

Flexible mating system in distylous populations of *Psychotria carthagenensis* Jacq. (Rubiaceae) in Brazilian Cerrado

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Received: 22 March 2011 / Accepted: 9 November 2011
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Abstract In family Rubiaceae distyly is very common, and large variation in heterostyly characteristics has been previously documented. Analysis of these variations, even within a species, is very useful to our understanding of the evolutionary process that caused this polymorphism. For this reason, the goal of this study is to investigate the floral morphology and diallelic incompatibility system of three populations of *Psychotria carthagenensis*. The three studied populations of *P. carthagenensis* occur in forest fragments in protected areas in an urban matrix in the Municipality of Campo Grande, Mato Grosso do Sul State, Brazil. Reciprocal position of style length and stamen height was found in populations, and in general short-styled flowers were larger than long-styled flowers. All populations presented 1:1 morph ratio between short-styled

and long-styled flowers. Regarding breeding system, flowers of *P. carthagenensis* were self-compatible and compatible within plants of the same morph, and there was no pollen limitation in the populations in any case. In only one of the populations there were differences in the extent of compatibility between morphs, with the long-styled morph being more self-compatible than the short-styled morph. The reproductive strategy of these populations can be advantageous in case of fluctuation of pollinator activity.

Keywords Distyly · Floral polymorphism · Reciprocal herkogamy · Self-incompatibility system

Introduction

Heterostyly is a morphological and genetic plant polymorphism in which populations are composed of different floral morphs that differ reciprocally in the height of their stigmas and anthers (Ganders 1979). Distylous species are characterized by long-styled flowers with short stamens and short-styled flowers with long stamens (Barrett and Richards 1990). This sexual polymorphism has evolved independently in at least 28 animal-pollinated angiosperm families through convergent selective pressure associated with cross-pollination (Barrett 2002). Heterostyly has traditionally been seen as a mechanism to improve legitimate pollination and reduce pollen wastage (Darwin 1877; Lloyd and Webb 1992a, b).

Heterostylous species usually possess a sporophytic diallelic incompatibility system, which prevents self- and intramorph fertilization (Ganders 1979), and a set of ancillary characters which mainly involve differences between morphs in pollen, style, and stigma or corolla

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features (Vuilleumier 1967; Ganders 1979; Barrett and Richards 1990; Dulberger 1992). The association between morphological (reciprocal herkogamy) and physiological (incompatibility system) characteristics is very interesting to study, since the proposed evolutionary models for heterostyly include different orders in their origin and relationships. Thus, Charlesworth and Charlesworth (1979) proposed that acquisition of a very simple, diallelic incompatibility system would be the first necessary step in the evolutionary process towards acquisition of heterostyly and a heteromorphic incompatibility system, being linked to some extent with reciprocal herkogamy in this model. Contrarily, Lloyd and Webb (1992a) proposed that incompatibility reactions would evolve after emergence of reciprocal herkogamy as a result of pollen specialization for legitimate pollination from a herkogamous ancestor via stigma-height polymorphism, in this case without the need for a link between the two. For this reason, descriptions of such characteristics in plant species and populations can be very useful for future evolutionary studies. Variations in morphological and/or incompatibility patterns can be found among congeneric species and even among populations (Li and Johnston 2001; Castro et al. 2007; Sakai and Wright 2008; Ferrero et al. 2011a, b). In addition, cases of variation in the incompatibility system within the same genus have already been reported [e.g., in *Narcissus* (Pérez-Barrales et al. 2006) or *Lithodora* (Ferrero et al. 2011c)], or even a breakdown of the incompatibility system associated to reproductive assurance (e.g., Barrett and Shore 1987; Schoen et al. 1997).

Moreover, lack of heteromorphic incompatibility means that plants can be pollinated by pollen of plants of the same morph. Taking into account the genetic inheritance of this polymorphism (Lewis and Jones 1992), deviations from a state of equilibrium in which both morphs present the same proportion (isoplethy) can occur. Basic variations in morph proportion include monomorphic as population with a single morph (pin or thrum population) and anisoplethy as population with morphs in a proportion different from 1:1 (Ganders 1979).

In family Rubiaceae, heterostyly is very common, mainly in subfamily Rubioideae and within tribe Psychotrieae (Barrett and Richards 1990). South America contains nearly one-third of total species of Rubiaceae in the world (Chiquieri et al. 2004). Among the Rubiaceae occurring in Neotropical regions, *Psychotria* has been well studied in relation to floral biology and breeding systems. Previous studies have described the genus as distylous, although there is not a perfect reciprocal position between anthers and styles in all cases. Moreover, there is a broad spectrum of breeding systems in the genus, in which different degrees of self-compatibility can be observed (Bawa and Beach 1983; Faivre and McDade 2001; Castro and Araujo

2004; Castro et al. 2004; Teixeira and Machado 2004; Rossi et al. 2005; Pereira et al. 2006; Sakai and Wright 2008).

Psychotria carthagenensis is an understory shrub, 2–3 m high, with distribution covering from Costa Rica to Argentina (Delprete et al. 2005). In Brazil, previous studies addressed the floral morphology and breeding systems of *P. carthagenensis*, describing homostylous populations in the northeast of the country (Demetrio and Machado 2005), self-incompatibility in the middle west (Pereira 2007; Koch et al. 2010), and also self-compatibility in the southeast (Consolaro et al. 2011). Nonetheless, all the cited studies focused on a single population, thus preventing interpopulation comparisons. Moreover, some of such studied populations were anisoplethic (i.e., presented a proportion of morphs different from 1:1) (Pereira 2007) or even monomorphic (Consolaro et al. 2011). All these studies open the question of whether variations in morphology are associated with changes in incompatibility system type, as described for other distylous genera (Ferrero et al. 2011c). Thus, the goal of this study is to investigate the breeding system by hand pollination, the floral morphology, and the proportion of morphs in three populations of *P. carthagenensis* occurring in urban forest fragments. These were analyzed to increase the information available for future studies on the definition of the status of the species and to analyze whether the incompatibility and morphological heterostylous characteristics are associated or not.

Materials and methods

Study areas

The present study was performed in Campo Grande Municipality, Mato Grosso do Sul State, Brazil, in the Brazilian Cerrado biome domain. The three chosen populations of *P. carthagenensis* are located in forest fragments in protected areas in an urban matrix: one in a state park (Parque Estadual do Prosa, PEP, 20°27'00"S, 54°33'46"W, comprising 135 ha) and two in biological reserves (Reserva Biológica da Universidade Federal do Mato Grosso do Sul, UFMS, 20°29'58"S, 54°36'50"W, comprising 35 ha; Reserva Natural da Empresa Brasileira de Pesquisa Agropecuária—Gado de Corte, EMBRAPA, 20°25'41"S, 54°43'03"W, extension 175 ha). The average distance among populations is 12.5 km. In all areas, *P. carthagenensis* is present in moist soils and near to small watercourses. The climate in the region is Tropical Savanna (Aw type cf. Köppen 1948), characterized by a pronounced dry season from May to September and a rainy season from October to April. Mean annual precipitation is 1,532 mm, and annual mean temperatures range between 20°C and

22°C (EMBRAPA-CNPGC 1985). In this region, *P. carthagenensis* flowers between October and December. The experiments and data collection were conducted in 2008 and 2009 during the blooming period of *P. carthagenensis*.

Floral morphometrics

Psychotria carthagenensis has tubular white flowers arranged on terminal inflorescences. Flowers have a pentalobed calyx and corolla. Flowers started to open at ca. 0700 h, when the stigma is receptive and pollen and nectar are also available to floral visitors, and wilted at dusk. The ovary has two ovules, and the fruits are red drupaceous and bear one or two nutlets (R.R. Faria pers. comm.). In each population of *P. carthagenensis*, 102 flowers (51 per morph, 1 flower per individual) were randomly collected, and conserved in 70% alcohol. In the laboratory, measures of floral structures were carried out using a digital caliper to the nearest 0.01 mm. Traits measured were: (1) corolla length, (2) diameter of corolla opening, (3) anther length, (4) anther height, (5) style length, and (6) ovary length. The herkogamy distance (separation between stamen height and style length) was calculated afterwards (Fig. 1). All measures are presented as means of the coefficient of variation and the arithmetic average. The reciprocity between the sexual whorls of the two morphs was calculated for each of the three populations of *P. carthagenensis* using the index of Sánchez et al. (2008). This index is based on comparison of the position of every single anther of each flower with the stigmas of all flowers of the opposite morph in a population. When reciprocity is perfect, the value of the index is zero. Values depart from zero when reciprocity is not perfect, and are modulated by the average standard deviation of height gaps, so the greater the dispersion, the greater the departure from zero (for a complete description, see Sánchez et al. 2008; computational software available at <http://webs.uvigo.es/plantecology/software.es.html>).

Scatter plots were made to show the variation in style length and anther height. Plots summarize the individuals in the populations sampled, ordered by increasing style length. The other measurements were analyzed by two-way analysis of variance (ANOVA) with morph as fixed factor and population as random factor.

Morph ratio

The morph ratio of flowers in the three populations of *P. carthagenensis* was assessed in ten 10 × 10 m² quadrants distributed at random. Within the quadrants, all isolated flowering individuals of each morph, separated by more than 50 cm from other individuals of the same species, were counted. This separation distance was established after a procedure to identify clonal growth. This

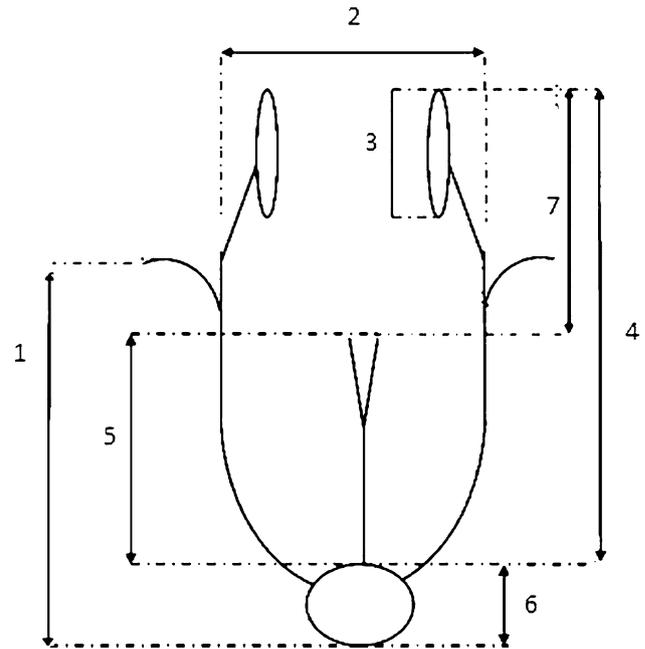


Fig. 1 Morphometric measurements carried out in flowers of *Psychotria carthagenensis*: 1 corolla length, 2 corolla opening, 3 stamen length, 4 anther height, 5 style length, 6 ovary length, and 7 herkogamy distance separation (calculated afterwards). Adapted from García-Robledo and Mora (2007)

procedure consists in excavations around a focal plant to identify connections between ramets. In all observations ($n = 10$, per population), ramets were not observed beyond 30 cm. The morph ratio was tested with standard chi-square tests against the expectation of 1:1 with alpha level of 5%.

Breeding system

To investigate the reproductive system of *P. carthagenensis*, the following treatments were employed: (1) intermorph hand cross-pollination ($L \times S$ or $S \times L$), (2) intramorph hand cross-pollination ($L \times L$ or $S \times S$), (3) obligatory autogamy, and (4) agamospermy (bagged emasculated flowers). Furthermore, we observed natural fruit formation in (5) spontaneous self-pollinated flowers (which were only bagged but not hand pollinated) and (6) control flowers (unbagged flowers during anthesis). Hand crossed flowers (treatments 1, 2, and 3) were emasculated before pollinations, and to avoid herbivory, all treated inflorescences were bagged at the end of experiments. After 4–5 weeks, fruit production was recorded in order to calculate fruit set. In all treatments, the flowers treated were tagged with colored lines and the whole inflorescences were bagged because of the small size of the flowers. In addition, other flowers and buds present in the inflorescence were removed. The six treatments were

conducted in a single plant and replicated in 20 plants per morph in each population.

Flower anthesis in *P. carthagenensis* begins approximately between 0500 and 0600 h, in both morphs. At this time, the corolla lobes are totally separated and perpendicularly positioned in relation to the floral axis, with reproductive structures exposed. Stigmatic receptivity begins when the lobes are opened. Thus, hand pollinations were carried out between 0800 and 1300 h, using pollen extracted from anthers of recently opened flowers. Flower senescence occurs between 1700 and 1800 h of the same day (Koch et al. 2010).

For each morph and population, several reproductive indices were calculated. These indices were calculated as the ratio between fruit set obtained in the following treatments: ISI (self-incompatibility index), from obligatory autogamy/intermorph crosses; ISS (spontaneous self-pollination index), from spontaneous autogamy/obligatory autogamy; and RE (reproductive efficacy index) from control/intermorph crosses. For all these indices, we considered values similar to or lower than 0.25 as indicative of self-incompatibility (for ISI), spontaneity in self-pollination (for IAS), and pollen limitation (for RE) (Sobrevilla and Arroyo 1982).

To test differences among treatments, a *G* test was performed between autogamy, intra- and intermorph crosses, and between control and intermorph crosses with alpha level of 5% (Sokal and Rolf 1995).

Results

Floral morphometrics

Mean values and coefficients of variation (CV) of the measurements of flower traits for the three populations are given in Table 1. In general, short-styled flowers were significantly larger than long-styled ones and flowers from EMBRAPA larger than those from UFMS. The highest value of reciprocity between floral whorls was found in PEP (indicated by the lowest index value), whereas the lowest was recorded in EMBRAPA (Tables 1, 2). The scatter plots show separation between style and stamens in the three studied populations (Fig. 2).

Morph ratio

A total of 234 individuals in UFMS, 226 in PEP, and 375 in EMBRAPA populations were sampled (Table 3). The proportion of morphs in all populations was isoplethic, i.e., not differing from 1:1 proportion. There was no significant difference from expectations (1:1) in the chi-square analysis.

Table 1 Corolla length, corolla opening, anther length, anther height, stigma height, herkogamy distance, and reciprocity values obtained for the three studied populations of *Psychotria carthagenensis*

Population	Corolla length (mm)		Corolla opening (mm)		Anther length (mm)		Anther height (mm)		Stigma height (mm)		Ovary length (mm)		Herkogamy distance (mm)		Reciprocity
	L	S	L	S	L	S	L	S	L	S	L	S	L	S	
EMBRAPA	5.017 (12.7)	5.375 (12.2)	1.600 (14.9)	1.775 (28.1)	1.007 (10.5)	1.178 (13.0)	4.652 (9.7)	7.094 (0.13)	6.493 (10.1)	4.454 (14.3)	1.223 (14.7)	1.208 (14.5)	1.854 (30.9)	2.504 (26.1)	0.025
PEP	4.901 (15.0)	5.337 (12.1)	1.811 (24.6)	1.880 (11.8)	0.849 (11.6)	1.128 (14.8)	4.434 (13.6)	7.446 (11.4)	7.119 (12.9)	4.579 (11.5)	1.070 (16.9)	1.072 (14.8)	2.336 (28.4)	2.570 (35.4)	0.020
UFMS	4.536 (11.4)	4.884 (13.9)	1.660 (15.6)	1.652 (14.5)	1.059 (11.0)	1.219 (12.1)	4.128 (11.7)	6.711 (11.7)	6.507 (12.9)	4.236 (13.4)	1.106 (15.0)	1.064 (15.6)	2.434 (23.4)	2.716 (22.6)	0.024

Values for long-styled (L, n = 51 per population) and short-styled (S, n = 51 per population) flowers are given in mm as arithmetic mean (coefficient of variation), except in the case of index of reciprocity

Table 2 Results of two-way ANOVA for comparison of corolla length, corolla opening, anther length, ovary length, and herkogamy distance for long-styled ($n = 51$ per population) and short-styled flowers ($n = 51$ per population), and populations of *Psychotria carthagenensis*

	Corolla length (mm)			Corolla opening (mm)			Anther length (mm)			Ovary length (mm)			Herkogamy (mm)			
	df	MS	F	P	MS	F	P	MS	F	P	MS	F	P	MS	F	P
Morph	1	11.066	26.277	**	0.466	4.120	*	3.169	176.378	**	0.026	0.897	n.s.	11.562	26.014	**
Population	2	6.957	16.520	**	1.053	9.312	**	0.605	33.676	**	0.65	22.108	**	4.197	9.444	**
Mor × pop	2	0.06	0.142	n.s.	0.215	1.899	n.s.	0.11	6.097	**	0.013	0.434	n.s.	1.318	2.966	*
Error		0.421			0.113			0.018			0.029			0.444		

Values differ significantly at * $P < 0.05$ and ** $P < 0.01$; n.s. nonsignificant difference

Fig. 2 Variation in style length (plus symbols) and mean anther height (filled circles) in three populations of *Psychotria carthagenensis*, Mato Grosso do Sul, Brazil. Plots summarize individuals in sampled populations, ordered by increasing style length

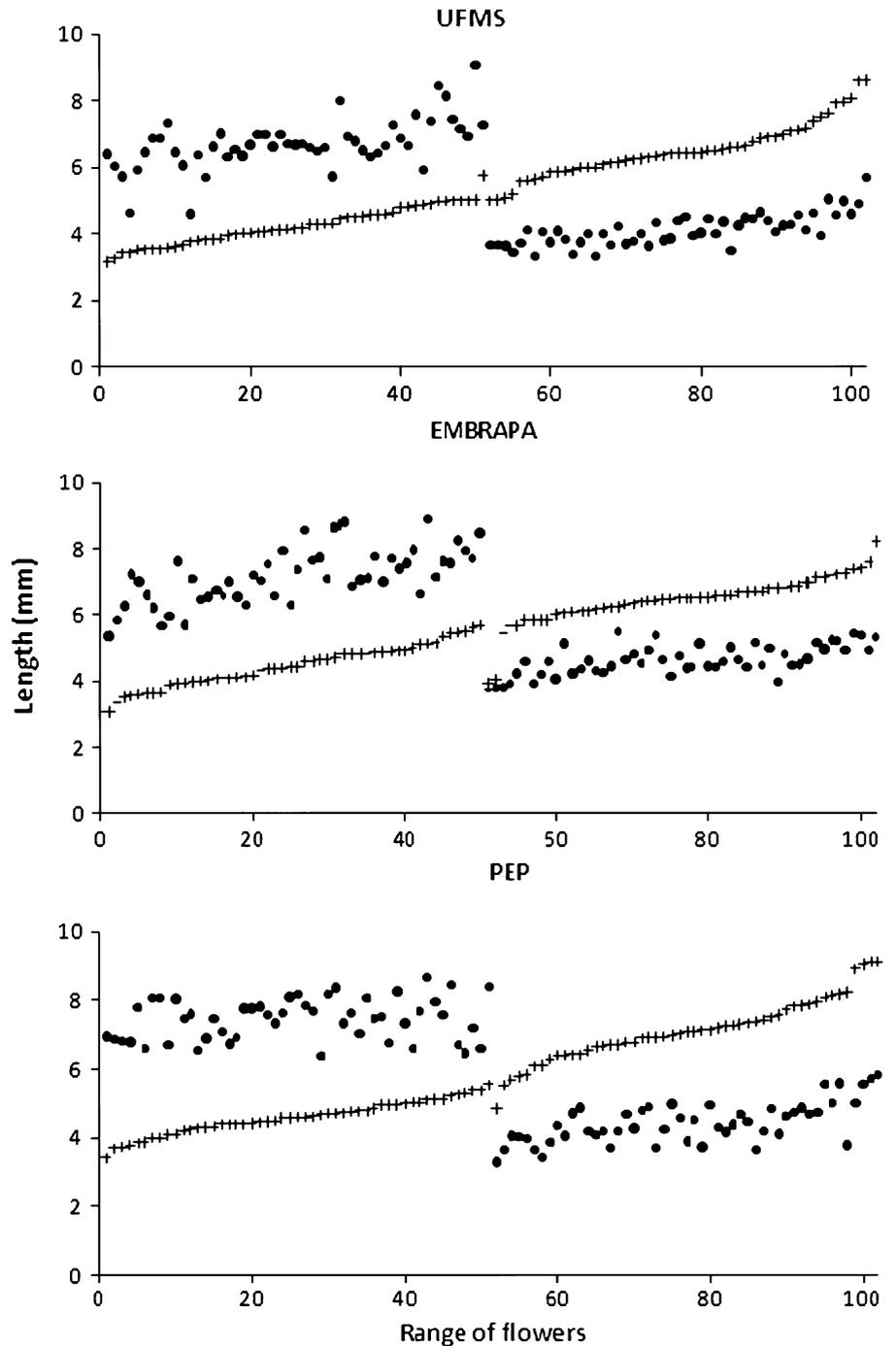


Table 3 Abundance of long- (*L*) and short-styled (*S*) individuals, and values of chi-square test for deviations from isoplethy in the morph ratio for each of the three studied populations of *Psychotria carthagenensis*, Mato Grosso do Sul, Brazil

	<i>S</i> abundance	<i>L</i> abundance	Chi-square
PEP	126	100	$\chi^2 = 2.991$, n.s.
UFMS	127	107	$\chi^2 = 1.709$, n.s.
EMBRAPA	198	177	$\chi^2 = 1.776$, n.s.

Breeding system

Results of hand pollination experiments are shown in Fig. 3. Agamospermy treatment produced no fruits except for 5% of long-styled individuals in EMBRAPA. In other hand treatments, values of fruit set ranged between 5% and 45%. Regarding the heteromorphic incompatibility system, there were no significant differences when comparing treatments of autogamy, intra- and intermorphs (except in population PEP; Table 4). Fruit set on control flowers of the three populations ranged between 1% and 30%. Regarding pollen limitation, there was no difference between control and intermorph crosses among all populations, or between morphs or among treatments (Table 4). Studied populations varied according to the calculated reproductive indices (Sobrevilla and Arroyo 1982). For the ISI index, values of 1.50 in PEP, 1.50 in UFMS, and 0.71 in EMBRAPA were found. The ISS index was 0.08 in PEP, 0.40 in UFMS, and 0.25 in EMBRAPA. Finally, for the RE index, a value of 0.38 was obtained for PEP, 0.43 for UFMS, and 1.38 for EMBRAPA. The three populations can be considered as self-compatible, and individuals from EMBRAPA and PEP present the capacity of spontaneously self-pollination (ISI and ISS indices similar to or lower than 0.25). None of the studied populations showed evidence of pollen limitation (RE higher than 0.25).

Discussion

In this study, variations in breeding system and floral morphology at morph and population levels are reported for three isoplethic populations of *P. carthagenensis*, a heterostylous plant species that is widely distributed in the Neotropical region.

Evolution of heterostyly usually implies acquisition of all of its related features: reciprocal herkogamy, self-incompatibility, and ancillary characters (Lloyd and Webb 1992a, b), with all of them being present in the final state. For this reason, description of species where different combinations of these characteristics are present becomes crucial to improve understanding of this kind of floral polymorphism. In fact, such species have been largely

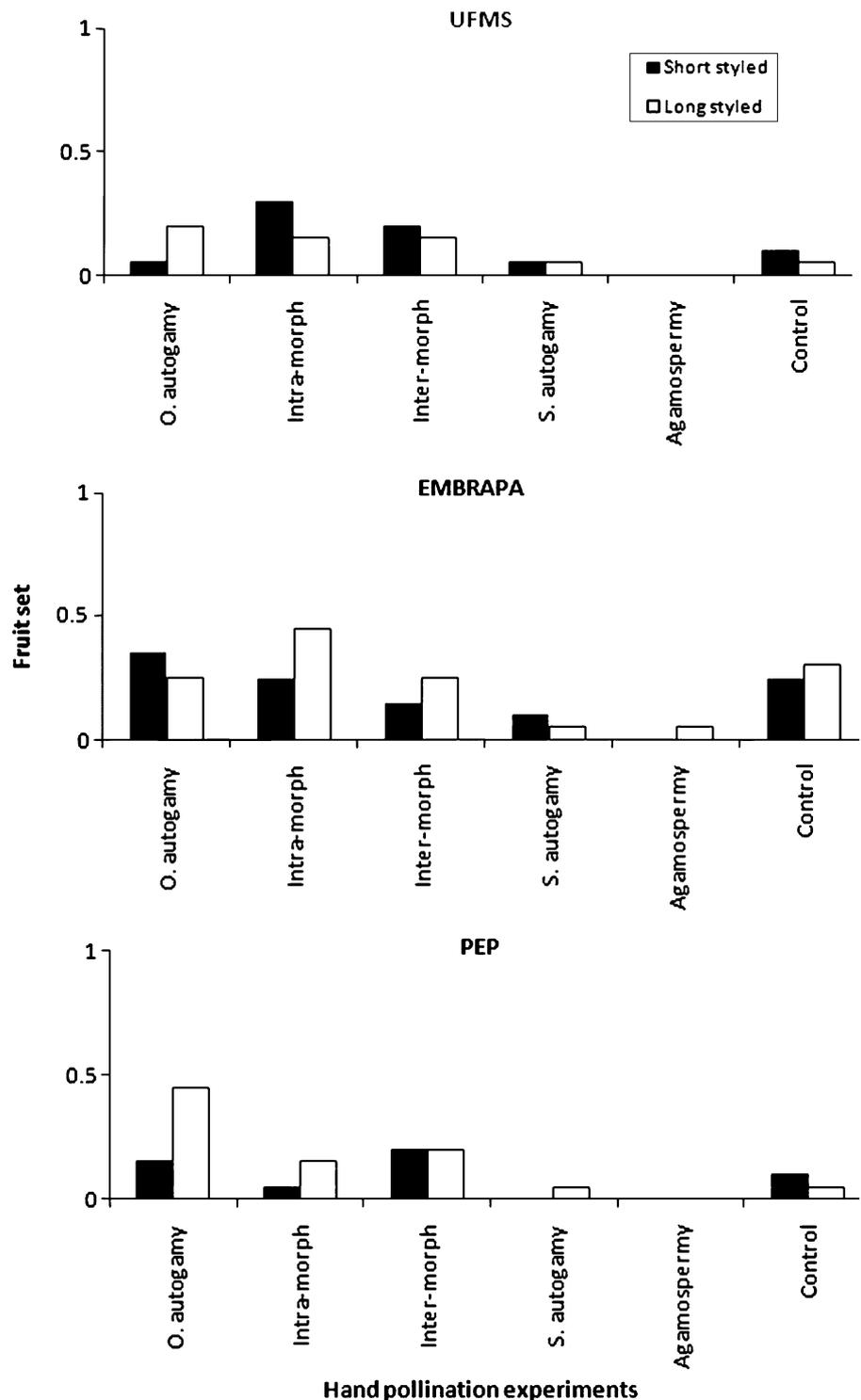
characterized as anomalous heteromorphic (Barrett and Richards 1990). Nowadays, different types of stylar polymorphism have been described. Distyly means a reciprocal position between sexual whorls of both morphs, whereas stigma dimorphism consists in two morphs within the population that differ in the position of styles (above or below stamens) but present the same height for the anthers. Taking into account the current classification, the analyzed populations of *P. carthagenensis* can be defined as distylous.

In Rubiaceae, the incompatibility system has traditionally been associated with reciprocal herkogamy (e.g., Bawa and Beach 1983). In genus *Psychotria*, a wide variety of breeding systems in which incompatibility is also linked to reciprocal position of sexual whorls has been reported (Castro and Araujo 2004; Castro et al. 2004; Pereira et al. 2006; Sakai and Wright 2008). In the studied populations of *P. carthagenensis* reciprocal herkogamy is not linked to the incompatibility system, as previously found by Consolaro et al. (2011). Similar cases, in which the incompatibility systems seem not to be linked to genes responsible for style polymorphisms, have been found in other taxa (Barrett and Harder 2005; Ferrero et al. 2011c). Because of the hand pollination results, it seems reasonable that the mating system in these populations would be modified due to self-fertilization (Barrett and Richards 1990; Van Rossum et al. 2006). A flexible mating system takes advantage of high pollinator activity, by producing largely outcrossed progeny, but does not sacrifice fecundity when pollinators are less frequent (Barrett and Cruzan 1994).

Reciprocity is important for maintenance of heterostyly in plant populations because it has been proposed to favor allogamous pollinations between morphs, increasing disassortative pollen transfer and reducing pollen waste (Lloyd and Webb 1992a, b; Barrett 2002). As a consequence of efficient disassortative mating and because of the negative frequency-dependent selection, the frequencies of morphs should be equal (Charlesworth and Charlesworth 1979). A balanced proportion of morphs at population level does not seem to be a rule for *P. carthagenensis* (Pereira 2007; Consolaro et al. 2011), even with deviations to monomorphism (Demetrio and Machado 2005). However, we found that the populations present the same proportion of morphs, which can be explained in the presence of efficient pollinator fauna (Pérez-Barrales et al. 2006). Such a pattern has already been found in the same species (Koch et al. 2010).

In the present study, high reciprocity was found in the studied populations of *P. carthagenensis*. In addition, there was a general pattern for short-styled flowers to be larger. This pattern has already been shown by other authors (see review in Ganders 1979) and is frequent in Rubiaceae (Favre and McDade 2001; Castro and Araujo 2004; Castro et al. 2004), although it is not a rule (Consolaro 2008).

Fig. 3 Fruit set after hand pollination treatments: O. autogamy (hand self-pollination), intramorph (intramorph hand cross-pollination), intermorph (intermorph hand cross-pollination), S. autogamy (spontaneous self-pollinated flowers), agamospermy (bagged and emasculated flowers), and control (without any treatment and free access to floral visitors) in three populations of *Psychotria carthagenensis*, Mato Grosso do Sul, Brazil ($n = 20$ flowers per treatment/morph/population)



It has been associated with the result of the ontogenetic structural arrangement on stamens and styles (Dulberger 1992; Richards and Barrett 1992; Faivre 2000).

In this study two different aspects related to heterostyly, which are very important when analyzing the evolution of this polymorphism, were analyzed. In light of the present

results, there seems to be a lack of association between the SI and the reciprocal herkogamy, which makes this genus an interesting subject for study of the evolutionary pathway to heterostyly. *P. carthagenensis* appears to be an intermediate stage in the evolution of heterostyly, and the reciprocal herkogamy appears to be effective in promoting

Table 4 Results for the *G* test between autogamy, intramorph and intermorph crosses; between control and intermorph crosses; and between autogamy and intramorph crosses for *Psychotria carthagenensis* in the three studied populations

	Autogamy versus intramorph versus intermorph			Control versus intermorph		
	<i>G</i> test	<i>df</i>	<i>P</i>	<i>G</i> test	<i>df</i>	<i>P</i>
EMBRAPA	3.83	2	0.16	0.121	1	0.73
UFMS	1.78	2	0.41	0.080	1	0.78
PEP	6.90	2	0.03	0.249	1	0.62

In all cases degrees of freedom and *P* value are shown

disassortative mating in the studied populations even in the absence of an incompatibility system. In addition, this study increases the information available for future studies aimed at elucidating the evolution of this polymorphism.

Acknowledgments The authors thank V. Pott for identifying the plants, P. Menezes and V.A. Laura for facilitating access to the PEP and EMBRAPA reserves, respectively, and M.R. Sigrist, A.P.L. Lemke, and C.C. Castro for helpful comments. Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (FUNDECT)/Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) provided R.R.F. with a doctoral fellowship (41/100.271/2006); CAPES provided R.R.F. with a grant in the Sandwich Doctorate Program (1964/10-0); FUNDECT provided financial support for the study (23/200.288/2008). L.N. was supported by the Spanish Dirección General de Investigación, Ciencia y Tecnología (DGICYT) through grants CGL2009-10466, the Xunta de Galicia through grant INCITE09-3103009PR, FEDER funds from the European Union and projects AECID A/023710/09, and CYTED 409AC0369, and the work of V.F. was supported by the Fundación Ramón Areces.

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