



UNIVERSIDADE FEDERAL DE UBERLÂNDIA  
INSTITUTO DE BIOLOGIA  
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA VEGETAL



## **Inacurácia em espécies distílicas de Rubiaceae**

Renata Trevizan Telles de Souza

Orientador: Prof. Dr. Paulo Eugênio Alves Macedo de Oliveira

UBERLÂNDIA - MG

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Dissertação apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do título de Mestre em Biologia Vegetal.

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## **Inacurácia em espécies distílicas de Rubiaceae**

Renata Trevizan Telles de Souza

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*“Eu faço parte dos que acham que a ciência é belíssima”*

Marie Curie

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## Resumo

TELLES, R.T. 2019. **Inacurácia em espécies distílicas de Rubiaceae**. Dissertação de mestrado. Universidade Federal de Uberlândia. Programa de Pós-Graduação em Biologia Vegetal. 69p.

As angiospermas apresentam diferentes estratégias para reduzir a autopolinização e favorecer a polinização cruzada. A heterostilia é um polimorfismo floral que envolve estruturas reprodutivas femininas e masculinas colocadas em posição recíproca em morfos florais distintos, sendo vista como um mecanismo facilitador da polinização cruzada em indivíduos de uma espécie. Entretanto, fatores ambientais podem afetar o sistema de polinização, causando variações e até a quebra do polimorfismo. As variações na posição das estruturas reprodutivas entre os morfos podem afetar os níveis de reciprocidade, a dispersão direcional do pólen legítimo e o sucesso reprodutivo das plantas. As plantas heterostílicas estão representadas em 28 Angiospermas e as Rubiaceae abrigam o maior número de grupos distílicos entre as plantas com flores. O objetivo do estudo foi investigar variações morfológicas em populações de espécies distílicas de Rubiaceae utilizando o índice de inacurácia e ainda, buscar entender padrões e fatores que influenciam na imprecisão a nível populacional e intra-individual. Para entender a variação ao nível populacional foram compiladas informações sobre a inacurácia de espécies de Rubiaceae e testados os fatores que podem afetar esses valores. Além disso, foi observada a variação intra-individual de algumas espécies de Cerrado, entendendo que a variação dentro do indivíduo é um componente importante da seleção para a variação populacional. No capítulo um encontramos uma correlação positiva da inacurácia nos órgãos reprodutivos de ambos os morfos. Por outro lado, os vieses maladaptativos (desvio do ótimo) de órgãos altos e baixos foram positivamente relacionado entre si. Apontamos o viés maladaptativo dos órgãos baixos e o estigma do morfo brevistilo como sendo os principais contribuintes para a inacurácia total. Além disso, demonstramos que o tamanho da corola é o único fator que influencia nos valores de inacurácia dos órgãos reprodutivos nos morfos. Assim, nosso estudo apontou padrões e tendências em plantas distílicas com base em uma ampla amostragem de populações. No segundo capítulo, observamos que a variação intra-individual ocorre principalmente no tamanho dos órgãos baixos dos morfos e na altura do estigma brevistilo. Assim, sugerimos que a imprecisão pode variar de maneira diferente no nível da flor e isso pode estar relacionado à diferente efetividade de cada órgão sexual em cada morfo e as diferentes pressões seletivas que condicionam essa imprecisão.

Palavras-chave: polinização, polimorfismos florais, heterostilia, imprecisão entre morfos, variação intra-individual.

### Abstract

TELLES. R.T. 2019. **Inaccuracy in distylous species of Rubiaceae**. Dissertation. Universidade Federal de Uberlândia. Programa de Pós-Graduação em Biologia Vegetal. 69p.

Angiosperms have largely evolved very specialized reproductive strategies to benefit from efficient cross-pollination and cross-fertilization. The Heterostyly is a floral polymorphism that involves female and male reproductive structures in reciprocal position in distinct floral morphs, been seen as a mechanism that promotes cross-pollination in plant species. However, environmental factors can affect the pollination system causing variations and the breakdown of the polymorphism. Variations in the reproductive structures position affect the reciprocity level between morphs, the dispersion of legitimate pollen, and the reproductive success of the species. The heterostylous plants are represented in 28 Angiosperms and the Rubiaceae have the larger number of distyly groups among the flowering plants. The objective of the study was to investigate morphological variations in populations of distylous species of Rubiaceae using the inaccuracy index and understand patterns and factors that influence imprecision at the population and intra-individual level. To understand the variation at the population level, information of the inaccuracy in species of Rubiaceae were compiled and the factors that affected these values were tested. In addition, it was observed the intra-individual variation in some Rubiaceae species of the Cerrado, to understand the variation within the individual as an important component of for the evolution of population variation. The study pointed out patterns and trends in distylous plants based on a wide sampling of populations. In chapter one, we found that the inaccuracy of organs of both morphs was positively related among then. On the other hand, maladaptive bias (mean departure from optimum) of high and low organs were positively related to each other. We point out the maladaptive bias on low organs and thrum stigma as the main contributors to total inaccuracy. We demonstrate that corolla size is the only factor influencing inaccuracy on reproductive organs of both morphs. In the second chapter, we observed that the intra-individual variation occurs mainly in the low organs size and height of stigma of thrum morphs. Therefore, we suggest that the imprecision may vary differently at the flower level and this may be related to the different effectiveness of each sexual organ in each morph and the different selective pressures conditioning such imprecision.

Key words: pollination, floral polymorphisms, heterostyly, imprecision between morphs, intra-individual variation.

## Introdução geral

As Angiospermas em sua maioria são hermafroditas, tendo órgãos femininos e masculinos em uma mesma flor. Dessa maneira, as possibilidades de autopolinização e autofecundação são grandes. A autopolinização reduz a quantidade de material disponível para a recombinação genética, diminuindo a variabilidade genética de populações (Percival 1965; Oliveira & Maruyama 2014; Barrett & Harder 2017). Diferentes estratégias para reduzir a autopolinização e favorecer a polinização cruzada evoluíram nas plantas com flores, incluindo a separação espacial de órgãos sexuais femininos e masculinos dentro de uma flor, como a hercogamia (Lloyd, Webb & Dulberger 1990). Um tipo especial de hercogamia ocorre em espécies heterostílicas, onde as estruturas reprodutivas femininas e masculinas são colocadas em posição recíproca em tipos florais distintos (Ganders 1979; Barrett 2002; Barrett 2010; Keller *et al.* 2014).

A heterostilia envolve a ocorrência de morfos florais que produzem flores com estruturas sexuais de tamanhos e posições diferentes, sendo vista como um mecanismo que potencializa a polinização cruzada em populações (Ganders 1979; Barrett *et al.*, 2000). Está presente em 28 famílias das Angiospermas, em 199 gêneros e 15 ordens, sendo que Rubiaceae é a família mais representativa (Ganders, 1979; Hamilton, 1990; Barrett e Richards, 1992; Naiki 2012). O polimorfismo ocorre em 416 espécies de Rubiaceae pertencentes a 31 gêneros. Destas, 122 espécies são da tribo Psychotrieae (Jung-Mendaçolli & Melhem, 1995), mas também há ocorrência em outras tribos da família.

Mesmo tendo sido descoberta mais cedo, a heterostilia foi investigada mais intensamente pelos pesquisadores Charles Darwin e Hildebrand no século XIX (Ganders, 1979). Em 1877, Charles Darwin publicou o livro chamado “*The different*

*forms of flowers on plants of the same species*”, onde escreveu sobre as diferentes formas de flores em plantas de uma mesma espécie e também sobre a origem, função e evolução dos polimorfismos florais e seu significado funcional como mecanismo facilitador da polinização cruzada (Barrett 2010). Desde lá, o assunto tem interessado muitos naturalistas, ecologistas evolutivos e geneticistas (Ganders 1979; Lloyd, Webb & Dulberger 1990; Endress 1994; Barrett 2010). Darwin sugeriu em seu livro que a posição recíproca entre estigmas e anteras em morfos florais opostos facilitaria a ocorrência de polinizações legítimas (inter-morfo) mediadas pelos animais polinizadores (disassortative pollination), isto é, polinizações entre anteras de um morfo floral e estigmas no nível equivalente do outro morfo floral (Dulberger 1992; Keller *et al.* 2014). A disposição das estruturas reprodutivas reduziria o desperdício de pólen decorrente de polinizações ilegítimas (intra-morfo) e também da autopolinização, garantindo dessa forma um maior sucesso reprodutivo para a planta (Dulberger 1992).

A distília é a forma mais comum encontrada da heterostilia, onde apresenta um morfo com flores com pistilo longo e estames curtos (morfo longistilo/pin) e o outro com pistilo curto e estames longos (morfo brevistilo/thrum) (Hamilton 1990; Ganders 1979; Barrett *et al.* 2000; Faivre 2002). Para populações distílicas em equilíbrio espera-se que os morfos ocorram em igual proporção, situação conhecida como isopleτία (Ganders 1979; Hamilton 1990; Barrett *et al.*, 2000). Além disso, as plantas distílicas também apresentam características auxiliares, como diferenças no tamanho da corola, na estrutura das papilas estigmáticas, tamanhos das anteras e tamanho do grão de pólen entre os morfos (Ganders 1979; Barrett 2010) e também a presença de um mecanismo fisiológico de auto-incompatibilidade e incompatibilidade intramorfo (Barrett & Shore 2008).

Uma vez que as plantas distílicas dependem de um vetor biótico para a

manutenção e eficiente reprodução, o sistema pode ser afetado negativamente quando o serviço de polinização é interrompido, e isso ocorre principalmente por processos de perturbação antrópica, como a fragmentação e degradação de habitats naturais, causando mudanças na estrutura da paisagem e influenciando a ação dos polinizadores (Kremen *et al.* 2007). Diante dessas situações, as condições ambientais favorecem a autopolinização e essas plantas podem sofrer transições para se adaptar ao ambiente alterado. Entre as derivações dentro do sistema observam-se alterações nos níveis de reciprocidade, perda da igual proporção de morfos na população (anisopletia), quebra no sistema de incompatibilidade, homostilia (estruturas sexuais em uma mesma posição - perda da hercogamia) e o monomorfismo (apenas um dos morfos presentes na população) (Hamilton 1990; Ganders 1979; Ferrero *et al.* 2011; Yuan *et al.* 2017).

A caracterização da distilia, assim como as derivações ocorrentes no sistema, podem ser avaliadas por cálculos relacionados à hercogamia recíproca e à precisão no processo de transferência de pólen. A reciprocidade entre os morfos pode ser calculada pela inacurácia das flores, através de um modelo matemático baseado no conceito de imprecisão adaptativa desenvolvido recentemente por Armbruster *et al.* (2017). O valor da inacurácia é definido com base nas posições das anteras e estigmas em toda população, sendo considerada reciprocidade perfeita quando o valor de inacurácia é igual à zero (Armbruster *et al.* 2017). Nesse sentido, o índice tem o objetivo de verificar a probabilidade de polinização legítima e precisa entre os morfos compatíveis e observar possíveis variações, avaliando assim o sucesso reprodutivo das espécies (Armbruster *et al.* 2017).

Como variações na posição das estruturas reprodutivas entre os morfos podem afetar a reciprocidade, a dispersão direcional do pólen legítimo e consequentemente o sucesso reprodutivo das plantas, o objetivo dessa dissertação de mestrado foi investigar

e entender variações morfológicas em populações de espécies distílicas utilizando o índice de inacurácia para avaliar o nível de reciprocidade e relacionar as imprecisões na posição das estruturas reprodutivas e ainda, buscar fatores que influenciam na variação da inacurácia floral nas populações. Nesse sentido, o primeiro capítulo buscou avaliar a distribuição de variação dentro de populações de espécies distílicas de Rubiaceae a partir de dados disponíveis na literatura, usando como modelos *Psychotria* e *Palicourea*, dois gêneros bastante representativos em número de espécies distílicas. Neste capítulo também buscamos entender padrões e fatores que influenciam na inacurácia floral nas populações. O segundo capítulo buscou compreender os padrões de variação floral intra-individual em populações de quatro espécies distílicas de Rubiaceae do Cerrado brasileiro e discutir quais as causas de variações dentro do indivíduo.

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## Capítulo 1

### Variation of inaccuracy patterns across distylous populations of Psychotrieae (Rubiaceae)

Renata Trevizan Telles, João C.F. Cardoso, Paulo E. Oliveira

#### Abstract

Angiosperms have largely evolved very specialized reproductive strategies to benefit from efficient cross-pollination and cross-fertilization. Heterostyly is a floral polymorphism that is thought to promote disassortative pollination between morphs. Variations in the reciprocal herkogamy cause the inefficiency pollen transfer by pollinators affecting the species' reproductive system. Among the derivations in the system is observed the variations of reciprocity, variations in morph ratios, homostily and monomorphism formations. Through literature review we investigated the distribution and variation of reciprocity among Rubiaceae distylous populations based on their two larger representative distylous genera *Psychotria* and *Palicourea*. We aimed to understand how the different imprecision components in the flower relate to and differ from one another. We also which were the factors genus, morph ratio and corolla length that influence the populations-level imprecision. We investigated 97 populations of distylous species and verified how the factors are related. Inaccuracy of organs of both morphs was positively related among them. The maladaptive bias of high and low organs was positively related to each other. We point out which are the most contributive components to the total inaccuracy, which were maladaptive bias on low organs followed by stigmas of thrum. In addition, we demonstrate that corolla size is the only factor determining inaccuracy on reproductive organs of both morphs. This is the first study to test the total and decomposed inaccuracy metrics to a large number of populations. Our study is important because it points out patterns and trends in distylous plants based on a wide sampling of populations.

Key words: distyly, herkogamy, pollination, *Psychotria*, *Palicourea*.



## Introduction

Pollination is an essential ecosystem service, which ensures fruits/seeds production and reproduction to most flowering plants. For allowing pollen flow and fertilization of basically sessile plants, the pollination mechanism is an important factor to ensure mating and genetic variation in these organisms (Proctor *et al.* 1996; Daily 1997; Kremen *et al.* 2007; Rech *et al.* 2014). Approximately 90% Angiosperms depend on animal pollinators to fertilization (Ollerton *et al.* 2011). Several factors are taken into account to optimize the pollination process. Cross-fecundation is most common type of mating system in the flowering plants, providing greater possibilities of recombination and maintaining genetic variability (Percival 1965; Raven 2001; Oliveira and Maruyama 2014; Barrett and Harder 2017).

Angiosperms have largely evolved very specialized reproductive strategies to benefit from efficient cross-pollination and cross-fertilization (Barrett, 2010; Barrett and Harder 2017). These strategies may involve morphological, genetic and physiological mechanisms. The Heterostyly is a floral polymorphism that has evolved independently in 28 Angiosperms families (Barrett and Shore 2008; Naiki 2012). Populations are composed of two (distyly) or three (tristyly) floral morphs (Ganders 1979; Lloyd and Webb 1992; Harder *et al.* 2000). Plants with distyly, the main form of heterostyly, present two floral morphs with different sexual structure positions: thrum flowers (stigma below the anthers) and pin flowers (stigma above the anthers). It has been seen as a mechanism that promotes cross-pollination in plant species (Ganders 1979; Barrett & Richards 1990; Barrett 1992; Barrett *et al.*, 2000; Castro & Oliveira 2002; Barrett 2010).

The flower morphology is an important factor in distyly because it would assure the expected reciprocal position of the sexual structures – reciprocal herkogamy (HR),

in other words, the positions of stigmas and anthers would coincide with those of flowers of a different morph (Barrett 2002; Barrett 2010; Keller *et al.* 2014). This promotes a directional pollen flow (disassortative between-morph pollination), which affects the pollination precision and optimize the cross-fertilization, because the pollen from the anthers of a morph is deposited on the part of the pollinator body that will come into contact with the stigma of the opposite morph, thus performing a legitimate intermorph pollination (Barrett 2002; Keller *et al.* 2014). Distyly is usually accompanied by ancillary characteristics as corolla size, stigmatic papillae structure, anther size, and the differences in pollen grain size between morphs (Ganders 1979; Barrett & Richards 1990). In addition, it is characterized by a self and intra-morph incompatibility mechanism (Ganders 1979; Barrett and Shore 2008) and morph ratios are commonly 1:1 in the population (isopleth) (Ganders 1979).

Despite established in some groups, variations within the distylous populations are common (Sobrevila *et al.* 1983; Faivre and McDade 2001; Santos *et al.* 2008; Coelho *et al.* 2003; Pereira *et al.* 2006; Consolaro *et al.* 2009; Costa *et al.* 2017; Rodrigues *et al.* 2013; Sugawara *et al.* 2013; Brys and Jacquemyn 2015; Sá *et al.* 2016) and reasons for these variations are less well defined. Variations are observed when the environment is under ecological pressure or disturbances, such as the habitats fragmentation (Consolaro *et al.* 2011). The distyly system is changed when the ecosystem service is affected (Ganders 1979; Ree 1997). The pollination service is interrupted when processes of anthropic disturbance occur, such as the fragmentation of natural habitats, causing changes in the landscape structure and influencing the pollination activity (Kremen *et al.* 2007).

Variations in the reciprocal herkogamy cause the inefficiency pollen transfer by pollinators affecting the species' reproductive system. Among the derivations in the

system is observed the variations of reciprocity, variations in morph ratios (anisoplethy), homostily and monomorphism (Hamilton 1990; Ganders 1979; Ferrero *et al.* 2011; Yuan *et al.* 2017). The homostily is a common derivation in distylous plants where flowers have anthers and stigma at the same height and the monomorphism is characterized for the populations with only one morph (either thrum or pin) (Barrett 2010). The presence of the atypical populations may convert a self-incompatible breeding system into a self-compatible one, leading to species' rapid speciation (Sobrevila *et al.* 1983; Consolaro *et al.* 2009). But even before these drastic changes in distyly, changes in the ancillary features, including isoplethy and reciprocal herkogamy may indicate pressures affecting breeding effectiveness.

Distyly characterization, as well as the derivations in the system, can be evaluated by morphometric evaluation of floral features associated with reciprocal herkogamy. The morphs reciprocity can be calculated through the inaccuracy of flowers, using a mathematical model based on the adaptive imprecision concept recently developed by Armbruster *et al.* (2017). The larger correspondence of reproductive organs between different morphs, the greater the probability of legitimate pollen transfer (intermorph). If the anthers and stigmas are at the same (optimal) position, inaccuracy will be zero and pollination should be precise/effective (Armbruster *et al.* 2017). Thus, the objective of this measurement is to assess the likelihood of legitimate pollinations between compatible morphs and consequently species reproductive success (Armbruster *et al.* 2017).

Since variations in distylous system influence the reproductive structures position, affect the reciprocity between morphs, the dispersion of legitimate pollen, and consequently the reproductive success of the species, the objective of our study was investigate the morphological variation in distylous populations using the inaccuracy

index to evaluate the reciprocity level and relate imprecisions in the sexual organs position. Studies in the current literature are commonly isolated cases, usually dealing with individual populations and sampling limitations. Therefore, there are few studies showing patterns among various populations (Armbruster *et al.* 2017). We thus investigated the distribution and variation of reciprocity among Rubiaceae distylous populations based on two of its largest and most representative distylous genera: *Psychotria* and *Palicourea*. To do so, we revised literature gathering studies related to this topic. From the results, we aim to understand how the different imprecision components of inaccuracy in the flower relate to and differ from one another. We also test which are the factors (genus, morph ratio and corolla length) that influence the populations-level imprecision.

## Material and Methods

### *Literature survey*

We conducted the review using the search Google Academic ([scholar.google.com.br](http://scholar.google.com.br)) and the Capes Scientific Journals gateway (<http://www.periodicos.capes.gov.br/>). The key words used were *Palicourea*, *Psychotria*, *heterostyly*, *distyly*. We considered studies with populations of species that presented typical distyly. The data collected included taxonomic information (species of *Palicourea* and *Psychotria*), source type (paper, personal data or doctoral thesis), reference, species, the ratio between pin and thrum morphs and the morphometry measurements as the means and standard deviation (SD) of stigma height, anther height and corolla length of pin and thrum morphs.

We standardized morphometry measurements to mm. For the study by Sá *et al.* (2016), which reported 95% confidence intervals, SD was calculated following the protocol suggested by Higgins and Deeks (2008), which is based on sample size and the

difference between the upper and lower limits. For the study by Faria *et al.* 2012, we calculated SD based on the coefficients of variation reported.

### *Inaccuracy calculations*

We did not calculate inaccuracy indexes for studies: (1) that did not report measurements of reproductive organs; (2) involved homostylous, pin- and or thrum-monomorphic populations; (3) had homostylous co-occurring along with thrum and/or pin morphs (e.g. Consolaro *et al.* (2009); Sá *et.al.* (2018)); (4) with the presence of a single thrum morph in the population (against 151 pin; i.e. Consolaro *et al.* (2011)).

We used the adaptive inaccuracy index introduced by Armbruster *et al.* (2017) as a measure of reciprocity between morphs. As the values of inaccuracy increase, reciprocity decreases. Inaccuracy is calculated based on populations' means and variances in anthers and stigmas height in each morph (Armbruster *et al.* 2017). Whenever necessary, we converted the SD values of studies into variance. As we were dealing with distinct populations/species, instead of an absolute scale, we mean<sup>2</sup> standardized the total inaccuracy by the average organ height to generate a proportional value (as suggested by Armbruster *et al.* 2017). We partitioned the independent contributions of imprecisions (variances on stigmas anthers of each morph) and maladaptive bias (departure from optimum reciprocity of high and low organs) for each case study. To generate equitability among values, we standardized the variance of an organ by its respective mean<sup>2</sup> (e.g. mean<sup>2</sup> standardized imprecision of pin anthers:  $\text{pin\_anther\_variance}/(\text{pin\_anther\_mean})^2 * 100$ ) and the maladaptive bias by the mean of respective organs averages (e.g. mean<sup>2</sup> standardized maladaptive bias of low organs:  $\text{low\_maladaptive\_bias}/((\text{pin\_anther\_mean} + \text{thrum\_stigma\_mean})/2)^2 * 100$ ). We called

these as “decomposed inaccuracies” along the manuscript. All analyses were carried out using mean-corrected values.

### *Statistical analyses*

We ran a principal component analysis (PCA) on  $\log(x+0.01)$  corrected indexes (including total inaccuracy and decomposed inaccuracies) to create dimensionality reduction, evaluate associations between variables and reveal the most contributive significant ones. We used the package FactoMineR version 1.41 (Husson *et al.* 2018) and specified a covariance matrix because our variables were on the same scale (Abdi and Williams 2010). Genus was treated as a supplementary variable for plotting.

We investigated differences among inaccuracy values of the (i) decomposed inaccuracies (six levels: stigmas of pin and thrum, anthers of pin and thrum, maladaptive bias of high and low organs) and (ii) genus (two levels: *Palicourea* and *Psychotria*) through a LMM (linear mixed-effects model) using the packages *lme4* version 1.1-19 (Bates *et al.* 2018) and *car* version 3.0-2 (Fox *et al.* 2018). Species and number of study were treated as random effects. We applied  $\log(x+0.01)$  to the response variable in order to improve residuals distributions. After finding significative differences among the decomposed inaccuracies (see Results), we created a hierarchical tree structuring groups according to their means using the package *factorMerger* version 0.3.6 (Sitko *et al.* 2018). We chose this method instead of traditional pairwise *post hoc* tests due to our large number of levels (six). The merging algorithm divides groups into non-overlapping clusters based on given significance level according to the likelihood ratio test statistic. We ran the analysis based on a ‘multi-dimensional gaussian’ model using the ‘adaptive’ method (Sitko *et al.* 2018).

Afterwards, we ran separated LMMs for all inaccuracy indexes (total and

decomposed inaccuracies). We investigated the effects of (i) genus, (ii) the ratio between pin and thrum morphs found on populations (a measure of isopleth), and (iii) the mean between thrum and pin corolla lengths (a measure of flower size). The response variables were  $\log(x+0.01)$  corrected and corolla was  $\log$  corrected to improve residual distribution. Species was treated as random effect. If any model was found significant, we then calculated the proportion of variance explained by the fixed effect alone using the marginal  $R^2$ , and by the full model (fixed and random effects), using the conditional  $R^2$  (respectively  $R^2_m$  and  $R^2_c$ ; *sensu* Nakagawa and Schielzeth 2013) through the package *MuMIn* version 1.42.1 (Bartoń 2018). Preliminary data exploration was performed following Zuur *et al.* (2010) and models were validated by inspecting homogeneity of fitted vs. residual values plots, quantile-quantile plots, histograms and Cook's distance (Zuur *et al.* 2009). Analyses were conducted in R statistical environment version 3.5.1 (R Core Team 2018).

## Results

We gathered information on 97 distylous populations from 69 research papers, 10 doctoral thesis and 18 authors' personal data (Table S1). These comprised 39 *Palicourea* (12 species) and 58 *Psychotria* (31 species) populations.

As a result of the PCA analyses carried out for the data pooled together, the first two PC axes on indexes accounted for 78.73% variance (Fig. 1). The PC1 axis explained 50.94% of variance, with stigmas of thrum, anthers of thrum, stigmas of pin and anthers of pin as the most contributive inaccuracies, respectively with 24.12, 20.97, 20.93 and 20.63 % of variance explanation in this axis. The second PC axis explained 27.79% of data variance, with maladaptive bias of low organs being the most important

(59.49% of PC2). This variable was also the most important when considering total contribution to PC1 and PC2. *Palicourea* and *Psychotria* had a high overlap according to inaccuracy indexes, showing a lack of differences between these two genera (see further analysis). Overall, the four inaccuracy indexes related to organ imprecision (stigmas and anthers of both morphs) were highly correlated among them. On the other hand, maladaptive bias on high and low organs were mainly correlated to each other, while total inaccuracy was less correlated to other indexes and explained a small portion of the variance of the first two PC axes.

Inaccuracy values did not differ between the two genera ( $\chi^2 = 0.54$ ;  $df = 1$ ;  $p = 0.462$ ). However, there were significant differences among the decomposed indexes ( $\chi^2 = 42.97$ ;  $df = 5$ ;  $p < 0.001$ ;  $R^2m = 0.053$ ;  $R^2c = 0.228$ ). The *post hoc* hierarchical tree had three final clusters (Fig. 2; Table 1). The first node bifurcation splits maladaptive bias of low organs (inaccuracy mean  $\pm$  SD:  $6.64 \pm 11.35$ ), the level with the highest values, from the remaining groups. The second node separates stigmas of thrum ( $1.76 \pm 2.63$ ), group with the second highest values, from those remaining. All other pairwise comparisons on remaining nodes were not significant. Thus, anther of pins ( $0.98 \pm 0.97$ ), the stigma of pins ( $0.80 \pm 0.78$ ), anther of thrums ( $0.88 \pm 0.93$ ) and maladaptive bias on high organs ( $1.40 \pm 2.31$ ) we're not significantly different from each other.

Considering the explanation of each of the inaccuracies, there were no effects of genus or morph ratios (Table 2). However, there was a consistent effect of corolla length across inaccuracies related to all organ imprecisions (Fig. 3; Table 2). Anthers of pin ( $R^2m$ : 0.176;  $R^2c$ : 0.451) and thrum ( $R^2m$ : 0.121;  $R^2c$ : 0.429) and stigmas of pin ( $R^2m$ : 0.096;  $R^2c$ : 0.284) and thrum ( $R^2m$ : 0.130;  $R^2c$ : 0.363) showed a decrease in inaccuracy as flower size increased. On the other hand, corolla size had no effect on total inaccuracy or any of the maladaptive biases.



## Discussion

Using the information on 97 populations we showed the trends of inaccuracy of the reproductive organs in distylous species of Rubiaceae. In both morphs, the inaccuracy was positively related among reproductive organs. On the other hand, maladaptive bias of high and low organs were also positively related to each other. We point out which are the most contributive factors to the total inaccuracy, which were maladaptive bias on low organs followed by stigmas of thrum. Finally, there was no effect of genus or morph ratios, but the corolla size was the only factor which determined the inaccuracy on reproductive organs of both morphs.

### *Inaccuracy correlation*

The correlation of the distinct components of inaccuracy may show how they behave across several populations. The stigmas of thrum, anthers of thrum, stigmas of pin and anthers of pin inaccuracies presented a high positive correlation but had no correlation with the maladaptive bias inaccuracies. On the other hand, maladaptive low and maladaptive high correlated with each other. We suggest that the correlation among indexes is an important pattern to maintain the balance of distylous system in the populations. In distylous plants, the reciprocal position of anthers and stigmas is the main characteristics that promote the disassortative pollination among morphs and limit selfing and sexual interference (Barrett and Shore 2008; Barrett 2010; Keller *et al.* 2014; Jacquemyn *et al.* 2015). Variations in the inaccuracies of organs cause deviations in the level of reciprocal position and can lead to ineffective pollen flow and pollen limitation, which can affect the reproductive production in one or both morphs types (Faivre and McDade, 2001; Jacquemyn *et al.* 2015; Jacquemyn *et al.* 2018). Since all the populations studied are typically distylous, any change of the reproductive organs positions would result in collapse and a possible breakdown in the system. Finally, the

*Palicourea* and *Psychotria* genera presented high overlap, suggesting a similarity among the two according to the indexes. We were expecting differences in the inaccuracy between the *Palicourea* and *Psychotria* because they are taxonomically different. They have differences in the floral traits, for example, *Palicourea* is defined by corollas with gibbous swelling at the base and brightly colored. On the other hand, *Psychotria* have the corolla straight at base and usually is white or greenish (Nepokroeff *et al.* 1999). They also have differences in the type of pollinators, being that the *Psychotria* is usually pollinated by bees and *Palicourea* by hummingbirds (Nepokroeff *et al.* 1999). Nevertheless, our study pointed out the stabilizing selection between genera.

#### *Inaccuracy differences*

We found inaccuracy values significantly different among the decomposed indexes in the populations. The maladaptive bias of low organs was significantly different, it means, they have higher inaccuracy in relation to others decomposed indexes. The thrum stigma inaccuracy also differed from decomposed indexes. Inaccuracies values were smaller for the pin anther, thrum anther, pin stigma, and maladaptive high. In our results, we found that low organs position (pin anthers and thrum stigma) were less precise in the populations, while high organs position (pin stigma and thrum anthers) had greater precision between reciprocal organs/structures. Studies have discussed the function of the morphs within distylous plants populations and have demonstrated that exposed reproductive structures (high organs) are more efficient exporting pollen and capturing pollen than those inside the corolla tube (low organs) (Keller *et al.* 2014). In the long term this situation may lead to a functional dioecy or even to dioecy itself, with thrum anther specialized in exporting and pin stigmas in receiving pollen (Watanabe *et al.* 2013; Keller *et al.* 2014). In the studied

populations, the pin morph stigma, thrum anther and maladaptive high presented low values of inaccuracy, which demonstrate greater reciprocity between morphs. In contrast, pin anther, thrum stigma and maladaptive low showed higher inaccuracy values, thus, a lower reciprocity. This may indicate high organs inaccuracy is restricted by the need of effective pollen flow between high organs, while low organs would be less constrained in size/position since they might contribute less to reproductive success. These patterns can explain why the functional dioecy sometimes occurs. However, reproductive success of isoplectic and truly distylous populations, such as the studies here, seldom show between morph differences in reproductive success (e.g. Machado et al. 2010 to the *Palicourea rigida*).

#### *Factors influencing the inaccuracy*

The inaccuracies did not differ between *Palicourea* and *Psychotria* neither was affected by morph ratios. However, our results did show a relationship between flower size and inaccuracy. The increase in corolla size seems to reduce stigma and anthers inaccuracies both in thrum and pin flowers, and this leads to an increase also in the reciprocity between morphs. Tubular corollas in distylous plants are considered to improve the pollen deposition in specific parts of the insect's mouth, thus promoting precision in pollination (Herrera 1995; Ganders, 1979). Plants with long corolla tube are adapted to morphologically more specialized pollinators with a long tongue. Long-tongued pollinators reach the nectar at the bottom of the corolla and increase the chance to have contact with the reproductive structures. Consequently, these plants with long corolla tubes achieve higher reproduction than plants with shorter corolla tubes (Herrera 1995; Huang *et al.* 2016). This directional selection may favor long corolla tubes in the populations. (Huang *et al.* 2016). In addition, long corolla tube can also act as a visual attraction to lure more specialized pollinators, and may promote pollination

effectiveness (Gómez *et al.* 2016). The difference in flower size between morphs is an ancillary characteristic noted in distylous plants, where commonly the corolla length is greater in the thrum morph. It has already been noted in several studies (Sobrevila *et al.* 1983; Ganders 1979; Hamilton 1989; Ree 1997; Contreras and Ornelas 1999; Faivre and McDade 2001; Castro and Araújo 2004; Castro *et al.* 2004; Rossi *et al.* 2005; Mendonça *et al.* 2005; Pereira *et al.* 2006; Hernández *et al.* 2007; Fonseca *et al.* 2008; Consolaro *et al.* 2009; Machado *et al.* 2010; Valois-Cuesta *et al.* 2010; Hernández-Ramírez 2012; Martén-Rodríguez *et al.* 2013; Watanabe *et al.* 2014; Gusmán *et al.* 2015). Since distylous flowers present stamens united to the petals (epipetalous stamens), the corolla length influences directly anthers height. The selection in the corolla size places the anthers in the correct position with the opposite stigma morph, suggesting that corolla length influences the reciprocal reproductive structures from one morph to another (Dulberger 1992).

### **Final considerations**

In this study, we used inaccuracy indexes to evaluate the distribution and variation of reciprocity among 97 populations of distylous Rubiaceae, and observed the factors that might influence such population-level imprecision. This is the first study to test the total and decomposed inaccuracy metrics to a large number of populations. We verified how they are related. Inaccuracy of organs of both morphs was positively related among them. On the other hand, maladaptive bias of high and low organs was positively related to each other. We point out which are the most contributive indexes to the total inaccuracy, which were maladaptive bias on low organs followed by stigmas of thrum. Finally, we demonstrate that corolla size is the only factor determining inaccuracy on reproductive organs of both morphs. Our study is important because it

points out patterns and trends in Rubiaceae distylous plants based on a wide sampling of populations.

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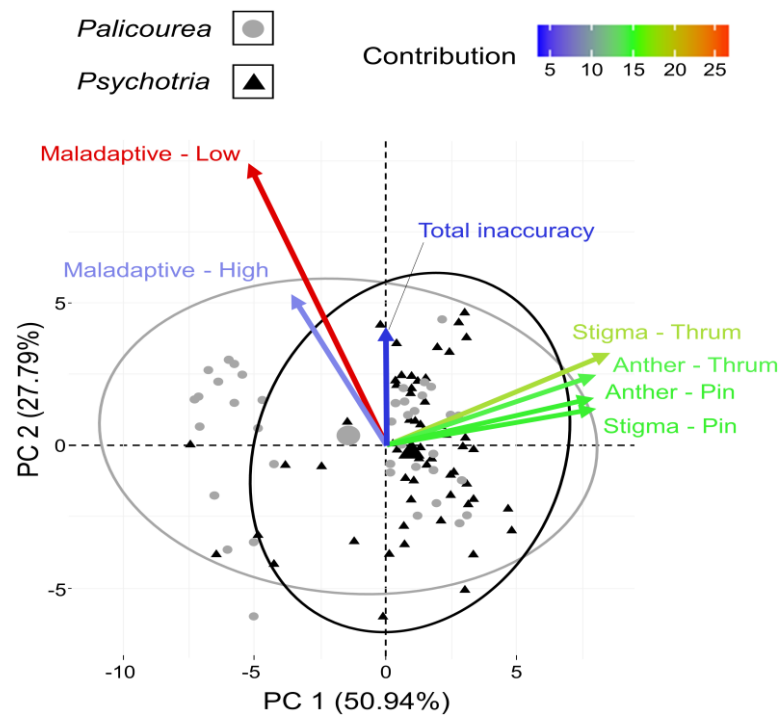
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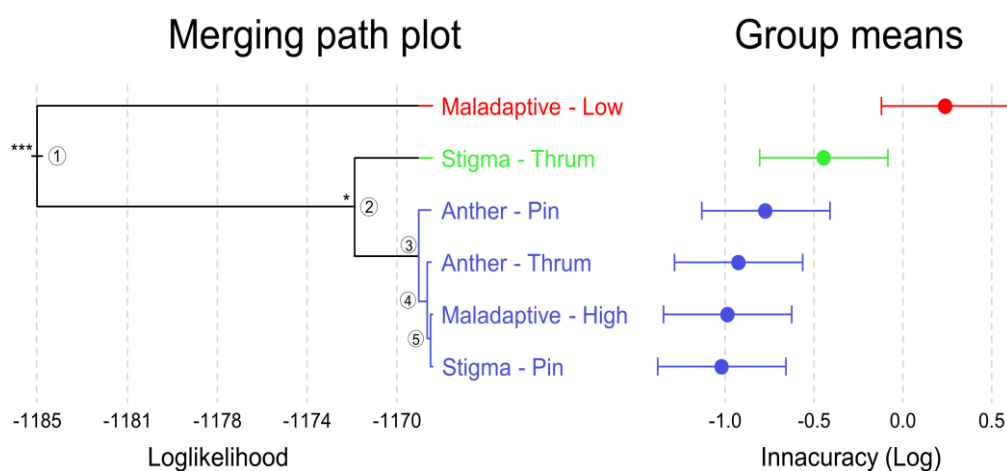
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### Figure and table captions

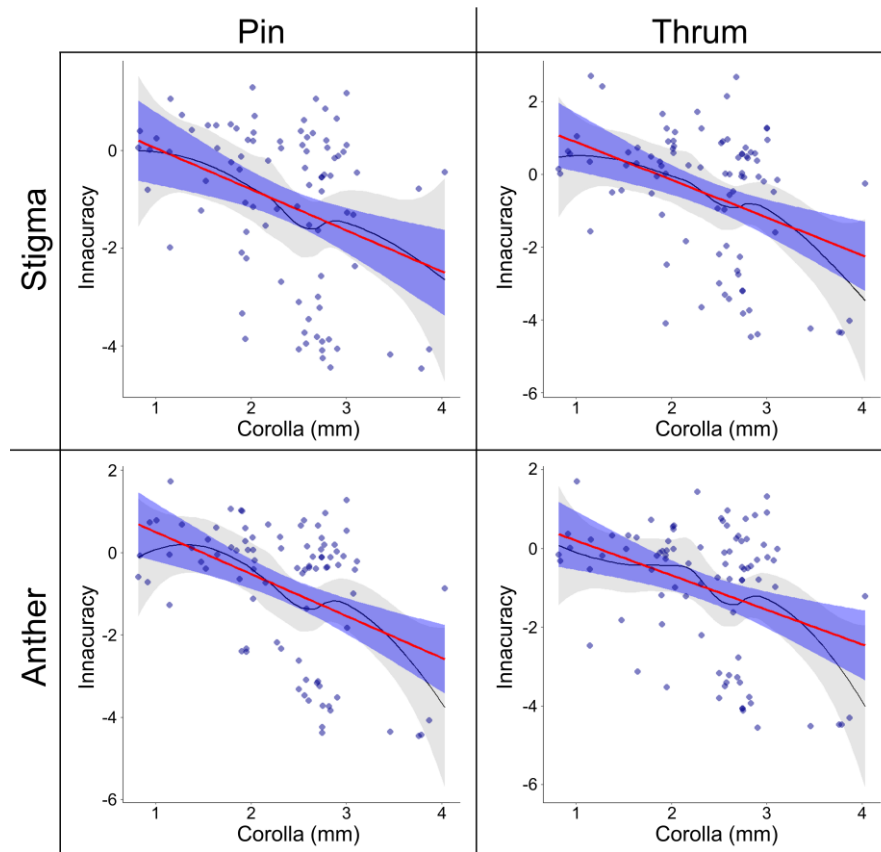


**Fig. 1.** PCA biplot demonstrating variables contributions according to the two genera along the first two PCs axes. Contributions are expressed in percentages and ellipses comprise 0.95 confidence intervals.



**Fig. 2.** Merging path plot alongside group means for the decomposed inaccurrencies. Distinct colors display the three final clusters. Symbols indicate significant differences at 0.001 (\*\*\*) and 0.05 (\*) levels. The

exact values for each node bifurcation number are available in Table 1. Points in the graph display means and line segments are 95% CI.



**Fig. 3.** Inaccuracy in relation to corolla size (both in log scale) for all combinations between morphs and organs. Linear regression line and its 95% CI are expressed respectively in red and blue shades. Loess line and its 95% CI are respectively in black and grey.

**Table 1.** Loglikelihood and p-values (in decreasing order of significance) of pairwise comparisons on node bifurcations. The numbers of pairwise comparisons are available in Fig. 2. Significant p-values are expressed in bold.

Pairwise comparison	Loglikelihood	p-value
1	-1185.21	< <b>0.001</b>
2	-1171.67	<b>0.02</b>
3	-1168.90	0.34
4	-1168.44	0.73
5	-1168.38	0.90

**Table 2.** Results for tests on each of the inaccuracy indexes according to the three predictor variables (df = 1 in all cases). Significant p-values are expressed in bold.

Inaccuracy	Variables					
	Genus		Pin/Thrum ratio		Corolla size	
	$\chi^2$	p	$\chi^2$	p	$\chi^2$	p
Total inaccuracy	0.01	0.917	0.26	0.613	1.76	0.184
Pin morph						
Stigma imprecision	0.12	0.734	0.64	0.423	4.02	<b>0.045</b>
Anther imprecision	0.01	0.913	1.69	0.194	8.00	<b>0.005</b>
Thrum morph						
Stigma imprecision	0.54	0.463	0.44	0.505	7.49	<b>0.006</b>
Anther imprecision	0.19	0.661	1.34	0.248	4.06	<b>0.044</b>
Maladaptive bias						
High organs	0.01	0.906	0.97	0.324	0.46	0.496
Low organs	0.18	0.668	0.88	0.347	0.32	0.571

## Capítulo 2

### Intra-individual variation in distylous populations

Renata Trevizan Telles, João C.F. Cardoso, Paulo E. Oliveira

#### Abstract

Distyly is a floral polymorphism composed of two morphs that have reproductive structures at corresponding heights - reciprocal herkogamy. The size of floral traits associated with reciprocal herkogamy is important for precise pollen deposition and affective functioning of distyly. Each individual possesses a unique genetic constitution, which responds differently according to the environmental conditions generating morphological variation. The intra-individual imprecision on floral traits results from a developmental instability and from environmental effects. These factors can be the main contributors to the imprecision at the population level. This work aimed to understand the intra-individual variation patterns among distylous populations and what are the causes of morphological imprecision. The distylous species studied were *Declieuxia fruticosa*, *Palicourea coriacea*, *P. marcgravii* and *P. rigida*. All studied populations were isoplectic, without differences in morph ratio. The four species were also typically distylous. Corollas were longer in thrum morphs of *D. fruticosa* and *P. marcgravii*. Anther length was larger in thrum morphs of all species, except *P. coriacea*. In all species, stigmas were longer in thrum morphs. The best model had morph and organ type explaining intra-individual imprecision of the height of reproductive whorls. Low organs had more intra-individual imprecision in height than high organs. On the other hand, thrum morphs had more height imprecision than pin ones. Model selection related to intra-individual imprecision in length had sexual organ as the variable with higher relative importance. There was no variation between species. This suggest mean that this pattern is generalized to distylous species. The reciprocal herkogamy between the morphs allows pollinators performance to be more efficient, providing optimal pollen transfer between the thrum and pin individuals, ensuring a fit between the place of deposition of the pollen grains on the body of the pollinators with the stigma that should receive the correct pollen grain. Therefore, the greater imprecision in the low organs may be related to the stigma structure in thrum flowers, which has a larger size and would allow a greater variation. Regarding the high organs where the imprecision was lower, the stigma of pin flowers has a smaller size, which may select for greater position/size accuracy for correct pollen deposition and hence pollination success. The variation at intra-individual level is a novel approach to study distyly and any conclusion should be made carefully. However, the results pointed to important trends, showing that intra-individual imprecision may vary among different flower levels. This may be related to the different effectiveness of each sexual organ in each morph and distinct selective pressures conditioning such imprecision.

Keywords: distyly, floral polymorphisms, reciprocal herkogamy, within-individual variation.

## Introduction

Different levels of biological organization occur in nature. For instance, they may be an individual organism, a population (organisms of the same species in an area), a community (set of populations that interact), an ecosystem (wherein the communities connect) or even the biosphere as a whole (understanding all the ecosystems of Earth) (Eomnicki 2018). These levels, from lower to higher, form an ecological hierarchy. Since the representatives that make up the hierarchical levels are not static in nature, there may be biologically significant variations at the ecosystems, communities, species, populations, and even at the individual level (Herrera 2009). Considering the genetic diversity and phenotypic plasticity of populations, natural selection operates in the range of characteristics and the adequacy of each individual. Thus, the different levels of variation are related to the evolution of species (Hensen *et al.* 2006; Jung *et al.* 2010; Sobral *et al.* 2013). Variations also occur within a given species. For instance, intraspecific variations can be interpopulational (between populations), interindividual (between individuals), and even intraindividual (within a single individual).

Regarding variations that have some ecological and evolutionary significance, the individual level has been considered the minor in the hierarchy, and often overlooked (Herrera 2009). Each individual has a unique genetic constitution, which respond differently according to the environmental conditions and can lead to higher or lower intra-individual variation (Albert 2010). This is caused by several factors, including the environmental ones such as temperature, light availability, wind velocity, or resources availability (Herrera 2009). For instance, these may cause deviations in leaf and/or floral development among the different parts of a plant (Herrera 2009).

Plants are modular organisms, it is common the emergence of trait intra-individual variations in multiple components of the same organ, such as in leaves,

flowers, fruits, and seeds, since these ‘repeated’ structures may be expressed differentially from one another (Herrera 2009). Within-plant variations can be ecologically relevant not only for plants themselves but also for animals interacting with them, such as pollinators, affecting the interactions with these mutualists (Herrera 2009; Austen *et al.* 2015). In this sense, intra-individual floral variability can affect plant fitness, influencing the visitation frequency and pollination success (Dai *et al.* 2016).

Cross-pollination is commonly found among angiosperms, providing greater possibilities of recombination and genetic variability maintenance (Barrett and Harder 2017). For this, biotic vectors play a crucial role transferring pollen between flowers/individuals, which afterwards leads to fertilization and formation of a new viable individual (Barrett and Harder 2017). The behavior of pollinators induces strong selective pressure on plants, influencing characteristics such as flower morphology, size, color, chemistry, phenology, among others (Herrera 2009; Sobral *et al.* 2013; Arceo-Gómez *et al.* 2017; DeAngelis *et al.* 2018). In this sense, the floral traits are indispensable to promote the precision of pollination, in which the correspondent height of reproductive whorls among flowers is one of the main factors for the pollination efficiency (Dai *et al.* 2016). Studies relating intra-individual flower variation and pollination have been rarely approached (Herrera 2009). Even though, some have discussed the variation in intra-individual floral morphology (e.g. Williams and Conner, 2001; Ishii and Morinaga, 2005; Bateman and Rudall, 2006; Zywiec *et al.*, 2012; Dai *et al.*, 2016). For instance, Arceo-Gómez *et al.* (2017) studied intra-individual herkogamy (stigma-anther separation) variation in *Ipomoea wolcottiana* (Convolvulaceae) and related it to the selection to prevent self-pollen deposition. Nevertheless, the intra-individual variation has been less discussed among floral systems (*sensu* Cardoso *et al.* 2018), especially concerning floral polymorphisms. These polymorphisms optimize



reproductive success, acting as mechanisms to promote self- and/or cross-pollination (Cardoso *et al.* 2018). For instance, distyly is a genetically controlled floral polymorphism composed of two floral morphs that differ in stigma and anther heights in the same population (Ganders 1979; Barrett *et al.* 2000; Barrett and Shore 2008; Barrett 2010). In distylous populations, the intra-individual variation is a new approach to be investigated and, although the polymorphism is a population characteristic, individuals may express characters in different ways.

Due to the dependence of pollen transfer between different morphs for fruit formation, the relationship between distylous plants and pollinators is fundamental for the polymorphism maintenance (Ganders 1979). Accordingly, the imbalance of this interaction can lead to the failure of the system (Barrett and Shore 2008; Barrett 2010). Variations of floral structures at population level may affect the distyly system, influencing the species reproduction and maintenance (Armbruster *et al.* 2017). In addition, variations at the level of individuals may also occur, being considered as a result of developmental instability and microenvironmental effects (Armbruster *et al.* 2017). A greater intra-individual variation could be related to higher imprecision, since it deviates from optimal heights (Armbruster *et al.* 2017). Explaining the ecological causes of floral diversification is of great interest, but few studies focused on the effects of intra-individual variations. Based on this novel approach, we aimed to understand the floral within-individual variation patterns in distylous populations of some Rubiaceae and discuss which are their causes.

## Material and Methods

### *Studied sites and species*

The study was conducted in the Panga Ecological Reserve (Panga; 409.5 ha; 19°09'S; 48°24'W), in the Natural Reserve of Clube Caça e Pesca Itororó (CCPIU; 127 ha; 18°55'S; 48°17'W), both in Uberlândia, Minas Gerais, Brazil, and in the Caldas Novas State Park (PESCAN; 12.500 ha; 17°45'S; 48°41'W), Caldas Novas, Goiás, Brazil. The areas are dominated Cerrado, the Brazilian Neotropical Savanna plant formations, presenting several phytophysionomies of the biome.

The distylous species studied were *Declieuxia fruticosa* (Willd. ex Roem. & Schult.) Kuntze (Panga and CCPIU), *Palicourea coriacea* (Cham.) K.Schum, *Palicourea marcgravii* A.St.-Hil. and *Palicourea rigida* Kunth (PESCAN) (Fig 1). *Declieuxia fruticosa* is found in open cerrado areas such as in *sensu stricto* cerrado phytophysionomies (Panga) and along the ecotone between vereda and *sensu stricto* cerrado (CCPIU). It has an herb and shrub life form/habit, with white and purple flowers, short tube, and sweet odor, with nectar and pollen as reward (Calió 2015; Matias *et al.* 2016). It is a self-/intramorph-incompatible species pollinated primarily by bees. *Palicourea rigida* and *P. coriacea* are typically found together in areas of *sensu stricto* cerrado and more open phytophysionomies (Silva 1995; Machado 2010). On the other hand, *P. marcgravii* occurs in forest groves (Magalhães and Maimoni-Rodella 2012). These plants present brightly colored odorless flowers with tubular corolla and nectar as reward. They are pollinated primarily by hummingbirds, although bees can be occasional pollinators (Taylor 1997).

### Procedures

To verify if populations of the four species were isopletic (same proportion between morphs in the population), we identified and counted individuals of each morphs for each species in the given areas. For the *Palicourea* spp., we selected 20 individuals of each species, 10 of each morph. In *Declieuxia fruticosa*, we surveyed 21 and 19 individuals in Panga and 19 and 20 at CCPIU, respectively of thrum (stigmas below anthers) and pin (stigmas above anthers) morphs. For all species, each individual had from one to four flowers collected. The measurements taken from flowers were: corolla, style, stigma and anther lengths and heights of stigma and anther.

As suggested by Armbruster *et al.* (2017), imprecision at the level of individuals can be measured as the variance in the positions of anther and stigma in flowers of the same plant. As we were dealing with flowers of distinct morphs and species, the use of the raw variance values could entail some biases related to the difference in organs sizes and hinders comparison. To deal with this, intra-individual imprecision was computed as the coefficient of variation (ratio between standard deviation and mean, multiplied by 100), a measure of relative variability. As variances/coefficients of variation are influenced by sample size, we standardized to use only individuals with at least three flowers. Individuals with fewer flowers were removed from the analyses and the ones with more had three flowers randomly selected using the *sample* function available in the base package of the R software version 3.5.1 (R Core Team 2018). The final sample sizes were: *D. fruticosa*: 34 thrum, 38 pin; *P. coriacea*: 10 thrum, 10 pin; *P. marcgravii*: 10 thrum, 10 pin; and *P. rigida*: 10 thrum, 9 pin.

### *Statistical analyses*

We assessed if populations were isopletic using/by chi-square goodness of fit tests, expecting equal proportions for each morph. For each species, we compared if the distinct measurements (lengths of corolla, anther, stigma and style and heights of anther and stigma) differed between morphs using LMMs (linear mixed-effects models) having individual and area (in the case of *D. fruticosa*) as random effects. We built models using the *lme4* R-package version 1.1-17 (Bates *et al.* 2018) and accessed their significance through the *lmerTest* R-package version 3.0-0 (Kuznetsova *et al.* 2018). To avoid type I error due to multiple testing, the p-values within each species were corrected using the Benjamini and Hochberg (1995) (BH) procedure, through the *p.adjust* function in R software stats package.

We tested the influence of species (four levels), morph (two levels: thrum and pin), sexual organ (two levels: anther and stigma), organ type (two levels: high and low) and average corolla length factors in determining the values of intra-individual imprecision related to the height of reproductive whorls. The natural logarithm of the response variable was used to improve residuals distribution. We conducted multi-model dredging based on the full linear model and all combinatory candidate sub-models, including the null one, through the R-package *MuMIn* (Bartoń 2018). We based on the Akaike information criterion values corrected for small sample sizes (AICc) and the AICc likelihood weights (AICcw) to infer the plausibilities of the 32 possible models (Table S3; Table S5). Afterwards, we used the difference between the AICc value of the best model (with the lowest value) and all others ( $\Delta\text{AICc}$ ) to rank models. Every model that had a  $\Delta\text{AICc} \leq 2.0$  was considered parsimonious (*sensu* Burnham and Anderson 2002). A second model selection (with the same predictor variables) was then

performed having the natural logarithm of intra-individual imprecision related to the length of reproductive whorls as response variable.

After finding models containing morph and organ type as the most parsimonious determining intra-individual imprecision in height (the first model selection; see Results section), we further explored these results by creating an interaction term between them (four levels: thrum high [anther], thrum low [stigma], pin high [stigma] and pin low [anther]). We then test for differences among these by building a hierarchical tree through the R-package *factorMerger* (Sitko *et al.* 2018). The procedure divides groups into non-overlapping clusters based on given significance level according to the likelihood ratio test statistic. We ran the merging algorithm based on a ‘multi-dimensional gaussian’ model using the ‘adaptive’ method.

We conducted data exploration following the protocol introduced by Zuur *et al.* (2010) while models were validated by inspecting homogeneity of fitted vs. residual values plots, quantile-quantile plots, histograms and Cook's distance (Zuur *et al.* 2009). All analyses were conducted in R statistical software version 3.5.1 (R Core Team 2018).

## Results

All studied populations were isopletic, without significant differences between thrum and pin morphs (Table S1). Populations of the four species were also typically distylous, with thrum morphs having significant high anthers and low stigmas and vice versa for pin morphs, without overlap between morphs (Table S2; Fig. S1). Corollas were longer in thrum morphs of *D. fruticosa* and *P. marcgravii*. Anther length was larger in thrum morphs of all species, except *P. coriacea*. In all species, stigmas were longer in thrum morphs while styles were longer in pin morphs.

Considering model selection on the potential factors explaining intra-individual

imprecision of the height of reproductive whorls, organ type and morph had the highest values of relative variable importance (respectively 0.64 and 0.51), followed by sexual organ (0.18), species (0.16) and average corolla length (0.08). The best model had morph and organ type as predictor variables while the following two had each of these predictors solely (Table 1; Table S3). The fourth parsimonious model was the null model. All the remaining models had more than 2.0 AICc units of difference when compared to the best one. The hierarchical tree with the interaction between morph and organ type had only two final clusters (Fig. 2). The first node bifurcation ( $p < 0.001$ ) splits stigmas of thrum ( $6.18 \pm 4.12$ ), which had the highest values, from a cluster containing anthers of thrum ( $4.57 \pm 3.04$ ), anthers of pin ( $4.42 \pm 3.16$ ) and stigmas of pin ( $4.24 \pm 2.64$ ). All remaining pairwise comparisons on the other nodes were not significant (Fig. 2; Table S4).

Model selection related to intra-individual variation in length had sexual organ as the variable with higher relative importance (1.00), followed by morph (0.20), organ type (0.18), species (0.17) and corolla length (0.17). The most parsimonious model had only sexual organ as predictor (Table 1; Table S5). Stigmas ( $10.82 \pm 6.56$ ) had 106.49% more intra-individual imprecision in length than anthers ( $5.24 \pm 4.40$ ) (Fig. 3). All following models had a  $\Delta\text{AICc} \geq 2.0$ .

## Discussion

### *Typical distyly*

The distyly promotes reproductive success by means of cross-pollination (Hamilton 1990; Ganders 1979; Barrett 1992; Lloyd and Webb 1992). In addition, the populations usually have an incompatibility system that prevents self and intramorph germination of pollen tubes (Barrett 2002; Barrett and Shore 2008) and an equal

proportion of morphs occurrence within the population (Hamilton 1990; Ganders 1979). The isopleth found in all species studied here shows equilibrium between morphs in the populations.

The size of floral traits associated with reciprocal herkogamy is important for precise pollen deposition and effective functioning of distyly and this feature has important functional consequences for mating behavior and plant fitness (Hamilton 1990; Barrett 2002; Kálmán *et al.* 2007). The presence of morphs allows pollinators performance to be more efficient, providing optimal pollen transfer between thrum and pin individuals, ensuring a fit between the place of deposition of the pollen grains on the body of the pollinators with the stigma that should receive the correct pollen grain (disassortative pollination) (Barrett and Shore 2008; Keller *et al.* 2014). All the studied populations had typical distyly, with thrum and pin morphs showing the classical arrangement of reproductive whorls. This suggests that between-morph pollen transfer may occur efficiently in the systems studied. The longer corollas of thrum morphs of some species (*D. fruticosa* and *P. marcgravii*.) were also reported by other studies (Sobrevila *et al.* 1983; Ganders 1979; Hamilton 1989; Ree 1997; Contreras and Ornelas 1999; Faivre and McDade 2001; Castro and Araújo 2004; Castro *et al.* 2004; Rossi *et al.* 2005; Mendonça *et al.* 2005; Pereira *et al.* 2006; Hernández *et al.* 2007; Fonseca *et al.* 2008; Consolaro *et al.* 2009; Machado *et al.* 2010; Valois-Cuesta *et al.* 2010; Hernández-Ramírez 2012; Martén-Rodríguez *et al.* 2013; Watanabe *et al.* 2014; Gusmán *et al.* 2015) and are probably related to the epipetalous stamens (Dulberger, 1992). Consequently the corolla length places the anthers in correct position with the opposite morph stigma influencing the reciprocal reproductive structures height (Dulberger 1992; Keller *et al.* 2014).

The larger anther length found in thrum morphs of most species (*D. fruticosa*, *P. marcgravii* and *P. rigida*) is also common in other Rubiaceae distylous species (e.g. Castro *et al.* 2004; Coelho *et al.* 2004; Pereira *et al.* 2006). It may be related to the specialization of thrum morphs in pollen donation (see topic below). Also may be related with pollen size that is usually larger in the thrum morph (Ganders 1979).

Finally, all species had stigmas longer in thrum morphs, which is also often found in distylous populations (see topic below). It may be related to the thrum morph pollen self-interference. With a larger area, it is more likely that the pollen of the pin morph have the opportunity to germinate and cross-pollination to occur (Ree 1997; Barrett 2002). We suggest that this trait may be especially important in explaining intra-individual imprecision patterns (see topic below).

#### *Individual-level imprecision in height*

When compared to other plant structures such as leaves and fruits, the floral morphology has much lower variation (Herrera 2009). This occurs because flowers function is linked to pollinator's attraction and pollen effective transfer, so interaction and pollination maintain a stabilized pattern in the flower morphology (Herrera 2009). Selection favors the matching between organs, which is related to reciprocal herkogamy and disassortative pollination in distylous plants (Barrett 2010). Imprecision may be then considered as a departure from reciprocity, which potentially hinders optimal pollination. Therefore, intra-individual variation in floral traits may be associated with the decline of reproductive success, especially in distylous populations (Armbruster *et al.* 2017). It may also be associated with less constrained pollination requirements due to breakdown of self-incompatibility and atypical distyly (Consolaro *et al.* 2011).



Our results demonstrate that intra-individual imprecision in distylous populations are not evenly distributed across organs and morphs. We found that the thrum morph stigma had higher intra-individual imprecision in height when compared to other reproductive whorls. Thus, we suggest that stigmas of thrum are less successful in their function. Since they are dependent on pollen flow from pin anthers, and these are intra-individually more precise, thrum stigmas higher intra-individual imprecision may harm reproduction of such morph. Variations within the distyly system are common, with several cases of homostyly and pin- and thrum-monomorphism (e.g. Consolaro *et al.* 2009; Consolaro *et al.* 2011; Watanabe *et al.* 2014; Sá *et al.* 2016). The asymmetric flow of pollen affects reproductive success and is one of the main causes of departures from typical distyly (Hamilton 1990; Ganders 1979; Barrett 1992; Lloyd and Webb 1992; Barrett 2010). Thus, we may even speculate that the higher imprecision and smaller pollen receipt of thrum stigmas may, in the long term, lead to some sort of atypical distyly such as pin-monomorphism, commonly found in Rubiaceae (e.g. Sobrevila *et al.* 1983; Faivre & McDade 2001; Rodrigues *et al.* 2013; Sá *et al.* 2016; Costa *et al.* 2017). As mentioned, this may be mediated by the acquisition of other characteristics such as loss of self-incompatibility (Lloyd and Webb 1992).

So far, we have considered intra-individual imprecision/variation in distylous populations as a negative trait (following Armbruster *et al.* 2017), since it deviates from the pollination optimal height. However, we suggest that it may be seen otherwise. Since the animals can exercise selective pressures on plant characteristics (Bronstein *et al.* 2006; Arceo-Gómez *et al.* 2017), pollination service may provide a possible explanation for the variation of the floral traits (Bronstein *et al.* 2006; Arceo-Gómez *et al.* 2017). Individual flowers may receive visits from several pollinator types (Arceo-Gómez *et al.* 2017), as described for the studied species (Pereira *et al.* 2006; Consolaro

*et al.* 2009; Machado *et al.* 2010; Matias *et al.* 2016). A larger intra-individual variation in thrum stigma height may be advantageous, since various pollinator groups with different morphological features may efficiently deposit pollen in different ways and heights. Another possible explanation is that the stigma of thrum morph would be more flexible to the variation because it is inside the floral tube. Thus, it will be accessed by pollinator anyway, since it is in the way to nectar access. Contrarily, the pin morph stigma varies less. Since it is exposed, selection favors its positioning on an optimal height.

Accordingly, several studies (including ours) have shown that populations of distylous Rubiaceae species have stigmas of thrum morphs that are longer than those of pin morphs (e.g. Sobrevila *et al.* 1983; Ree 1997; Faivre *et.al.* 2001; Hernandez *et.al.* 2003; Castro *et.al.* 2004; Coelho *et.al.* 2004; Rossi *et.al.* 2005; Mendonça *et al.* 2006; Pereira *et al.* 2006; Hernandez *et.al.* 2007; Virillo *et. al.* 2007; Santos *et.al.* 2008; Consolaro *et. al.* 2009; Machado *et.al.* 2010; Koch *et.al.* 2010; Valois-Cuesta *et.al.* 2012; Hernández-Ramírez 2012; Martén-Rodríguez *et.al.* 2013; Guzmán *et.al.* 2015; Sá *et. al.* 2016). This common characteristic is related to the fact that the thrum stigma has a large surface to receive pollen grains, since this morph suffers more with pollen self-interference (Ree 1997; Barrett 2002). Thereby, the higher height imprecision in thrum low organs may be related to the stigma structure in this morph, which has a bigger size and would allow for more variation. Thus, larger stigmas are related to an increased probability of pollen deposition and to a less strict selection on an intra-individual optimal height. Regarding the scenario of the opposite morph, the stigma of pin flowers has a smaller size, which would require greater intra-individual accuracy in the pollination for correct deposition of pollen and hence reproductive success.

Several studies have discussed the function of each morph within a distyly population and have demonstrated that exposed male structures (i.e. thrum morph anthers) are more efficient exporting pollen than those inside the corolla tube (i.e. pin morph anthers). Accordingly, exposed female structures (i.e. pin morph stigmas) are more efficiently in pollen capture than those inside the corolla tube (i.e. thrum morph stigmas) (Watanabe *et al.* 2013; Keller *et al.* 2014). In the long term and depending on the circumstances, this situation may lead to a functional dioecy or even to dioecy itself, with thrum anther specialized in exporting and pin stigmas in receiving pollen (Barrett 2002; Keller *et al.* 2014). In our results, we found smaller intra-individual variation in height in pin morph stigmas and thrum morph anthers. Therefore, this can show greater reciprocity between the most exposed male and female flower structures, as pointed in other studies (e.g. Watanabe *et al.* 2013; Keller *et al.* (2014). Thus, higher intra-individual variation of thrum stigmas would be a way to compensate the less efficient pollen flow, increasing the chance of deposition by occupying a greater range of heights in the same individual.

#### *Individual-level imprecision in size*

In addition to differences related to height, we also found that intra-individual variation may differ according to size of the organ. The stigmas, regardless of morph, had higher intra-individual imprecision in size than anthers. Making a parallel with height (Armbruster *et al.* 2017), this may be seen as negative. However, unlike height, a higher variation in size would indicate a larger range of lengths within the plant, and it is not necessarily related to the optimal height and reciprocal herkogamy. Since stigmas are related to pollen receipt, this may be related to the fact that a higher size variation would increase the chance of deposition within the same individual. In this sense, the

same plant has flowers with larger and smaller stigma areas, indicating that some flowers are more likely to have female success. Thus, higher intra-individual imprecision in height may compensate deviations from optimal height.

### **Final considerations**

Intra-individual variation is an important approach related to the ecology and evolution of species, with several implications yet to be understood. Selection occurs at the level of individuals and their variation may play an important role on this process. The study of distyly at the individual level is on its beginning and any conclusions should be made carefully. Our results show that intra-individual imprecision may vary differently at the flower level. This may be related to the different effectiveness of each sexual organ in each morph and the different selective pressures conditioning such imprecision. Our study inaugurates a new avenue in the study of heterostyly, and further research is necessary to corroborate our results and uncover other patterns.

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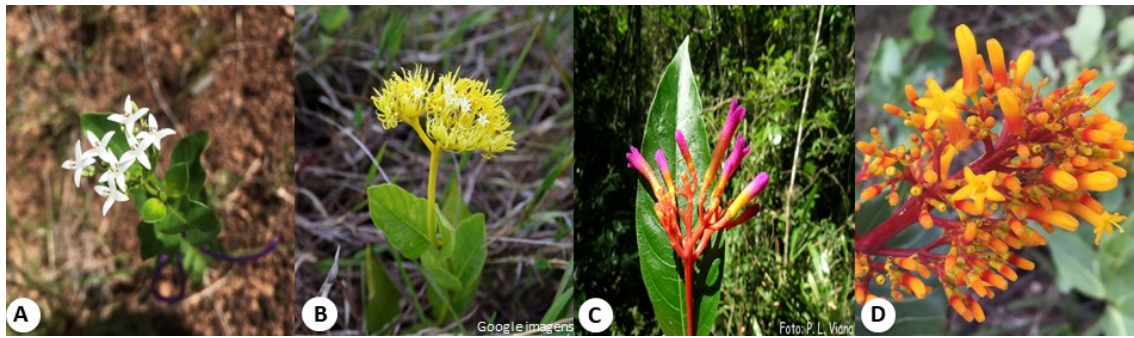
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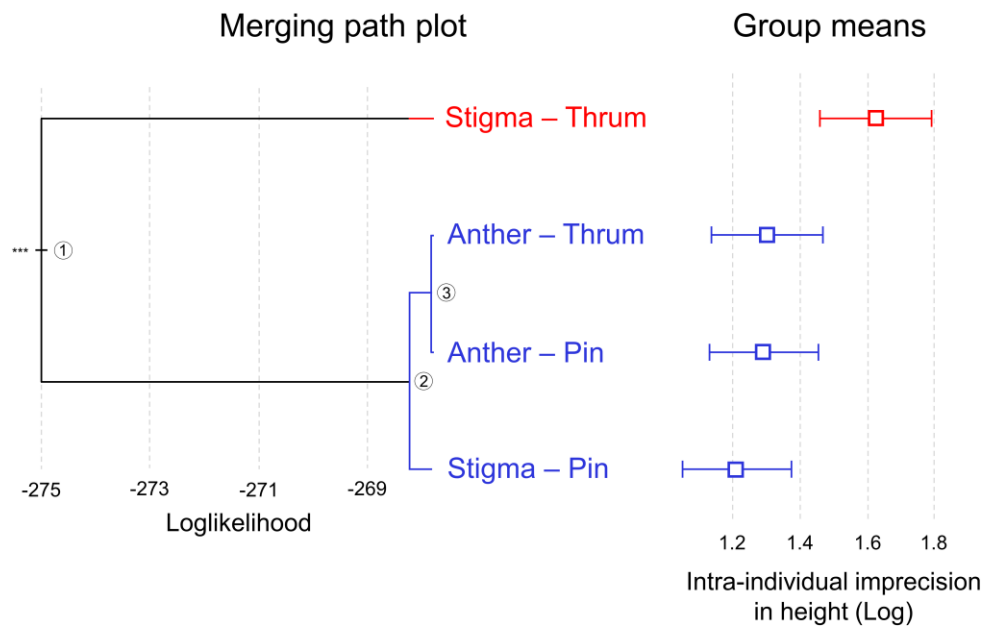
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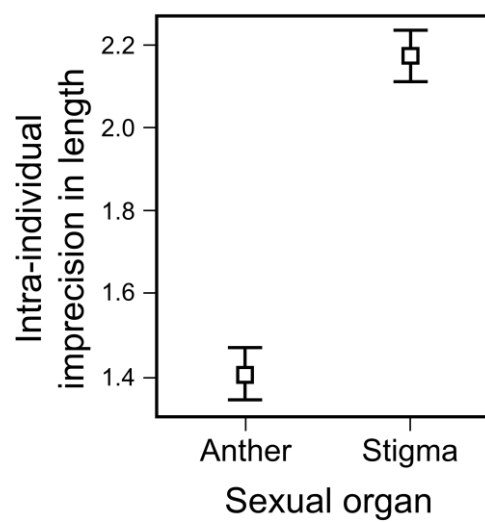
## Figures and tables captions



**Fig 1.** The distylous species studied (A) *Declieuxia fruticosa* (Willd. ex Roem. & Schult.) Kuntze (Panga and CCPIU), (B) *Palicourea coriacea* (Cham.) K.Schum, (C) *Palicourea marcgravii* A.St.-Hil. and (D) *Palicourea rigida* Kunth (PESCAN).



**Fig 2.** Merging path plot (based on loglikelihood) alongside group means for intra-individual imprecision related to the height of reproductive whorls. Different colors display the two final clusters. Symbol (\*\*\*) indicate significant differences at 0.001 level. The exact values for each node bifurcation number are available in Table S4. White squares in the graph display means and line segments are 95% CI.



**Fig 3.** Intra-individual imprecision in length according to sexual organ. y axis is on logarithmic scale.

White squares and line segments indicate respectively means and 95% CI.

**Table1.** Results from model selections for intra-individual imprecision related to the height and length of reproductive whorls having species, morph, sexual organ, organ type and average corolla length as independent variables. Models are ranked according to their fit based on AICcw (likelihood weights) and AICc.  $\Delta AICc \leq 2.0$  are expressed in bold. The first six best models for each model selection are shown and the complete results are available in Tables S3 and S5.

Model	Weight	AICc	$\Delta AICc$
<i>Height</i>			
Morph+Organ type	0.24	555.43	<b>0.00</b>
Organ type	0.20	555.74	<b>0.31</b>
Morph	0.13	556.56	<b>1.13</b>
Null model	0.11	556.88	<b>1.45</b>
Morph+Sexual organ+Organ type	0.05	558.43	3.00
Sexual organ+Organ type	0.05	558.72	3.29
<i>Length</i>			
Sexual organ	0.45	581.54	<b>0.00</b>
Sexual organ+Species	0.11	584.35	2.82
Morph+Sexual organ	0.10	584.52	2.98
Sexual organ+Organ type	0.10	584.57	3.03
Corolla+Sexual organ	0.10	584.57	3.04
Corolla+Morph+Sexual organ	0.04	586.52	4.98

## Supplementary material

**Table S1.** Number of thrum and pin morph individuals found of the four Rubiaceae species and the values of statistics.

Species	Thrum	Pin	$\chi^2$	p-value
<i>D. fruticosa</i>				
Panga	106	126	1.72	0.19
CCPIU	111	108	0.04	0.84
<i>P. coriácea</i>	25	26	0.02	0.89
<i>P. marcgravii</i>	17	18	0.03	0.87
<i>P. rigida</i>	10	15	1.00	0.32



**Table S2.** Floral traits mean $\pm$ SD of thrum and pin morph flowers of the four Rubiaceae species. Significant p-values (BH corrected for each species) are expressed in bold.

Species	Mean $\pm$ SD		Statistics	
	Thrum	Pin	F	p-value
<i>D. fruticosa</i>				
Corolla lenght	4.31 $\pm$ 0.51	3.65 $\pm$ 0.36	56.68	< <b>0.001</b>
Anther lenght	1.22 $\pm$ 0.16	0.95 $\pm$ 0.09	86.08	< <b>0.001</b>
Stigma lenght	0.88 $\pm$ 0.16	0.79 $\pm$ 0.16	8.41	<b>0.005</b>
Style lenght	2.74 $\pm$ 0.61	4.98 $\pm$ 0.46	383.08	< <b>0.001</b>
Anther height	5.72 $\pm$ 0.68	3.10 $\pm$ 0.32	511.58	< <b>0.001</b>
Stigma height	3.35 $\pm$ 0.55	5.49 $\pm$ 0.49	371.36	< <b>0.001</b>
<i>P. coriacea</i>				
Corolla lenght	12.98 $\pm$ 1.58	12.38 $\pm$ 0.89	1.14	0.30
Anther lenght	2.83 $\pm$ 0.35	2.63 $\pm$ 0.20	3.20	0.11
Stigma lenght	2.75 $\pm$ 0.66	1.31 $\pm$ 0.31	45.51	< <b>0.001</b>
Style lenght	6.42 $\pm$ 1.23	9.27 $\pm$ 1.25	30.18	< <b>0.001</b>
Anther height	13.02 $\pm$ 1.87	10.38 $\pm$ 0.81	19.11	<b>0.001</b>
Stigma height	9.18 $\pm$ 1.44	10.58 $\pm$ 1.11	6.88	<b>0.02</b>
<i>P. marcgravii</i>				
Corolla lenght	24.05 $\pm$ 1.63	19.24 $\pm$ 0.98	98.84	< <b>0.001</b>
Anther lenght	4.16 $\pm$ 0.31	3.62 $\pm$ 0.18	41.51	< <b>0.001</b>
Stigma lenght	5.72 $\pm$ 1.11	0.78 $\pm$ 0.17	381.55	< <b>0.001</b>
Style lenght	8.15 $\pm$ 1.14	16.11 $\pm$ 0.91	430.81	< <b>0.001</b>
Anther height	18.76 $\pm$ 1.60	14.74 $\pm$ 0.88	85.77	< <b>0.001</b>
Stigma height	13.88 $\pm$ 1.08	16.89 $\pm$ 0.86	68.51	< <b>0.001</b>
<i>P. rigida</i>				
Corolla lenght	16.15 $\pm$ 1.46	16.85 $\pm$ 1.45	1.45	0.24
Anther lenght	3.59 $\pm$ 0.36	3.19 $\pm$ 0.34	7.38	<b>0.017</b>
Stigma lenght	4.10 $\pm$ 1.03	0.93 $\pm$ 0.18	111.81	< <b>0.001</b>
Style lenght	6.94 $\pm$ 1.07	15.63 $\pm$ 1.73	211.19	< <b>0.001</b>
Anther height	18.42 $\pm$ 1.43	13.87 $\pm$ 1.16	65.97	< <b>0.001</b>
Stigma height	11.05 $\pm$ 1.38	16.66 $\pm$ 1.71	72.64	< <b>0.001</b>

**Table S3.** Results from model selection for intra-individual imprecision related to the height of reproductive whorls. Models are ranked according to their fit based on AICcw (likelihood weights) and AICc.  $\Delta AICc \leq 2.0$  are expressed in bold.

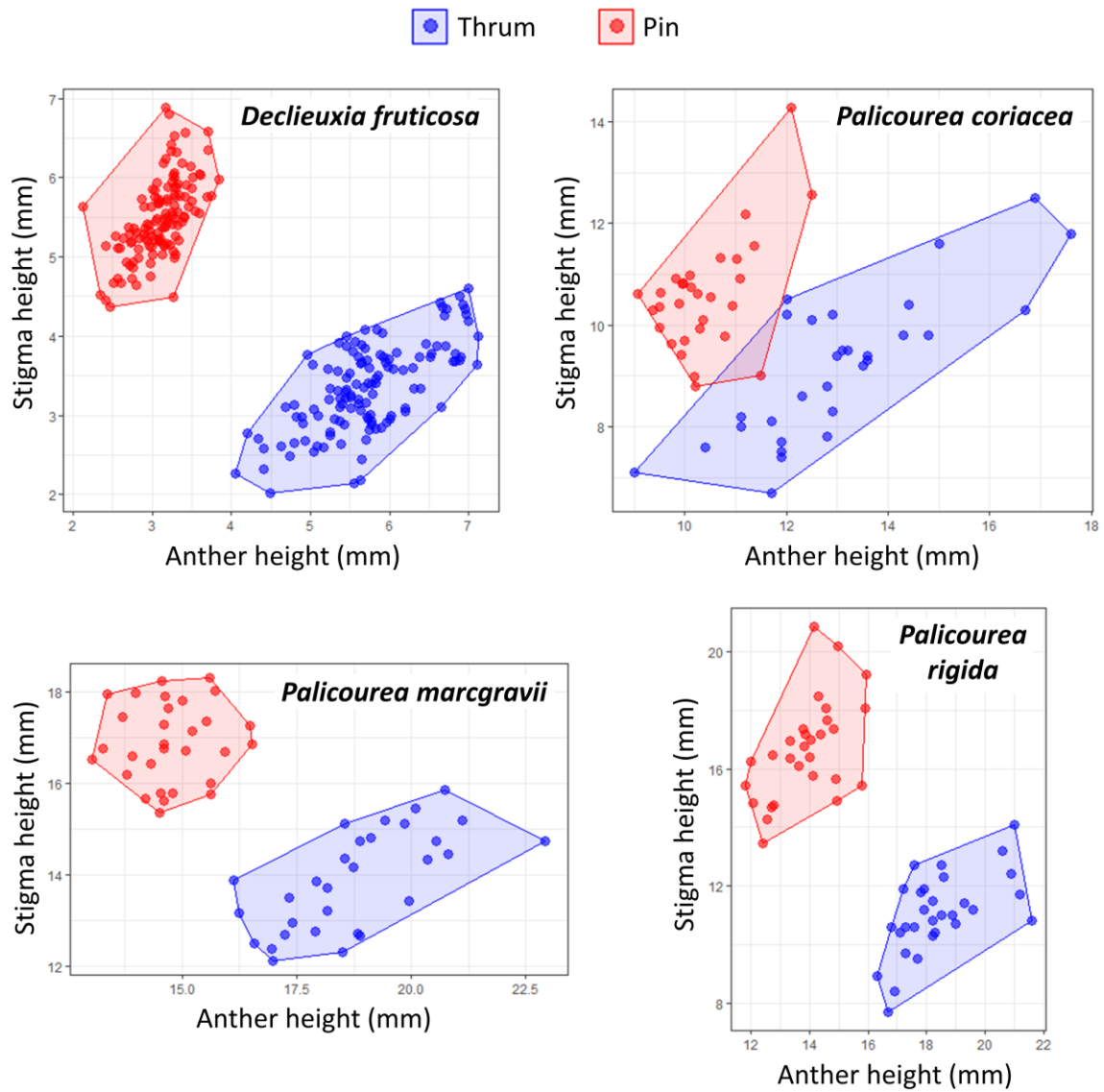
Model	Weight	AICc	$\Delta AICc$
Morph+Organ type	0.24	555.43	<b>0.00</b>
Organ type	0.20	555.74	<b>0.31</b>
Morph	0.13	556.56	<b>1.13</b>
Null model	0.11	556.88	<b>1.45</b>
Morph+Sexual organ+Organ type	0.05	558.43	3.00
Sexual organ+Organ type	0.05	558.72	3.29
Corolla+Species+Organ type	0.03	559.49	4.06
Morph+Sexual organ	0.03	559.77	4.34
Morph+Species+Organ type	0.03	559.83	4.40
Sexual organ	0.02	560.08	4.65
Species+Organ type	0.02	560.26	4.83
Corolla+Species	0.02	560.57	5.13
Morph+Species	0.02	560.91	5.48
Species	0.01	561.35	5.92
Corolla+Sexual organ+Species+Organ type	0.01	562.54	7.11
Corolla+Morph+Species+Organ type	0.01	562.76	7.33
Morph+Sexual organ+Species+Organ type	0.01	562.88	7.45
Sexual organ+Species+Organ type	0.00	563.29	7.86
Corolla+Morph+Species	0.00	563.82	8.39
Corolla+Sexual organ+Species	0.00	563.83	8.40
Morph+Sexual organ+Species	0.00	564.17	8.74
Sexual organ+Species	0.00	564.60	9.17
Corolla+Organ type	0.00	565.34	9.91
Corolla+Morph+Organ type	0.00	565.42	9.99
Full model	0.00	565.82	10.39
Corolla	0.00	566.47	11.04
Corolla+Morph	0.00	566.53	11.10
Corolla+Morph+Sexual organ+Species	0.00	567.10	11.67
Corolla+Sexual organ+Organ type	0.00	568.34	12.91
Corolla+Morph+Sexual organ+Organ type	0.00	568.44	13.01
Corolla+Sexual organ	0.00	569.69	14.26
Corolla+Morph+Sexual organ	0.00	569.76	14.33

**Table S4.** Loglikelihood and p-values (in decreasing order of significance) of pairwise comparisons on node bifurcations. Numbers of pairwise comparisons are available in Fig. 1. Significant p-values are expressed in bold.

Pairwise comparison	Loglikelihood	p-value
1	-274.5986	< <b>0.001</b>
2	-268.07	0.39
3	-267.71	0.94

**Table S5.** Results from model selection for intra-individual imprecision related to the length of reproductive whorls. Models are ranked according to their fit based on AICcw (likelihood weights) and AICc.  $\Delta AICc \leq 2.0$  are expressed in bold.

Model	Weight	AICc	$\Delta AICc$
Sexual organ	0.45	581.54	<b>0.00</b>
Sexual organ+Species	0.11	584.35	2.82
Morph+Sexual organ	0.10	584.52	2.98
Sexual organ+Organ type	0.10	584.57	3.03
Corolla+Sexual organ	0.10	584.57	3.04
Corolla+Morph+Sexual organ	0.04	586.52	4.98
Morph+Sexual organ+Species	0.03	587.03	5.50
Sexual organ+Species+Organ type	0.02	587.51	5.97
Morph+Sexual organ+Organ type	0.02	587.55	6.02
Corolla+Sexual organ+Organ type	0.02	587.68	6.15
Corolla+Morph+Sexual organ+Organ type	0.01	589.62	8.09
Morph+Sexual organ+Species+Organ type	0.01	590.19	8.65
Corolla+Sexual organ+Species	0.00	591.40	9.87
Corolla+Morph+Sexual organ+Species	0.00	593.33	11.79
Corolla+Sexual organ+Species+Organ type	0.00	594.58	13.04
Full model	0.00	596.50	14.97
Null model	0.00	639.82	58.28
Morph	0.00	642.75	61.22
Organ type	0.00	643.38	61.84
Species	0.00	644.25	62.71
Corolla	0.00	644.31	62.77
Morph+Organ type	0.00	646.32	64.78
Corolla+Morph	0.00	646.40	64.87
Morph+Species	0.00	646.96	65.42
Species+Organ type	0.00	647.92	66.38
Corolla+Organ type	0.00	647.93	66.40
Corolla+Morph+Organ type	0.00	650.04	68.50
Morph+Species+Organ type	0.00	650.63	69.10
Corolla+Species	0.00	651.03	69.49
Corolla+Morph+Species	0.00	653.07	71.54
Corolla+Species+Organ type	0.00	654.72	73.18
Corolla+Morph+Species+Organ type	0.00	656.77	75.23



**Fig S1.** Heights of stigmas and anthers (in mm) according to floral morph for the four studied species. Polygons indicate the area occupied by each morph by means of the connection of the most extreme points. Scales of axes have the same aspect ratio.

### Considerações Finais

Esse é o primeiro estudo que a inacurácia total e decomposta são testadas para um grande número de populações, apontando padrões e tendências em plantas distílicas de Rubiaceae com base em uma ampla amostragem de populações. Uma correlação positiva da inacurácia entre órgãos reprodutivos e viéses maladaptativos de ambos os morfos foi encontrada. O Viés maladaptativo dos órgãos baixos e o estigma do morfo brevistilo foram apontados como os principais contribuintes da inacurácia total. Também, mostramos que tamanho da corola foi o único fator que determina a inacurácia dos órgãos reprodutivos nos morfos.

O estudo em variação floral intra-individual tem grande relevância, visto que a variação dentro do indivíduo é um componente importante da seleção para a variação populacional. Nossos resultados apontam tendências importantes, mostrando que a imprecisão intra-individual pode variar de maneira diferente no nível da flor. Isso pode estar relacionado à diferente efetividade de cada órgão sexual em cada morfo floral e as diferentes pressões seletivas condicionam tal imprecisão. Nosso estudo inaugura uma nova abordagem no estudo da heterostilia, e mais pesquisas são necessárias para corroborar nossos resultados e descobrir outros padrões.

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