. *New Phytologist*, **214:** 1368-1380. https://doi.org/10.1111/nph.14453

Capítulo I - Phylogenetic reconstruction of distyly and its breakdown in species of *Psychotria* L. and *Palicourea* Aublet (Rubiaceae)

Supplementary data

Supplementary table 1. Refences of the DNA sequences and of the breeding system data of the species used for the phylogenetic analysis.

Genbank reference number	Species	Breeding system reference
EU145363.1	*Faramea multiflora*	Consolaro, 2009
KJ804878.1	*Palicourea alpina*	Tanner, 1982
AF149321.1	*Palicourea calophlebia*	Taylor ,1989
AF149320.1	*Palicourea corymbifera*	Santos, 2016
AF149322.1	*Palicourea crocea*	Pers. obs.
AF149324.1	*Palicourea fendleri*	Lau & Bosque, 2003
AF072010.1	*Palicourea guianensis*	Taylor, 1997
AF072009.1	*Palicourea lasiorrhachis*	Feinsinger & Busby, 1987
AF149335.1	*Palicourea macrobotrys*	Consolaro *et al*., 2009
KC480539.1	*Palicourea macrocalyx*	Taylor, 1989
KC480540.1	*Palicourea montivaga*	Taylor, 1989
AF072008.1	*Palicourea padifolia*	Ree, 1997
AF149337.1	*Palicourea petiolaris*	Sobrevilla *et al*., 1983
AF149338.1	*Palicourea pittieri*	Sakai & Wright, 2008
AF149342.1	*Palicourea rigida*	Machado *et al*., 2010
EF667969.1	*Psychotria acuminata*	Bawa & Beach, 1983
AF072001.1	*Psychotria brachiata*	Faivre & McDade, 2001
AF072004.1	*Psychotria brachybotrya*	Faivre & Mcdade, 2001
AF072053.1	*Psychotria brasiliensis*	Silva & Vieira, 2015
AF072005.1	*Psychotria capitata*	Faivre & Mcdade, 2001
KC480533.1	*Psychotria carthagenensis*	Consolaro *et al*., 2011
KJ804900.1	*Psychotria cephalophora*	Watanabe *et al*., 2015
AF072051.1	*Psychotria chagrensis*	Faivre *and* McDade, 2001
AF072006.1	*Psychotria deflexa*	Sá *et al*., 2016
AF072011.1	*Psychotria elata*	Silva & Segura, 2015
AY350670.1	*Psychotria grandiflora*	Shomer, 1978
KJ804909.1	*Psychotria grandis*	Sakai & Wright, 2001
AY350664.1	*Psychotria hatheway*	Sohmer, 1978
AF034907.1	*Psychotria hexandra*	Sohmer, 1977
AF034906.1	*Psychotria hobdyi*	Sohmer, 1976
EF667970.1	*Psychotria hoffmannseggiana*	Sá *et al*., 2016
AF072047.1	*Psychotria horizontalis*	Sakai & Wright, 2008
AF072052.1	*Psychotria limonensis*	Sakai & Wright, 2008
AF072025.1	*Psychotria manillensis*	Watanabe *et al*., 2015
AF072040.1	*Psychotria mapourioides*	Pers. obs.
AF072049.1	*Psychotria marginata*	Sakai & Wright, 2008
AF072048.1	*Psychotria micrantha*	Sakai & Wright, 2008
AF072013.1	*Psychotria microdon*	Pers. obs.
AF072046.1	*Psychotria nervosa*	Hernandes Ramires, 2012
AF071998.1	*Psychotria pittieri*	Sakai & Wright, 2008

Capítulo I - Phylogenetic reconstruction of distyly and its breakdown in species of *Psychotria* L. and *Palicourea* Aublet (Rubiaceae)

AF071993.1	*Psychotria poeppigiana*	Coelho *et al*., 2003
AF071997.1	*Psychotria pubescens*	Sakai & Wright, 2008
AF071995.1	*Psychotria racemosa*	Sakai & Wright, 2008
AF072035.1	*Psychotria rubra*	Yang, 1998
AF072036.1	*Psychotria serpens*	Sugawara, 2013
AF072050.1	*Psychotria tenuifolia*	Sakai & Wright, 2008
AF149407.1	Psychotria trichophoroides	Sá *et al*., 2016
FJ208620.1	*Psychotria viridis*	Pers. obs.

Capítulo II

Pollination imprecision and phenotypic selection in monomorphic and distylous *Psychotria* L. **(Rubiaceae) species**

Introduction

Distylous plants have two floral morphs in their populations, in which, plants exhibit long-styled flowers (long stigmas placed above anthers height) or short-styled flowers (short stigmas placed below anthers height), there is also reciprocal placement of the sexual organs between the two floral morphs and a heteromorphic system of incompatibility (Ganders, 1979; Hamilton, 1990). Nevertheless, this breeding system commonly present breakdowns, absence of one of the floral morphs (monomorphism) or herkogamy loss (homostyly) are the most reported transitions in heterostylous plants (Hamilton, 1990; Barrett, 1990). Studies with the genetic control of distyly for some taxa proposed that floral morphology and incompatibility system are jointly controlled by a supergene (Dowrick, 1956; Muenchow, 1981, Barrett & Richards, 1990; Brennan, 2017). In this model, one of the genes determines the androecial height, the second gene regulates the pollen grain size and the third gene controls pistil height, stigmatic papillae size and incompatibility reaction (Barrett & Richards, 1990). But, distyly have evolved independently in many family plants (Ganders, 1979; Naiki, 2012) and its genetic control was not studied for most of the heterostylous taxa. There are evidences that the incompatibility reactions and the morphological traits of the breeding system may have developmental and functional independence or even that they are developmentally and physiologically distinct process among the floral morphs (Ernst, 1955; Ganders, 1979; Dulberger, 1992; Perez-Barrales *et al.*, 2006; Consolaro *et al.*, 2011; Ferrero *et al.*, 2012; Santos-Gally *et al.*, 2013; Rodrigues & Consolaro, 2013).

Distyly is proposed since Darwin (1887) studies as a mechanism for the promotion of precision in cross-pollination. Precision or imprecision in pollination can be estimated in flowering plants (inaccuracy *sensu* Hansen *et al.*, 2006; Armbruster *et al.*, 2009). This imprecision can be understood as the departure from the optimum caused by variation among phenotypic targets. And can be estimated as adaptive inaccuracy (composed by trait deviance and adaptive imprecision), in different plant pollination systems

(Armbruster *et al.*, 2009). In distylous plants, pollination imprecision can also represent a measure of reciprocity among floral morphs of heterostylous species (Armbruster *et al.*, 2017). In distylous and non distylous hermaphrodite flowers, there is an intra-floral sexual conflict between avoiding within-flower interference and achieving precision in pollination, which is known as "herkogamy dilemma", that is reduced in herkogamous and dichogamous flowers (Barrett, 2002; Armbruster *et al.*, 2014). In distylous plants, reciprocal herkogamy is considered a mechanism for the promotion of outbreeding through disassortative breeding (Lewis & Jones, 1992; Hernandez & Ornellas, 2007). But, the pressures for the selection of reciprocal herkogamy are different of the proposed for approach herkogamy in flowers. Avoidance of self-pollination and promotion of outcross pollination may be the major selective pressures responsible for the evolution of the approach herkogamous flowers (Webb & Lloyd, 1986). Otherwise, reciprocal herkogamy works as a facilitation mechanism of legitimate pollination and prevention of pollen wastage (Baker, 1964; Lloyd & Yates, 1982; Webb & Lloyd, 1986; Charlesworth & Charlesworth, 1979; Ganders, 1979). Even though, the reciprocal placement of male and female sexual organs may also be selected for avoidance of mutual interference between male and female functions within the flower. Thus, heterostyly is another type of escape from the herkogamy dilemma, where having two forms of flowers and intramorph incompatibility promotes precision in pollination, expecting this way high pollination precision in species with strong reciprocal herkogamy (Sanchez *et al.*, 2010, Armbruster *et al.* 2009).

In these evolutionary transitions from distyly, it is predicted that is unlikely that distyly is more efficient in outbreeding than monomorphic adaptations of self-compatible species (Ganders, 1979). Despite heteromorphic plants pursuit adjusted integration of male and female traits of the floral morphs to ensure disassortative mating (Lewis & Jones, 1992), the male and female floral traits of long and short-styled flowers of distylous plants could have different developmental patterns (Cohen *et al.*, 2012), and the functional significance of each floral character may differ between the different floral morphologies (Yeo, 1975) with possible effects in pollination precision. On the other hand, approach herkogamy in monomorphic flowers may restrict self-interference itself, but, requires cross-pollination between anthers and stigmas placed at different levels of height, but this placement of stigmas above the anthers height promotes sequential touching of sexual organs with pollinators body and is also efficient in cross pollination (Webb & Lloyd,

1986). No studies have evaluated differences in pollination precision, in floral integration and selection on traits across species with distyly and species with breeding systems derived from distyly (eg.: monomorphism, homostyly) in the tropics. In this study, we aim to evaluate pollination imprecision and phenotypic selection two tropical species of *Psychotria* L. with distinct breeding system strategies, monomorphism and distyly. We hypothesize that: i) pollination imprecision is lower in distylous flowers than in monomorphic species ii) populations of *Psychotria* of both species with lower inaccuracy will present higher fitness; iii) spatial separation of male and female sexual organs in flowers (herkogamy) will have higher effect in plant fitness than traits related to pollinator attraction (corolla morphology).

Material and methods

Species system:

The study was developed in four tropical semidecidous forests in Minas Gerais, Central Brazil (fig.1). We sampled three populations of each species. *P. prunifolia* in Irara, São José and Uberaba forests in the cities of Uberlândia and Uberaba, Minas Gerais, Brazil. For *P. trichophoroides* we collected samples in Cruzeiro, Irara and São José forests (fig. 1). The region is characterized by Tropical Savanna Climate (Aw/As) under Köppen climate classification (Köpen, 1884). There are two dominant seasons throughout the year: a hot wet season from October to March, and a dry cold one, from April to September. The studied species, *Psychotria prunifolia* (Kunth) Steryerm. and *P. trichophoroides* Mull. Arg., (Fig. 2) are shrubs that occurs at the understory of the study forests areas. *P. prunifolia* is a monomorphic self-compatible species with white flowers with approach herkogamy, pollinated mainly by *Epicharis flava* (Apidae), *Euglossa* sp. *and Bombus* sp. bees (Sá *et al.*, 2016). *P. trichophoroides* also has white flowers, but the flowers are distylous with heteromorphic incompatibility and are mainly pollinated by *Epicharis* sp. and *Euglossa* sp. bees.

Floral morphology

We realized field sampling during November and December of 2015 and January of 2016, when the species were blooming. For each population of *P. prunifolia* we collected three flowers in 25 random plants, while for *P. trichophoroides* we collected three flowers per plant in 50 random individuals, 25 plants of each floral morph. Flowers were collected

and fixed in 70% alcohol in the field and photographed in the laboratory. Using the image analysis software ImageJ (Schindelin *et al.* 2015), we measured the corolla height (from its base to the petals tip), corolla entrance width, style length, stigma height (style length + half of the stigmatic lobules length), stigmatic lobes length (sum of the length of the two lobules), filament length, anther height (filament length + half of anther length) and anther length. Spatial separation between sexual organs (herkogamy) was calculated for each species at the flower level, (n = 75 flowers for *P. prunifolia* and N = 75 flowers per morph for *P. trichophoroides* in each population). We estimated herkogamy calculating stigma anther separation (|stigma height – anther height|).
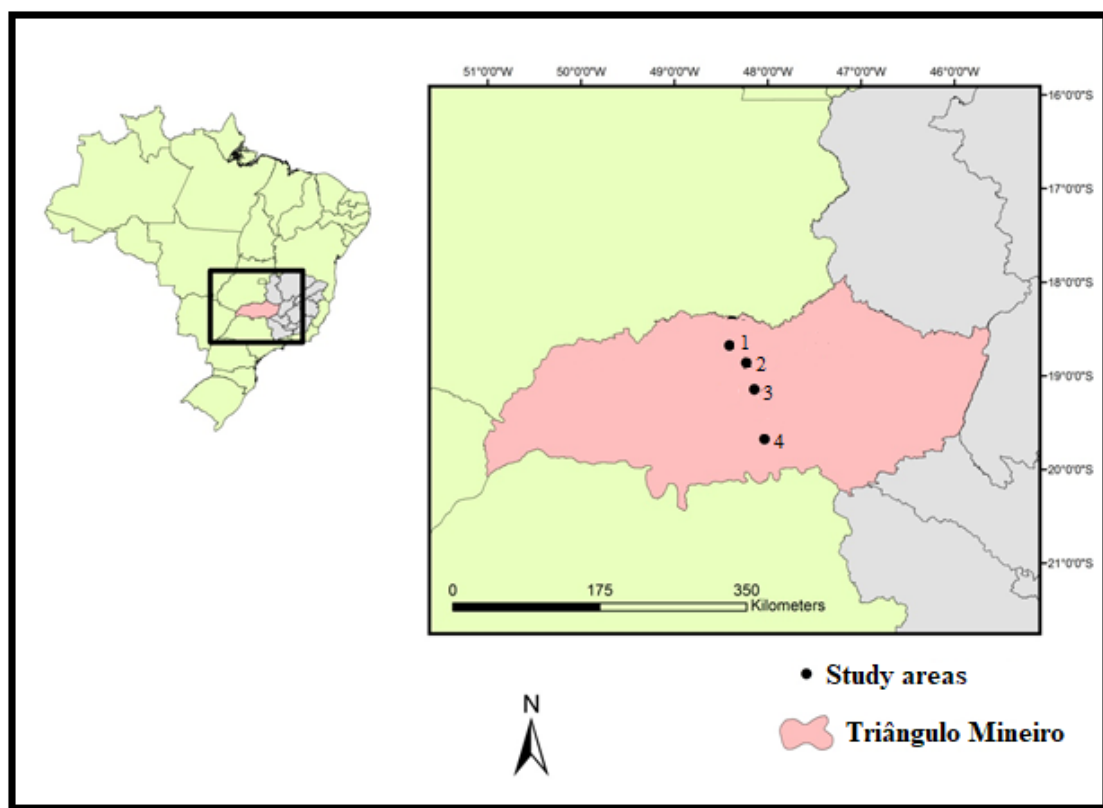
Figure 1: Study areas for sampling of breeding system data of populations of *Psychotria prunifolia* (Kunth) Steyerm. and *Psychotria trichophoroides* Mull. Arg in the Triângulo Mineiro, Minas Gerais, Brasil. 1- Cruzeiro; 3- São José; 3- Irara; 4- Uberaba.

Fitness sampling

We estimate female fitness (natural fruit set) in 25 plants of *P. prunifolia* and 50 of *P. trichophoroides* (25 per morph). We marked 3 random flowers in each plant, and 60 days after that, we recorded the presence and absence of fruits. We also measured female fitness through natural pollen load on stigmas in 25 plants of *P. prunifolia* and 50 of *P. trichophoroides* (25 per morph). At the end of the flower anthesis (4:00 pm), we collected

3 random flowers in each sample plant, stored them in microtubes and conducted in refrigerated bags to the laboratory. Then, we carefully removed the stigma from the flowers, fixed them in Fucsin glycerol jelly, and counted the number of pollen grains on the stigma under light microscopy. *P. trichophoroides* has pollen size dimorphism in the floral morphs (pers, obs.), therefore, the pollen grains can be easily discriminated from each other. We followed the same methods used by García-Robledo (2008) with Rubiaceae pollen grains, to estimate stigmatic pollen load. We photographed the pollen grains, and then measured and counted the number of compatible pollen grains in each stigma. Pollen grains that overlap in size and pollen from another species were excluded from the data to perform the analysis. In some study areas, the species were locally sympatric, and their blossoms overlapped making it impossible to distinguish among pollen grain types.

Figure 1: A – Flowers of *P. prunifolia*; B- Pin flowers of *Psychotria trichophoroides*; C- Thrum flower of *P. trichophoroides*.

Pollination imprecision estimating

We estimated pollination inaccuracy (a measure of pollination imprecision) in different levels. Inaccuracy at the population level (Hansen *et al.*, 2006; Armbruster *et al.*, 2009; Armbruster *et al.*, 2017) was estimated using the mean of stigma and anther height of the flowers (n=3) collected in one same plant of each population of the study (n= 25 plants for the monomorphic populations and n= 25 plants per morph for *P. trichophoroides*. We also estimated inaccuracy at the flower level (n = 225 for *P. prunifolia* and n = 225 flowers per morph for *P. trichophoroides* in each population of the study). Inaccuracy at both levels was estimated using the equation proposed by Armbruster (2004, 2009, Armbruster *et al*., 2017), using trait variance (O^2) and maladaptation bias (deviation from the trait mean (μ) from the optimum (Θ):

$$\text{Inaccuracy} = O^2 + (\mu - \Theta)^2$$

$$\text{High organs inaccuracy} = (A-S)^2 + VA + VS \text{ scaled by long stigma height}$$

$$\text{Short organs inaccuracy} = (a-s)^2 + Va + Vs \text{ scaled by short stigma height}$$

$$\text{Male organs inaccuracy} = (A-S)^2 + VA + VS \text{ scaled by anthers height}$$

$$\text{Female organs inaccuracy} = (a-s)^2 + Va + Vs \text{ scaled by stigma height}$$

$$\text{Flower inaccuracy} = (\text{flower stigma height - mean of population anthers height})^2 + \text{Vpopulation anthers height})$$

Where A represents the mean height of the high anthers, S the mean height of the high stigmas, a: mean height of the low anthers, s the mean height of the low stigmas and V the variance of these sexual organs.

Population data and mean of the traits of the three flowers collected in each sampled plant were pooled to perform the analysis comparing male and female inaccuracies in *P. prunifolia* and high and low inaccuracy for *P. trichophoroides*. The values of inaccuracy represent units of mm^2 of the traits. The total inaccuracies of male and female organs for *P. prunifolia* and low and high organs of *P. trichophoroides* are decomposed in percentages of maladaptive bias^2 (the square of the departure of the trait mean from the optimum), variance of the anthers and variance of the stigmas (Armbruster *et al.*, 2017).

To compare inaccuracy across populations and species we used the average mean of organs height to standardize the inaccuracies, which allow to compare pollination imprecision across species and populations (for details see Armbruster *et al.*, 2017).

Effect of inaccuracy on species fitness

To test the effect of inaccuracy in the stigmatic pollen load and in the fruit set of the species we performed General Linear Models (GLM), using binomial distribution with a logit-link function for the fruit set and Poisson distribution for the natural pollen load on stigmas of the species. In the models, we considered the female fitness measures (fruit set and natural pollen load) as dependent variables and the inaccuracies (female, low and high) as predictors in the analysis. Population data were pooled together to run the analysis (n=75 flowers in *P. prunifolia* and n= 75 flowers per morph in *P. trichophoroides*).

Phenotypic selection modelling

We used hierarchical statistics methods and structural equation models (Grace, 2006) to evaluate phenotypic selection in flowers of the two *Psychotria* species. A set of 4 candidate models were built using the variables that were thought to influence the probability of fruit set and the number of pollen grains on the stigma of flowers. We used the following variables to build the models: corolla length, corolla entrance width, stigma lobules length, anther length, approach herkogamy, stigma height and anther height. (tab. 1). We used General Linear Mixed Models (GLMMs), performing the analysis combining variables of spatial positioning of sexual organs and pollinator attraction described in Table 1. In all the models, we considered the fitness measures (fruit set and natural pollen load) as dependent variable, flower morphology variables as fixed predictors and flowers nested within plant as random factors in the analysis. In the distylous species *P. trichophoroides* we model floral integration and phenotypic selection in pin and thrum male and female fitness using GLMMs with binomial and Poisson distributions, respectively. The structural equation models also allowed to evaluate integration among traits that were considered as independent variables in the analysis (Armbruster *et al.*, 2005, Grace, 2006). To evaluate which of the models provide the highest empirical

support for phenotypic selection in fitness, we used the Akaike's Information Criterion (AIC) values and the differences in the AIC among the models (ΔAIC). The models with ΔAIC < 2 have substantial empirical support, while those between 4 and 7 have less support and those with values > 10 have no support (Burnham & Anderson, 2004). The models were fitted in R statistics (Team R, 2013) using the Stats (Team R, 2013) and the lme4 (Bates *et al.*, 2014) packages.

Table 1. Variables of 4 candidate models to evaluate floral integration and phenotypic selection at the flower and plant levels in two *Psychotria* species with different breeding systems.

Models	Variables
Adjustment to pollinator attraction and morphology	corolla height and corolla width
Herkogamy (self-interference avoidance)	*P. prunifolia*: approach herkogamy
	P. trichophoroides: pin herkogamy, thrum herkogamy
Full model	corolla height, corolla entrance width and approach herkogamy,

Results

Pollination imprecision

We find no evidences that distylous flowers are more precise in pollination than monomorphic flowers of *Psychotria* species. Both the *Psychotria* species had low imprecision in spatial positioning of stigmas and anthers. The mean-squared standardized inaccuracy in all populations was lower than 1% (tab. 2). But, total inaccuracy was higher in distylous *P. trichophoroides* than in monomorphic *P. prunifolia* (tab. 2). In *P. prunifolia*, male inaccuracy had higher contribution to total population inaccuracy than female inaccuracy. Moreover, trait deviation (anther and stigma heights) from the

optimum point of pollen placement/dispatch (stigma and anther height) was the factor with the highest contribution for the variations in pollination imprecision of monomorphic populations of *Psychotria* prunifolia (tab. 2). The distylous *P. trichophoroides* populations had higher inaccuracy at the low-level organs (pin anthers, thrum stigma) than at the high-level organs (thrum anthers and pin stigma) (tab. 2). However, the maladaptive bias (trait height deviation from optimality in pollen arrive/dispatch) in these populations was very reduced, and stigma and anther height variances were the factors which contributed to the inaccuracy in the distylous populations (tab. 2).

Capítulo II: Pollination imprecision and phenotypic selection in monomorphic and distylous *Psychotria* L. (Rubiaceae) species

Table 2 Inaccuracy and its different components of monomorphic populations of *P. prunifolia* and distylous populations of *P. trichophoroides*. Percentages of decomposed components of inaccuracy are presented in parentheses.

Pop.	Organ type	Inaccuracy	Maladaptive bias^2	Anther variance	Stigma variance	Total inaccuracy	Mean^2 standardized total inaccuracy
Psychotria prunifolia							
	Female	1.08 (42.52)	8.8 (30.05)	0.75 (2.56)	2.9 (9.9)	2.54	0.025
Irara	Male	1.46 (57.48)					
	Female	0.9 (42.65)	6.01 (30.66)	0.99 (5.05)	1.36 (6.94)	2.11	0.032
Sao Jose	Male	1.22 (57.82)					
	Female	0.57 (45.24)	3.31 (27.08)	1.22 (9.98)	1 (8.18)	1.26	0.016
Uberaba	Male	0.7 (55.56)					
P. trichophoroides							
	Low	4.19 (59.27)	0.0009 (0.072)	1.8 (25.46)	2.39 (33.8)	7.07	0.075
Cruzeiro	High	2.88 (40.73)	0.13 (0.2)	1.14 (16.1)	1.61 (22.77)		
	Low	1.18 (54.38)	0.29 (0.31)	0.63 (29.03)	0.26 (11.98)	2.17	0.018
Irara	High	0.99 (45.62)	0.46 (0.48)	0.19 (8.75)	0.34 (15.67)		
	Low	4.09 (55.05)	1.05 (1.12)	1.43 (19.25)	1.61 (21.67)	7.43	0.069
Sao Jose	High	3.34 (44.95)	0.04 (0.11)	2.07 (27.86)	1.23 (16.55)		

There was no effect on male or female inaccuracy in the probability of fruit set and in the number of pollen grains on the stigma of flowers of *P. prunifolia* (tab. 3). In *P. trichophoroides* low level inaccuracy did not affect the probability of fruit set and the number of compatible flowers in the stigma. However, flowers with reduced inaccuracy in the high-level organs presented higher number of compatible pollen grains on the stigma (tab. 3). The slope of the regressions indicates a moderate effect of imprecision in pollination in the number of compatible pollen grains on the stigma *P. trichophoroides* (tab 3).

Table 3 Slopes [estimate] of the generalized linear models of the effect of inaccuracy in the probability of fruit set and in the number of pollen grains on the stigma flowers of *Psychotria* species.

|	Fruit set probability			Number of pollen grains on the stigma		
Psychotria prunifolia	Slopes (β)	*t*	*P*	Slopes (β)	*z*	*P*
Male inaccuracy	-0.008	-1.1	0.27	0.004	0.61	0.54
Female inaccuracy	-0.001	-0.35	0.73	-0.005	1.45	0.14
Psychotria trichophoroides						
Low organs inaccuracy	0.06	2.24	0.03	-0.01	-1.06	0.29
High organs inaccuracy	0.005	0.14	0.88	-0.14	-7.06	< 0.001

For *P. prunifolia* approach herkogamy was a better predictor to explain the probability of fruit set than traits related to pollinators attraction. (Fig. 1, sup. data I). Flowers with reduction in stigma-anther separation had lower probability of fruit set success, although, this effect was not significant ($y= 13.85 – 0.28x$, $\chi^2 = 0.1$, $p = 0.74$). However, the number

of pollen grains on the stigma of *P. prunifolia* was influenced by traits responsible for pollinator attraction, corolla height and corolla entrance width. However, only the corolla width did have significant effect on the number of pollen grains on the stigma (Fig. 2). Flowers with narrower corollas entrance had lower number of pollen grains on their stigmas. Although, these effect size of corolla width was similar in *P. prunifolia* and *P. trichophoroides*. Integration among the traits related to pollinators attraction (height and width of corolla of the flowers) was weak and not significant for either species (Fig.2).

Different models explained the fruit set and number of compatible pollen grains on stigma of pin and thrum flowers of *P. trichophoroides* (Fig. I, sup. data I). The fruit set of pin flowers was better explained by approach herkogamy (y = 1,36 – 0.31x. χ^2 = 3.09, p = 0.29) than by corolla traits. Even with no statistical significance, pin flowers showed a tendency to lower fruit set in flowers with reduced approach herkogamy. However, the model with traits related to pollinator attraction (corolla's height and width) explained better the fruit set of thrum flowers than the approach herkogamy models (fig 2). This model had statistical significance (χ^2 = 19.28, p < 0.001), and indicated that thrum flowers with narrower corollas entrance may present lower probability of fruit set.

The number of compatible pollen grains on the stigma of pin flowers was explained by the approach herkogamy model, but this effect was not strong and not statistically significant when compared to the herkogamy model (y = 2.41 – 0,03x, χ^2 = 0.0002, p = 0.98). Nevertheless, in thrum flowers, traits related to attraction of pollinators better explained the number of compatible pollen grains on the stigma than the herkogamy model (Fig. 2). The model was statistically significant and indicated that a reduction in corolla width may also cause reduction in the number of compatible pollen grains in thrum flowers.

P. prunifolia
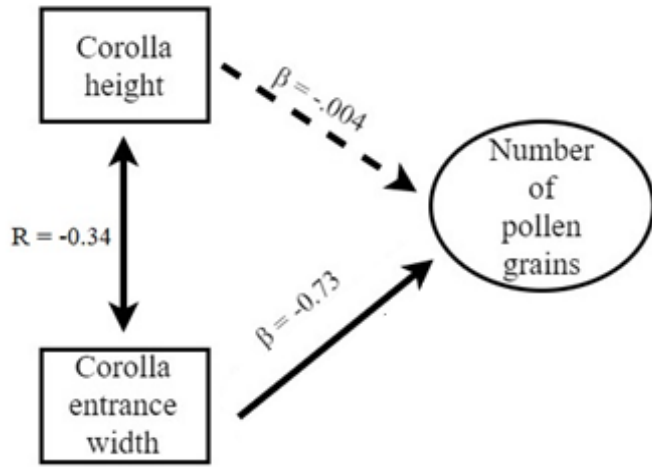
P. trichophoroides
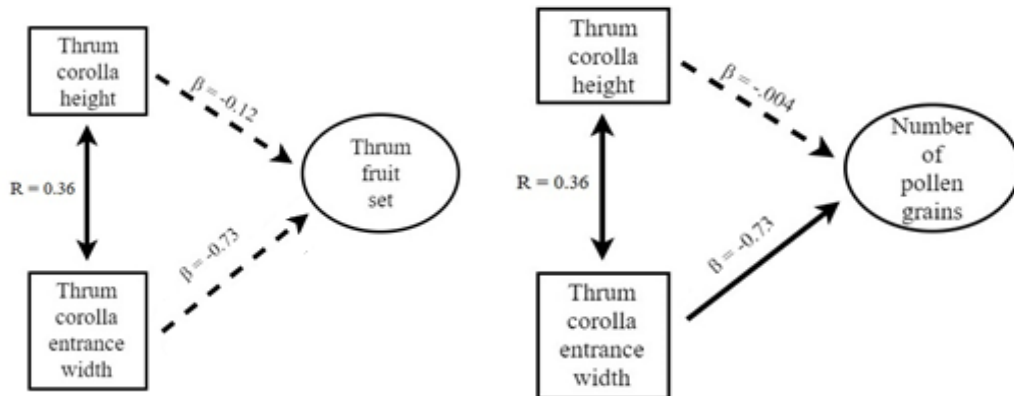
Figure 2. Structural models of phenotypic selection on female fitness of monomorphic and distylous *Psychotria* L. (Rubiaceae) species. Dashed arrows in the models represents non-significant probability at the 0.05 level while solid arrows indicate significant probabilities at the 0.05 level. In the pathway diagrams "R" represent the correlation coefficient of the variables and "β" indicates the slopes off the effect of the explaining variables in the dependent variable.

Discussion

Both the *Psychotria* species presented pollination precision and the estimated inaccuracy values were similar across populations and species. Darwin (1877) suggests that the presence of reciprocal herkogamy in plants as a mechanism to promote accuracy in cross-pollination. The main difference in inaccuracy of monomorphic and heterostylous flowers in *Psychotria* species, is that reciprocity (inaccuracy *sensu* Armbruster *et al.*, 2017) strongly reduced deviation from the optimum (maladaptation bias) in *P. trichophoroides*, and, the imprecision in pollination results from low-level organs height variance. Meanwhile, *P. prunifolia* inaccuracy occurs mostly due stigma and anthers height deviation from the optimal pollen receipt and dispatch position (adaptive bias). So, different factors influenced floral precision in monomorphic and distylous species and may represent different genetic response of the flowers to phenotypic selection (Armbruster, 2014).

There was no evidence of effect of pollination imprecision on female fitness. Our results agree with Ganders (1979) prediction, that distyly is not more efficient in outbreeding than adaptations for outcrossing in monomorphic self-compatible species. Herkogamy is widely distributed across plant families (Webb & Lloyd, 1986), and one of its types, movement herkogamy, can break the effects of this dilemma in *Parnasia* flowers (Armbruster *et al.*, 2014). Therefore, it seems that monomorphic and distylous *Psychotria* species can also solve herkogamy dilemma. Since approach herkogamy prevents self-interference in *P. prunifolia*, the species rely on pollinators for reproduction and there is no evidence of a self-pollen deposition mechanism (Sá *et al.*, 2016), and the species present low inaccuracy in pollination (< 0.5 %). The higher departure from the optimal position for pollen placement and dispatch in *P. prunifolia* than in *P. trichophoroides* may represent variations in floral parts that lack integration as observed in *Linum* species (Armbruster *et al.*, 2009). However, sequential contact of stigma and anthers in *P. prunifolia* flowers occurs associated with high precision in pollination and the species can avoid of self-interference with approach herkogamy. Furthermore, inaccuracy in *P. trichophoroides* was lower (0.5%), when compared to distylous *Primula veris*, *P. elatior* e *P. vulgaris*, in which, imprecision was higher than 1.5% (Armbruster *et al.*, 2017) and distylous *Linum* species (> 5% Armbruster *et al.*, 2009). Seems that the different adaptions for reproduction promote precision in pollination in both *Psychotria* species.

In classical heterostylous systems as *Primula* and *Linum* that occurs in temperate climate areas, transitions from heterostyly to other breeding system strategies seems to affect precision in pollination, but in tropical distylous *Psychotria* species there were no differences in pollination precision among monomorphic and distylous flowers. Despite these finds, there is no evidences of greater stability of floral polymorphisms in tropical than in temperate zones in Rubiaceae (Ferrero *et al.*, 2012), and actually heterostylous groups display more variation in distylous traits in the tropics than in temperate climate areas (Barrett & Richards, 1990). It seems that distinct pressures led to different deviations from ancestral distyly in the Rubiaceae in islands and temperate climate areas, as loss of herkogamy with entirely populations presenting homostyly, dioecy and monoecy (Nakamura *et al.*, 2007; Naiki & Kato, 2011; Zhou *et al.*, 2012; Nagawaka & Naiki, 2014). Thus, breeding system in this plant family is flexible with many evolutionary variations to ensure reproduction, so that the variations found in our results represent an example that deviations in floral polymorphisms can occur in the tropics without loss of precision in pollination.

Despite no evidence of phenotypic selection in herkogamy or floral traits related to pollinators attraction in our study, approach herkogamy seems to play an important role in the reproduction of distylous and monomorphic species. Approach herkogamy showed a tendency to have an effect in the fruit set of monomorphic *P. prunifolia* and of fruit set and pollen grains arrival in pin flowers of *P. trichophoroides*. The reciprocal herkogamy mechanism of pollen transference associated with self and intramorph physiological incompatibility can eliminate self-interference in distylous *P. trichophoroides* (Sá *et al.* 2016). Thus, canalization of different pressures with negative frequency dependent selection may be the responsible of selection and maintenance of distyly in plants. Meanwhile, monomorphism may have selection in other traits to have precision in cross-pollen arrival and export and promote self-interference avoidance.

Pollen grains arrival in *P. prunifolia* and in pin flowers of *P. trichophoroides* were better predicted by corolla morphology than by spatial separation of sexual organs within the flowers. In the phenotypic selection models, corolla height had very reduced effect on stigmatic pollen load, while narrow corolla on flowers had negative effect in the number of pollen grains on the stigma. In distylous plants, narrower corolla may restrict access to pollinators to low level organs of thrum and pin flowers (Beach & Bawa, 1980). And, parts of corolla in tubular distylous flowers are considered mechanisms to enhance

positioning of pollen in insect mouth parts (Ganders 1979), thus promoting precision in pollination. Selection on these attractive traits is hypothesized to be linked more strongly to male fertility than to female fertility (Burd & Callahan, 2000). Although our results reflect effects only in female fitness, both species present no strategies for self-pollen deposition (Sá *et al.*, 2016) and both floral morphs in *P. trichophoroides* had similar number of incompatible pollen grains (sup. data II). Non-legitimate/incompatible pollen grains can have negative effect on reproduction, and female fitness can be affected by reduction in the number of ovules available for cross pollination by obstruction in the deposition of outcrossing pollen on stigmas (Shore & Barrett 1984, Barrett & Glover, 1985, Barrett *et al.*, 1996, Sage *et al.*, 1999). But, probably due few ovules availability, only two in *Psychotria* species, a small number of compatible or cross-pollen grains are required for reproduction, and precision in pollen receipt and dispatch can easily ensure reproduction by cross-pollination.

Even with different breeding system strategies both species had similar pollination precision. Differently from temperate distylous species, our study in the tropics reveal that the species with transition toward monomorphism and self-compatibility had similar pollination precision when compared to the distylous species. The main distinction in the precision in pollination of the *Psychotria* species, is that reciprocal herkogamy promoted reduction in maladaptation in positioning of floral sexual organs for pollen arrival and dispatch compared to the species with approach herkogamy. Inaccuracy has no effect in the fitness of both species. Traits related to pollination attraction (corolla morphology) and spatial separation of sexual organs had no considerable effect on species female fitness, and floral herkogamy was a better predictor for fruit set than corolla traits. However, the number of pollen grains on the stigma were better predicted by corolla morphology than by spatial separation of sexual organs. Pressures for disassortative mating versus assortative mating can be factors that influences maintenance and breakdown of distyly. But, herkogamy (reciprocal and approach) and corolla morphology (height and width) play different roles in phenotypic selection on fitness of *Psychotria* species.

References

Armbruster WS, Pélabon C, Hansen TF, Mulder CH. 2004. Floral integration, modularity, and accuracy. In: Pigliucci M, Preston *K. eds*. *Phenotypic integration: studying the ecology and evolution of complex phenotypes. New* York: Oxford University Press, 23–79.

Armbruster WS, Pélabon C, Hansen TF, Bolstad GH. **2009**. Macroevolutionary patterns of pollination accuracy: a comparison of three genera. *The New phytologist* **183**: 600–17. https://doi.org/10.1111/j.1469-8137.2009.02930.x

Armbruster WS. **2014**. Floral specialization and angiosperm diversity: phenotypic divergence, fitness trade-offs and realized pollination accuracy. *AoB plants* **6**: 1–24. https://doi.org/10.1093/aobpla/plu003

Armbruster WS, Corbet SA, Vey AJM, Liu SJ, Huang SQ. 2014. In the right place at the right time: *Parnassia* resolves the herkogamy dilemma by accurate repositioning of stamens and stigmas. *Annals of Botany* **113:** 97–103. https://doi.org/10.1093/aob/mct261

Armbruster WS, Bolstad GH, Hansen TF, Keller B, Conti E, Pélabon C. 2017. The measure and mismeasure of reciprocity in heterostylous flowers. *New Phytologist* **215:** 906–917. https://doi.org/10.1111/nph.14604

Baker, HG. 1964. Variation in style length in relation to outbreeding in *Mirabilis* (Nyctaginaceae). *Evolution* **18:** 507-509. https://doi.org/10.1111/j.1558-5646.1964.tb01627.x

Barrett SCH, Glover DE. 1985. On the Darwinian hypothesis of the adaptive significance of tristyly. *Evolution* **39:** 766–774. https://doi.org/10.1111/j.1558-5646.1985.tb00418.x

Barrett SCH, Richards JH. 1990. Heterostyly in Tropical plants. Memoirs of The New York Botanical Garden **55:** 35–61.

Barrett SCH, Harder LD, Worley A. 1996. The comparative biology of pollination and mating in flowering plants. *Philosophical Transactions of the Royal Society* Ser. B. **351:** 1271–1280. https://doi.org/10.1098/rstb.1996.0110

Barrett, SCH 2002. The evolution of plant sexual diversity. *Nature Reviews Genetics* **3:** 274–284. https://doi.org/10.1038/nrg776

Bates D, Mächler M, Bolker B, Walker S. 2014. Fitting linear mixed-effects models using lme4. arXiv preprint arXiv:1406.5823. https://doi.org/10.18637/jss.v067.i01

Beach JH, Bawa KS. 1980. Role of pollinators in the evolution of dioecy from distyly. *Evolution* **34:** 1138–1142. https://doi.org/10.1111/j.1558-5646.1980.tb04055.x

Brennan AC. 2017. Distyly supergenes as a model to understand the evolution of genetic architecture. *American Journal of Botany* **104:** 5–7. https://doi.org/10.3732/ajb.1600363

Burd M, Callahan HS. 2000. What does the male function hypothesis claim? *Journal of evolutionary biology* **13:** 735–742. https://doi.org/10.1046/j.1420-9101.2000.00220.x

Burnham, KP, Anderson DR. 2004. Multimodel inference: understanding AIC and BIC in model selection. *Sociological Methods and Research* **33:** 261–304. https://doi.org/10.1177/0049124104268644

Charlesworth D, Charlesworth B. 1979. A Model for the Evolution of Distyly. *The American Naturalist* **114:** 467. https://doi.org/10.1086/283496

Cohen JI, Litt A, Davis JI. 2012. Comparative floral development in *Lithospermum* (Boraginaceae) and implications for the evolution and development of heterostyly. *American Journal of Botany* **99:** 797–805. https://doi.org/10.3732/ajb.1100329

Consolaro H, Silva SCS, Oliveira PE. 2011. Breakdown of distyly and pin-monomorphism in *Psychotria carthagenensis* Jacq. (Rubiaceae). Plant Species Biology **26:** 24–32. https://doi.org/10.1111/j.1442-1984.2010.00300.x

Darwin C. 1877. *The Different Forms of Flowers on Plants of the Same Species*. London: John Murray. https://doi.org/10.5962/bhl.title.46988

Dowrick VPJ. 1956. Heterostyly and homostyly in *Primula obconica*. Heredity **10:** 219–236. https://doi.org/10.1038/hdy.1956.19

Dulberger, R. 1992. Floral Polymorphism and Their Functional Significance in the Heterostylous Syndrome. In: Barrett, SCH., *Ed., Evolution and Function of Heterostyly*, Springer Verlag, New York, 41-84. https://doi.org/10.1007/978-3-642-86656-2_3

Ernst A. 1955. Self-fertility in monomorphic Primulas. Genetica **27:** 391–448. https://doi.org/10.1007/BF01664170

Ferrero V, Chapela I, Arroyo J, Navarro L. 2011. Reciprocal style polymorphisms are not easily categorised: the case of heterostyly in *Lithodora* and *Glandora* (Boraginaceae). *Plant biology* 13 Suppl **1:** 7–18. https://doi.org/10.1111/j.1438-8677.2009.00307.x

Ferrero V, Rojas D, Vale A, Navarro L. 2012. Delving into the loss of heterostyly in Rubiaceae: Is there a similar trend in tropical and non-tropical climate zones? *Perspectives in Plant Ecology, Evolution and Systematics* **14:** 161–167. https://doi.org/10.1016/j.ppees.2011.11.005

Ganders FR. 1979. The biology of heterostyly. *New Zealand Journal of Botany* **17:** 607–635. https://doi.org/10.1080/0028825X.1979.10432574

García-Robledo C. 2008. Asymmetry in pollen flow promotes gender specialization in morphs of the distylous neotropical herb *Arcytophyllum lavarum* (Rubiaceae). *Evolutionary Ecology* **22:** 743–755. https://doi.org/10.1007/s10682-007-9198-0

Grace, JB. 2006. *Structural equation modelling and natural systems*. Cambridge University Press. https://doi.org/10.1017/CBO9780511617799

Hamilton CW. 1990. Variations on a Distylous theme in mesoamericam *Psychotria* subgenus *Psychotria* (Rubiaceae). *Memoirs of the New York Botanical Garden* **55:** 62–75.

Hansen TF, Carter AJR, Pelabon C. 2006. On adaptive accuracy and precision in natural populations. American Naturalist **168:** 168–181. https://doi.org/10.1086/505768

Hernández a, Ornelas JF. 2007. Development of distylous flowers and investment of biomass in male and female function in *Palicourea padifolia* (Rubiaceae). Plant biology **9:** 694–704. https://doi.org/10.1055/s-2007-965238

Köppen, W. 1884. Die Wärmezonen der Erde, nach der Dauer der heissen, gemässigten und kalten Zeit und nach der Wirkung der Wärme auf die organische Welt betrachtet. *Meteorologische Zeitschrift* **1:** 5-226.

Lewis, D, Jones DA. 1992. The genetics of heterostyly. In S. C. H. Barrett [ed.], *Evolution and function of heterostyly*, 41–84. Monographs on theoretical and applied genetics, Springer-Verlag, Berlin, Germany. https://doi.org/10.1007/978-3-642-86656-2_5

Lloyd, DG, Yates, J. 1982. Intrasexual selection and the segregation of pollen and stigmas in hermaphrodite plants, exemplified by *Wahlenbergia albomarginata* (Campanulaceae). *Evolution* **36:** 903-913. https://doi.org/10.1111/j.1558-5646.1982.tb05462.x

Muenchow, G. 1981. An S-locus model for the distyly supergene. *American Naturalist* **118**:756-760. https://doi.org/10.1086/283866

Naiki A. **2012**. Heterostyly and the possibility of its breakdown by polyploidization. *Plant Species Biology* **27:** 3–29. https://doi.org/10.1111/j.1442-1984.2011.00363.x

Naiki A, Kato M. 1999. Pollination system and evolution of dioecy from distyly in *Mussaenda parviflora* (Rubiaceae). *Plant Species Biology*, **14** 217-227. https://doi.org/10.1046/j.1442-1984.1999.00021.x

Nakagawa M., Naiki A. 2014. Breeding system and seed production in pin-monomorphic populations of a distylous perennial, *Ophiorrhiza japonica* (Rubiaceae) on Iriomote Island, Japan. *Acta Phytotaxonomica et Geobotanica* **65:** 43–48.

Nakamura K, Denda T, Kameshima O, Yokota M.. 2007. Breakdown of distyly in a tetraploid variety of *Ophiorrhiza japonica* (Rubiaceae) and its phylogenetic analysis. *Journal of plant research* **120:** 501–9. https://doi.org/10.1007/s10265-007-0089-9

Pérez-Barrales, R., Vargas, P., Arroyo, J., 2006. New evidence for the Darwinian hypothesis of heterostyly: breeding systems and pollinators in *Narcissus* sect, Apodanthi. *New Phytologist*. **171:** 553–567. https://doi.org/10.1111/j.1469-8137.2006.01819.x

Rodrigues EB, Consolaro H. **2013**. Atypical distyly in *Psychotria goyazensis* Mull. Arg. (Rubiaceae), an intramorph self-compatible species. *Acta Botanica Brasilica* **27**: 155–161. https://doi.org/10.1590/S0102-33062013000100016

Sá T, Furtado MT, Ferrero V, Pérez-Barrales R, Rodrigues EB, dos Santos IG, Consolaro H. **2016**. Floral biology, reciprocal herkogamy and breeding system in four *Psychotria* species (Rubiaceae) in Brazil. *Botanical Journal of the Linnean Society* **182**: 689–707. https://doi.org/10.1111/boj.12476

Sage TL, Strumas F, Cole WW, Barrett SC. **1999**. Differential ovule development following self- and cross-pollination: the basis of self-sterility in *Narcissus triandrus* (Amaryllidaceae). *American journal of botany* **86**: 855–70. https://doi.org/10.2307/2656706

Sánchez JM, Ferrero V, Arroyo J, Navarro L. **2010**. Patterns of style polymorphism in five species of the South African genus *Nivenia* (Iridaceae). *Annals of botany* **106**: 321–31. https://doi.org/10.1093/aob/mcq111

Santos-Gally R, Gonzalez-Voyer A, Arroyo J. **2013**. Deconstructing heterostyly: the evolutionary role of incompatibility system, pollinators, and floral architecture. *Evolution; international journal of organic evolution* **67**: 2072–82. https://doi.org/10.1111/evo.12087

Schindelin, J., Rueden, C. T., Hiner, M. C., & Eliceiri, K. W. 2015. The ImageJ ecosystem: An open platform for biomedical image analysis. *Molecular reproduction and development*, **82:** 518-529. https://doi.org/10.1002/mrd.22489

Shore JS, Barrett SCH. 1984. The effect of pollination intensity and incompatible pollen on seed set in *Turnera ulmifolia* (Turneraceae). *Canadian. Journal of Botany* **62:** 1298–1303. https://doi.org/10.1139/b84-175

Team, R. (2013). R development core team. *R A Language Environment for Statistic al Computing* **55:** 275-286.

Webb CJ, Lloyd DG. **1986**. The avoidance of interference between the presentation of pollen and stigmas in angiosperms II. herkogamy. *New Zealand Journal of Botany* **24:** 163–178. https://doi.org/10.1080/0028825X.1986.10409726

Yeo PF. **1975**. Some Aspects of heterostyly. *New Phytologist* **75**: 147–153. https://doi.org/10.1111/j.1469-8137.1975.tb01381.x

Zhou W, Barrett SCH, Wang H, Li D-Z. **2012**. Loss of floral polymorphism in heterostylous *Luculia pinceana* (Rubiaceae): a molecular phylogeographic perspective. *Molecular ecology* **21**: 4631–45.

Supplementary data I

Supplementary table I: Statistical models used to test phenotypic selection on floral traits of Psychotria species.

Species	Model	Level	Log likelihood	AIC	Δ AIC	Chi-square value	p value
Psychotria prunifolia							
Full model	fruit set ~ corolla height + corolla entrance width+ approach herkogamy	Fruit set	-28.34	68.69	4.26	0.0001	0.99
		Number of pollen grains	-953.96	1921	1.1	0.34	0.56
Adjustment to pollinator attraction and morphology	fruit set ~ corolla height + corolla entrance width	Fruit set	-28.18	66.36	1.93	0.07	0.79
		Number of pollen grains	-954.13	1918.3	0	4.71	0.03
	fruit set ~ approach herkogamy	Fruit set	-28.217	64.43	0	0.1	0.74

Self - interference avoidance		Number of pollen grains	-953.96	1919.9	1.6	0.22	0.64
P. trichophoroides							
Full model	~ pin corolla height + pin corolla entrance width + pin approach herkogamy	Fruit set	-140.82	293.65	3.73	2.71	0.1
		Number of pollen grains	-897.69	1827.9	9.1	0.04	0.84
Adjustment to pollinator attraction and morphology	~ pin corolla height + pin corolla entrance width	Fruit set	-142.18	294.36	4.44	0.0001	0.99
		Number of pollen grains	-897.71	1822.5	3.7	1.68	0.19
Self - interference avoidance	~ pin corolla height + pin approach herkogamy	Fruit set	-140.96	289.92	0	3.11	0.08
		Number of pollen grains	-898.55	1818.8	0	0.0002	0.98
Full model		Fruit set	-152.13	314.27	3.49	0.02	0.89

	~ thrum corolla height + thrum corolla entrance width + thrum approach herkogamy	Number of pollen grains	-887.88	1787.8	2	0.46	0.49
Adjustment to pollinator attraction and morphology	~ thrum corolla height + thrum corolla entrance width	Fruit set	-150.39	310.78	0	3.49	< 0.001
		Number of pollen grains	-887.91	1785.8	0	6.06	0.01
Self - interference avoidance	~ thrum corolla height + thrum approach herkogamy	Fruit set	-152.15	312.29	1.51	0.11	0.74
		Number of pollen grains	-890.94	1789.9	4.1	0.05	0.82

Supplementary data II

Psychotria trichophoroides
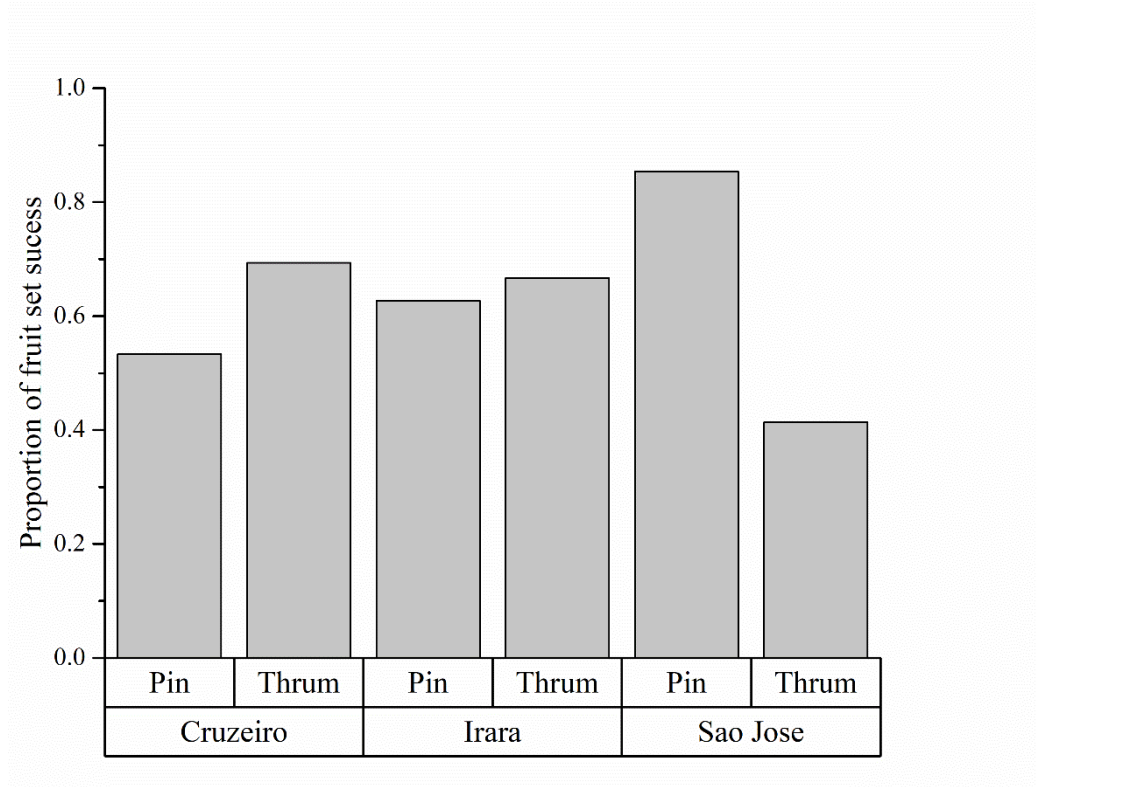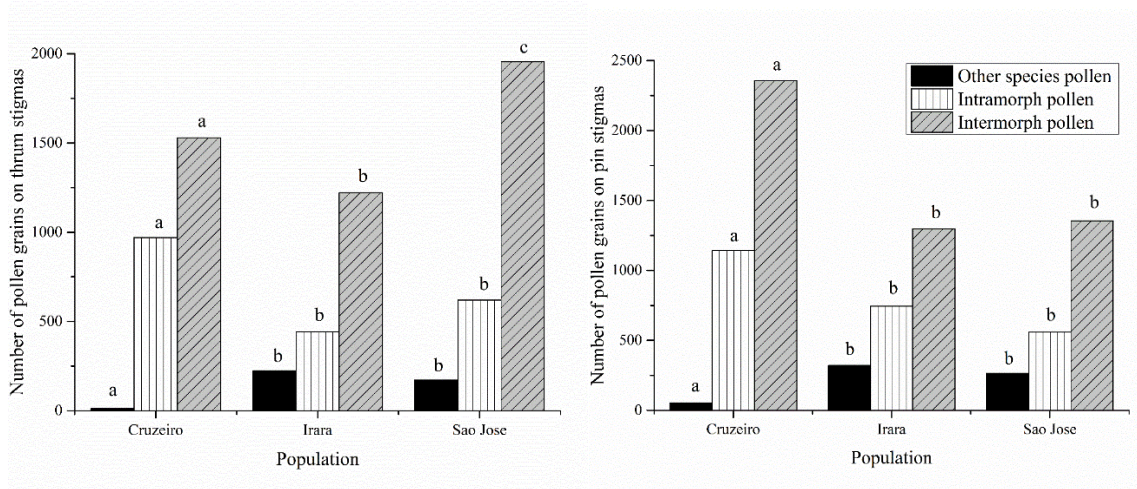
Psychotria prunifolia
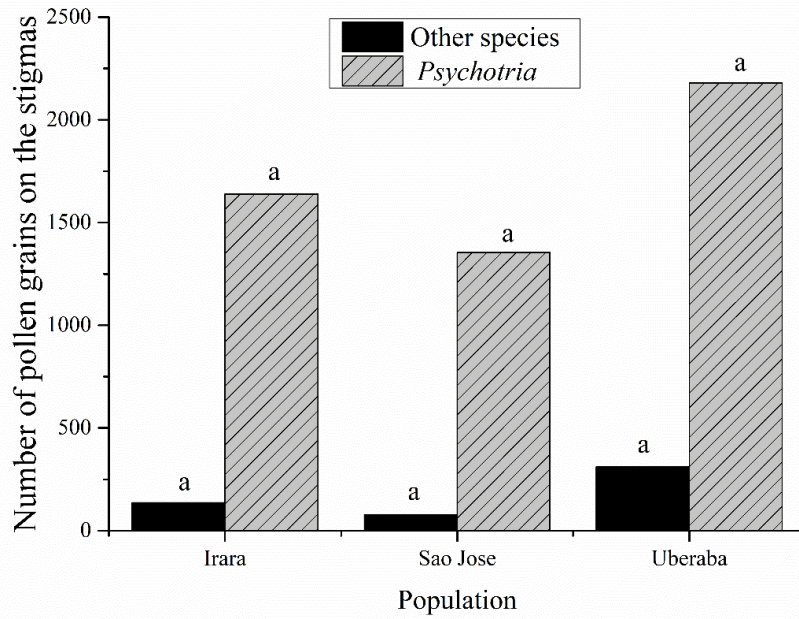
Supplementary figure I: Stigmatic pollen grains and natural fruit set of monomorphic and distylous of *Psychotria* L. species. Different letters indicate differences at the 0.05 level among the number pollen grains across populations of both the species

Capítulo III

Pollination precision, phenotypic integration and breakdown of distyly in *Psychotria carthagenensis* **Jacq. (Rubiaceae)**

Introduction

Distyly is a floral dimorphism in which plants present reciprocal placement in the heights of stigmas and anthers among the long-styled morph (flowers with stigmas placed above the anthers) and the short-styled morph (flowers with stigmas placed below the anthers), the floral morphs also pursuit self and intramorph self-incompatibility (Ganders, 1979; Barrett 1992; Barrett, 2010). Plant populations with distyly usually present a ratio of 1:1 among the floral morphs, the maintenance of this balanced ratio of both the phenotypes is mediated by negative frequency-dependent selection which result in disassortative mating in plants with heteromorphic incompatibility (Ganders, 1979; Heuch, 1979; Eckert *et al.*, 1996). However, several of the plant groups with distyly present breakdown in the mating system (Barrett & Shore, 2008). These breakdowns include morphological and physiological variations, as herkogamy loss (homostyly), loss of one of the floral morphs (monomorphism with herkogamy), loss of self-incompatibility and transitions towards gender specialization (monoecy and dioecy) (Ganders, 1979; Graham & Barrett, 1994; Truyens *et al.*, 2005, Mast *et al.*, 2006; Schoen *et al.*, 1997; Pérez-Barrales *et al.*, 2010; Watanabe *et al.*, 2013). The occurrence of self-compatibility has been documented in several heterostylous taxa, and, studies with these species considered the loss of heteromorphic incompatibility as an evidence of the first steps in the breakdown of distyly towards other self-compatible breeding systems strategies (Dulberger 1992, Schou & Philipp 1983, Ornduff 1988, Richards & Koptur 1993, Negron-Ortiz 1996). The variations in morphological and physiological features of distylous plants may occur in different ecological levels, among species of a genera, flowers of an individual plant, among plants of a population and across populations of a species (Baker, 1966; Ganders, 1979; Hamilton, 1990; Faivre & Mcdade, 2001; Sakai & Wright, 2007; Consolaro *et al.*,

2011; Zhou *et al*., 2012; Sá *et al*., 2016) and even among the distylous floral morphs (Bawa & Beach, 1983).

Pollinators' morphology (eg. short-tongued or long-tongued) and their interaction with floral resources (nectar and/or pollen) are pointed as important selective pressures for the breakdown of distyly and for the evolution of other breeding systems from distyly (Beach & Bawa, 1980; Santos-Gally *et al*., 2013). The occurrence of monomorphism in species that are commonly distylous is attributed to the absence of long tongued-pollinators in some plant populations, cause they are able to touch both heights of sexual organs and better promote pollen transfer in distylous morphs than short-tongued pollinators that may touch only higher sexual organs (Barrett e*t al*., 1989). And, even in the evolution of dioecy from distyly is hypothesized to have occurred due to changes in the pollinator fauna (Beach & Bawa, 1980; Valois-Cuesta *et al*., 2012) Watanabe *et al*., 2013).

Usually, theses transition of distyly towards monomorphism and homostyly are associated with the loss of self-incompatibility (Ganders, 1979; Barrett, 1992). Population with biased morph ratio are also considered deviations from the classical distylous system, occurring in association with loss of self-incompatibility and in populations with assortative pollen flow among the floral morphs (Nishihiro &Washitani, 2000; Kéry *et al*., 2003; Shibayama & Kadono, 2003). Therefore, differences in the pollinator morphology and pollination service may generate differences in floral morphology and changes in the phenotypic integration between truly distylous and distyly derived breeding system (Pérez-Barrales *et al*., 2007). The absence of plants of one of the floral morphs may lead to low availability of compatible pollen grains for reproduction and, as a consequence, plant populations may present reduction in their reproductive success (Matsumura & Washitani, 2000; Shibayama & Kadono, 2003; Brys *et al*., 2004), or an increase in the negative effects of genetic drift. (Richman & Kohn, 1996).

Strategies in the placement of sexual organs in hermaphrodite flowers face the herkogamy dilemma, a conflict between avoiding intrafloral sexual interference and promote accurate positioning of anthers and stigma with regard to where they contact the pollinators bodies (Armbruster *et al*., 2014; Opedal *et al*., 2017). The breakdown of distyly usually involves changes in the herkogamy (homostyly and/or switch from distylous reciprocal herkogamy to monomorphism with approach herkogamy) and loss of self-incompatibility (Ganders,

1979; Barrett, 1992). However, the stability of reciprocal herkogamy and the maintenance of distyly requires suitable environmental conditions and a pollination service that promotes disassortative pollen flow among the floral morphs (Ganders 1979). Among the floral traits, it has been suggested that herkogamy (spatial separation of male and female sexual organs in hermaphrodite flowers) is one of the first traits to evolve after changes in the breeding environment of the species (Mitchell & Ashman 2008; Bodbyl Roels & Kelly 2011), and that changes in the degree of herkogamy do not occur as easily as changes in the length of the pistil or stamens (Opedal *et al.*, 2017).

The integration of phenotypic traits, like flower's morphology, results from developmental, physiological, and historical effects (Armbruster *et al.*, 2014). The adaptive integration of floral traits should lead to efficient pollen transfer in flowers mediated by the pollination service (Stebbins, 1974; Faegri & van der Pijl, 1966), and, the structure of this integration may affect the accuracy and effectiveness of the pollination function (Armbruster *et al.*, 2004). Berg's hypothesis (1960) predict that plants with specialized breeding systems strategies present stronger phenotypic integration than non-specialized breeding systems. The distylous flowers with reciprocal herkogamy and heteromorphic self-incompatibility are hypothesized since Darwin (1877) and further studies (Ganders, 1979; Barrett, 1992; Armbruster *et al.*, 2009; Kissling & Barrett, 2013) as a mechanism for the promotion of precision in cross-pollination. However, monomorphic adaptations derived from distyly may be as efficient for reproduction as adaptations of the typical distylous plants (Ganders, 1979).

Few studies have assessed changes in floral integration after the transition from outcrossing to selfing (Anderson and Busch, 2006). We are unaware of studies investigating changes influenced by pollinators visits, precision in pollen receipt and dispatch, and the integration of floral traits across populations with breeding system transitions. In our study we investigated the relative imprecision in pollination and the phenotypic integration in populations of *Psychotria carthagenensis* with different degrees of morphological and physiological breakdown of distyly. We further investigated if there is an effect of phenotypic integration of floral traits in the imprecision in pollination and tested if populations with biased morph ratio presented differences in visitation rate of short-tongued and long-tongued pollinators.

Capítulo III: Pollination precision, phenotypic integration and breakdown of distyly in *Psychotria carthagenensis* Jacq. (Rubiaceae)

Material and methods

Species and sampled areas

Psychotria carthagenensis Jacq. is a shrub species that occurs in Neotropical forests areas with moist/humid soils or near watersheds from Costa Rica to Argentina (Fig. 1). In Brazil, the species presents ample occurrence in forests of Amazon, Atlantic forest and Cerrado (Delprete, 1999). *P. carthagenensis* presents a great morphological variation in vegetative and reproductive traits (Klein *et al*., 2005; Koch *et al*., 2010, Consolaro *et al.,* 2011, Faria *et al* 2012). We sampled 10 populations in the Brazilian states of Minas Gerais, Goiás, Mato Grosso, Mato Grosso do Sul and Distrito Federal (tab. 1; fig. 2). In some of these areas, studies on the breeding system of the species have already been performed (Koch *et al.*, 2010; Consolaro *et al.*, 2011; Faria *et al.*, 2012) and part of the data of these studies were used in our analysis.

Morph ratio and pollinators visitation rate

In all the study areas we performed random walks of about 60-80 minutes in the forests recording the occurrence of the floral morphs in each plant of the species. We compared the number of both floral morphs in the populations with the expected 1:1 ratio maintained in typical distylous populations by frequency dependent selection using chi-square tests. We realized 30 hours of observation per floral morph in the populations (Panga, Jataí and Água Fria) in different number of plants in each one of the populations (Tab). In the populations Embrapa-MS and UFMS we used the pollination observations data (tab. 1) extracted from Faria *et al*. with (2017). We considered bees, flies and wasps as short-tongued pollinators and moths and butterflies as long-tongued pollinators.

Capítulo III: Pollination precision, phenotypic integration and breakdown of distyly in *Psychotria carthagenensis* Jacq. (Rubiaceae)
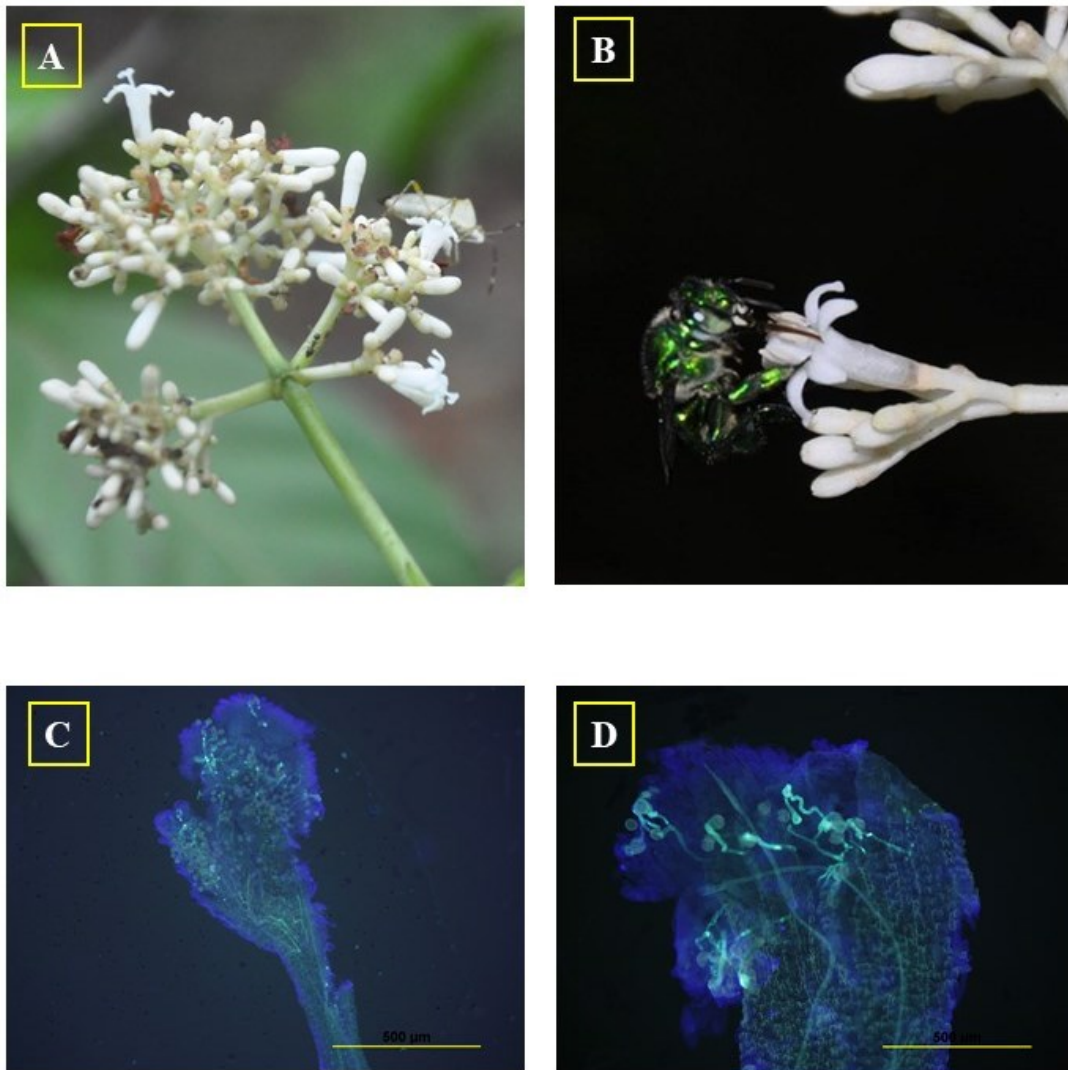
Figure 1: A: Long-style flowers of *Psychotria carthagenensis* Jacq.; **B:** Short-styled flower of *P. carthagenensis* visited by Euglossine bee; **C:** Pollen tube growth interruption in long-styled stigma 4 hours after self-pollination in the population Jataí ; **D:** Pollen tube grow interruption in short-styled flower 4 hours after intramorph-pollination in the population Água Fria.

Self-incompatibility experiments

Data from presence or absence of self-incompatibility were obtained from Koch *et al*. (2010) and Faria *et al*. (2012) for the populations Tangará, Embrapa MS and UFMS. We

tested the presence of self-incompatibility in in the populations of Agua Fria, Jataí, Panga and Uberaba. The flower buds in pre-anthesis were isolated and bagged. When the buds opened, hand pollination experiments were applied: self-pollination (exposure to pollen from the same flower, n = 10 per morph in each population), intramorph pollination (exposure to pollen from flowers of different plants of the same morph, n = 10); and intermorph cross-pollination (exposure to pollen from flowers of flowers of the other morph) (n = 41). We did not expose flowers from Panga and Uberaba populations to intermorph cross-pollination because they were monomorphic.
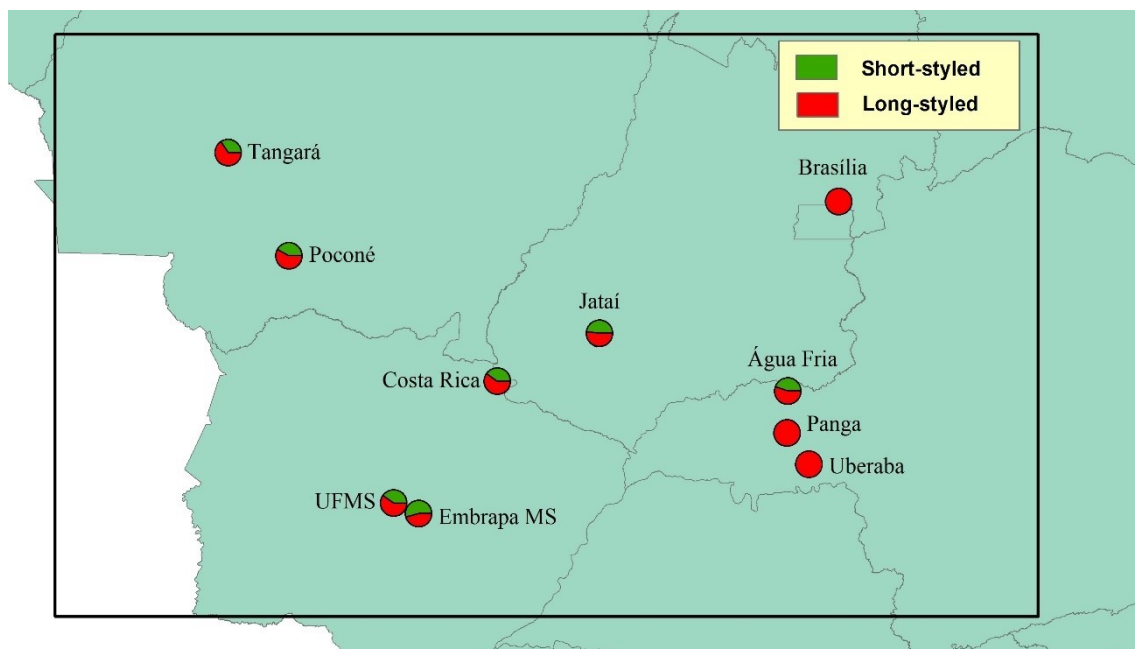
Figure 2. Areas of the study and morph ratio in populations of *Psychotria carthagenensis* Jacq. (Rubiaceae).

Pollen tube growth was observed to describe whether the species were self-incompatible or not. The styles from plants of the different morphs were collected and fixed in 70% alcohol ca. 4 hours after the application of hand pollination treatments. Pollen tube growth was observed using aniline blue staining and epifluorescence microscopy as described by Martin (1959, Maruyama *et al*., 2016). Pollen tube with excessive deposition of calosis

and growth interruption were considered as evidences of presence of self-incompatibility (fig. 1) (Bawa & Beach, 1983, Maruyama *et al.*, 2016)

Floral morphology and pollination imprecision estimation

In all the 10 populations we collected one flower per plant, at random. The sample size varied across populations (tab. 2). The flowers were collected, fixed in 70% alcohol, and after that, in the laboratory, we dissected and photographed each flower. Using an image analysis software (ImageJ) we measured the corolla height (from its base to the petals), corolla entrance width, style length, stigma height (style length + half of the stigmatic lobules length), stigmatic lobes length (sum of the length of the two lobules), filament length, anther height (filament length + half of anther length) and anther length.

Pollination imprecision at the population level was estimated using the inaccuracy measure (Hansen *et al.*, 2006; Armbruster *et al.*, 2009; Armbruster *et al.*, 2017). Inaccuracy for low organs (long-styled anther height and short styled stigma height) and high organs (long-styled stigma height and short-styled anthers height) were estimated using the equation proposed by Armbruster (2004, 2009) and Armbruster *et al.* (2017), which uses trait variance and maladaptation bias (deviation from the trait mean from the optimum) to estimate pollination imprecision. These measures can also be used as an estimation of reciprocity in distylous populations (Armbruster *et al.*, (2017). For monomorphic populations we estimated the pollination imprecision of male and female sexual organs (Hansen *et al.*, 2006; Armbruster *et al.*, 2009)

$$\text{Inaccuracy} = \sigma^2 + (\mu - \Theta)^2$$

$$\text{High organs inaccuracy} = (A-S)^2 + VA + VS \text{ scaled by long stigma height}$$

$$\text{Short organs inaccuracy} = (a-s)^2 + Va + Vs \text{ scaled by short stigma height}$$

$$\text{Male organs inaccuracy} = (A-S)^2 + VA + VS \text{ scaled by anthers height}$$

$$\text{Female organs inaccuracy} = (a-s)^2 + Va + Vs \text{ scaled by stigma height}$$

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Where A represents the mean height of the high anthers, S the mean height of the high stigmas, a: mean height of the low anthers, s the mean height of the low stigmas and V the variance of these sexual organs. The values of inaccuracy represent units of mm^2 of the traits. To compare inaccuracy across populations and species we used the average mean of organs height to standardize the inaccuracies, which allow to compare pollination imprecision across species and populations (Armbruster *et al.*, 2017).

Phenotypic integration estimates

We estimated the phenotypic integration index (Wagner, 1984) using the eigenvalue of a Principal Component Analysis of the floral morphometric data (Corolla length, corolla entrance width, style length, stigma length, filament length, anther length). These values represent the amount correlation of the morphological variables. Total integration was estimated for the distylous populations as the average of thrum and pin flowers integration. The structure of the phenotypic correlations among the floral traits of the different populations of *P. carthagenensis* were obtained from the eigenvalues of the eigenvectors of the principal component analysis.

Effect of phenotypic integration in pollination imprecision

We used Pearson's correlation analysis to test the effect of phenotypic integration in the pollination imprecision of the populations. We used the estimated imprecision values (mean squared inaccuracy) as dependent variable and the phenotypic integration index of the populations as predictor. Both the variables were log transformed to achieve normality in the distribution of data. The analysis was performed in R statistical software (R Team, 2013).

Results

Morph ratio, pollinators visits frequency and self-incompatibility

The populations of Água Fria, Costa Rica, Embrapa MS e Jataí presented balanced morph ratio (Fig 1; Tab. 1). The populations of Brasília, Panga, and Uberaba presented pin monomorphism, i.e. there were only plants with flowers with approach herkogamy that looked like the morphology of the long-styled flowers of distylous populations. All the

other populations (Poconé, Tangará and UFMS) presented biased morph ratio, with higher number of long-styled plants (Fig 1; tab. 1). The monomorphic populations occurred in the east part of the sampled areas. However, there were distylous populations nearby monomorphic populations. In all the populations we conducted pollination observations there were higher number of visits by short-tongued pollinators (bees, wasps, and flies) than by long-tongued pollinators (moths, and butterflies) (tab.1). The populations Embrapa MS, UFMS, and Tangará exhibit self and intramorph compatibility (Koch *et al*. 2010, Faria *et al*., 2012) (tab.1). However, the populations of Água Fria and Jataí presented pollen tube interruption (fig. 2) in the hand pollination experiments of self-pollination and intramorph pollination. The monomorphic populations of Panga and Uberaba were self-compatible with pollen tubes of hand self-pollination experiments growing down to the ovarian.

Imprecision in pollination

The standardized imprecision in pollination were similar in the distylous populations, with values lower than 0.2%, with a range in total inaccuracy in these populations from $0.02 - 0.1$ mm^2. But, the monomorphic populations of Brasília, Panga and Uberaba presented standardized imprecision in pollination higher than 0.1% and total inaccuracy vary from $2.83 - 4.57$ mm^2. The deviation from optimal placement for receive and dispatch pollen grains (maladaptive bias) were higher in monomorphic populations. And the variances of low and high sexual organs were similar in each population.

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Table 1: Morph ratio, presence/absence of self-incompatibility and pollinators frequency in populations of *Psychotria carthagenensis* Jacq. (Rubiaceae) with distyly and morphological and physiological breakdowns of distyly.

Population	N of long-styled plants	N of short-styled plants	X^2	P	System of incompatibility	Pollinators frequency	
						Short-tongued	*Long-tongued*
Água fria	38	31	0.71	0.39	Self-incompatible	78.6	21.4
Costa Rica	36	24	0.13	0.71	-	-	-
Embrapa MS	50	39	1.36	0.24	Self-compatible*	99.5*	0.5*
Jataí	47	44	0.09	0.75	Self-incompatible	60	40
Poconé	58	42	3.72	0.05	-	-	-
Tangará	25	13	3.79	0.05	Self-compatible**		-
UFMS	65	44	4.04	0.04	Self-compatible*	98.3*	2.7*
Brasília	11	-	-	-	-	-	-
Panga	37	-	-	-	Self-compatible	78.05	21.95
Uberaba	11	-	-	-	Self-compatible		-

Table 2. Pollination imprecision (inaccuracy) in populations of *Psychotria carthagenensis* Jacq. (Rubiaceae) with distyly and morphological and physiological breakdowns of distyly.

Population	Number of flowers	Inaccuracy		Maladaptive bias^2	Anther variance	Stigma variance	Total inaccuracy (mm^2)	Mean^2 standardized total inaccuracy (%)
Água Fria	45	Tall	0.2	0.01	0.49	0.62	0.42	0.02
		Low	0.22	0.2	0.20	0.31		
Costa Rica	45	Tall	0.4	0.29	1.49	0.66	0.85	0.03
		Low	0.45	0.53	0.4	0.5		
Embrapa M	33	Tall	0.18	0.001	0.27	0.84	0.38	0.02
		Low	0.2	0.04	0.38	0.28		
Jataí	45	Tall	0.26	0.37	0.41	0.75	0.78	0.03
		Low	0.52	0.56	0.34	0.62		
Poconé	30	Tall	0.24	0.05	0.33	0.4	0.56	0.1
		Low	0.32	0.07	0.19	0.2		
Tangará	30	Tall	0.76	1.9	1.96	1.26	1.03	0.04
		Low	0.27	0.28	0.18	0.49		
UFMS	30	Tall	0.21	0.06	0.7	0.72	0.62	0.02
		Low	0.41	0.09	1.05	0.56		

Brasília	30	Female	1.83	4.11	0.08	0.26	2.83	0.24
		Male	1					
Panga	32	Female	3;02	6.54	0.27	1.29	4.57	0.29
		Male	1.55					
Uberaba	29	Female	1.39	3.76	0.18	0.51	2.25	0.13
		Male	0.86					

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Phenotypic integration of floral traits

The index of phenotypic integration of monomorphic populations with approach herkogamy were lower than the values of the distylous populations with reciprocal herkogamy (tab. 3). In all the distylous populations the integration of the floral traits of long-styled flowers were higher than in the short-styled flowers (tab. 3).

Effect of phenotypic in pollination imprecision

We found a significant relationship between imprecision in pollination and phenotypic integration of floral traits (fig. 3). Populations with low pollination imprecision presented high phenotypic integration (fig. 3). The effect of pollination imprecision was moderately strong and had statistically significance (Pearson's R = - 0.77, n = 10; $F_{(9,8)}$ = 12.33, p = 0.008).

Table 3. Phenotypic integration of populations of *Psychotria carthagenensis* Jacq. (Rubiaceae) with distyly and morphological and physiological breakdowns of distyly. *: data from Faria *et al*. 2012. **: data from Koch *et al*. 2010).

Population	Morph		Average phenotypic integration index
Água fria	S	34.08%	58%
	L	47.85%	
Costa Rica	S	34.53%	54.55%
	L	40.04%	
Embrapa MS	S	29.95%	46.09%
	L	32.28%	
Jataí	S	34.58%	53.32%
	L	37.48%	
Poconé	S	39.59%	56.31%
	L	33.44%	
Tangará	S	34.54%	54.77%

	L	40.47%	
UFMS	S	43.32%	66.06%
	L	45.49%	
Brasília	L		31.22%
Panga	L		37.21%
Uberaba	L		39.20%
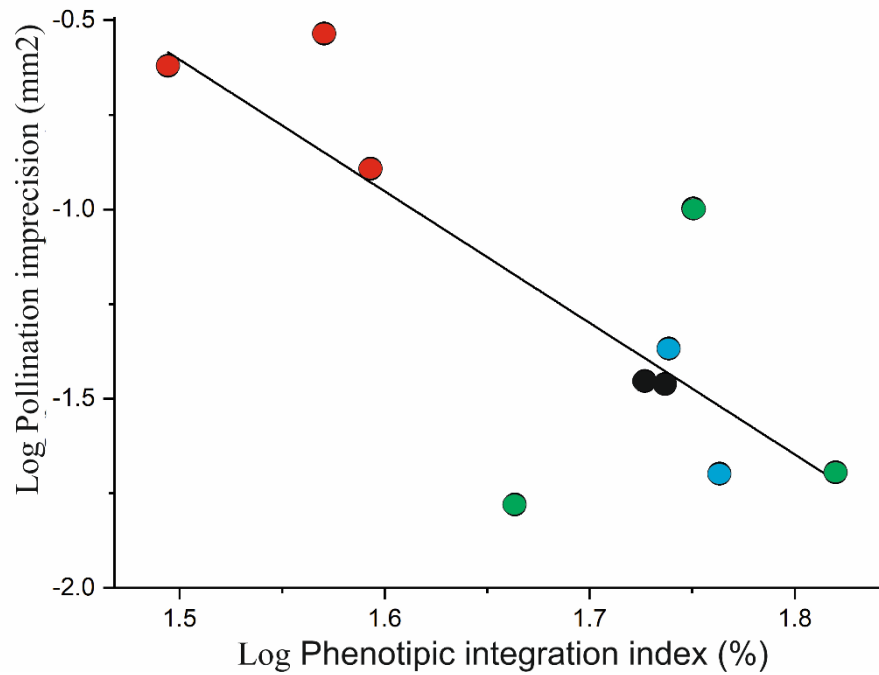
Figure 3. Pearson's correlation analysis of the effect of phenotypic integration of floral traits in the pollination imprecision of populations of *Psychotria carthagenensis* Jacq. (Rubiaceae) with distyly and morphological and physiological breakdowns of distyly. Red dots represent monomorphic populations with approach herkogamy, black dots with unknown system of incompatibility status, green dots represent populations with distylous morphs with self-compatibility and blue dots populations with distylous morphs and self-incompatibility

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Discussion

The breeding system results of our study in *Psychotria carthagenensis* indicated the presence of populations with typical distyly (distyly with balanced morph ratio and possibly heteromorphic incompatibility), self-compatible populations with distyly, populations with biased morph ratio, and populations with pin-monomorphic flowers. There was also physiological breakdown of distyly (loss of self-incompatibility) without occurrence of morphological breakdown (homostyly and monomorphism) in *P carthagenensis*. Several studies predict a connection between the genetic control of the morphological features of distylous flowers and of the heteromorphic self-incompatibility (Dowrick, 1956; Ganders, 1979; Muenchow, 1981). Nevertheless, Vuilleumier (1967) predicted independence among the genetic control of morphological and physiological traits of plants with distyly. The occurrence of populations with both floral morphs with self-compatibility in typically distylous populations of *P. carthagenensis* corroborates the prediction of independence in the control of the floral morphology and incompatibility system. Meanwhile, the proximity in the occurrence of monomorphic and distylous populations may indicate the importance of founder effects and differences in the performance of the floral morphs of *P. carthagenensis* in colonize and establish in new habitats in the breakdown of distyly at the population level.

Founder effects and stochastic pressures may also lead to breakdown of distyly and may indicates non-stability of these breeding system in a species (Barrett, 1993, Eckert and Barrett, 1995; Hodgins & Barrett, 2008). These evolutionary transitions to other breeding system strategies may have harmful effects for plants populations, as loss of genetic diversity (Meeus *et al.*, 2012), reduced population size (Aguilar *et al.*, 2008) or even a reduction in pollination precision in species with relaxed floral polymorphisms compared to the precision of species with distyly (Thompson *et al.*, 2012; Ferrero *et al.*, 2017). The bias the morph ratio in the populations of *P. carthagenensis* can also represent a step in the evolutionary breakdown of distyly or even failures of the pollinators in promote disassortative pollen flow in these populations. The mixing of assortative and disassortative reproduction in *Narcissus* (Amaryllidaceae) was associated with bias in the morph ratio or even the fixation of monomorphism (Baker *et al.*, 2000). Moreover, in other *Narcissus* species, shifts in the pollinators morphology were related with deviations from the balanced morph ratio (Arroyo & Dafni, 1995; Pérez-Barrales & Arroyo, 2010;

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Santos-Gally *et al*., 2013). However, in *P. carthagenensis* the frequency of visits of short-tongued pollinators predominated above the number of visits of short-tongued pollinators in the populations, and there were no evidences of shifts in the morphology of the pollinator fauna across monomorphic and distylous populations,

Reciprocal herkogamy of distylous populations was more precise in pollination than the approach herkogamy of populations with monomorphism. The occurrence of the short-styled floral morph with reciprocal placement of anthers and stigma reduced the maladaptive bias in the distylous populations. A departure from the positioning of sexual organs for pollen placement and reception of a millimetre may represent risks for the species reproduction with effects in intrafloral sexual interference and in promotion of cross-pollination (Armbruster *et al*., 2004). In *Luculia pinceana* (Rubiaceae), populations that lack self-incompatibility achieved disassortative breeding due the maintenance of reciprocity in the floral morphs (Zhou *et al*., 2012). And, in *Narcissus assoanus* (Amaryllidaceae) there were enhancing of reciprocity with increasing in frequency of the short-styled morph (Thompson *et al*., 2012). However, in our study, populations with biased morph ratio had similar values of imprecision in pollination compared to the distylous populations with balanced morph ratio. Although the local absence of partners for reproduction did not affect the precision in populations of *P. carthagenensis*, the monomorphic populations with approach herkogamy was not as efficient in pollination precision as the reciprocal herkogamy of distylous populations.

The floral integration of the long-styled flowers of monomorphic populations and the long-styled flowers of distylous populations was similar. And, the occurrence of the short-styled morph seems to enhance the phenotypic integration in the distylous of populations *P. carthagenensis*. The floral integration of the species was correlated with the pollination imprecision, populations with low phenotypic integration of traits had high pollination imprecision. As predicted by Berg (1960) and discussed by Ordano *et al*. (2008) floral traits with specialization in promoting outcrossing present high phenotypic integration. Usually, the breakdown of self-incompatibility affects the pattern of floral integration in plants (Ordano *et al.*, 2008, Sozenski *et al*., 2010, Fornoni *et al*., 2016). Likewise, for *P. carthagenensis* floral integration was also related with pollination precision in other distylous species. In species of *Lithodora* and *Glandora* (Boraginaceae) high phenotypic integration of floral traits was also associated with high reciprocity among floral morphs

(pollination precision) (Ferrero *et al*., 2011). Nevertheless, in *P. carthagenensis* seems that the loss of self-incompatibility it is not associated with reduction in phenotypic integration of floral traits. Otherwise, the occurrence of monomorphism seems to be related with reduction in phenotypic integration of flowers and in pollination imprecision. Thus, in *Psychotria carthagenensis* and in other plants species, reciprocal herkogamy may have and additive effect in the promotion of floral integration and precision in cross-pollination in flowers.

Distyly and its reciprocal herkogamy seem to be advantageous over approach herkogamy of monomorphic populations of *Psychotria carthagenensis*. Distyly in the populations is an important mechanism for enhancing precision in cross-pollination. The occurrence of two floral phenotypes in the populations allowed high phenotypic integration among their floral traits and play an important role in the specialization for cross-pollination in the species. Although there is no information about differential fitness or effects of self-compatibility and of monomorphism in the reproduction of *P. carthagenensis*, these morphological and physiological breakdowns of distyly in this species were probably influenced by local pressures during the establishment of these populations and linked with pollinators efficiency rather whether they were short or long-tongued. *Psychotria carthagenensis* represent an example of how plants breeding system are versatile in their strategies for reproduction and how morphological and physiological evolutionary transitions from distyly are shaped to ensure reproduction.

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References

Aguilar R, Quesada M, Ashworth L, Herrerias-Diego Y, Lobo J. **2008**. Genetic consequences of habitat fragmentation in plant populations: Susceptible signals in plant traits and methodological approaches. *Molecular Ecology* **17**: 5177–5188. https://doi.org/10.1111/j.1365-294X.2008.03971.x

Anderson I, Busch J. 2006. Relaxed pollinator-mediated selection weakens floral integration in self-compatible taxa of *Leavenworthia* (Brassicaceae). *American Journal of Botany* **93:** 860–867. https://doi.org/10.3732/ajb.93.6.860

Armbruster WS, Pélabon C, Hansen TF, Mulder CH. 2004. Floral integration, modularity, and accuracy. In: Pigliucci M, Preston *K. eds. Phenotypic integration: studying the ecology and evolution of complex phenotypes. New* York: Oxford University Press, 23–79**.**

Armbruster WS, Pélabon C, Hansen TF, Bolstad GH. **2009**. Macroevolutionary patterns of pollination accuracy: a comparison of three genera. *The New phytologist* **183**: 600–17. https://doi.org/10.1111/j.1469-8137.2009.02930.x

Armbruster WS. **2014**. Floral specialization and angiosperm diversity: phenotypic divergence, fitness trade-offs and realized pollination accuracy. *AoB plants* **6**: 1–24. https://doi.org/10.1093/aobpla/plu003

Armbruster WS, Corbet SA, Vey AJM, Liu SJ, Huang SQ. 2014. In the right place at the right time: *Parnassia* resolves the herkogamy dilemma by accurate repositioning of stamens and stigmas. *Annals of Botany* **113:** 97–103. https://doi.org/10.1093/aob/mct261

Armbruster WS, Bolstad GH, Hansen TF, Keller B, Conti E, Pélabon C. 2017. The measure and mismeasure of reciprocity in heterostylous flowers. *New Phytologist* **215:** 906–917. https://doi.org/10.1111/nph.14604

Arroyo J, Dafni A. 1995. Variations in habitat, season, flower traits and pollinators in dimorphic *Narcissus tazetta* L.(Amaryllidaceae) in Israel. *New Phytologist*, **129:** 135-145. https://doi.org/10.1111/j.1469-8137.1995.tb03017.x

Baker, HG. 1966. The evolution, functioning and breakdown of heteromorphic incompatibility systems. I. The Plumbaginaceae. *Evolution*, **20:** 349-368. https://doi.org/10.1111/j.1558-5646.1966.tb03371.x

Baker AM, Thompson JD, Barrett SCH. **2000**. Evolution and maintenance of stigma-height dimorphism in *Narcissus*. I. Floral variation and style-morph ratios. *Heredity* **84**: 502–513. https://doi.org/10.1046/j.1365-2540.2000.00651.x

Barrett SCH. 1989. The evolutionary breakdown of heterostyly. In: *The Evolutionary Ecology of Plants* (Eds. Y. Linhart & J. Bock), pp.151–169. Westview Press, Boulder, Colorado. https://doi.org/10.1201/9780429310720-10

Barrett SCH. 1992. Heterostylous genetic polymorphisms: model systems for evolutionary analysis**.** In: *Evolution and function of heterostyly* (S.C.H. Barret, ed.) Monographs on theoretical and applied genetics. Springer-Verlag, Berlin. https://doi.org/10.1007/978-3-642-86656-2_1

Barrett SCH. 1993. The evolutionary biology of tristyly. In: *Oxford Surveys in Evolutionary Biology* (Eds. D. Futuyma & J. Antonovics), Vol. **9**: pp. 283–326. Oxford University Press, Oxford, U.K.

Barrett SCH, Shore JS. 2008. New insights on heterostyly: Comparative biology, ecology and genetics. In: *Self-Incompatibility in Flowering Plants: Evolution, Diversity and Mechanisms*. (Ed. V. Franklin-Tong). Springer-Verlag, Berlin. pp. 3–32. https://doi.org/10.1007/978-3-540-68486-2_1

Barrett SCH. 2010. Darwin's legacy: the forms, function and sexual diversity of flowers. *Philosophical Transactions of the Royal Society of London Ser. B.* **365**: 351–368. https://doi.org/10.1098/rstb.2009.0212

Bawa KS, Beach JH. **1983**. Self-Incompatibility in the Rubiaceae of a Tropical Lowland Wet Forest. *American Journal of Botany* **70**: 1281–1288. https://doi.org/10.1002/j.1537-2197.1983.tb07917.x

Beach JH, Bawa KS. **1980**. Role of pollinators in the evolution of dioecy from distyly. *Evolution* **34**: 1138–1142. https://doi.org/10.1111/j.1558-5646.1980.tb04055.x

Berg RL. 1960. The ecological significance of correlation pleiades. *Evolution*, **14:** 171-180. https://doi.org/10.1111/j.1558-5646.1960.tb03076.x

Bodbyl Roels SA, Kelly JK. 2011. Rapid evolution caused by pollinator loss in *Mimulus guttatus*. *Evolution*, **65:** 2541-2552. https://doi.org/10.1111/j.1558-5646.2011.01326.x

Brys R, Jacquemyn H, Endels P, Van Rossum F, Hermy M, Triest L, De Bruyn L, Blust GDE. **2004**. Reduced reproductive success in small populations of the self-incompatible *Primula vulgaris*. *Journal of Ecology* **92**: 5–14. https://doi.org/10.1046/j.0022-0477.2004.00840.x

Busch JW, Schoen DJ. 2008. The evolution of self-incompatibility when mates are limiting. *Trends in plant science*, **13:** 128-136. https://doi.org/10.1016/j.tplants.2008.01.002

Consolaro H, Silva SCS, Oliveira PE. **2011**. Breakdown of distyly and pin-monomorphism in *Psychotria carthagenensis* Jacq. (Rubiaceae). *Plant Species Biology* **26**: 24–32. https://doi.org/10.1111/j.1442-1984.2010.00300.x

Darwin C. 1877 *The different forms of flowers on plants of the same species*. London, UK: John Murray. https://doi.org/10.5962/bhl.title.46988

Delprete PG. 1999. Rondeletieae (Rubiaceae): Part I (Rustia, Tresanthera, Condaminea, Picardaea, Pogonopus, Chimarrhis, Dioicodendron, Molopanthera, Dolichodelphys, and Parachimarrhis). *Flora Neotropica*, 1-225.

Dowrick J. **1956**. Heterostyly and homostyly in *Primula obconica*. *Heredity* **10**: 219–236. https://doi.org/10.1038/hdy.1956.19

Dulberger R. **1992**. Floral polymorphisms and their functional significance in the heterostylous syndrome. In: *Evolution and function of heterostyly*. Monographs on theoretical and applied genetics. 41–84. https://doi.org/10.1007/978-3-642-86656-2_3

Eckert CG, Barrett SCH. 1995. Style morph ratios in tristylous *Decodon verticillatus* (Lythraceae): selection vs. historical contingency. *Ecology* **76**: 1051–1066. https://doi.org/10.2307/1940915

Eckert CG, Manicacci D, Barrett SCH. **1996**. Genetic drift and founder effect in native versus introduced populations of an invading plant, *Lythrum salicaria* (Lythraceae). *Evolution* **50**: 1512–1519. https://doi.org/10.1111/j.1558-5646.1996.tb03924.x

Faegri K, Van der Pijl, L. 2013. *Principles of pollination ecology*. Elsevier.

Faivre AE, McDade LA. 2001. Population-level variation in the expression of heterostyly in three species of Rubiaceae: does reciprocal placement of anthers and stigmas characterize heterostyly? *American journal of botany* **88:** 841-853. https://doi.org/10.2307/2657036

Faria RR, Ferrero V, Navarro L, Araujo AC. **2011**. Flexible breeding system in distylous populations of *Psychotria carthagenensis* Jacq. (Rubiaceae) in Brazilian Cerrado. *Plant Systematics and Evolution* **298**: 619–627. https://doi.org/10.1007/s00606-011-0571-7

Ferrero V, Chapela I, Arroyo J, Navarro L. 2011. Reciprocal style polymorphisms are not easily categorised: the case of heterostyly in *Lithodora* and *Glandora* (Boraginaceae). *Plant biology* 13 Suppl **1:** 7–18. https://doi.org/10.1111/j.1438-8677.2009.00307.x

Ferrero V, Barrett SCH, Rojas D, Arroyo J, Navarro L. 2017. Associations between sex-organ deployment and morph bias in related heterostylous taxa with different stylar polymorphisms. *American journal of botany*, **104:** 50-61. https://doi.org/10.3732/ajb.1600345

Fornoni J, Ordano MPIR, Boege K, Domínguez CA. 2015. A comparison of floral integration between selfing and outcrossing species: A meta-analysis. *Annals of Botany* **117:** 299–306. https://doi.org/10.1093/aob/mcv166

Ganders FR. **1979**. The biology of heterostyly. *New Zealand Journal of Botany* **17**: 607–635. https://doi.org/10.1080/0028825X.1979.10432574

Graham SW, Barrett SCH. **2004**. Phylogenetic reconstruction of the evolution of stylar polymorphisms in *Narcissus* (Amaryllidaceae). *American Journal of Botany* **91**: 1007–1021. https://doi.org/10.3732/ajb.91.7.1007

Hamilton CW. **1990**. Variations on a Distylous theme in mesoamericam *Psychotria* subgenus *Psychotria* (Rubiaceae). *Memoirs of the New York Botanical Garden* **55**: 62–75.

Hansen TF, Carter AJR, Pelabon C. 2006. On adaptive accuracy and precision in natural populations. American Naturalist **168:** 168–181. https://doi.org/10.1086/505768

Heuch I. 1979. Equilibrium populations of heterostylous plants. *Theoretical Population Biology*, **15:** 43-57. https://doi.org/10.1016/0040-5809(79)90026-1

Hodgins KA, Barrett SCH. 2008. Natural selection on floral traits through male and female function in wild populations of the heterostylous daffodil *Narcissus triandrus*. *Evolution* **62**: 1751– 1763. https://doi.org/10.1111/j.1558-5646.2008.00404.x

Kéry M, Matthies D, Schmid B. **2003**. Demographic stochasticity in population fragments of the declining distylous perennial *Primula veris* (Primulaceae). *Basic and Applied Ecology* **4**: 197–206. https://doi.org/10.1078/1439-1791-00142

Kissling J, Barrett SCH. **2013**. Variation and evolution of herkogamy in *Exochaenium* (Gentianaceae): Implications for the evolution of distyly. *Annals of Botany* **112**: 95–102. https://doi.org/10.1093/aob/mct097

Koch AK, Silva PC Da, Silva CA. **2010**. Biologia reprodutiva de Psychotria carthagenensis (Rubiaceae), espécie distílica de fragmento florestal de mata ciliar, Centro-Oeste do Brasil. *Rodriguésia* **61**: 551–558. https://doi.org/10.1590/2175-7860201061314

Klein DE, Freitas L, Da Cunha M. **2009**. Self-incompatibility in a distylous species of Rubiaceae: is there a single incompatibility response of the morphs? *Sexual plant reproduction* **22**: 121–31. https://doi.org/10.1007/s00497-009-0097-0

Martin FW. 1959. Staining and observing pollen tubes in the style by means of fluorescence. *Stain technology*, **34:** 125-128. https://doi.org/10.3109/10520295909114663

Mast AR, Kelso S, Conti E. **2006**. Are any primroses (*Primula*) primitively monomorphic? *The New phytologist* **171**: 605–16. https://doi.org/10.1111/j.1469-8137.2006.01700.x

Maruyama PK, Justino DG, Oliveira PE. 2016. Does intraspecific behavioural variation of pollinator species influence pollination? A quantitative study with hummingbirds and a Neotropical shrub. *Plant Biology*, **18:** 913-919. https://doi.org/10.1111/plb.12492

Matsumura C, Washitani I. **2000**. Effects of population size and pollinator limitation on seed-set of *Primula sieboldii* populations in a fragmented landscape. *Ecological Research* **15**: 307–322. https://doi.org/10.1046/j.1440-1703.2000.00350.x

Meeus S, Honnay O, Brys R, Jacquemyn H. **2012**. Biased morph ratios and skewed breeding success contribute to loss of genetic diversity in the distylous *Pulmonaria officinalis*. *Annals of Botany* **109**: 227–235. https://doi.org/10.1093/aob/mcr272

Mitchell RJ, Ashman TL. 2008. Predicting evolutionary consequences of pollinator declines: the long and short of floral evolution. *New Phytologist*, **17:** 576-579. https://doi.org/10.1111/j.1469-8137.2008.02354.x

Muenchow, G. 1981. An S-locus model for the distyly supergene. *The American Naturalist*, **118:** 756-760. https://doi.org/10.1086/283866

Negron-Ortiz V. **1996**. Reproductive biology of *Ernodea* (Rubiaceae-Spermacoceae) in the Bahamas and Puerto Rico. *Second International Rubiaceae Conference, Proceedings* **7**: 403–412.

Nishihiro J, Washitani I, Thomson JD, Thomson BA. **2000**. Patterns and consequences of stigma height variation in a natural population of a distylous plant, *Primula sieboldii*. *Functional Ecology* **14**: 502–512. https://doi.org/10.1046/j.1365-2435.2000.00449.x

Opedal ØH, Bolstad GH, Hansen TF, Armbruster WS, Pélabon C. **2017**. The evolvability of herkogamy: Quantifying the evolutionary potential of a composite trait. *Evolution* **71**: 1572–1586. https://doi.org/10.1111/evo.13258

Ordano M, Fornoni J, Boege K, Dominguez D. 2008. The adaptive value of phenotypic floral integration. *New Phytologist* **179:** 1183–1192. https://doi.org/10.1111/j.1469-8137.2008.02523.x

Ornduff, R. 1988. Distyly and incompatibility in *Villarsia congestiflora* (Menyanthaceae), with comparative remarks on *V. capitata*. *Plant systematics and evolution*, **159:** 81-83. https://doi.org/10.1007/BF00937426

Pérez-Barrales R, Arroyo J, Scott Armbruster W. **2007**. Differences in pollinator faunas may generate geographic differences in floral morphology and integration in *Narcissus papyraceus* (Amaryllidaceae). *Oikos* **116**: 1904–1918. https://doi.org/10.1111/j.0030-1299.2007.15994.x

Pérez-Barrales R, Arroyo J. **2010**. Pollinator shifts and the loss of style polymorphism in *Narcissus papyraceus* (Amaryllidaceae). *Journal of evolutionary biology* **23**: 1117–28. https://doi.org/10.1111/j.1420-9101.2010.01988.x

Richards J, Koptur S. **1993**. Floral Variation and distyly in *Guetarda scabra* (Rubiaceae). *American journal of botany* **80**: 31–40. https://doi.org/10.1002/j.1537-2197.1993.tb13764.x

Richman AD, Kohn JR. **1996**. Learning from rejection: The evolutionary biology of single-locus incompatibility. *Trends in Ecology and Evolution* **11**: 497–502. https://doi.org/10.1016/S0169-5347(96)10051-3

Rodrigues EB, Consolaro H. **2013**. Atypical distyly in *Psychotria goyazensis* Mull. Arg. (Rubiaceae), an intramorph self-compatible species. *Acta Botanica Brasilica* **27**: 155–161. https://doi.org/10.1590/S0102-33062013000100016

Sá T, Furtado MT, Ferrero V, Pérez-Barrales R, Rodrigues EB, dos Santos IG, Consolaro H. **2016**. Floral biology, reciprocal herkogamy and breeding system in four *Psychotria* species (Rubiaceae) in Brazil. *Botanical Journal of the Linnean Society* **182**: 689–707. https://doi.org/10.1111/boj.12476

Sakai S, Wright SJ. **2008**. Reproductive ecology of 21 coexisting *Psychotria* species (Rubiaceae): when is heterostyly lost? *Biological Journal of the Linnean Society* **93**: 125–134. https://doi.org/10.1111/j.1095-8312.2007.00890.x

Santos-Gally R, Gonzalez-Voyer A, Arroyo J. **2013**. Deconstructing heterostyly: the evolutionary role of incompatibility system, pollinators, and floral architecture.

Evolution; international journal of organic evolution **67**: 2072–82. https://doi.org/10.1111/evo.12087

Schindelin, J., Rueden, C. T., Hiner, M. C., & Eliceiri, K. W. 2015. The ImageJ ecosystem: An open platform for biomedical image analysis. *Molecular reproduction and development*, **82:** 518-529. https://doi.org/10.1002/mrd.22489

Sosenski P, Fornoni J, Molina-Freaner FE, Weller SG, Domínguez C a. **2010**. Changes in sexual organ reciprocity and phenotypic floral integration during the tristyly-distyly transition in *Oxalis alpina*. *The New phytologist* **185**: 829–40. https://doi.org/10.1111/j.1469-8137.2009.03105.x

Stebbins GL. 1974. *Flowering plants. Evolution above the species level*. Belknap Press, Cambridge, MA. https://doi.org/10.4159/harvard.9780674864856

Shibayama Y, Kadono Y. **2003**. Floral morph composition and pollen limitation in the seed set of *Nymphoides indica* populations. *Ecological Research* **18**: 725–737. https://doi.org/10.1111/j.1440-1703.2003.00591.x

Team, R. (2013). R development core team. *R A Language Environment for Statistic al Computing* **55:** 275-286.

Thompson JD, Cesaro AC, Arroyo J. 2012. Morph ratio variation and sex organ reciprocity in style-dimorphic *Narcissus assoanus*. *International Journal of Plant Sciences*, *173*(8), 885-893. https://doi.org/10.1086/667231

Truyens S, Arbo MM, Shore JS. **2005**. Phylogenetic relationships, chromosome and breeding system evolution in *Turnera* (Turneraceae): Inferences from its sequence data. *American Journal of Botany* **92**: 1749–1758. https://doi.org/10.3732/ajb.92.10.1749

Valois-Cuesta H, Soriano PJ, Ornelas JF. **2012**. Gender specialization in *Palicourea demissa* (Rubiaceae), a distylous, hummingbird-pollinated treelet. *Plant Systematics and Evolution* **298**: 975–984.

Vuilleumier, BS. 1967. The origin and evolutionary development of heterostyly in the angiosperms. *Evolution*, **21:** 210-226. https://doi.org/10.1007/s00606-012-0607-7

Wagner, GP 1984. On the eigenvalue distribution of genetic and phenotypic dispersion matrices: evidence for a non-random organization of quantitative character variation. *Journal of Mathematical Biology* **21:** 77-95. https://doi.org/10.1007/BF00275224

Watanabe K, Shimizu A, Sugawara T. **2013**. Dioecy derived from distyly and pollination in *Psychotria rubra* (Rubiaceae) occurring in the Ryukyu Islands, Japan. *Plant Species Biology*. https://doi.org/10.1111/1442-1984.12013

Zhou W, Barrett SCH, Wang H, Li D-Z. **2012**. Loss of floral polymorphism in heterostylous *Luculia pinceana* (Rubiaceae): a molecular phylogeographic perspective. *Molecular ecology* **21**: 4631–45. https://doi.org/10.1111/j.1365-294X.2012.05707.x

Zhou W, Barrett SCH, Li HD, Wu ZK, Wang XJ, Wang H, Li DZ. 2017. Phylogeographic insights on the evolutionary breakdown of heterostyly. *New Phytologist*, **214:** 1368-1380. https://doi.org/10.1111/nph.14453

Considerações finais

Este estudo aponta que os gêneros *Psychotria* L. e *Palicourea* Aubl. apresentam diversas estratégias reprodutivas derivadas da distilia. Monomorfismo é a transição evolutiva maia encontrada nos gêneros, seguido pela homostilia e pelas especializações de gênero dioicia e monoicia. Transições evolutivas no sistema reprodutivo foram reportadas entre espécies co-gêneres de *Psychotria* L. e também entre populações de *Psychotria carthagenensis*. Demonstrando que a distilia não é um sistema reprodutivo estável e que espécies e populações podem responder diferentemente a pressões seletivas locais com variações morfológicas e fisiológicas para assegurar a reprodução.

Essas variações evolutivas no sistema reprodutivo de espécies de *Psychotria* também podem apresentar diferentes efeitos na eficiência em polinização e na integração de caracteres do fenótipo das flores. Ao nível intraespecífico espécies com monomorfismo foram tão precisas quanto espécies distílicas e a morfologia da corola é um melhor preditor para o fitness feminino de espécies de *Psychotria* do que a separação espacial dos órgãos sexuais dentro da flor (hercogamia). Já ao nível interpopulacional, populações monomórficas apresentaram maior imprecisão na polinização e menor integração floral do que populações distílicas e a também foi maior em populações distílicas comparada a de populações monomórficas e houve uma forte relação entre integração fenotípica e precisão em polinização em flores de *Psychotria*. Ao nível interespecífico e interpopulacional condições favoráveis a autopolinização e habitats insulares ou isolados parecem ser os fatores responsáveis pela perda local de um dos morfos florais e mesmo em sua fixação nas espécies ou nas populações com transições evolutivas no sistema reprodutivo. As estratégias reprodutivas de *Psychotria* e *Palicourea* parecem assegurar a reprodução dessas espécies, porém, pouco se sabe a respeito da diversidade genética dessas espécies com diferentes formas de reprodução ou também qual a sequência evolutiva da quebra das características morfológicas e fisiológicas da distilia em um grupo de espécies tão grande quanto os desses gêneros. Elucidar essas questões permitiriam uma maior compreensão dos fatores atuantes na evolução de estratégias reprodutivas em grupos de plantas que apresentam alta diversidade.