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1 Floral biology, reciprocal herkogamy and breeding system in *four* *Psychotria* L. (Rubiaceae)

2 species in Brazil.

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Abstract:

Distylous species present two floral morphs with reciprocal positions of sexual whorls, which function to promote cross-pollination. Additionally, most distylous species present an incompatibility system linked to the discrete variation of anthers and stigmas. Here we provide new data on heterostyly, reproductive biology and pollination in four *Psychotria* species from the Cerrado Region (Brazil). *Psychotria deflexa*, *P. nitidula* and *P. trichophoroides* fitted the distylous morphological syndrome, whereas *P. prunifolia* was monomorphic and self-compatible. Reciprocity varied across species, being the upper whorl of anthers and stigmas more reciprocal than the lower whorl. *Psychotria nitidula* presented a heteromorphic incompatibility system while, *P. deflexa* and *P. trichophoroides* showed partial self-compatibility. *Psychotria prunifolia* and *P. trichophoroides* were visited by large bees, and exhibited lower reproductive efficacy than *P. nitidula* and *P. deflexa*, visited by wasps and small bees. Our results provide new evidence that the morphological distylous syndrome not always corresponds with the heteromorphic incompatibility, and additional mechanisms (e.g. disassortative-pollen transfer) may operate to maintain the polymorphism and isoplethy of species with different incompatibility system. This highlights the value of Rubiaceae to understand the ecology and evolution of heterostyly.

Key words: Cerrado – heterostyly–reproductive biology - incompatibility system – isoplethy-pollination ecology.

55 Introduction

56 Heterostyly is a sex polymorphism in plants that has evolved in at least 28 families of
57 angiosperms (Barrett & Shore, 2008), and its function has been interpreted as a
58 mechanism for the promotion of cross-pollination and avoidance of selfing (Barrett,
59 Jesson & Baker, 2000). These functions are attained by morphological and
60 physiological adaptations, namely reciprocal herkogamy and incompatibility system
61 (Ganders, 1979; Barrett, 2002). Populations of heterostylous taxa include two (distyly)
62 or three (tristyly) floral morphs with reciprocal placement of anthers and stigma
63 (Darwin, 1877; Barrett, 2002). This morphological variation usually represents the
64 necessary condition to classify a species as heterostylous, but most heterostylous taxa
65 also present a sporophytic and heteromorphic incompatibility system associated with
66 the polymorphism (Trick & Heizmann, 1992). With this incompatibility system, only
67 cross-pollinations between morphs result in fertilization of ovules and production of
68 seeds (Philip & Schou, 1981; Barrett *et al.*, 1997; Takayama & Isogai, 2005; Pérez-
69 Barrales, Vargas & Arroyo, 2006; Barrett & Shore, 2008; Klein, Freitas & Da Cunha,
70 2009; Zhou *et al.*, 2015).

71 Theoretical models on the function of floral polymorphisms predict that
72 negative-frequency dependent selection, combined with the mode of inheritance of
73 heterostyly (a supergene with simple Mendelian inheritance, where the locus controlling
74 the morph expression is associated to the incompatibility system locus, Lewis & Jones,
75 1992) maintain the heterostylus populations at equilibrium (e.g. isoplethy; Fisher, 1930;
76 Heuch, 1979; Pannell, Dorken & Eppley, 2005). Essentially, isoplethy is maintained
77 when rates of disassortative mating (mating between flowers of different morphs) are
78 greater than the rates of assortative mating (mating between flowers of similar morph).
79 This can be achieved morphologically (e.g. high reciprocity between morphs and

efficient pollinators) as well as by reinforcement of mating patterns by the action of the heteromorphic incompatibility system (Sakai & Wright, 2008; Zhou *et al.*, 2015).

Population deviations from isoplethy are usually associated with atypical morphologies such as homostylous (flowers with stigmas and anthers at the same height) and self-compatible flowers (Gardens, 1979; Barrett & Richards, 1990; Brys *et al.*, 2008; Consolaro *et al.*, 2009; Zhou *et al.*, 2012). This usually occurs under unpredictable pollination environments or with founder effects, as a mechanism for reproductive assurance (Schoen *et al.*, 1997; Mast, Kelso & Conti, 2006).

Despite the functional interpretation, the extent to which the morphological heterostylous syndrome contributes to disassortative mating is still unresolved. Estimates of pollen flow in species with pollen dimorphism have provided high rates of assortative pollen transfer in many species (Stone & Thomson, 1994; Lau & Bosque, 2003; Hernandez & Ornelas, 2007; Keller, Thomson & Conti, 2014). Assortative pollen transfer may be associated to low precision in the placement of anthers, which can result in pollen flow asymmetries (Glover & Barrett, 1986; Barrett & Shore, 1987; Mal & Lovett-Doust, 1997). This is not surprising because many heterostylous species deviate from the expected perfect match between reciprocal whorls (Faivre & McDade, 2001; Pérez, Vargas & Arroyo, 2004; Kálmán *et al.*, 2007; Sánchez *et al.*, 2010; Ferrero *et al.*, 2011; Keller, De Vos & Conti, 2012). Reciprocity may be influenced by floral development, particularly in species with epipetalous flowers, where the anthers are merged to the corolla, and the growth of the corolla tube determines the position of the anthers (Faivre 2000, Faivre & McDade 2001). In heterostylous flowers, the epipetalous condition can influence the reciprocal herkogamy and pollen transfer in two ways. Firstly, it affects herkogamy in L- and S- flowers, and therefore the likelihood of self-pollination and pollen flow among flowers of the same morph (Cesaro *et al.*, 2004,

Pérez-Barrales & Arroyo, 2010). Secondly, it affects reciprocity between morphs because the elongation of the filaments, and hence the position of the anthers, is directly influenced by flower tube length (Richards & Koptur, 1993; Faivre, 2000; Faivre & McDade, 2001; Ferrero *et al.*, 2009a, Pérez-Barrales *et al.*, 2014). Furthermore, when the incompatibility system is not associated with the floral morphs, and intra-morph and inter-morph crosses are all compatible (Philipp & Schou, 1981; Barrett *et al.*, 1997; Pérez-Barrales *et al.*, 2006; Ferrero *et al.*, 2012; Zhou *et al.*, 2015), the pollinator community can strongly influence mating patterns (Pérez-Barrales & Arroyo, 2010; Simón-Porcar, Santos-Gally & Arroyo, 2014), which results in variation in the morph ratio across populations (Pérez-Barrales, Arroyo & Armbruster, 2007; Pérez-Barrales *et al.*, 2009; Santos-Gally, Vargas & Arroyo, 2012).

Rubiaceae includes more heterostylous species than any other plant family (Dulberger, 1992). The family is divided into three monophyletic subfamilies (Rubioideae, Ixoroideae and Cinchonoideae), and they all include distylous species, although distyly commonly occurs in the subfamily Rubioideae (Faivre & McDade, 2001; Bremer & Eriksson, 2009; Ferrero *et al.*, 2012). Within Rubioideae, *Psychotria* L. is the most important genus, and heterostyly appears to be an ancestral condition, as shown at the family level in a phylogenetic context (Robbrecht, 1988; Hamilton, 1990; Ferrero *et al.*, 2012). At the genus level, *Psychotria* includes substantial variation in the expression of the polymorphism across species and populations, both physiologically (variation in incompatibility system) and morphologically (variation in the matching between reciprocal organs) (Sakai & Wright, 2008; Consolaro, Silva & Oliveira, 2011; Faria *et al.*, 2012; Naiki, 2012; Rodrigues & Consolaro, 2013).

In this paper, we studied four species from the Brazilian Cerrado Biome to increase the current knowledge of the floral biology and variation of the heterostylous

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3 130 syndrome in *Psychotria*. Specifically, we aimed at (i) describing morph ratio variation
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5 131 in the populations of the study species, (ii) detecting differences between morphs in
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8 132 floral traits and describing reciprocity, including a test to evaluate the effect of the
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10 133 corolla tube length on the position of anthers and stigmas, and therefore the effects on
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12 134 reciprocity, (iii) characterizing the incompatibility system and reproductive parameters,
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14 135 as well as the level at which the incompatibility reaction occurs (stigma or style level)
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16 136 and, (iv) describing the main floral visitors. We compared our results with the available
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18 137 information in other Rubiaceae species, underlying the possible ecological and genetic
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20 138 causes behind the observed variation.
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24 139 Material and Methods

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26 140 *Study area* - The study was carried out in the city of Catalão, located in the Cerrado
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28 141 Region of the SE of the state of Goiás (Brazil), between December 2011 and January
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30 142 2013, in two protected fragments of a tropical semi-deciduous forest. The first fragment
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32 143 corresponded to Parque Municipal da Mata do Setor Santa Cruz (18°09'S, 47°55'W), an
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34 144 urban forest with an area of 28.48 hectares. The second fragment corresponded to Pasto
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36 145 do Pedrinho (18°16'S, 47°95'W), an area of 54 hectares formed by a typical Cerrado
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38 146 savannah vegetation, with a mixture of shrubs, pastures and forest. The study sites have
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40 147 an annual average rainfall of 1236 mm, with 89% of the rainfall occurring between
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42 148 October and March (Vieira, Coutinho & Rocha, 2012). The climate is of AW type, with
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44 149 two distinct seasons: a cold and dry season from May to September and another, hot and
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46 150 wet, from October to April (Koppen, 1948).
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52 151 *Studied species* – The four species of *Psychotria* (*Heteropsychotria*) studied were *P.*
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54 152 *deflexa* DC., *P. nitidula* Cham. & Schltdl, and *P. trichophoroides* Müll. Arg., all
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56 153 dimorphic for style length, and the monomorphic *P. prunifolia* (Kunt) Steyerm.
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58 154 *Psychotria nitidula* presents a shrubby habit, whereas the other species are sub-shrubs.
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155 Flowering occurs between October and January, but flowering peak differs across
156 species (Sá, 2013); hence, fieldwork was conducted at the flowering peak of every
157 species to optimize the efforts of the data collection (see details below). Mata do Sector
158 Santa Cruz included the species *Psychotria deflexa*, *P. nitidula* and *P. prunifolia*,
159 whereas *P. trichophoroides* occurred at Pasto do Pedrinho. Because these two patches
160 are relatively small, embedded in an urban environment, and several km apart from the
161 closest natural area (ca. 3 Km), we considered that every species studied was
162 represented by one population. All species were common and equally abundant in the
163 study areas, with the exception *P. deflexa*, which is less common. *Psychotria deflexa*, *P.*
164 *nitidula*, and *P. trichophoroides* were distributed in a heterogeneous way; *P. prunifolia*
165 usually appeared grouped in clusters (H. Consolaro, personal communication).
166 Vouchers of the species were deposited in the herbarium of the Federal University of
167 Goiás (Goiânia, Brazil), with the following voucher numbers: *P. deflexa* (47,920), *P.*
168 *nitidula* (43,241), *P. prunifolia* (43,236) and *P. trichophoroides* (47,921).
169 *Floral biology* – Data on anthesis, floral longevity, pollen release and stigma receptivity
170 were collected for all species. Five flowers were tagged before opening in 10
171 individuals of each morph, totaling 100 flowers in the dimorphic species, and 50 in the
172 monomorphic species. The start of anthesis was considered to occur when the corolla
173 lobes began to separate and floral longevity was considered the period between the
174 opening of flowers until they turned dark and displayed a fragile appearance. The
175 release of pollen was determined by the observation of pollen grains after anthers
176 dehiscence, and the stigma was considered receptive once the stigmatic lobes were fully
177 separated (Coelho & Barbosa, 2003; Consolaro, Silva & Oliveira, 2005).
178 *Morph ratio, flower morphology, categorization of the style polymorphism and*
179 *reciprocity measurements*– Morph ratio was investigated in a long transect of at least

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3 180 800 m, which represented the maximum fragment length for the two fragments. Floral
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6 181 morphs from all plants found within 5 m. at both sides along the transect was recorded
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8 182 (Consolaro *et al.*, 2009; Consolaro *et al.*, 2011). These data were collected at the
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10 183 flowering peak of each species to ensure that most of flowering individuals were
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13 184 included. Departures from the expected 1:1 morph ratio were analyzed with a Chi-
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15 185 square test performed with the R statistical software (R Development Core Team,
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17 186 2009). Four to five flowers in 10 individual plants per morph were collected and fixed
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19 187 in 70% ethanol for floral measurements (see below). Flowers were classified either as
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22 188 L-morph or S-morph when the stigmatic lobes were placed above or below the anther
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24 189 level respectively. Floral measurements were obtained with a digital caliper (error = $\pm$
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26 190 0.01 mm) and the traits measured were: 1) anther height (from the basis of the corolla to
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28 191 the middle of the anther); 2) style length (from the basis of the corolla to the basis of the
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30 192 stigma); 3) stigmatic lobes length (from the basis of the stigma to its apex); 4) stigma
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32 193 height, estimated as the sum of style length and length of the stigmatic lobes divided by
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34 194 2 to avoid overestimation of the height (the stigma in *Psychotria* species is bifid and the
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36 195 stigmatic lobes are fully separated during anthesis); 5) corolla tube length and 6)
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38 196 herkogamy, measured as the absolute value of the difference between anther length and
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41 197 stigma height. For dimorphic species, generalized linear mixed models were used to
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43 198 detect differences between morphs for all traits. In these models, the response variable
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45 199 was the floral trait, the fixed factor was the morph and the random factor was the
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47 200 individual plant. For the monomorphic *P. prunifolia*, a paired t-test stratified per
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49 201 individual was applied to compare anther height and stigma height. To control for the
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51 202 lack of independence, a bootstrap analysis (n = 20000 permutations) was implemented
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53 203 to detect significant differences (Manly, 1998). These analyses were conducted with
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55 204 SPSS version 22.0 (IBM Corp, 2013).
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The results of the above analyses were used to categorize the type of polymorphism of the species as described in Ferrero *et al.* (2009b). Specifically, the categories considered were stigma height dimorphism, i.e., two morphs with anthers at the same height and different position of the stigma; distyly, i.e., two morphs showing reciprocal placement of anthers and stigmas in the two morphs; homostyly, i.e., when the length of the style equals that of the anthers in the flowers; and approach herkogamy, i.e., when the stigma is placed above the anthers.

Reciprocal placement of anthers and stigma was further studied with the reciprocity index developed by Sánchez, Ferrero & Navarro (2008; 2013) and by studying the influence of corolla tube length on the herkogamy of the two floral morphs (Faivre & McDade, 2001). The reciprocity index compares the relative position of the sexual organs (anthers and stigmas) of each flower in relation to the reciprocal organ in the flowers of the opposite morph. The index varies between 0 and 1 (values closer to 1 correspond to higher reciprocity) and it provides an overall estimate of how reciprocal the two floral morphs are. [The index can be calculated using RECIPROCITY, a simple macro for Excel](#). A full description of the index and the software for the development of the analysis is available in <http://webs.uvigo.es/plantecology/software.es.html>.

The approach used by Faivre & McDade (2001) aims at understanding the effect of corolla tube length on anther and stigma height. From a theoretical perspective, the reciprocal placement of anthers and stigmas should result in similar herkogamy between L- and S-flowers (Barrett, 2002). However, flowers in the Rubiaceae family are epipetalous, and the filaments are fused with the flower tube. Hence, the position of anthers can be influenced by developmental processes unrelated to the function of the polymorphism but to the length of the corolla, which may differ between morphs (Faivre & McDade, 2001; Conner, 2002; Santos-Gally *et al.*, 2012; Pérez-Barrales *et*

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3 230 *al.*, 2014; Santos-Gally *et al.*, 2015). We studied this with a generalized linear mixed
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5 231 model in which the height of the organ was the response variable, the fixed factor was
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7 232 the organ type (anther, stigma), corolla tube length was included as covariate, and
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9 233 individual plant was included as a random factor (see Faivre & McDade, 2001 for
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11 234 details). To evaluate whether the slopes of the regression line for anther height and
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13 235 stigma height of the same plant differed, the interaction term morph x corolla tube
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15 236 length was included. In this analysis, a regression slope between corolla tube length and
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17 237 the organ type that statistically differs from zero can be interpreted as a significant
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19 238 relationship between the length of the corolla tube and the corresponding floral whorls
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21 239 (stamens and style). When the slopes for anther height and stigma height are significant
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23 240 but unequal, the height of the sexual organs of the flowers (stamens or style) increases
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25 241 more in one than in the other. When the slopes of the linear regressions are equivalent
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27 242 and significant, the effect of the corolla on the height of the floral sexual organs is
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29 243 similar (Faivre & McDade, 2001). Finally, to understand whether departures from the
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31 244 expected reciprocal placement of anthers and stigmas between morphs are caused by the
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33 245 lack of accurate placement of the upper level organs (L-stigma and S-anthers) or the
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35 246 lower level organs (S-stigmas and L-anthers), a general linear mixed model was used
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37 247 where the height of reciprocal organs was the response variable, the fixed factor was
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39 248 morph type, corolla tube length was included as covariate, and individual plant was
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41 249 included as a random factor. To evaluate whether the slopes of the regression line for
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43 250 anther height and stigma height between morphs differed, the interaction term morph x
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45 251 corolla tube length was included (see details above).
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48 252 *Breeding system* – In order to describe the breeding system of the species, hand-
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51 253 pollinations were conducted to study pollen tube growth and fruit formation. Rubiaceae
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58 254 flowers produce two ovules, and fruit production is a good proxy to describe the
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breeding system (Bawa & Beach, 1983; Castro, Oliveira & Alves, 2004). The experiments were conducted in natural conditions, and data on fruit formation, rather than seed formation, was collected to avoid data loss as a result of herbivory or fruit predation. Due to the capituliform morphology in all species (except for *P. nitidula*), the experiments were performed per inflorescence (i.e., 2-6 flowers) in 8-10 individuals per morph and treatment. We bagged several flower buds per plant and randomly assigned one of the following treatments: (1) intra-morph cross-pollination (L-morph x L-morph or S-morph x S-morph); (2) inter-morph cross-pollination (L-morph x S-morph or S-morph x L-morph); (3) obligatory self-pollination (flowers were hand-pollinated using pollen from the same flower); (4) spontaneous self-pollination (flowers were just bagged) and, (5) control flowers (flowers exposed to open natural pollination). Inflorescences were protected from pollinators and herbivores using mesh bags during the experiment, except for the control treatment. The inter-morph cross-pollination treatment was not performed in *P. prunifolia* as the species is monomorphic. Statistical comparisons between treatments were based on fruit set (number of fruits/number of flowers). For each species, the effects of pollination treatment, morph and the interaction term were explored with a generalized linear mixed model, where treatment and morph were included as fixed factors, and individual plants were included as a random factor to account for within-subject variability. Fruit set was modeled with a binomial distribution using the logit link function. We used LS-means to detect significant differences between treatments. Analyses were conducted with SPSS version 22.0 (IBM Corp, 2013).

To describe the degree of incompatibility in the four species, a self-incompatibility index was calculated (ISI) as the ratio between the number of fruits formed after the hand self-pollination treatment and the intermorph hand cross-

pollination treatment (Zapata & Arroyo, 1978). Values of this index lower than 0.25 indicate that the species is self-incompatible (Bullock, 1985). The reproductive efficacy (RE) was estimated as the ratio between fruit production obtained from the control pollinations and fruit production resulting from the inter-morph hand cross-pollination treatment (Zapata & Arroyo, 1978).

Pollen tube growth was observed to describe whether the incompatibility reaction takes place in the stigma or along the style. Specifically, hand-pollination treatments (1) to (3) were performed, and the styles were collected and fixed in 70% alcohol four and eight hours after the application of the hand-pollinations. The time of collection was determined in relation to the floral longevity of the species (see above). For each species, 20 flowers (10 per schedule – 4h and 8h) were hand pollinated in 10 individual plants per morph (two flowers per individual). Pollen-tube growth was observed using epi-fluorescence microscopy as described by Martin (1959). Stigmas and styles were softened with 9 N sodium hydroxide for 15 min at 60°C, washed in distilled water and placed overnight in 0.1% (w/v) aniline blue prepared in 0.1 N potassium phosphate (Dafni, Kevan & Husband, 2005). They were then placed on a microscope slide with a drop of 50% glycerin, squashed beneath a coverslip and observed using an epifluorescence microscope. We also reviewed the published research on incompatibility system and place of incompatibility (along the stigma-style) in Rubiaceae using the databases Web of Science, Scopus, Sciello. The following keywords were used: incompatibility system, incompatibility reaction, Rubiaceae, distyly, polymorphism, pollen tube. We completed the information using the information gathered from Brazilian Theses and Dissertations.

Floral visitors – Observations of pollinators were made by two observers at the flowering peak of every species. The observations were conducted between 5:30 and

17:30 h in four patches that included only one *Psychotria* species, and the number of visits was recorded. Six hours of observations were performed at each patch, accumulating a total of 24 h of observations per species. Patches presented similar number of L-morph and S-morph plants. The insect visitors were photographed, collected, and subsequently identified by specialists. The specimens were deposited in the Integrated Laboratory of Zoology, Ecology and Botany (Lizeb) of de Federal University of Goiás/Campus Catalão (Brazil).

Results

Floral Biology - All species presented white, tubular gamopetalous corollas, with pentamerous and actinomorphic flowers and five stamens per flower. The calyx of the species was greenish, gamosepalous and pentamerous. *Psychotria prunifolia* and *P. trichophoroides* presented terminal, capituliform inflorescences whereas *P. nitidula* and *P. deflexa* displayed flowers in terminal, paniculate inflorescences (Figure 1). In all species anthesis started at 4:00-5:00 h and, immediately after anthesis, the stigmatic lobes were receptive and the anthers started to release pollen. In all species, flowers lasted approximately 12 h.

Morph ratio, flower morphology, categorization of the style polymorphism and reciprocity measurements – Morph ratio in *P. deflexa*, *P. nitidula*, and *P. trichophoroides* did not depart from the expected 1:1. The proportions of L:S plants were 21:26 for *P. deflexa* (Chi-square=0.53, $P=0.46$), 24:20 for *P. nitidula* (Chi-square=0.36, $P=0.54$), and 50:47 for *P. trichophoroides* (Chi-square=0.09, $P=0.76$). Approximately 75 individuals of *P. prunifolia* were surveyed, and all the flowers assessed displayed similar morphology, with stigmatic lobes slightly above the anthers. Table 1 summarizes mean $\pm$ standard deviation for all floral measurements. The

comparisons between morphs for the three sexually dimorphic species showed that floral morphs did not differ for corolla tube length. In contrast, differences between morphs in anther-height, style-length and stigma-height were statistically significant (Table 1). This suggests that *P. deflexa*, *P. nitidula* and *P. trichophoroides* can be classified as distylous (Ferrero *et al.*, 2009b). Both herkogamy and stigmatic lobes were larger in S-flowers than in L-flowers, and these differences were statistically significant (Table 1). The analysis of *P. prunifolia* showed that stigma-height was larger than anther-height (Table 1), and this difference was statistically significant (mean difference (95% CI): 0.9 (0.47, 1.24), $t=4.046$, d.f.=49, $P=0.004$). Based on the classification by Ferrero *et al.* (2009a), this species can be classified as approach herkogamous.

The reciprocity index as proposed by Sánchez *et al.* (2013) was larger in *P. nitidula*, followed by *P. trichophoroides*, with values of 0.85 and 0.88 respectively; in contrast, *P. deflexa* presented the lowest reciprocity value of 0.46.

The analysis to evaluate the effect of the length of the corolla tube on anther and stigma height within morph revealed different patterns across species. For all the heterostylous species and the monomorphic species, there was a positive and significant relationship between corolla tube length and stigma height for the L-morph; in contrast, only *P. nitidula* and *P. prunifolia* showed a significant relationship between corolla tube length and anther height (Table 2, Figure 2). The results for the analysis of S-flowers revealed different trends. The slopes between corolla tube length and anther height were positive and statistically significant for all species, whereas only in *P. deflexa* was there a positive and statistically significant relationship between corolla tube length and stigma height (Table 2, Figure 2). The slopes of the regression of anther height and stigma height on corolla tube length for L-flowers were only statistically different in *P. trichophoroides* (Table 2). For this species, stigma height increased more rapidly than

anther height (see Figure 2). For S-morph flowers, differences in slopes were statistically significant for *P. nitidula* and for *P. trichophoroides* (Table 2). For *P. nitidula*, anther height increased more rapidly than stigma height; in contrast, in *P. trichophoroides* anther height showed a positive relationship with corolla tube length whereas the relationship was negative between stigma height and corolla tube length (Table 2, Figure 2).

The analysis to evaluate the level to which corolla tube length influences the reciprocal position of upper and lower organs showed that for all the distylous *Psychotria* species, the slopes for the upper organs (i.e. L stigma height and S anther height) were not statistically different (*P. deflexa*: $F_{1,78} = 3.637$, $P = 0.06$; *P. nitidula*: $F_{1,78} = 0.533$, $P = 0.46$; *P. trichophoroides*: $F_{1,78} = 0.004$, $P = 0.95$, see Table 2 for slope values). For the lower organs, the slope of L anther height and S stigma height on corolla tube length was not statistically different for *P. trichophoroides* ($F_{1,78} = 2.299$, $P = 0.13$), whereas differences for *P. deflexa* and *P. nitidula* were detected ($F_{1,78} = 4.315$, $P = 0.05$ and $F_{1,78} = 11.854$, $P < 0.001$ respectively).

Breeding system – Values for the fruit set after hand-pollinations for each species are summarized in Table 3. There were not significant differences in fruit set between morphs for any species, neither was the interaction term treatment x morph significant, which indicates that both L- and S-flowers responded similarly to all treatments (Table 4). Significant differences in fruit set were found between control and intermorph pollinations for *P. deflexa* and *P. nitidula*. This suggests that these two species present the typical, heteromorphic incompatibility system of heterostylous taxa (Table 3, Figure 3). These species showed ISI values equal to zero, which indicates that the species are self-incompatible (values lower than 0.25 indicate the presence of a self-incompatibility system; Bullock, 1985; Table 3). In *P. trichophoroides* there were not statistically

380 significant differences between the self-pollination treatments and the control, and fruit
 381 set was higher in the intermorph crosses compared with all the other crosses. The L-
 382 morph of this species showed an ISI value of 0.30 (Table 3), suggesting that the species
 383 presents a certain level of self-compatibility. *Psychotria prunifolia* did not show
 384 differences in fruit set between the self-pollination treatments (treatments 3 and 4, see
 385 above); however, fruit set after hand self-pollination was lower than the control and
 386 intramorph crosses (Table 3). The ISI index value indicates that this species is self-
 387 compatible (ISI = 0.75). *Psychotria nitidula* showed the largest RE values (Table 3);
 388 across species and morphs, RE values were larger in the L-morph rather than in the S-
 389 morph.

390 The observations of pollen-tube growth in the three heterostylous species
 391 showed that pollen tubes from legitimate hand-pollinations reached the ovary in the first
 392 4 h. The description of the site where the incompatibility reaction occurred is
 393 summarized in Table 5. For most of intramorph and self-pollinations, the interruption of
 394 the pollen tube occurred in the first 4 h. In the S-morph, pollen tubes stopped at the
 395 stigma level, in contrast, the interruption of pollen tubes occurred along the style in the
 396 L-morph. In *P. deflexa* and *P. trichophoroides*, some of the pollen tubes from
 397 illegitimate pollinations reached the style further down after 8 h. In *P. nitidula*, the
 398 pollen tubes produced after illegitimate pollination stopped at the stigma or the upper
 399 area of the style in both morphs. In *P. prunifolia*, the pollen tubes reached the end of the
 400 style after 4 h for both the self- and cross-pollination.

401 Table 6 includes the data about the location (stigma or style) of the
 402 incompatibility reaction across different Rubiaceae species gathered from the literature
 403 review. Most of the information available corresponds to distylous species, with the
 404 exception of some homostylous *Psychotria* species. The data show substantial variation

in the place where the incompatibility reaction occurs, occurring both at the stigma and style level, being the variation greater for the L- than the S-morph.

Floral visitors- Table 7 summarises the observations of floral visitors and number of visits. Insect activity started at 5:00 h, ended at 17:30 h, and the peak of activity occurred between 9:00 and 10:00 h. *Psychotria nitidula* received the largest number of visits (1992), whereas *P. trichophoroides* had the lowest (102). Wasps of genus *Pepsis* were the main visitors of *P. deflexa* (90 visits, 36% of total visits). The honeybee *Apis mellifera* was the most common pollinator of *P. nitidula* (1794 visits, 90% of total visits) and it was also frequent in *P. deflexa*. *Psychotria trichophoroides* and *P. prunifolia* were visited mostly by bees of the genus *Epicharis* [128 (51%) and 53 (52% of total visits) respectively].

Discussion

Reciprocal herkogamy is considered the main feature to classify a species as heterostylous (Barrett, 1992; Faivre & McDade, 2001), and it involves the complementary placement of anthers and stigmas in flowers of different morphs (Ganders, 1979). In addition, heterostylous species commonly present a heteromorphic incompatibility system that prevents selfing and fertilization among plants of the same morph (Barrett, 2002). These morphological and physiological characteristics were considered linked in the early literature (Darwin, 1877; Ganders, 1979; Barrett, 1992), but research on various plant groups has shown that the morphological syndrome and incompatibility system are not always genetically associated (Pérez-Barrales *et al.*, 2006; Ferrero *et al.*, 2012; Santos-Gally, Gonzalez-Voyer & Arroyo, 2013a). In the present study, we described variation in flower morphology, reciprocity and the place of the incompatibility reaction in several heterostylous *Psychotria* species, a genus with substantial variation in the expression of the polymorphism (Faivre & McDade, 2001;

Oliveira, 2008; Sakai & Wright, 2008; Consolaro *et al.*, 2011; Rodrigues & Consolaro, 2013).

Variation in flower morphology, reciprocity and morph ratio

We confirmed that one species was monomorphic whereas the populations studied of the other species presented two floral morphs. Theoretical expectations on the morphological variation in heterostylous species predict that reciprocal placement of anthers and stigmas occur when the herkogamy values of the L-morph and S-morph are similar (Darwin, 1877; Charlesworth & Charlesworth, 1979; Lloyd & Webb, 1992a). The dimorphic *Psychotria* species in this study fitted the morphological syndrome since they present reciprocal herkogamy, although their degree of reciprocity varied and was not completely accurate (e.g. reciprocity in *P. deflexa* was approximately half the values obtained for *P. trichophoroides* and *P. nitidula*). We also found that herkogamy was larger for the S-morph than the L-morph in all the species, as previously found in *Psychotria* (Hamilton, 1990; Faivre & McDade, 2001; H. Consolaro; R. Perez-Barrales; P.E. Oliveira & J. Hay, unpubl. data) and other Rubiaceae species (Richards & Koptur, 1993; Ree, 1997; Faivre & McDade, 2001; Consolaro *et al.*, 2009).

To better understand departures from the expected reciprocity, we explored the influence of corolla tube length on the position of anthers and stigmas (Faivre, 2000; Faivre & McDade, 2001; Conner, 2002; Ordano *et al.*, 2008; Bissell & Diggle, 2010; Santos-Gally *et al.*, 2013a; Pérez-Barrales *et al.*, 2014; Santos-Gally *et al.*, 2015), and detected interesting patterns. The rate of change of anthers and stigmas was similar in the monomorphic species, and in the L-morph of the distylous species, except for *P. trichophoroides*. In contrast, the slopes in the S-morphs were only similar in *P. deflexa*. Furthermore, we tested the hypothesis that the regression slope between corolla tube length and anther height should be similar to the slope between corolla tube length and

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3 455 stigma height of the reciprocal organ (Faivre & McDade, 2001). We detected a similar
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5 456 positive relationship between corolla tube length and the position of the upper organs
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8 457 (i.e. stigma height in L-flowers, anther height in S-flowers). In contrast, this relationship
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10 458 was not significant for the lower organs, with the exception of the short style of *P.*
11
12 459 *deflexa* and short anthers of *P. nitidula*. These trends suggest that the upper organs are
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14 460 more reciprocal than the lower organs. In *Psychotria*, the anthers are physically linked
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16 461 to the corolla, and a strong relationship between corolla tube length and anther position
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18 462 should be expected regardless of the morph (Faivre, 2000; Faivre & McDade, 2001;
19
20 463 Conner, 2002; Pérez-Barrales *et al.*, 2014). A significant relationship between the height
21
22 464 of the sexual organs and the length of the corolla has been reported in other Rubiaceae
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24 465 species such as *Bouvardia ternifolia* (Cav.) Schltdl., *Palicourea fendleri* Standl.,
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26 466 *Guettarda scabra* (L.) Lam., *Psychotria chiapensis* Standl. and *Psychotria poeppigiana*
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28 467 Mull. Arg. (Sobrevila, Ramirez & Enrech, 1983; Richards & Koptur, 1993; Faivre,
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30 468 2000; Faivre & McDade, 2001), as well as in the family Primulaceae, also with
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32 469 epipetalous stamens (Nishihiro *et al.*, 2000; Webster & Gilmartin, 2006; Kálmán *et al.*,
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34 470 2007). In contrast, in the distylous species *Amsinckia* Lehm. (Boraginaceae) this
35
36 471 relationship was not significant (Li & Johnston, 2010). In *Narcissus* L.
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38 472 (*Amaryllidaceae*), floral tube length and the epipetalous stamens were considered
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40 473 important traits in the evolution of style dimorphism and heterostyly (Santos-Gally *et*
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42 474 *al.*, 2013b). The length of the flower tube influences the position of the sexual organs,
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44 475 and this relationship differs among populations and species depending on the historical
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46 476 and/or ecological context, as proved in *Narcissus* (Pérez-Barrales *et al.*, 2007; Santos-
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48 477 Gally *et al.*, 2013a; Pérez-Barrales *et al.*, 2014; Santos-Gally *et al.*, 2015). In
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50 478 *Psychotria*, future research should address whether variations in herkogamy, upper and
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52 479 lower organ reciprocity patterns result from developmental constraints (Faivre, 2000;
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Conner, 2002; Bissell & Diggle, 2008; 2010; Pérez-Barrales *et al.*, 2014), pollinator-mediated natural selection (Pérez-Barrales & Arroyo, 2010; Santos-Gally *et al.*, 2013a) or both (Armbruster & Muchhala, 2009; Armbruster *et al.*, 2009a).

An important result of our study consist of the maintenance of the 1:1 ratio in the populations of the species studied, regardless the variation in reciprocity. The four *Psychotria* species are perennials and, as expected, morph ratios have been consistent in these patches in the later years (Sá & Consolaro, personal observations). The heteromorphic incompatibility system usually allows maintenance of the 1:1 morph ratio, as is probably the case in *P. deflexa* and *P. nitidula*. However, when the incompatibility system does not operate fully, the maintenance of isoplethy depends on high rates of intermorph mating promoted by efficient pollinators (Lloyd & Webb, 1992b; Thompson, Barrett & Baker, 2003; Cesaro *et al.*, 2004; Pérez-Barrales & Arroyo, 2010). *Psychotria trichophoroides* presented certain level of self-compatibility, and it could be possible that pollinators play an important role in maintaining the polymorphism and the 1:1 morph ratio (see discussion below).

Differences in breeding system

Distylous species typically present a diallelic incompatibility system (Barrett & Shore, 2008). Within Rubiaceae, *Psychotria* is perhaps the genus with the greatest variation in the incompatibility system, with *ca.* 9.5% of the species with deviations to self-compatibility (see Table 6 for a comparison with other Rubiaceae species). The species studied here differed in the degree of self-compatibility: *P. nitidula* presented the expected heteromorphic incompatibility, while the other species displayed certain level of self-compatibility. We did not find significant differences among the treatments intermorph, intramorph and spontaneous self-pollination in *P. deflexa*, but fruit production was low for intramorph and self-pollinations. Furthermore, this species

displayed the lowest fruit production in the control treatment (open natural pollination, see Figure 3), despite its similar habitat, flower morphology and pollination ecology to the other species. Pollen limitation or resource allocation are the two main hypotheses usually invoked when attempting to explain the low production of fruits and seeds per flower (Stephenson, 1981; Sutherland, 1986; Charlesworth, 1989). The low reproductive success in *P. deflexa* may be caused by pollen limitation, although further investigation is needed to understand whether this is associated to low visitation rates or inefficient pollen transfer between morphs. *Psychotria trichophoroides* appeared to be partially self-compatible and presented intra-morph incompatibility. In heterostylous species of Rubiaceae, self-compatibility often occurs in monomorphic species (Sobrevila *et al.*, 1983; Consolaro *et al.*, 2005; Sakai & Wright, 2008; Consolaro *et al.*, 2011; Rodrigues & Consolaro, 2013). *Psychotria prunifolia* was a self-compatible, monomorphic and herkogamous species. In the absence of a phylogeny, it is unclear whether the self-compatibility and monomorphic condition of this species is ancestral condition to distyly (Lloyd & Webb, 1992b), or a derived condition resulting from the supergene recombination (Ganders, 1975; Hamilton, 1990). Transition to monomorphism in heterostylous plant groups is associated with the loss of self-incompatibility. This has been shown in some families such as Amaryllidaceae (*Narcissus*, Pérez-Barrales *et al.*, 2006), Linaceae (*Linum* L., Nicholls 1986; McDill *et al.*, 2009) or Primulaceae (*Primula* L., Ernst, 1955; Mast *et al.*, 2006). Once the incompatibility system is lost, very rarely is it regained, at least in its ancestral form (Barrett, 2013). Taken together, our results suggest that the incompatibility system in *Psychotria* is labile, and further work is required to clarify the evolutionary transitions from monomorphism to distyly and *vice versa* in the group.

529 In many Rubiaceae species, crosses between L-flowers tend to be more
 530 compatible than those between S-flowers, and the place of pollen-tube interruption
 531 appears to be similar to that of species with gametophytic incompatibility system. This
 532 has been described in *Hedyotis* L., *Pentas* Benth, and *Oldenlandia* L. (Bir-Bahadur,
 533 1966; Bir-Bahadur, 1968; Bir-Bahadur, 1970a, b, c; Ganders, 1979). Our results
 534 combined with those found in the literature review indicated that most pollen tubes
 535 from illegitimate crosses were interrupted in the stigma or upper part of the style (Table
 536 6), but some variation between morphs was detected with regards the site of disruption
 537 of pollen tubes. Furthermore, variation in the place of interruption was greater in the L-
 538 morph than the S-morph for the self- and intramorph- treatments, which could be
 539 caused by the presence of two or more distinct incompatibility mechanisms
 540 (sporophytic and gametophytic) in the family (Bawa & Beach, 1983). In many species,
 541 the incompatibility reaction occurs in the same site for both morphs (Baker, 1966;
 542 Dulberger, 1975; Murray, 1990; Wedderburn & Richards, 1990) and our review of 43
 543 species of Rubiaceae seems to indicate an absence of a common pattern in the variation
 544 of inhibition sites for pollen tubes between floral morphs (Table 6). However, an
 545 observed trend is the greater pollen-tube penetration for the L-morph compared with the
 546 S-morph (Wedderburn & Richards, 1990). This variation is also confirmed in the
 547 studied species and has been related to differential growth rates due to distinct pollen
 548 grain size (S-morph pollen grains are commonly larger, see Darwin, 1877; Dulberger,
 549 1992).

550 *Pollination context*

551 Only insects visited the flowers of the *Psychotria* species studied, and the most
 552 frequent visitors were wasps, flies and bees. *Psychotria prunifolia* and *P.*
 553 *trichophoroides* were visited mainly by large bees (*Epicharis cockerelli* and *E. flava*)

554 compared with *P. nitidula* and *P. deflexa*, whose visitors were wasps (*Pepsis* sp.) and
555 small bees (*Apis mellifera* and *Augochloropsis* sp1). The distylous floral syndrome
556 requires efficient cross-pollination between anthers and stigma at different levels
557 (Barrett, 2002), as well as a morphological match between the flower shape and the size
558 of the pollinator (Armbruster *et al.*, 1994; Campbell, Waser & Price, 1996;
559 Alexandersson & Johnson, 2002; Castellanos *et al.*, 2004; Armbruster *et al.*, 2006;
560 Armbruster *et al.*, 2009b). The maintenance of the 1:1 morph ratio in the partially self-
561 compatible *P. trichophoroides* can occur if large bees promote higher rates of
562 disassortative mating than assortative mating. *Psychotria deflexa* is a species with small
563 corollas (Table 1), but with the largest pollinator (Table 4). Based on the high rate of
564 RE in the L-morph, the low values in the S-morph and the low reciprocity of the
565 species, it could be predicted that the efficiency of pollinators differed between morphs.
566 However, the 1:1 morph ratio observed in *P. deflexa* suggests that the incompatibility
567 system, or perhaps differences between morphs in fecundity (Ree, 1997), maintain
568 isoplethy. Future research should address these hypotheses.

569 The *Psychotria* species in this work differed in flower morphology, reciprocity
570 and incompatibility system. This supports the hypothesis that distylous species can
571 follow different strategies to maintain the polymorphism and the isoplethic morph ratio.
572 *Psychotria nitidula* showed high reciprocity index, a high rate of reproductive efficacy
573 and a heteromorphic incompatibility system, as expected in typical distylous species.
574 *Psychotria deflexa* also showed a heteromorphic incompatibility system, but the low
575 reciprocity might affect pollen flow, which may be the cause of the asymmetries.
576 *Psychotria trichophoroides* presented a relaxed self-incompatibility system and high
577 reciprocity. *Psychotria prunifolia*, a monomorphic self-compatible species, has high
578 fruit production and high reproductive efficacy. Our results support the lack of

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3 579 correlation between distyly and the incompatibility system, which is more common than
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5 580 previously thought (Pérez-Barrales *et al.*, 2006; Ferrero *et al.*, 2012; Santos-Gally *et al.*,
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8 581 2013b). Future research should adopt a functional and an evolutionary perspective to
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10 582 understand the adaptive value of the morphological and physiological variation in
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12 583 relation to the pollination environment to fully understand the origin and maintenance
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15 584 of heterostyly. The family Rubiaceae, and the genus *Psychotria*, in particular, represents
16
17 585 an excellent plant group to address these questions.
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21
22 587 Acknowledgement
23

24 588 The authors thank Catalão City Council for permits to access the protected areas to
25
26 589 conduct the research. This research was supported by the grants of CNPq 474516/2013-
27
28
29 590 0 to HC, British Council to RPB and the postgraduate program from the University of
30
31 591 Uberlandia to support RPB and VF. Support was also provided by FAPEMIG—
32
33 592 Research Support Foundation of Minas Gerais (PACCS CRA-30058/12). The work of
34
35 593 V. Ferrero was supported by the Xunta de Galicia (I2C program). The English style was
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37 594 corrected by J. H. Peterkin.
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921 Figure Legends

922 **Figure 1.** Overview of flowers and inflorescences of *Psychotria* species. A and B: *P.*
923 *nitidula*, L-morph and S-morph, respectively. C and D: *P. deflexa*, L- and S-morph,
924 respectively. E and F: *P. trichophoroides*, L- and S-morph, respectively. Bars = 10 mm.

925 **Figure 2.** Relationship between anther height and stigma height with the corolla length
926 for L- and S-flowers in *Psychotria* species.

927 **Figure 3.** Mean $\pm$ standard error of fruit set in studied species of *Psychotria* after hand
928 pollination treatments. Pollination treatments were: intra-morph cross pollination (L $\times$ L,
929 S $\times$ S; intra), inter-morph cross pollination (L $\times$ S, S $\times$ L; inter), spontaneous self-
930 pollination (self-sp), self-pollination (self-pol). Results from flowers exposed to natural
931 pollinations are also shown (control). Since there were no statistical differences between
932 floral morphs within species, the results for both morphs are plotted together for each
933 taxon.

936 Table 1. Floral measurements (mean and 95% CI) for dimorphic (long-styled flowers L and short-styled flowers S) and monomorphic
937 *Psychotria* species and comparisons of floral measurements between morphs by means of a general linear mixed model (see Materials &
938 Methods for details) in two forest remnants in SE Goiás, Brazil. For all species, four to five flowers per individual were surveyed in ten long-
939 styled and ten short-styled plants. * P<0.05, ** P<0.01, ***P<0.001, n.s.= not significant.

	<i>P. nitidula</i>				<i>P. deflexa</i>				<i>P. trichophoroides</i>				<i>P. prunifolia</i>	
Floral trait (mm)	Morph	Mean	95% CI	F-value	Mean	95% CI	F-value	Mean	95% CI	F-value	Mean	95% CI	Mean	95% CI
Corolla length	L	6.77	(6.50, 7.04)	0.166 n.s.	3.05	(2.77, 3.34)	0.642 n.s.	9.97	(9.42, 10.51)	0.642 n.s.	10.21	(10.05, 10.37)		
	S	6.70	(6.43, 6.97)		3.31	(3.03, 3.60)		10.26	(9.71, 10.81)					
Anther height	L	5.37	(5.11, 5.63)	687.174 ***	2.62	(2.44, 2.80)	1010.962 ***	6.89	(6.68, 7.10)	1010.962 ***	8.72	(8.56, 8.90)		
	S	9.28	(9.67, 10.19)		5.68	(5.49, 5.86)		11.4	(11.19, 11.61)					
Style length	L	6.86	(6.63, 7.08)	876.67 ***	4.06	(3.81, 4.30)	541.507 ***	10.53	(10.16, 10.89)	541.507 ***	8.99	(8.56, 9.33)		
	S	2.37	(2.15, 2.60)		1.25	(1.00, 1.49)		4.78	(4.42, 5.15)					
Stigmatic lobes length	L	1.31	(1.21, 1.41)	106.995 ***	0.75	(0.58, 0.91)	67.791 ***	1.78	(1.59, 1.98)	67.791 ***	1.27	(1.21, 1.33)		
	S	1.98	(1.89, 2.08)		1.4	(1.24, 1.57)		2.85	(2.66, 4.04)					
Stigma height	L	7.51	(7.29, 7.360	751.708 ***	4.43	(4.21, 4.65)	531.519 ***	11.42	(11.08, 11.76)	531.519 ***	9.62	(9.19, 9.95)		
	S	3.37	(3.14, 3.59)		1.95	(1.73, 2.17)		6.21	(5.87, 6.55)					
Herkogamy	L	2.14	(1.93, 2.35)	964.96 ***	1.81	(1.58, 2.04)	7.099 *	4.53	(4.17, 4.89)	7.099 *	1.28	(0.98, 1.67)		

		2.04	4.90)
S	6.56 (6.35, 6.77)	3.72 (3.49, 3.96)	5.19 (4.82, 5.56)

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Table 2. Slope (estimate $\pm$ s.e.) of the regression between anther height and stigma height with the corolla length for L- and S-flowers of *Psychotria* species, and results for the interaction effect organ x corolla term of the generalized mixed linear model (see Materials and Methods for details) to evaluate the significance of those relationships (Faire & McDade, 2001). In bold are highlighted the statistically significant slopes between corolla length the organ.

Species	L-morph			S-morph		
	Slopes(β)			Slopes(β)		
	Anthers	Stigma	<i>F</i> <i>P</i>	Anthers	Stigma	<i>F</i> <i>P</i>
<i>Psychotria deflexa</i>	0.11 $\pm$ 0.09	0.39$\pm$0.15	1.85 0.18	0.82$\pm$0.12	0.48$\pm$0.13	2.67 0.11
<i>P. nitidula</i>	0.57$\pm$0.08	0.33$\pm$0.11	3.50 0.07	0.48$\pm$0.19	0.02 $\pm$ 0.12	4.93 0.02
<i>P. trichophoroides</i>	0.09 $\pm$ 0.06	0.35$\pm$0.09	6.38 0.01	0.34$\pm$0.10	-0.14 $\pm$ 0.08	17.99 0.0001
<i>P. prunifolia</i>	0.55$\pm$0.15	0.81$\pm$0.36	0.27 0.60			

948 Table 3. Results for fruit set after the hand-pollination treatments in *Psychotria* species and reproductive indices calculated using fruit set
949 values (see details in Materials and Methods). Number of flowers used for pollinations (in brackets). Inter-morph and intra-morph stands
950 for intra-morph and inter-morph hand-pollinations, control stands for open pollination, SSE and Self stands for spontaneous self-pollination
951 and hand self-pollination respectively. The reproductive indices are self-incompatibility index (ISI; [Zapata & Arroyo 1978](#)) and
952 reproductive efficacy ratio (RE; Zapata & Arroyo, 1978).
953

Species	Hand-pollinations					Index		
	Morph	Inter-morph	Intra-morph	Control	SSE	Self	ISI	RE
<i>P. deflexa</i>	L	0.171 (42)	0.033 (61)	0.122 (113)	0.030 (66)	0 (41)	0,00	0,73
	S	0.138 (67)	0 (73)	0.036 (167)	0 (27)	0 (39)	0,00	0,27
	L	0.827 (40)	0 (22)	0.800 (26)	0 (49)	0 (32)	0,00	0,97
<i>P. niidula</i>	S	0.876 (39)	0 (28)	0.801 (30)	0 (27)	0 (49)	0,00	0,92
	L	0.573 (47)	0 (34)	0.332 (102)	0.293 (47)	0.201 (40)	0,30	0,55
	S	0.656 (40)	0 (58)	0.193 (61)	0.255 (35)	0.125 (37)	0,12	0,30
<i>P. trichophoroides</i>	L	0.829 (35)	0.850 (39)	0.575 (27)	0.445 (49)	0,75	1,17	
	S	0.829 (35)	0.850 (39)	0.575 (27)	0.445 (49)	0,75	1,17	
	Monomorphic	-	0.829 (35)	0.850 (39)	0.575 (27)	0.445 (49)	0,75	1,17

Table 4. Results of the generalized mixed linear model analysis for the comparisons of the fruit set after different hand-pollination treatments (see Materials and Methods for details) in four *Psychotria* species. In *P. prunifolia*, only differences among treatments were considered since it is a monomorphic species.

	<i>P. nitidula</i>			<i>P. deflexa</i>			<i>P. prunifolia</i>			<i>P. trichophoroides</i>		
	<i>F</i>	df	<i>P</i>	<i>F</i>	df	<i>P</i>	<i>F</i>	Df	<i>P</i>	<i>F</i>	df	<i>P</i>
Morph	0.068	1	0.795	2.460	1	0.130	-	-	-	0	1	1
Treatment	26.875	4	0	3.029	4	0.038	7.162	3	0.001	7.485	4	0
Treatmen x Morph	0.161	4	0.957	0.756	4	0.564	-	-	-	1.046	4	0.397

Table 5. Pollen-tube growth in *Psychotria* species. The place where the pollen tubes stopped growing (stigma, upper- or mid style) was determined in sets of ten flowers 4 h and 8 h after hand pollinations. For each illegitimate treatment (self-pollination and intra-morph pollination) the place and percentage of pollen tubes stopping at that point after each hour period (4 h and 8 h, respectively) is provided. For the legitimate pollinations all the pollen tubes reached the lower part of the style (results not shown).

Species	Morph	Treatment	
		Self-pollination Place % at 4h, 8h	Intra-morph Place % at 4h, 8h
<i>P. deflexa</i>	L-morph	Upper style / Mid style 100, -/ -, 50	Upper style 100, 100
	S-morph	Stigma 100, 100	Stigma 100, 100
<i>P. nitidula</i>	L-morph	Stigma 100, 100	Upper style 100, 100
	S-morph	Stigma 100, 100	Stigma 100, 100
<i>P. prunifolia</i>	L-morph	Low style 100, 100	Low style 100, 100
<i>P. trichophoroides</i>	L-morph	Stigma 100, 80	Mid style 100, 70
	S-morph	Stigma 100, 100	Stigma 100, 100

Table 6. Summary of the incompatibility system and place of incompatibility reaction in the family Rubiaceae. Distily*= Presence of individuals homostily. Br=Brazil, Cr=Costa Rica. SC= Self-compatible, SI= Self-incompatible. Reference: 1) Bawa & Beach, 1983; 2) Castro & Araújo, 2004; 3) Castro *et al.*, 2004; 4) Coelho, 2013; 5) Coelho & Barbosa, 2004; 6) H. Consolaro, unpubl. data; 7) Consolaro *et al.*, 2009; 8) Faivre & McDade, 2001; 9) Faivre, 2002; 10) Jung-Mendacoli & Melhem, 1995; 11) Klein *et al.*, 2009; 12) Machado *et al.*, 2010; 13) Maruyama, Amorim & Oliveira, 2010; 14) Novo, 2010; 15) Oliveira, 2008; 16) Passos & Sazima, 1995; 17) Pereira *et al.*, 2006; 18) Rossi *et al.*, 2005; 19) Teixeira & Machado, 2004a,b.

Genus/Species	Number of Species	Polymorphism	L-interrupted	S-interrupted	L	S	Homology	Country	Reference
<i>Guettarda platypoda</i>	1	Distily	Style	Stigma	SC	SI	-	Br	14
<i>Sabicea cinerea</i>	1	Distily	Style	Stigma	SI	SI	-	Br	19
<i>Coussarea</i>	2	Distily	Stigma (50%)/Style (50%)	Style (25%)/Stigma (75%)	SI	SI	-	Cr	1
<i>Faramea</i>	3	Distily	Stigma	Stigma	SI	SI	-	Br and Cr	1, 6, 13
<i>Palicourea</i>	7	Distily*	Stigma (14.3%)/Style (85.7%)	Stigma (14.3%)/Style (85.7%)	SI	SI	SC	Br	7, 12, 17
<i>Psychotria</i>	25	Distily (96%)/Homostily (4%)	Style (75%)/Stigma (25%)	Style (88%)/Stigma (12%)	SI (90.5) SC (9.5)	SI (90.5) SC (9.5)	-	Br and Cr	1, 2, 3, 4, 5, 8, 10, 11, 15, 17, 19
<i>Rudgea</i>	2	Distily	Style (50%)/Stigma (50%)	Stigma	SI	SI	-	Br	1, 17
<i>Bouvardia ternifolia</i>	1	Distily	Style (50%)/Stigma (50%)	Stigma	-	-	-	Cr	8, 9

Manettia luteo-rubra 1 Distyl Style (50%)/Stigma (50%) Stigma SI SI - Br 16

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987 Table 7. Floral visitors and number of visits in four species of *Psychotria* species. The number of
988 visits corresponds to 24 h observation for each species.

Insect species	<i>P. deflexa</i>	<i>P. nitidula</i>	<i>P. prunifolia</i>	<i>P. trichophoroides</i>
<i>Aellopos titan</i>	0	0	20	0
<i>Apis mellifera</i>	10	1794	0	0
<i>Augochloropsis</i> sp1.	37	0	0	0
<i>Augochloropsis</i> sp2.	4	0	0	0
<i>Augochloropsis</i> sp3.	16	0	0	0
<i>Epicharis cockerelli</i>	0	0	0	33
<i>E. flava</i>	0	0	0	20
<i>E. rustica flava</i>	0	0	128	0
<i>Euglossa</i> sp1.	0	20	54	11
<i>Euglossa</i> sp2.	0	0	4	10
<i>Eulaema nigrita</i>	0	159	0	5
<i>Lophopedia pygmaea</i>	18	0	0	0
<i>Monoeca</i> sp.	0	0	10	0
<i>Paratetrapedia flaveola</i>	26	0	0	4
<i>Partamona rustica</i>	16	19	0	0
<i>Pepsis</i> sp.	90	0	0	0
<i>Pseudoaugochlora</i> sp.	0	0	7	5
<i>Ptiloglossa</i> sp.	15	0	0	0
<i>Rhathymus bicolor</i>	0	0	0	5
<i>Rhathymus</i> sp.	0	0	0	3

<i>Urbanus proteus</i>	0	0	29	0
Vespoidea sp.	19	0	0	6
TOTAL	251	1992	252	102

Figure 1. Overview of flowers and inflorescences of *Psychotria* species. A and B: *P. nitidula*, L-morph and S-morph, respectively. C and D: *P. deflexa*, L- and S-morph, respectively. E and F: *P. trichophoroides*, L- and S-morph, respectively. Bars = 10 mm.
225x399mm (300 x 300 DPI)

Psychotria nitidula

Psychotria deflexa

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Psychotria trichophoroides

Psychotria prunifolia

Treatment