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Pós-graduação
Botânica
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GEOGRAPHICAL PHENOTYPIC VARIATION AND REPRODUCTIVE SYSTEM OF A DISTYLOUS RUBIACEAE IN THE BRAZILIAN CERRADO

MARIA JÚLIA WALDEMARIN DORIA

Dissertação apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Mestre no Programa de Pós-Graduação em Ciências Biológicas Botânica, Área de concentração Morfologia e Diversidade Vegetal.

BOTUCATU – SP
2020

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FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSEMEIRE APARECIDA VICENTE-CRB 8/5651

Doria, Maria Júlia Waldemarin.
Geographical phenotypic variation and reproductive system of a distylous Rubiaceae in the brazilian cerrado / Maria Júlia Waldemarin Doria. - Botucatu, 2020

Dissertação (mestrado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Felipe Wanderley de Amorim
Coorientador: Camila Kissmann
Capes: 20303025

1. Rubiaceae. 2. Polinização. 3. Plantas - Reprodução.
4. Plantas dos cerrados.

Palavras-chave: Biologia da polinização; Distilia; Ecologia.

Dedico minha dissertação primeiramente a minha mãe, Lourdes, que por sua força, determinação e suporte me possibilitou essa enorme conquista. Ao meu pai, Antônio, por todo apoio, carinho e zelo que me permitiram trilhar meu caminho.

A toda minha família por todo o apoio.

A minha companheira Karla Polo, por todo o amor, companheirismo e apoio incondicional nessa jornada e em todas as outras que compartilharemos.

A minha melhor amiga Danielle Yamauchi, que me ensinou muito sobre a vida e sobre amizade. Obrigada por sempre estar ao meu lado.

Dedico a todos meus amigos, especialmente a Gabriel Fellipe, Fábio Henrique, Bruno Salata, Francislene Martins e todos os “Pererecos”.

A meu orientador Felipe Amorim, por acreditar em mim mesmo quando eu não consigo e por todo carinho e paciência.

A minha terapeuta, Karina Pani, pelo excelente auxilio profissional durante as maiores tempestades.

A todos que de alguma forma acrescentaram em minha vida e minha formação acadêmica.

AGRADECIMENTOS

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) - Brasil pelo apoio no presente trabalho.

Ao meu orientador Felipe W. Amorim por todo o acolhimento e orientação que possibilitaram a existência desse trabalho.

Aos colegas de trabalho Pedro A. Barbosa, Pablo Oliveira, Natalia Kano, Eduardo Dal Farra, Larissa Silva, Isabella Castro, Caio S. Ballarin, Leandro Hachuy Filho, Priscila S.Veiga, Diego Polizello e Murilo M. Giffu pela colaboração científica e técnica na elaboração e condução do trabalho.

A Amabílio Camargo e toda equipe da Expedição Veadeiros da Embrapa Cerrados, e a Hélder Consolaro e alunos por toda enorme contribuição na coleta de dados.

A Vinícius Lourenço Garcia de Brito pela grande colaboração científica, auxílio nas análises das cores e discussões.

E por fim a minha universidade casa, a Universidade Estadual Paulista “Júlio de Mesquita Filho” - UNESP e ao Instituto de Biociências de Botucatu – IBB sem os quais nenhuma etapa desse trabalho ou de minha formação pessoal ou profissional teria sido concretizada.

CONTENTS

ABSTRACT

Palicourea rigida is a Rubiaceae species widely distributed across the Brazilian Cerrado. The species presents distyly, a type of floral dimorphism comprised of two floral morphs that are associated with a genetic heteromorphic self-incompatibility that enables the reproduction only after pollen flow between the distinct floral morphs. *Palicourea rigida* is typically pollinated by hummingbirds and has flowers varying in the color spectrum between orange and red. The species, however, presents a phenotypic variation across its distribution, and this variation includes floral size, color, and life form. In the southernmost distribution of the Cerrado vegetation, *P. rigida* presents a phenotypic variation with a markedly different life form, flower size, and color. In this sense, considering the phenotypic variation in *Palicourea rigida* populations both in life form and floral traits, as well as its dependence on pollinators for sexual reproduction, in this study we described the changes in the floral morphology of different populations of *P. rigida* and tested if they are followed by alterations of the basal features of distyly, *i.e.*, breakdown of the heteromorphic self-incompatibility system, reciprocal hercogamy and isoplethy. Additionally, due to the change in flower color and size, we discussed the hypothesis of a potential pollinator shift from hummingbirds (ornithophily) to bees (melitophilly) in the dwarf with yellow flowed population as a possible outcome of pollinator-mediated selection. For this, we perform controlled pollination experiments in the southernmost dwarf population, analyzed the floral morphology, and evaluate floral color and pollinator perception. Our results showed that the basic features of distyly were maintained in the southernmost dwarf population, and flowers were significantly smaller, with a larger stigmatic lobe in both floral morphs. Our results reinforce the role of inaccuracy in legitimate pollen deposition, where the higher inaccuracy in high organs seems to be responsible for lower pollen deposition at that level. Besides that, we found a pollinator shift from hummingbirds to bees in the population, even with no cognitive difference for bees when exposed to *P. rigida* yellow and reddish flowers.

INTRODUCTION

Environmental heterogeneity is an important factor affecting the phenotype of species with broad distribution, because of the influence it plays on the local microevolutionary processes (Liao *et al*. 2016). Phenotypic variations may be the result of either the population response to local selective pressures under which they are subjected, *i.e.*, local adaptation, or the ability of a given genotype to express different phenotype according to the local environmental conditions, which allows acclimation, *i.e.*, phenotypic plasticity (Liao *et al*. 2016, de Villemereuil *et al*. 2018). Environmental conditions play a key role in the phenotypic plasticity and in local adaptation of species, which may affect traits related to survival and reproduction of species, such as plant phenology, floral morphology, variation and specificity of pollinators systems, as well as growth habits in plants (Ollerton *et al*. 2006; Etterson *et al*. 2016; Petry *et al*. 2016). Therefore, species with a broad distribution are subject to different selective pressures throughout their geographical range and may have different responses to the environmental conditions (Liao *et al*. 2016).

The Cerrado (the Neotropical savanna of Brazil) is one of the largest biomes in South America and is composed of a complex of plant formations. Like other savanna biomes worldwide, such as those in Africa and Australia, the Cerrado is also affected by environmental conditions, such as climate, soil, and fire events as well as by several anthropic disturbances (Ribeiro & Walter 2008). The effect of these factors can influence the structure, spatial distribution, and floristic composition of these habitats (Oliveira 2000; Ribeiro & Walter 2008). Recent studies on the Brazilian Cerrado and in species under different environmental pressures have shown contrasting forms of variations and anomalies in the common features of heterostylous (distylous or tristylous species) plant populations, such as the variations in the level of reciprocal hercogamy, self-compatibility, anisoplethy and in the frequency of monomorphic and homostylous populations in typically distylous species (Baker 1955; Bawa & Beach 1983; Sobrevila *et al*. 1983; Richards & Koptur 1993; Pailler *et al*. 1998; Faivre & McDade 2001; Coelho & Barbosa 2003; Consolaro *et al*. 2005; Toledo *et al*. 2007; Consolaro *et al*. 2009; Silva & Segura 2015).

Palicourea rigida Kunth. (Rubiaceae) is a distylous species, with a broad distribution in the Brazilian Cerrado. In the core distribution of *P. rigida* in the Cerrado of central Brazil, the species is a treelet (with a phanerophytic growth form) of about 1-1.5 meters in height (Fig. 1 A). Flower color varies from orange to reddish and the species is typically pollinated by hummingbirds (Fig. 2 A, but see Machado *et al*. 2010; Justino *et al*. 2011; Maruyama 2016). However, in the southernmost distribution of the Cerrado vegetation, *P. rigida* presents a notoriously phenotypic variation with a markedly reduced size (Fig. 1 C). In this region, growth form changes from phanerophyte to hemicryptophyte, *i.e.*, the species present underground stems with shoot apical meristems born at soil level, and plants are dwarfs. Flowers and inflorescences are also notoriously smaller and the color is quite distinct, being yellowish in tone (Fig. 2 B), a color spectrum which is associated with melittophily (Chittka & Waser 1997; Lunau *et al*. 1996; Dyer *et al*. 2016; Papiorek *et al*. 2016). Also, *P. rigida* also presents another extreme of the phenotype variation in which plants are trees reaching more than 2 meters in height, and flowers are reddish (Fig. 2 C).

Heterostyly, which is observed in *P. rigida*, is a floral polymorphism genetically determined that are present in at least 28 families of angiosperms and is a basal feature in the genus *Palicourea* (Vuilleumier 1967; Taylor 1997; Barrett & Shore 2008). Heterostylous species populations are comprised of plants with different floral morphs, which can be either distylous (two floral morphs) or tristylous (three floral morphs). Distylous species produce pin (long-style) and thrum (short-style) flowers, and the populations, in general, present a morph (*i.e.* pin : thrum) ratio of 1:1 due to the genetic control. Pin flowers have the stigma at a higher level than the anthers, and thrum flowers have the anthers at a higher level than the stigma. This conformation allows the level of low and high organs to be reciprocal between floral morphs (reciprocal hercogamy). Tristylous species, in turn, produce short-, mid- and long-style morphs that differ reciprocally in the heights at which stigmas and anthers are positioned within flowers (Lloyd &Webb 1992; Barrett 2013). This morphology is associated with a heteromorphic self-incompatibility system, which only enables fertilization in pollination crosses between plants of different morphs. As a result, heterostyly is expected to promote disassortative pollen transfer between different morphs optimizing cross-pollinations (Barrett 2019). Thus, due to the heteromorphic self-incompatibility system, isoplethy, and the reciprocal hercogamy, the reproduction of heterostylous species relies on legitimate cross-pollinations (*i.e.*, intermorph cross-pollinations) mediated by biotic pollinators (Ganders 1979; Barrett 2013).

Although heterostyly is followed by several basal features, variations within heterostylous populations are frequent and may arise as a consequence of pollinator availability and behavior, genetic mutations or recombination, environmental pressure or disturbances (Sobrevila *et al*. 1983; Washitani *et al*. 1994; Consolaro *et al*. 2011), as well as reduced population size (Kéry *et al*. 2000), clonal reproduction (Barrett 1977), geographic isolation or habitat fragmentation (Van Rossum *et al*. 2006). These factors may lead to the breakdown of the heteromorphic self-incompatibility system and the loss of heterostyly in distinct populations of the same species, often leading to the evolution of homostylous populations, or even to an alternative outcrossing system (Baker 1955; Charlesworth & Charlesworth 1979, Barrett and Shore 1987; Coelho & Barbosa 2003; Consolaro *et al*. 2005; Toledo *et al*. 2007; Barrett &

Shore 2008; Consolaro *et al*. 2009, Consolaro *et al*. 2011, Bramow *et al.* 2013; Rodrigues *et al.* 2013; Costa *et al*. 2017; Barrett 2019). Under these circumstances, heterostyly may represent a reproductive constraint for some plant populations, due to the absence of mates or actual pollinators. As a result, many species may suffer from a limitation in the pollination services (Sobrevila *et al*. 1983, Washitani *et al*. 1994; Bramow 2013).

Although abiotic conditions may affect floral morphology (Etterson *et al*. 2016), pollinator-mediated selection can also play a crucial role in the evolution of floral traits (Stebbins 1970; Agrawal 2001; Kearns *et al*. 1998; Klein *et al*. 2007; Aguilar *et al*. 2006; Menz *et al*. 2011; Schiestl & Johnson, 2013). Stebbins (1970) postulates the principle of the most effective pollinator, in which an animal group with a high frequency of visits and effectiveness as pollinators, can mediate the selection of floral traits that will increase plant fitness in a given location where the species has evolved. In this sense, some floral attributes subjected to phenotypic variation can be selected throughout the plant distribution due to a higher attractiveness of a given pollinator group, or a higher plant-pollinator morphological matching, which increases plant fitness, and can be fixed in the population (Anderson & Johnson 2008; Pauw *et al*. 2009). Whereas bees are excluded from flowers on the red spectrum since they lack visual conspicuity due to the characteristics of their visual system (Chittka & Waser 1997; Lunau *et al*. 1996; Dyer *et al*. 2016; Papiorek *et al*. 2016), and hummingbirds are associated with red flowers (Faegri & van der Pijl 1979; Lunau *et al*. 2011; Bergamo *et al*. 2016; Camargo *et al*. 2018), flowers in other color spectrum are more conspicuous to bees, improving bee pollination.

In this sense, considering the phenotypic variation in *Palicourea rigida* populations either in life form and floral morphology, as well as its dependence on pollinators for sexual reproduction, in this study we described the changes in the floral morphology of different populations of *P. rigida* and tested if they are followed by alterations of the basal features of distyly, *i.e.*, breakdown of the heteromorphic self-incompatibility system, reciprocal hercogamy and isoplethy. Additionally, due to the change in flower color and size, we discussed the hypothesis of a potential pollinator shift from hummingbirds (ornithophily) to bees (melitophilly) in the dwarf population presenting yellow flowers as a possible outcome of pollinator-mediated selection. This population is located at the southernmost distribution of *Palicourea rigida* in the Brazilian Cerrado, which presents consistent morphological alterations from the typical plant populations in the core region of the distribution of the species. To better understand the southernmost population, we also compare it with two populations, one presenting the usual phenotypic features described for the species, and a second representing another extreme of the phenotype distribution of *Palicourea rigida*, where the plants are trees with long-tubed reddish flowers.

Figure 1. Phenotypic variation of *Palicourea rigida* populations across its distribution in the Brazilian Cerrado: (A) Clube Caça e Pesca de Uberlândia, Uberlândia - MG; (B) Conservation Unit of Botucatu, Botucatu - SP, at the southernmost limit of the species distribution; (C) Chapada dos Veadeiros National Park, Alto Paraíso de Goiás - GO.

Figure 2. Phenotypic variation of *Palicourea rigida* flowers and inflorescences across its distribution in different sites of the Brazilian Cerrado: (A) Chapada dos Veadeiros National Park, Alto Paraíso de Goiás - GO; (B) Clube Caça e Pesca de Uberlândia, Uberlândia - MG; and (C) Conservation Unit of Botucatu, Botucatu - SP, at the southernmost limit of the species distribution.

MATERIAL AND METHODS

SPECIES CHARACTERIZATION AND STUDY SITES **-** *Palicourea rigida* is a Rubiaceae species distributed in the Brazilian Cerrado and disjunct areas of amazon savannah, found in 37% of the Cerrado areas until now (Taylor, 1997, Ratter *et al*. 2003). Flowers producing nectar are receptive for only 1 day and are grouped into thyrsoid inflorescence with zygomorphic flowers and the plant has characteristic large coriaceous leaves (Robbrecht 1988, Machado 2010). The blooming season occurs during the rainy season, varying throughout the distribution range of the species (Robbrecht 1988; Machado *et al*. 2010).

This work was conducted in three study sites between the years of 2017 and 2019. The first site, where plants are dwarfs, is a Cerrado area in the Conservation Unit of Botucatu (BTU), municipality of Botucatu, São Paulo state, Brazil (22°56'S, 48°27'O). The climate is warm-temperate with two distinct seasons, a cold dry winter (from March to September), and a hot wet summer (from October to April), Cwa according to the Köppen classification, and with an annual rainfall of 1336 mm (Köppen 1948; Alvares *et al*. 2013). The main study site is an integral protection conservation unit that comprehends a Cerrado vegetation characterized by shrub-tree vegetation, with sparsely distributed trees and treelets, and scattered shrubs and sub-shrubs (Ribeiro e Walter, 1998). The total area comprehends 33.8 hectares, located at an altitude of 860 meters a.s.l.. The second site, where the population presents the typical morphology of the species, is located at Serra de Caldas Novas State Park (Caldas), Caldas Novas, Goiás State, Brazil (17º43'S, 48º40'O) and has a total area of 12,500 hectares. Finally, the third site is located at Chapada dos Veadeiros National Park (CV), Alto Paraiso de Goiás, Goiás State, Brazil (47º30'S, 14º02'O) with an area of 240.611 ha, where *P. rigida* plants are trees. Serra de Caldas Novas State Park and Chapada dos Veadeiros National Park comprehend a Cerrado *strictu sensu*, with seasonal tropical climate with summer rains, Aw. according to Köppen classification, and with annual rainfall averaging about 1500 mm (Alvares *et al*. 2013).

FLORAL MORPHOMETRIC AND RECIPROCAL HERKOGAMY- To test the reciprocal hercogamy and also to evaluate the floral phenotypic variation among sites, we measured flowers from the three studied populations. For this, we collected 4-5 flowers per plant, totaling 1127 flowers (fixed in 70% alcohol solution) distributed in 238 plants sampled in three populations: 61 plants in Caldas, 100 in Chapada, and 77 in BTU. In these flowers we measured the length of the following structures using a digital caliper: 1) corolla tube length; 2) diameter of the corolla; 3) anther length; 4) anther height; 5) stigma length; 6) stigma height; 7) distance between stigma and anther heights. To avoid overestimation of the anther height, we measured from the bottom of the corolla until 2/3 of the anther length, and to avoid the stigma height overestimation we measured from the basis of the corolla until the middle of the stigma length. We used an analysis of variance (ANOVA) with Tukey's posthoc test for multiple comparisons among pairs of means to evaluate the phenotypic variances of floral verticiles among populations considering their floral morphs (Zuur *et al*. 2007).

To assess the hercogamy in the populations of *P. rigida*, we use the reciprocity index of Armbruster (2017), which are the most updated analyses for heterostyly measure. The index uses the measure of heights of the reproductive organs, anther, and stigma, to enable the estimation of the ability to achieve an inter-morph cross-pollinations and the relation of this ability to reproductive fitness (Armbruster *et al*. 2017).

The Armbruster's index assesses the adaptative inaccuracy (I), which measures the degree of maladaptation of a floral morph on a fitness scale that regards both the mean and variance of the phenotypic values of the morph (Armbruster *et al*. 2004; Hansen *et al*. 2006, Armbruster *et al*. 2017). We calculate the adaptive inaccuracy of low (pin anthers and thrum stigma) and high organs (thrum anthers and pin stigma), and the total inaccuracy (sum of both inaccuracies) to better evaluate the floral differences between the populations and floral morphs and the maladaptive bias, which is a result of the mismatch of the means of the anthers and stigma (the square of the departure of the trait mean from the optimum). Besides, we calculated the total mean inaccuracy (Mean^2) standardized by the squared mean of all anther and stigma heights in each population, which will be most used and will facilitate comparisons across different populations. In this test, the closer to zero the value obtained, the lower the inaccuracy, and the greater the reciprocity of sexual organs (Armbruster *et al*. 2017).

MATING SYSTEM AND ISOPLETY- To evaluate the reproductive system of the population of *P. rigida* in the southernmost distribution (BTU), we covered inflorescences with nylon mesh bags to avoid interference of floral visitors, and conducted the following controlled pollination experiments using 4 to 5 flowers per plant: 1) hand self-pollination, where we tested for self-incompatibility (n = 87 flowers from 19 individuals); 2) intra-morph cross-pollination, *i.e.,* crosses between distinct individuals of the same flower morph (n = 98 flowers in 19 plants); 3) inter-morph cross-pollinations, *i.e.,* crosses between distinct individuals of different flower morphs, (n = 98 flowers in 19 plants); 4) autogamy, flowers without pollination covered in nylon mesh bags (n = 181 flowers from 37 individuals); 5) Control, flowers in natural conditions naturally exposed to the floral visitors (n = 9866 in 79 plants, 4582pin and 5184 thrum).

Cross pollinations were carried out in both directions, using pollen of thrum flowers (anthers at a higher level than stigma) to pollinate the pin ones and vice versa. After the experiments, the flowers were isolated again to analyze the fruit set, which was assessed 30-60 days after pollination treatments. Also, we used a two-sample *t*-test to assess whether the proportion of fruits formed in natural conditions (control) differed between the floral morphs. The index of self-incompatibility (ISI) was calculated as the ratio between the proportion of fruits formed after hand self-pollination and hand cross-pollination. The index indicates that species with values under 0.25 are self-incompatible, values between 0.25 and 0.75 indicate self-incompatibility with some level of self-compatibility (pseudocompatibility), and values above 0.75 indicate self-compatibility (Bullock 1985, Lloyd & Schoen 1992). We also used an index to measure the reproductive efficacy (IRE) of the population. This index was calculated by dividing the average number of fruits per flowers hand-self-pollinated by the same following Inter-morph cross-pollination (*sensu* Zapata & Arroyo 1978). Index values above 0.66 indicate high reproductive efficiency of species due to a pollen flow promoted by pollinators (Zapata & Arroyo 1978) and values similar to or less than 0.25 indicate pollen limitation (Sobrevilla & Arroyo 1982). The reproductive system of Caldas population was assessed by Machado *et al*. (2010) and Maruyama *et al*. (2016).

To test for isoplethy, we quantify the number of individuals of each morph (*i.e.*, pin or thrum) in all studied populations. The occurrence of isoplethy in the populations was assessed with a Chi-square goodness of fit test, where the populations were considered isopletic when they have equal proportions for each morph. All analyses were performed using R software (Version 4.0.2; R. Core Team, 2020)

FLORAL COLOR, BEE PERCEPTION AND POLLINATION BIOLOGY- To assess the differences in flower color between different populations, we modeled the color of flowers according to the bumblebee visual system using newly opened flowers from 8 different individuals in BTU and 14 from Caldas (n= 1 - 5 flowers per individual). From each flower, we measured the spectral reflectance of the corolla (tip and base) by using a Jazz spectometre (Ocean Optics S2000, Ocean Optics, Dunedin, FL) relative to a calibrated white DH 2000-CAL (Ocean Optics) and black (light absence) sources. We assumed a standard green background (Chittka and Kevan 2005), a daylight illumination (D65; Wyszecki and Stiles 1982) and the spectral sensitivity of *Bombus terrestris* as surrogate, since the plant species are often visited by the sister species *Bombus morio*. We used the color hexagon model (Chittka 1992; Lunau *et al*. 2011) to predict the color discrimination capacity of bees to the plant species. We established the discrimination threshold of 0.09 hexagon units based on previous behavioral experiments (Dyer *et al*. 2008).

To estimate the ability of bumblebees to discriminate flower colors, we use linear mixed-effect models analysis (LMMs). The dependent variables were the mean Euclidian distance in hexagon units between BTU and Caldas, measured from the corolla tip or base, in both populations, we use as fixed factors the paired Euclidian distances measured, and the values of x- and y-axis of the color hexagon as the response variable. Individual plants were included as a random factor. To perform the LMM, we used the *lme4* package version 1.1-21 (Bates *et al*. 2019) and the *lmer* function. To determine the significance of each fixed factor (morphs and populations) and their interaction, a type II analysis of variance was conducted using the *Anova* function in the *car* package version 3.0-2 (Fox *et al*. 2019). As a reference, bumblebees can distinguish correctly by 60% between colors with 0.09 hexagon units of perceptual distance (Dyer, 2006). From these color loci, we also calculated the green contrast using the same color hexagon model (Chittka *et al*. 1994; Lunau *et al*. 2011). The green contrast is measured as the distance between the target color locus and the central point of the color hexagon representing the locus of the standard green background (Chittka, 1992).

To determine the pollinators of the BTU population, we performed 31 hours of focal observations between 07:00h and 18:00h, being 12 hours in 2017 and 18 hours in 2019. Observations were made in sessions of 30 minutes per plant during the flowering period of the species and floral visitors were photographed for identification. The pollinators of Caldas and

Chapada were assessed by Silva 1995, Machado *et al*. (2010), and Maruyama *et al*. (2016), but we also have conducted about 15 hours of observations at the Chapada site to record the floral visitors and the actual pollinators.

All analyses were performed using R software (Version 4.0.2; R. Core Team, 2020)

RESULTS

FLORAL MORPHOMETRIC AND RECIPROCAL HERKOGAMY - Floral morphology differed among the three analyzed populations in several parameters, varying among morphs and populations compared (Table 1, Fig. 3). The BTU population has the smallest mean corolla length in both floral morphs, while Chapada has the highest, BTU is also different in corolla length with both populations and pin and thrum morphs (F=4.6314, $P < 0.05$). Considering corolla diameter, BTU has also the smallest diameter in both morphs, while Chapada has the largest, BTU also differs from Chapada in both morphs ($F=0.4736$, $P < 0.05$), while when compared with Caldas, the diameter is only different in thrum morph ($F=0.4736$, $P < 0.05$). Regarding reproductive structures (Fig. 4), BTU has the smallest stigma heights in pin and thrum flowers and is also different from all populations in both floral morphs ($F=1.3811$, $P < 0.05$). The anther height, BTU is only different in thrum morphs with both populations ($F=9.3463$, $P > 0.05$). Floral dimorphism remained in all studied populations, with all measured organs differing between thrum and pin morphs within populations, with exception of anther length in the BTU population (Table 1).

Considering the Inaccuracy index (Armbruster *et al*. 2017), the standardized inaccuracy (Mean^2) of the BTU population is the highest (6.766) and the Caldas have the lowest inaccuracy (6.370). When we detach low and high organs inaccuracy, Chapada presents a contrasting difference between their low and high inaccuracy (Table 2), whereas BTU and Caldas present a more paired index. The highest Inaccuracy proportion in low organs was found in Caldas and for high organs in the Chapada population. Chapada presented the lowest inaccuracy proportion of low organs and Caldas has the lowest inaccuracy for high organs. The maladaptive bias has shown that BTU presented the higher maladaptation, while Chapada presented the lowest.

MATING SYSTEM AND ISOPLETY - Pollination treatments showed that the heteromorphic self-incompatibility in BTU remained, with only fruit formation after inter-morph cross-pollination treatments (Table 3), and as a result, the ISI and the IRE in the population were equal to zero, showing complete self-incompatibility (Table 3). Population fruit set in natural conditions was 37.98%, but thrum flowers presented a higher fruit set (41.53%) than pin flowers (33.98%; $t = 2.5196$, $p = 0.01$).

However, the Chapada deviated from the 1:1 ratio between pin and thrum plants ($\chi^2 = 5.76$; P = 0.0164), where 38 are pin and 62 are thrum.

FLORAL COLOR, BEE PERCEPTION AND POLLINATION BIOLOGY - Considering the *Palicourea rigida* flower's color discrimination by the bees between the populations, both corolla's tip and base were fully visible to the bee visual system (Fig 6). Although, we have found difference in tip color comparing both populations (Fig 7; t = -3.2334, P < 0.05), and also within population, comparing tip with base of the same population (BTU t = -2.8249 and Caldas t = -8.4804, P < 0.05). We also found that more than 95% of the bee receptors exited were green receptors, considering both parts of the corolla analyzed.

Considering color contrast, BTU presents a very low contrast between tip and base (t = -1.5015, $P > 0.05$), and Caldas population presented a much higher contrast between both corolla's parts analyzed (t = 15.852, $P < 0.05$). When we compared the contrast within populations, the tip (t = -4.4471, $P < 0.05$) and base (t = -2.994, $P < 0.05$) also differed between each other.

Palicourea rigida in BTU were visited by bees, wasps, and butterflies, but bees were the main pollinators of this population. We recorded a total of 121 visits in 31 hours of observations, of which 79 were from bees of the genus *Bombus*, *Xylocopa*, and *Centris*, 22 visits were made by small-sized bees (Meliponini and Halictidae), 15 from *Epicharis cockerelli* and five by butterflies. Large-sized bees were the main pollinators of *P. rigida* in the BTU population, particularly, *Bombus morio* which was the most frequent flower visitor performing 57.5% of the visits (Fig. 5A). In Chapada, we recorded bees (*Bombus*, *Xylocopa*, *Centris*, and *Trigona*) and hummingbirds visiting the *P. rigida* flowers (Fig. 5B). The bees were observed foraging all day long, but due to the long floral tube, most of them acted only as nectar robbers and occasional pollinators. Hummingbirds, in turn, were quite frequent, performing about five visits per hour per plant, visiting many flowers in each foraging bout.

Table 1. Floral morphometrics of *Palicourea rigida* in Chapada dos Veadeiros National Park, Alto Paraíso de Goiás - GO (Chapada); Serra de Caldas Novas State Park, Caldas Novas - GO (Caldas); and at Conservation Unit of Botucatu, Botucatu - SP (BTU) (mean ± standard deviation). N = number of individuals analyzed. Means followed by different letters indicate a statistical difference at P < 0.05 between morphs in each population. P values of t-test analysis for comparison among floral morphs.

Measured items (mm)	**Chapada**		**Caldas**		**BTU**	
	thrum N=62	pin N=38	thrum N=30	pin N=31	thrum N=41	pin N=36
Corolla length	13.86±1.31a	12.35±1.03b	13.45±0.98a	11.85±1.06b	11.60±1.18a	11.04±1.02b
Corolla diameter	4.15±0.26a	3.78±0.18b	3.83±0.22a	3.54±0.30b	3.71±0.36a	3.30±0.35b
Anther length	3.48±0.11a	3.28±0.11b	3.80±0.29a	3.38±0.29b	3.07±0.31a	2.96±0.28a
Anther height	15.42±2.15a	8.70±0.89b	14.79±1.20a	10.15±0.620b	14.31±2.25a	9.47±3.36b
Stigmatic lobe length	4.20±1.01a	0.96±0.05b	4.01±0.54a	0.72±0.22b	4.54±0.83a	3.31±0.45b
Stigma height	8.85±1.04a	15.31±2.28b	8.87±0.87a	14.79±1.05b	7.68±0.66a	13.69±1.59b

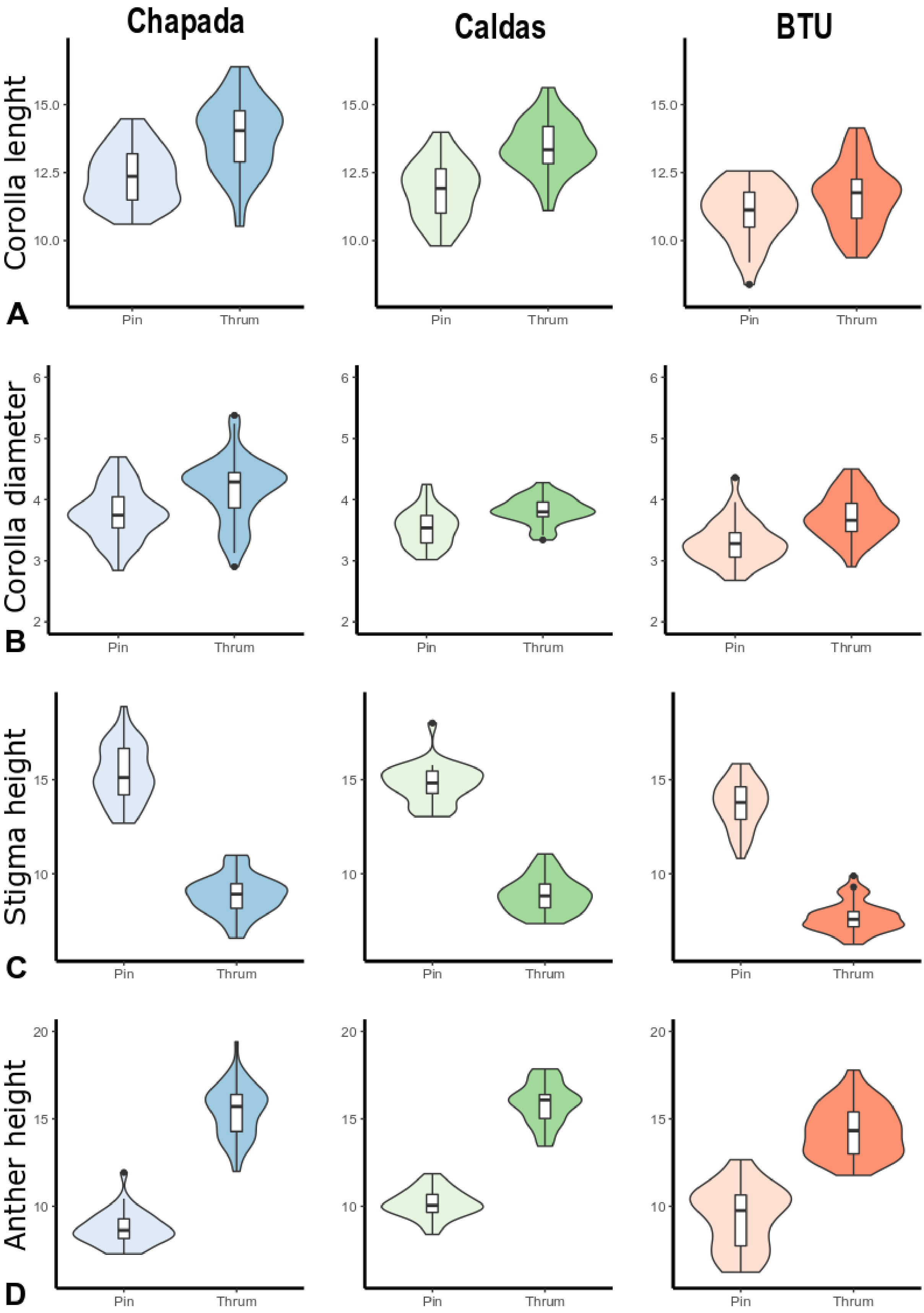

Figure 3. Variation of *Palicourea rigida* floral morphology across its distribution in the Brazilian *Cerrado* at Chapada dos Veadeiros National Park, Alto Paraíso de Goiás – GO (A); Serra de Caldas Novas State Park, Caldas Novas - GO (B); and Conservation Unit of Botucatu, Botucatu – SP (C). The measurements are in millimeters. Colors represent the density of the distribution of each population.

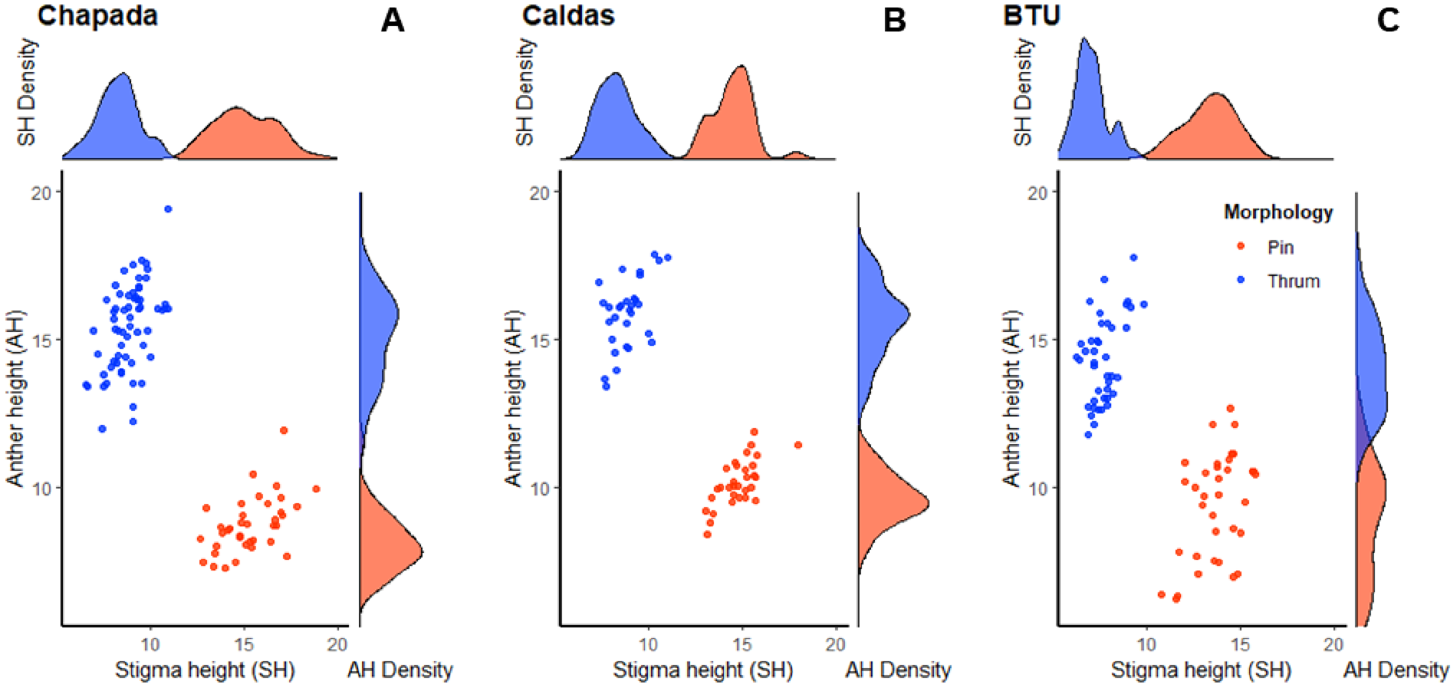

Figure 4. Distribution of anther and stigma heights across its distribution in the Brazilian Cerrado in different population of *Palicourea rigida* across its distribution in the Brazilian Cerrado: A) Chapada dos Veadeiros National Park, Alto Paraíso de Goiás - GO (Chapada), B) Serra de Caldas Novas State Park, Caldas Novas - GO (Caldas) and at C) Conservation Unit of Botucatu, Botucatu -SP (BTU). Height and length are in millimeters.

Table 2. Summary statistics of heterostyly reciprocity indexes calculated of *Palicourea rigida* populations in Chapada dos Veadeiros National Park, Alto Paraiso de Goiás - GO (Chapada), Serra de Caldas Novas State Park, Caldas Novas - GO (Caldas) and at Conservation Unit of Botucatu, Botucatu – SP (BTU).

Populations	**Inaccuracy Low**	**Inaccuracy High**	**Total inaccuracy**	**Mean^2**	**Maladaptive bias**
Chapada	1.950 (30.46%)	4.452 (69.54%)	6.402 (100%)	4.392	0.032
Caldas	3.138 (49.26%)	3.232 (50.74%)	6.370 (100%)	4.158	2.4305
BTU	3.138 (46.38%)	3.628 (53.62%)	6.766 (100%)	4.381	2.8265

Table 3. Fruit set of *Palicourea rigida* after experimental pollination treatments in a Cerrado area in Conservation Unit of Botucatu, Botucatu - SP. T: thrum; P: pin. ( ) = number of formed fruits.

Pollination treatments		Flowers	Fruits set (%)
Hand-self-pollination	T	48	0 (0)
	P	39	0 (0)
Inter-morph cross-pollination	T	48	4 (8.3)
	P	50	6 (12)
Intra-morph cross-pollination	T	52	0 (0)
	P	46	0 (0)
Autogamy	T	70	0 (0)
	P	111	0 (0)
Control	T	5184	2153 (41.5)
	P	4582	1556 (33.9)

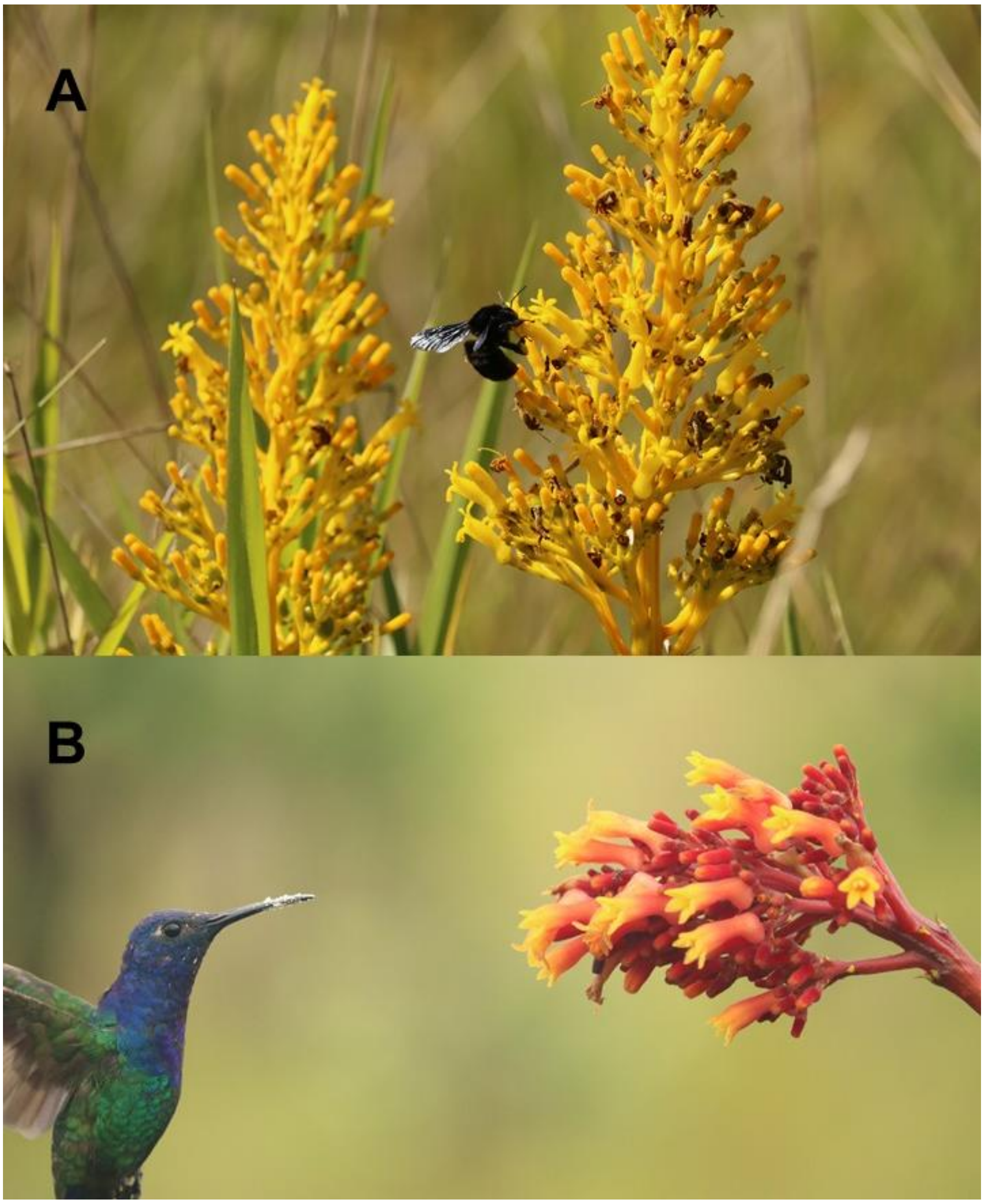

Figure 5. Pollinators visiting *Palicourea rigida* flowers **A-** *Bombus morio* visiting pin flowers at Conservation Unit of Botucatu, Botucatu - SP. **B-** *Eupetomena macroura* visiting pin flowers at Chapada dos Veadeiros National Park, Alto Paraíso de Goiás - GO

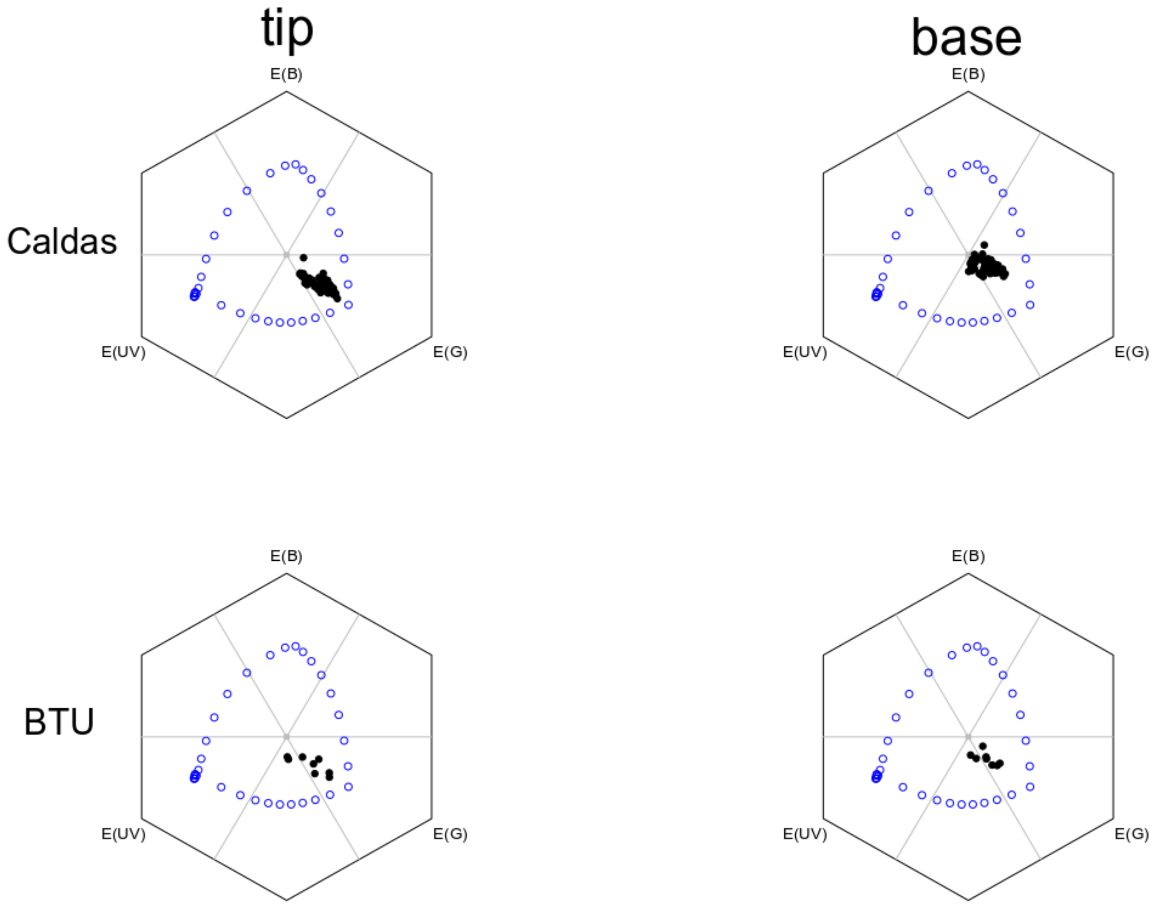

Figure 6. Color hexagon representation for bee vision of *Palicourea rigida* flowers, tip or base in two populations, Caldas and Botucatu. The grey central point in the visual spaces represents the achromatic center, and the vertices represent each bee photoreceptor: ultraviolet E(UV), blue E(B), and green E(G), the grey line represents the maximum sensitivity of each photoreceptor to a monochromatic light and the blue dots represents the bee visual detection. The excitation of ultraviolet, blue, and green photoreceptor types is indicated with respective black points in the hexagon.

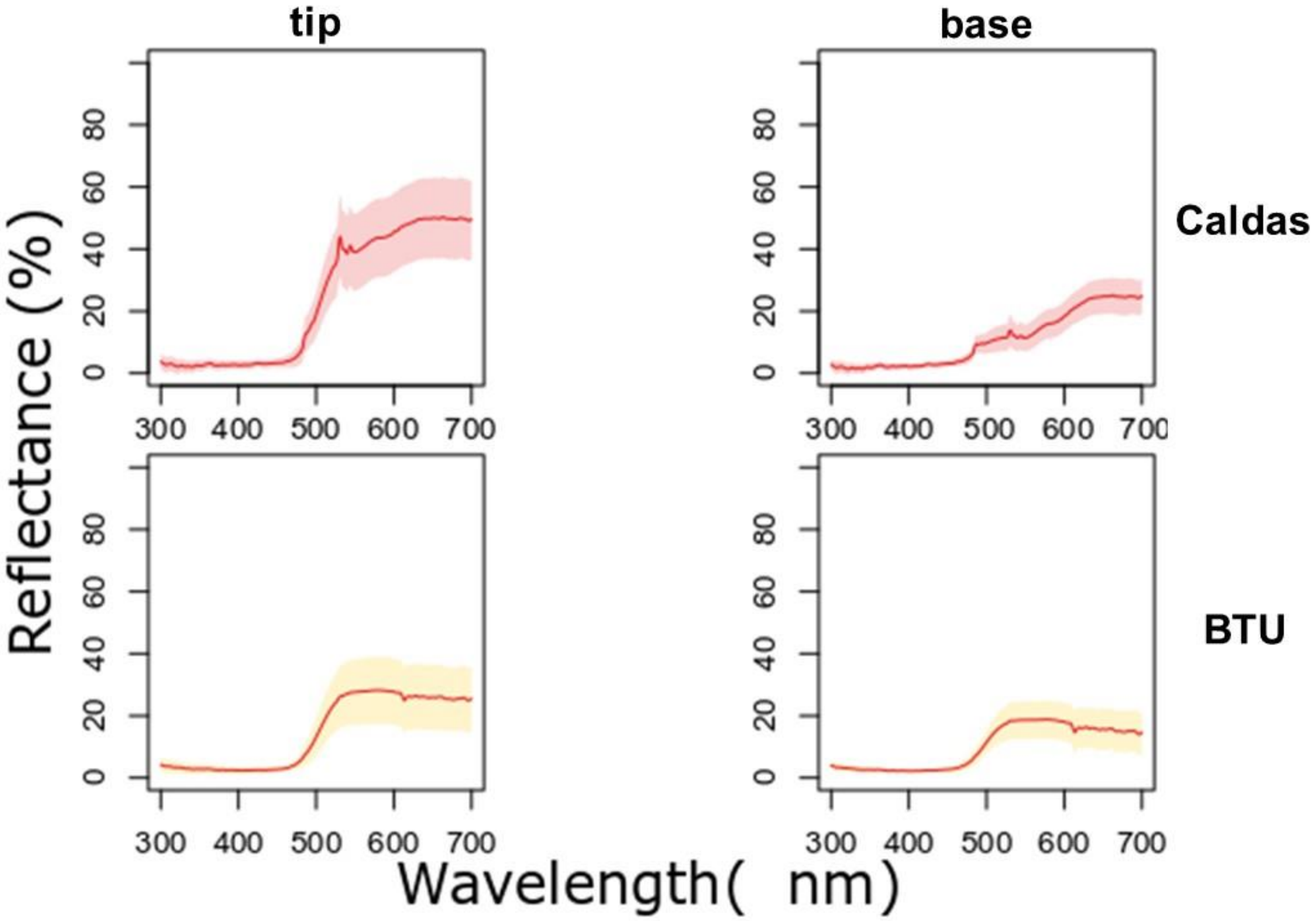

Figure 7. Spectral relative reflectance of *Palicourea rigida* flowers. The red line represents the mean reflectance, and the shaded area represents the standard variation on reflectance.

DISCUSSION

Our results show that the basal characteristics of distyly remained in the dwarf-population at the southernmost distribution of *Palicourea rigida* in Brazil, despite the contrasting morphology observed in this population in relation to the populations occurring in central Brazil. Despite that, the dwarf population presents the smallest corolla length, as well as, the smallest anther and stigma heights. The heteromorphic self-incompatibility and isoplethy were also maintained, which preserve the basal characteristic of heterostyly, which means that the species relies strictly on pollinators for reproduction. The efficiency of pollination depends on how functional pollinators groups get high- and low-level pollen deposited on different body parts, which can differ according to the morphology and length of buccal parts, body size and fit with the flower morphology (Ornelas et al., 2004; Massinga et al., 2005; Armbruster et al., 2006; Zhu et al., 2015; Deschepper et al., 2018). This population is visited and pollinated mainly by large-sized bees, such as *Bombus morio*, which have a much smaller buccal length when compared to hummingbirds, which act as the main pollinator of other *P. rigida* populations in Brazil. Thus, according to the Stebbins (1970) postulation of the principle of the most effective pollinator, an animal group with a high frequency of visits and effectiveness as pollinator can mediate the selection of floral traits that will increase plant fitness in a given location where the species has evolved. Hence, we suggest that the pollinator-mediated selection by bees had led to a decrease in the corolla tube length of the dwarf population of *P. rigida*. This kind of pollinator shift (hummingbird pollination to bee pollination), is relatively common, and the evidence for multiple reverse transitions from presumed ancestral hummingbird pollination to more derived bee or insect pollination are well documented in another plant family (Tripp & Manos 2008).

Furtado (2019) analyzed the inaccuracy of seven *Palicourea rigida* populations in the central distribution of the species in Brazil, and the dwarf population analyzed in this study presented an inaccuracy lower than the five populations studied. Also, taking into account the populations we have analyzed the dwarf population presents an inaccuracy lower than seven populations, in a total of 10, so far studied in Brazil. This shows that the dwarf population presents accurate reciprocity higher than most populations already analyzed. This can be corroborated by the high fruit set in natural conditions found in the dwarf population, which are within the expected for the hummingbird-pollinated populations (Machado *et al.* 2010), showing that bee pollination is as efficient as hummingbird pollination in this species. Hence, bees are very effective pollinators of the dwarf population, suggesting the occurrence of a pollinator shift in this southernmost population of *Palicourea rigida*.

Despite that, when comparing the floral morphology with the populations from Caldas and Chapada we noticed a difference between the main morphological parameters involved in reproduction, such as corolla size, diameter, and height of reproductive organs. The dwarf population has the smallest flower tube, with the lowest position of the reproductive organs, however, presents the largest stigmatic lobe of pin flowers among all populations, which is 3.4 and 4.6 times larger than the Chapada and Caldas populations, respectively. Raupp *et al.* (2020) have found that the *P. rigida* flowers in three populations studied presented greater reciprocity in low organs, which in association to the larger stigmas of thrum flowers, enable a greater legitimate pollen deposition on thrum flowers. The factors that could affect pin stigmas from receiving a higher amount of legitimate pollen are the shorter length of the stigmatic lobes, as well as the lower levels of reciprocity found in high organs (Furtado *et al.* 2020; Raupp *et al.* 2020). The average length previously found of pin stigmas was (1.10 mm), and in thrum ones (3.38 mm), which presents a ratio of 3.7 times larger (Raupp *et al.* 2020). Considering the populations that we analyzed, Chapada has the thrum stigma four times larger than the pin flowers, and in Caldas, this rate was 4.6 times larger, while in the dwarf population (BTU) this ratio is only 1.37. The dwarf population presents a mean pin stigma length of 3.31 mm and the average length of thrum was 4.54 mm. Thereby, the larger stigmatic lobe found in BTU, as a consequence, can increase the legitimate pollen deposition rate, once the larger stigmatic lobes, larger the surface for pollen reception (Furtado *et al*. 2020). With that, since the species rely on legitimate crosses for sexual reproduction (Ganders 1979; Barrett 2013), a greater legitimate pollen deposition could proportionate a higher reproduction rate in a population.

Our results show that even though the BTU population presents a much larger stigmatic lobe in comparison to the other studied populations (Machado *et al*. 2010), plants producing pin flowers do not have a higher fruit set such as we suggested. These results suggest that the lower levels of reciprocity in high organs (stigmas of pin flowers and anthers of the thrum ones) founded in all *P. rigida* populations analyzed so far, seems to more likely to cause low legitimate pollen deposition in high stigmas, leading to lower fruit set (see also Furtado *et al.* 2020, Raupp *et al*. 2020). Our results show that even with a larger stigmatic surface, the reproduction rate of pin flowers in the dwarf population was not significantly higher when compared with other *P. rigida* populations with a smaller stigmatic surface. Therefore, the level of inaccuracy observed in the high organs seems to play a more important role for fruit formation of pin flowers, than the size of the stigmatic lobes.

Moreover, regarding floral color and bee perception of *P. rigida* flowers, although the dwarf population presents a different corolla's color compared with Caldas population, such difference seems to do not play a significant role from the perspective of the bee visual system model used in this study (*i.e.* the *Bombus terrestris* visual system), once the flowers seem to be equally visible to the bee visual system model used, and the receptors that are excited when exposed to *P. rigida* flowers were the same in both populations, suggesting the flowers are essentially the same between populations, even the dwarf population presenting a marked yellowish color from the human visual system perspective (Chittka 1992; Lunau *et al*. 2011). Considering the color contrast, the dwarf population presented no contrast between tip and base, so flowers are homogeneously yellowish, while the Caldas population presents a contrasting color pattern between the tip and the base of the corolla. In this sense, reddish color may be an attribute selected by hummingbirds in the populations pollinated by these animals, while in the dwarf population in BTU, which is pollinated by bees, the yellowish color could represent an economy in term of pigment production. However, further analyses are needed to better understand the role of pollinators on this marked difference in the flower color of different populations of *P. rigida* across the Brazilian Cerrado.

Thereby, our results show a *Palicourea rigida* pollinated by bees, highlighting a pollinator-shift in the dwarf population of the species in its southernmost distribution, where bees are the main pollinators of the area and promote the reproductive success that maintains the heterostyly in the population. We suggest that the smaller size of *P. rigida* flowers observe in the dwarf population is a result of pollinator-mediated selection of floral traits driven by bees. We also elucidate the role of stigmatic surface in the legitimate pollen deposition, where the lower legitimate pollen deposition on high-level stigmas seems to be due to the unevenness of reciprocity between the low- and high organs level inaccuracy, rather than the smaller stigma in high organs. Thereby, our work helps to better understand the reproduction of distylous species and the role of pollinators in phenotypic variant species.

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