Pollinator availability, mating system and variation in flower morphology in a tropical savanna tree

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ABSTRACT

Widely distributed organisms face different ecological scenarios throughout their range, which can potentially lead to micro-evolutionary differentiation at specific localities. Mating systems of animal pollinated plants are supposed to evolve in response to the availability of local pollinators, with consequent changes in flower morphology. We tested the relationship among pollination , mating system, and flower morphology over a large spatial scale in Brazilian savannas using the tree *Curatella americana* (Dilleniaceae). We compared fruit set with and without pollinators in the field, and analyzed pollen tube growth from self- and cross-pollinated flowers in different populations. Populations with higher natural fruit set also had lower fruit set in bagged flowers, suggesting stronger barriers to self-fertilization. Furthermore, higher levels of autogamy in field experiments were associated with more pollen tubes reaching ovules in self-pollinated flowers. Morphometric studies of floral and leaf traits indicate closer-set reproductive organs, larger stigmas and smaller anthers in populations with more autogamy. We show that the spatial variation in mating system, flower morphology and pollination previously described for herbs also applies to long-lived, perennial tropical trees, thus reemphasizing that mating systems are a population-based attribute that vary according to the ecological scenario where the plants occur.

Keywords: Cerrado savanna, mating system, pollination biogeography, reproductive assurance, self-compatibility

Introduction

The interactions established between organisms can vary greatly throughout space and time (Thompson 2005). For instance, plants can interact with contrasting assemblages of pollinators throughout their geographical range (Gómez *et al*. 2014; Herrera 2005; Waser *et al*. 1996). The resulting pollination interactions can vary from specialist to generalist for both plants and animals, and may have strong implications for the evolution of plant mating systems (Gómez 2002; Dart *et al*. 2012; Barrett 2013). Historically, plant mating systems were considered an attribute of the

species (Brys & Jacquemyn 2011; Rosas-Guerrero *et al*. 2014), even though their lability between populations was pointed out over 40 years ago (Stebbins 1974). Therefore, the mating system may vary within a species' range due to trade-offs between genetic variability, promoted by cross-pollination, and reproductive assurance from self-pollination, which vary in response to different ecological scenarios throughout space and time (Barrett *et al*. 1989; Moeller 2006; Opedal *et al*. 2016). For example, some herb species with mixed mating systems show different levels of autogamy throughout their ranges (Kalisz & Vogler 2003; Moeller 2006).

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To rely on a mixed mating system, and oscillate between the two extremes of selfing and outcrossing as the environment demands, may be a consequence of the reproductive assurance hypothesis proposed by Darwin (1876), which was later re-elaborated (Baker & Stebbins 1965; Baker 1967). According to this hypothesis, mechanisms ensuring autogamy should be favoured in areas with low pollinator availability (Lloyd 1992; Herlihy & Eckert 2002). On the other hand, shorter-lived plants experience stronger selection for reproductive assurance (more prone to selfing), as they usually only experience one or few reproduction events early in life (Moeller *et al.* 2017).

Besides spatially structured processes, past climate dynamics and stability have also affected species distribution and diversity patterns (Svenning & Skov 2007; Cárdenas *et al.* 2011; Sandel *et al.* 2011; Kissling *et al.* 2012), population demography and genetic structure (Grazziotin *et al.* 2006; Cabanne *et al.* 2007), and the structure of mutualistic plant-pollinator assemblages (Dalsgaard *et al.* 2011; 2013). Therefore, just as the lack of reproductive partners during range expansion or small population size may currently favour individuals with higher levels of autogamy (Ivey & Carr 2012; Levin 2012; Hargreaves & Eckert 2013; Griffin & Willi 2014), this could have happened over geological time.

Changes in mating systems are usually associated with variation in a group of floral traits (Berg 1960; Armbruster *et al.* 1999). For example, transitions to higher levels of autogamy are usually related to reduction in flower size and herkogamy (the separation between male and female reproductive organs within a flower - Wyatt 1988), faster flower development time (Armbruster *et al.* 2002; Mazer *et al.* 2004), and flowering earlier in the season (Mazer *et al.* 2004; Martin & Willis 2007). Other flower traits, such as the reduction of rewards and attractiveness in the display (Ornduff 1969; Dudley *et al.* 2007), are hypothesised to follow the previously mentioned primary modifications. Most research has focused on herb species, which are known to consistently present lower outcrossing rates (Moeller *et al.* 2017). Therefore, the life history (annual, perennial, etc.) and growth form (herb, vine, tree, etc.) are the main predictors of outcrossing rates among flowering plants (Moeller *et al.* 2017). To the best of our knowledge, only one studied has described the geographical variation in the mating system and flower morphology for a non-herbaceous species, in this case a vine (Opedal *et al.* 2016).

In a recent and extensive review of mating system variation among populations, the authors described such scenario as a widespread phenomenon weakly related to phylogeny or pollination mode (Whitehead *et al.* 2018). They concluded that ecological studies about the processes generating interpopulational variations in mating systems are still needed. With the aim of clarifying how pollinator availability relates to mating system and flower morphology in perennial plants, we studied populations of a widely distributed Neotropical tree *Curatella americana* (Dilleniaceae) (Ratter *et al.* 2003). Focusing on populations in three disjunct regions of savanna in Brazil, we tested the following hypotheses: 1. Effective pollinator availability (mean visitation frequency of pollinators per flower) is positively correlated to natural fruit set and inversely related to autogamy; 2. Smaller populations, or those at the distribution edge, will have higher levels of autogamy; 3. Populations with smaller flowers and less herkogamy will have higher levels of autogamy.

Materials and methods

Study sites

We studied ten populations of the Neotropical savanna tree *Curatella americana* L. (Fig. 1), regarding pollinators, floral biology and morphology, mating system, and pollen tube growth under controlled pollination. Populations 5-10 (Fig. 1) were in the large continuous area of savanna (Cerrado) located in Central Brazil, hereafter called Southern populations. Populations 1-3 were in the disjunct area of Roraima state, hereafter referred to as Northern populations and population 4 was in a fragment of savanna surrounded by the Amazon forest, hereafter called Santarém. Populations 9 and 10 (Jatai and Caldas Novas; Fig. 1) belong to the Southern region, located at one of the southern edges of the *C. americana* distribution in South America, but are still connected to the large continuous area of the distribution. These two populations therefore experience different scenarios when compared to the disjunct areas, as they are edge populations with much greater area. Such difference is important to note when considering the findings of this study (see Results).

Floral biology and autogamy were studied in all populations, but morphology for the purpose of morphometric analysis was studied in five populations from all the regions (2, 3, 4, 5 and 6; Fig. 1). The closest populations were 100 km apart and the furthest were more than 2,700 km apart from each other.

Floral biology

Floral biology measurements sensitive to dehydration were carried out *in situ* or, in the case of herkogamy, from pictures of the flowers in standard position using a fixed holder connected to the camera (Canon EOS Kiss X5). Time, sequence, and duration of anthesis, flower longevity, stigma receptivity, odour emission (presence of osmophores), and pollen availability (time of pollen presentation) were observed and described following Dafni *et al.* (2005). Receptivity was assessed by dropping hydrogen peroxidase onto the stigmas and checking for bubbles. The location of osmophores was visually assessed after immersing flowers in a solution of neutral red for five minutes.

Flowers were collected from five populations (2, 3, 4, 5 and 6; Fig. 1) and fixed in 70 % alcohol for linear or size measurements in the lab. The length of stamens (total and anther) and size of pistil (total length -ovary+style+stigma- and stigma area) were measured using a digital calliper. Measurements of shape of petal, sepal, ovary, and leaf (as a control) were obtained from pictures and analysed using geometric morphometrics (Bookstein 1991). Landmarks and semi-landmarks were placed on scanned structures using the software TPS Dig (Rohlf 2010). Semi-landmarks were used to define the borders of the structures when a homologous landmark was not available (Fig. S1 in supplementary material). We used three leaves per individual and 10 individuals in each population to analyze leaf morphology. For flower measurements, we sampled one flower per individual and 15 individuals per population. Qualitative variations in flower morphology were observed and noted in the field.

Pollinator importance

To check for fruit set with (open, natural fruit set, or NF hereafter) and without pollinators (autogamy or AU hereafter), we bagged inflorescences (minimum 20 flowers each) from at least 10 individuals per population (AU) and marked and left an equal amount of flowers open to visitation (NF). We used selection of linear models to test the effect of pollinator visitation (measured as the mean number of large-sized bee visits per flower) on both natural pollination and autogamy treatments. Visitation rates were measured during the flowering time, observing one individual tree (or a branch in large flower displays) per day and counting the number of flowers and the frequency of visitation for ten minutes each half hour, throughout the flower's lifetime. In each population at least five individuals were observed, resulting in a minimum of 20 observation hours per population. Pollinator counting and observations were carried out at the peak of the flowering season in each population, and this period was different in each geographic region. Within the same region, observations were concentrated in periods of about ten days. Afterwards, we moved to the next population, and only returned to the populations to collect fruit after a month. Total number of visits to flowers from each individual plant was divided by the total number of flowers observed, to calculate the mean visitation rate per flower on each day. The population
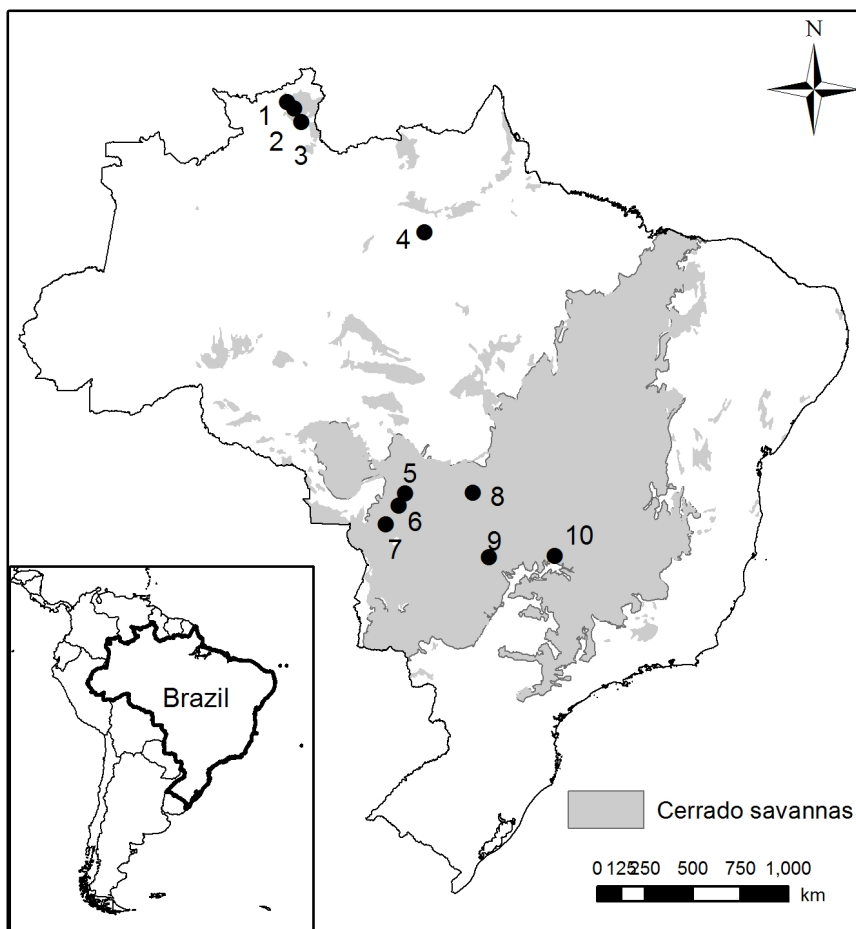
Figure 1. Map of the original area of Brazilian Cerrado (Neotropical Savanna) and indications of the studied populations. Populations 1, 2 and 3 are included in the region called Northern Cerrado, 4 is in Santarém, and 5-10 are in the Southern Cerrado region.

average of visitation rate for the observed flowers was then used to predict natural fruit set (NF) and autogamy (AU) in each area. During the other twenty minutes of observation we recorded visitors' behaviour on flowers and observed their potential as pollinators by looking for contact between insects and reproductive structures.

To check for possibile self-pollen deposition, we also bagged flowers one day before anthesis and then collected one stigma per flower as soon as the flower opened and another stigma four hours later. Each flower had two equal stigmas, which made the test perfectly paired. The stigmas were placed directly on a slide with stained glycerine jelly, covered with a cover slip, and pollen grains were counted under light microscopy. The difference in the number of pollen grains was tested by a paired t test. To test for possible accidental pollen deposition during manipulation, some stigmas were manipulated and the second stigma was removed 10 minutes later. Since the number of pollen grains on stigmas was not different between these two treatments, we assumed that accidental deposition had a minor effect and would not change the experimental results.

Pollen tubes

Pollen tubes were observed to identify possible mechanisms operating at the pistil level, related to the different pollination treatments in the studied populations. In order to check e pollen tube growth through the style, we collected ten hand-pollinated pistils (five cross and five self-pollinated) from ten individuals using exactly the same protocol in each population. The pistils were fixed in FAA (Formalin-Acetic acid-Alcohol) + ethanol 50 %, 24 hours after hand pollination. The preparation followed the technique proposed by Martin (1959) and pistils were stained with a solution of aniline blue. Since pistils were covered by trichomes, we pre-treated them in a solution of sodium hypochlorite (10 %) and distilled water to facilitate trichome removal. Even after such treatment it was still very difficult to count individual pollen tubes, therefore, results were assessed qualitatively through visual comparisons of the ten slides from each population under fluorescence microscopy.

Morphometric analysis

We separated the form of all the landmark configurations into shape and size using geometric morphometrics (Bookstein 1991). Size was measured as centroid size (CS), the square root of the sum of the squared distances of each landmark from the centroid, or gravity centre, of the landmark configuration. Size variation among populations and regions was tested using mixed models, comparing a set of three models: one with region, another with population, and a null model with only the intercept as a fixed effect.

Individual was included as a random effect in all models. Models were compared using AICc (Burnham & Anderson 2002).

To measure shape, all configurations were scaled to unit CS, and superimposed by a generalized least squares (GLS) Procrustes procedure. Given the presence of semi-landmarks, a sliding procedure minimizing the GLS residuals was used, as the exact location of the semi-landmarks along the structure outline was arbitrary. A mean shape was calculated and the differences between its landmarks and the landmarks of each individual were the residuals of the GLS procedure. To test for differences in shape among the regions, we ran a Procrustes Analysis of Variance (ANOVA) using a hierarchically nested design, with replications (repeated measurement of the landmarks from leaves, petals, etc.) nested within individuals, populations, and regions. Differences in shape among individuals, populations, and regions were visualized by doing a Principal Components Analysis on the superimposed shapes. All geometric morphometrics analysis were done using the geomorph package in R (Adams & Otarola-Castillo 2013).

For the univariate flower morphology measurements (number of stamens, stigma area, anther size, gynaecium, and androecia length) we used a one-way ANOVA, and when results were significant, the means were further differentiated using a Tukey multiple comparisons test (at p ≤0.05). All analyses were carried out using the R software environment (R Development Core Team 2014).

Results

Floral biology

Basic flower biology was observed in the individuals where the experiments were performed in all populations. In total, about 68,000 flowers were manipulated and at least 680,000 were observed. *Curatella americana* flowered from June to September in the Southern Cerrado (populations 5-10; Fig. 1), from September to October in Santarém (population 4), and from October to November in the Northern Cerrado (populations 1-3). Individual trees mass-flowered, producing all of their flowers within a short period of two weeks. Flowers are pentamerous, actinomorphic and open (plate type), grouped in panicles, white to the human eye, and UV absorbing (Fig. 2). Their odour is sweet and basic staining of the flowers indicated that osmophores are located at the margin of the petals. Flowers open between 04:30h and 06:30h and last for about seven hours. Pollen started to be released between 30 minutes to an hour after flower opening. The stigma was receptive for the entire period flowers were open. In the North, we registered one individual showing functional pistils and only rudiments of anthers without pollen, behaving as a female plant. On the other hand, in Central Brazil (Nova Xavantina) we found

two individuals with staminate and hermaphrodite flowers (andromonoecious).

Pollination and pollen tubes

Flowers of *C. americana* were visited by bees, flies, and beetles (Fig. 2, Tab. 1). Due to visitor behaviour (contact with sexual organs), mobility, and flight ranges observed in the field, large-sized bees (larger than *Apis mellifera*) were considered the main pollinators (for more details see AR Rech *et al*. unpubl. res.). The pollinator visitation probability was highest in the Southern populations (Tab. 2). Fruit set under natural pollination and visitation rate of main pollinators were positively correlated ($R2 = 0.49$, $P1,8 = 0.02$, Tab. 2). On the other hand, the relationship between autogamy and pollinator availability was negative and significant only when the two Southern edge populations were not considered ($R2 = 0.18$, $P1,8 = 0.61$ for all populations and $R2 = 0.65$, $P1,8 = 0.02$ for the restricted analysis). Similarly, the fruit set under natural pollination only negatively correlated to the level of autogamy when the same two populations were not considered ($r = -0.29$, $P8 = 0.40$ for all populations and $r = -0.78$, $P6 = 0.02$ for the restricted analysis). The two populations from the Southern edge had similar natural pollination fruit sets as the other populations in Central Brazil, but autogamy levels were like the populations from the North. Since the two Southern edge populations face a special biogeographical scenario, we ran the analysis with and without these populations and compared the results (see Materials and methods, and Discussion).
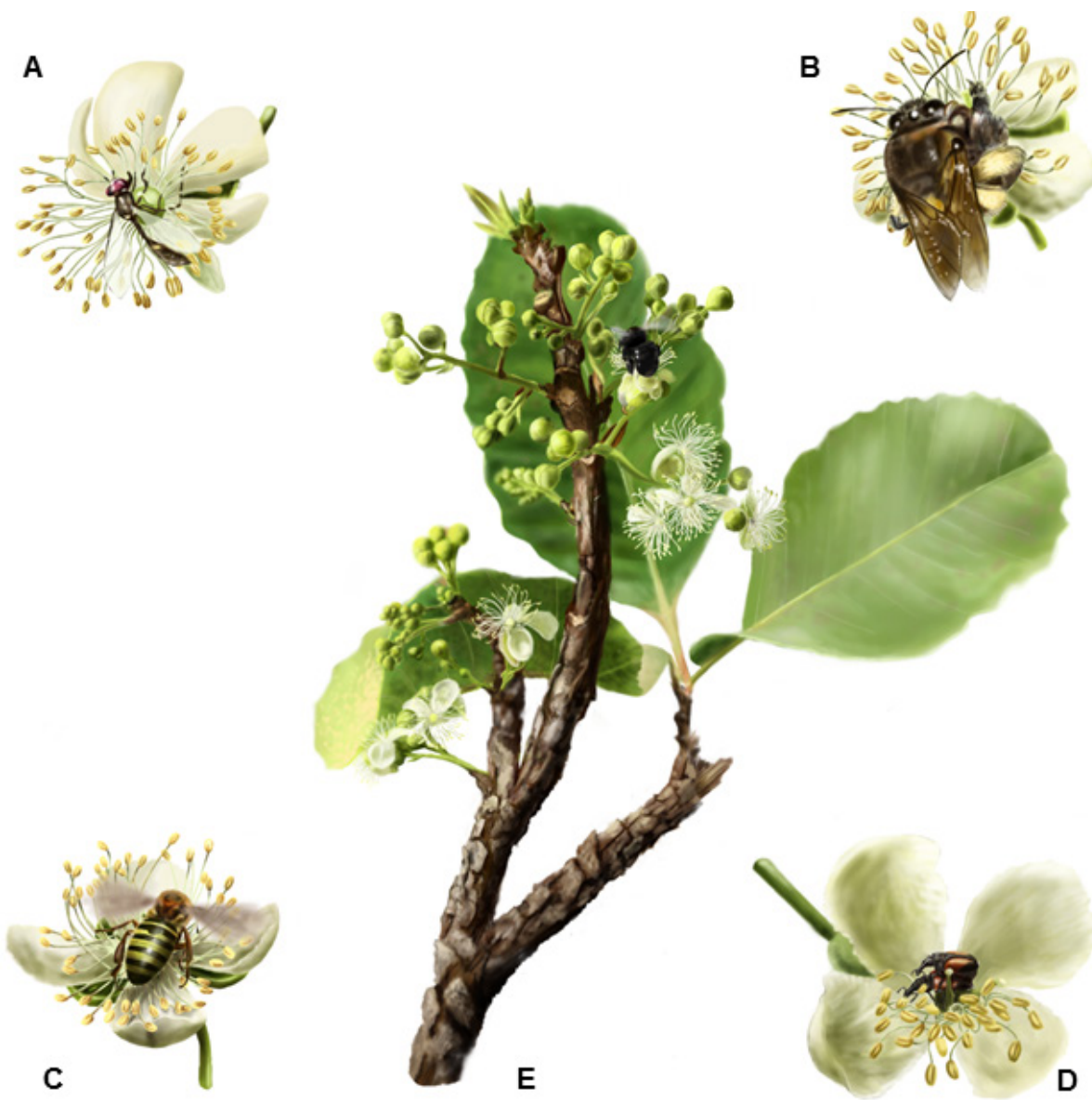
Figure 2. Inflorescence of *Curatella americana* and flower visitors in detail. **A.** Syrphidae (fly), **B.** *Ptiloglossa* sp. (large-bee), **C.** *Melipona quinquefasciata* (small-bee), **D.** Curculionidae beetle and **E.** Inflorescence with a *Bombus* cf. *morio* (large-sized bee) visiting the flower. Illustrated by Pedro Lorenzo.

Table 1. Flower visitors registered in the flowers of *Curatella americana* in the ten studied populations in Brazil. Curculionid Beetles were also observed but not quantified as the stay for the entire observation period without moving from the observed flower.

Number	Species	Number	Species
1	*Apis mellifera*	32	*Ptiloglossa* sp. 1
2	*Bombus* cf. *morio*	33	*Scaptotrigona* sp. 1
3	*Centris flavifrons*	34	*Scaptotrigona* sp. 2
4	*Centris fuscata*	35	*Scaptotrigona* sp. 3
5	*Colletidae* sp.	36	*Syrphidae* sp. 2
6	*Epicharis* sp.	37	*Syrphidae* sp.3
7	*Euglossa* sp.	38	*Syrphidae* sp1
8	*Eulaema nigrita*	39	*Tetragona clavipes*
9	*Exomalopsis* sp. 1	40	*Tetragona* sp. 1
10	*Frieseomellita doederleine*	41	*Trigona amazonensis*
11	*Frieseomellita portoi*	42	*Trigona branneri*
12	*Frieseomellita* sp.	43	*Trigona dallatorreana*
13	*Geotrigona mombuca*	44	*Trigona hyalinata*
14	*Halicitidae* sp.1	45	*Trigona meridionalis*
15	*Hesperidae* sp. 1	46	*Trigona pallens*
16	*Megachilidae* sp. 1	47	*Trigona recursa*
17	*Melipona fuliginosa*	48	*Trigona* sp. 1
18	*Melipona melanoventer*	49	*Trigona* sp. 2
19	*Melipona quinquefasciata*	50	*Trigona* sp. 2
20	*Melipona rufiventris*	51	*Trigona* sp. 3
21	*Melipona rufiventris*	52	*Trigona* sp. 4
22	*Melipona* sp. gr. *seminigra*	53	*Trigona* sp. aff. *pallens*
23	*Muscidae* sp. 1	54	*Trigona* sp. gr. *braueri*
24	*Muscidae* sp. 2	55	*Trigona* sp. gr. *chanchamayoensis*
25	*Muscidae* sp. 3	56	*Trigonisca vitrifrons*
26	*Ornidia obesa*	57	*Vespidae* sp. 1
27	*Oxaea flavescens*	58	*Vespidae* sp. 2
28	*Oxytrigona* sp.	59	*Xylocopa cearensis*
29	*Partamona ailyae*	60	*Xylocopa frontalis*
30	*Partamona auripenis*	61	*Xylocopa muscaria*
31	*Partamona vicina*	62	*Xylocopa* sp. 1

Table 2. Average proportion of fruit for each of the ten studied populations of *Curatella americana* in Brazil. Autogamy refers to bagged inflorescences with no pollinator access while natural pollination is the fruit set in exposed flowers and cross-pollination (cross) is the supplemented hand cross-pollination treatment. Single flower visitation (SFV) refers to the mean number of visits by large-sized bees to a single flower in each population per day. Population numbers follow Figure 1.

Region	North			Santarem	South					
Population	1	2	3	4	5	6	7	8	9	10
Autogamy	0.15	0.21	0.25	0.04	0.06	0.03	0.05	0.02	0.22	0.29
Natural pollination	0.31	0.12a	0.26	0.27	0.48	0.65	0.59	0.63	0.32	0.72
Cross-pollination	0.5	0.37	0.35	0.58	0.74	0.66	0.91	0.79	0.40	0.82
Single flower visitation	0	0	0	0.2	0.17	0.26	0.04	0.33	0.12	0.82

a Many fruits were destroyed by herbivores and could not be counted.

The amount of pollen on stigmas increased from 52 pollen grains at the beginning to 141 pollen grains per stigma at the end of the anthesis in bagged flowers (T8 = -4.96, p< 0.001), indicating the potential for spontaneous self-pollination in all populations. Nevertheless, the level of autogamy varied considerably among populations and individuals (Tab. 2). These differences in fruit set can be directly related to the way pollen tubes grew in the different treatments. Higher amounts of pollen tubes grew regularly and continuously through the styles of cross-pollinated flowers. Pollen tubes in self-pollinated flowers grew irregularly (strong callose reactions) and the difference between treatments (self- and cross-pollination) was clearly more contrasting in the Southern region than in the North (Fig. 3A-D). Most of the pollen tubes in self-pollinated flowers stopped growing in the first two thirds of the style. Therefore, we only saw pollen tubes in the self-pollination treatment reaching the ovules 24 hours after hand pollination in flowers from the Northern region (Fig. 3E-F).
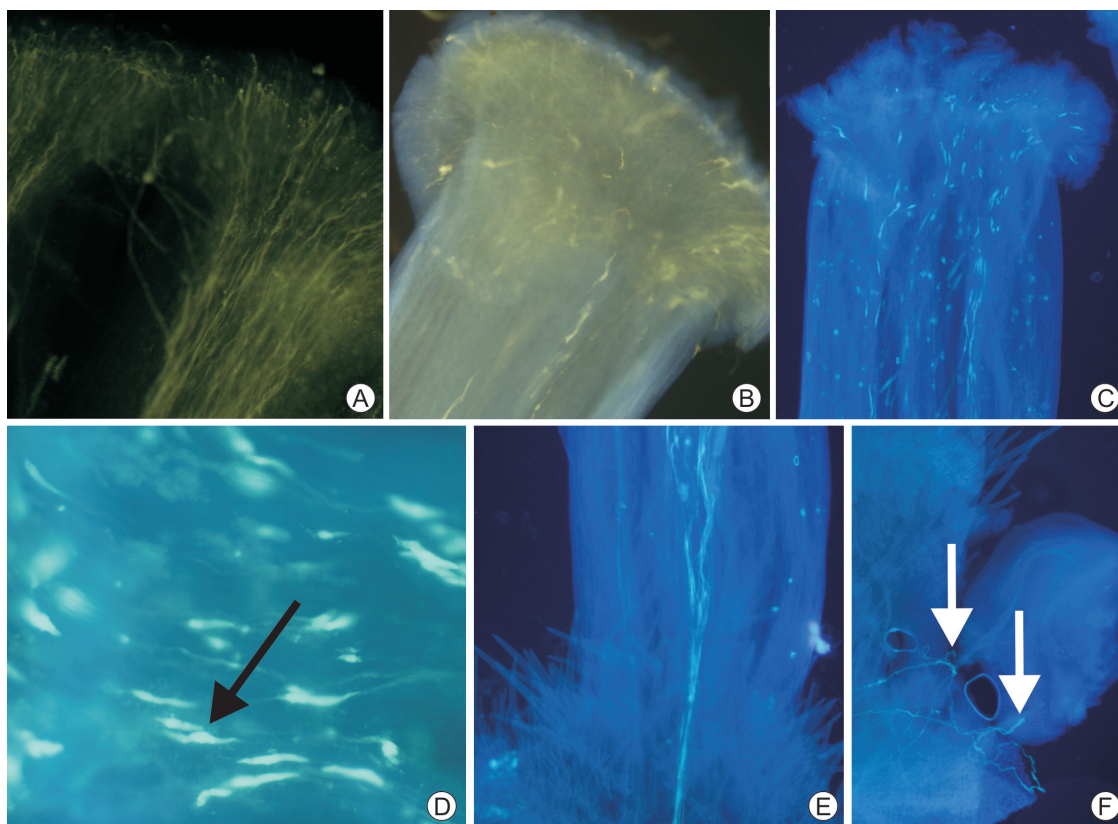
Figure 3. Pollen tube growth in flowers of *Curatella americana* after different pollination tests. **A-B.** Cross and self-pollination in one population from Southern Cerrado region (Cuiabá). **C.** Self-pollination in one population from the Northern region (Boa Vista). **D.** Self-incompatiblity reaction (black arrow) in one self-pollinated flower from Southern Cerrado region (Manso). **E.** pollen tube growing through the base of the style and **F.** reaching the ovule (white arrow) at the base of ovary in one population from the Northern Cerrado region (Boa Vista).

Morphometrics

The size (measured as centroid size) of petals, sepals, and leaves did not differ between regions, however, the linear lengths of anthers and gynoecium, and stigma area, varied significantly (Tab. 3 Fig. 4). For floral structures (petal and sepal), the null model including only individual size variation regardless of population and region was as likely as the more complex models. For leaves, the best model included differences among populations, but only the model with regions did not perform well, indicating a more idiosyncratic population-level variation in size instead of a geographical pattern. The number of stamens did not vary among regions (Tab. 3). Anthers were larger in the Southern region, while stigma area was larger in the North. Santarém was similar to Southern populations regarding anther size, and to the Northern populations regarding stigma area (Tab. 3, Fig. 4).

On the other hand, there were regional differences in shape for all traits considered, even though the proportion of variation among regions varied widely among structures (Tab 4). Although for the ovary, region was the main correlate of shape variation, for the other structures it had a minor role (Tab. 4 , Fig. 5). Population-level variation
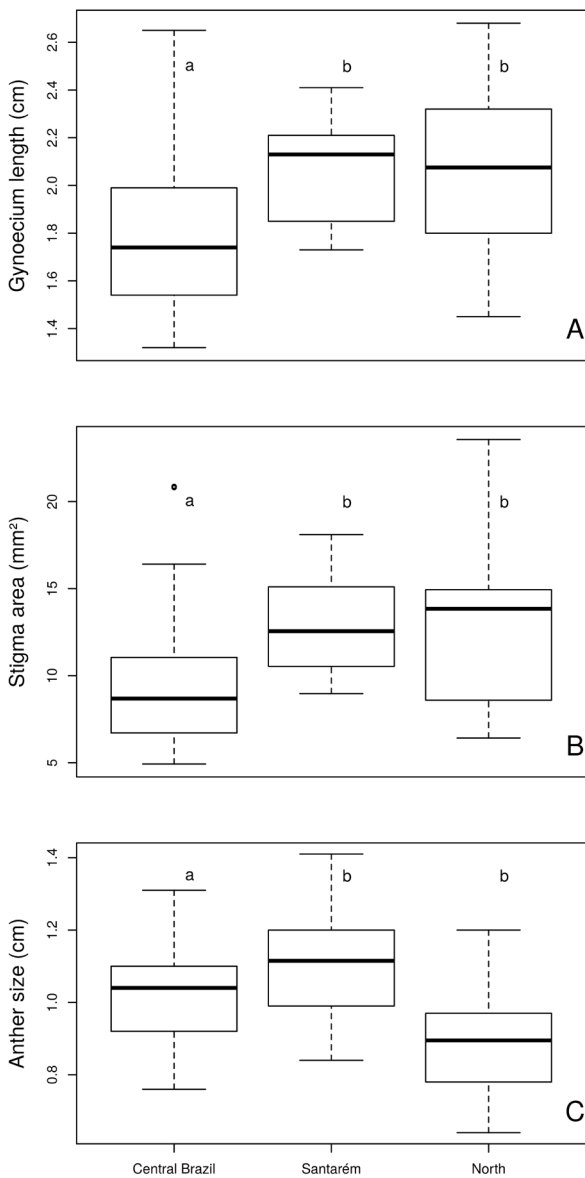
in shape within regions had a minor contribution to all structures, whereas individual variation was always high. Given the major role of regions in ovary shape variation, a clear geographic gradient was observed (Fig. 4), where northern populations presented the two stigmas close together, while in southern populations stigmas were further apart. Santarém, geographically located between populations from the other regions, had an intermediate ovary shape (Fig. 4 and Fig. S2 in supplementary material). For the other structures (leaves, petals, and sepals), no clear patterns of geographic variation were present (Fig. S2 in supplementary material).

Table 3. Analysis of Variance (ANOVA) for counted and linearly measured floral traits of *Curatella americana* from ten populations of three disjunct regions of Brazilian savanna (Southern Cerrado, Santarém and Northern Cerrado).
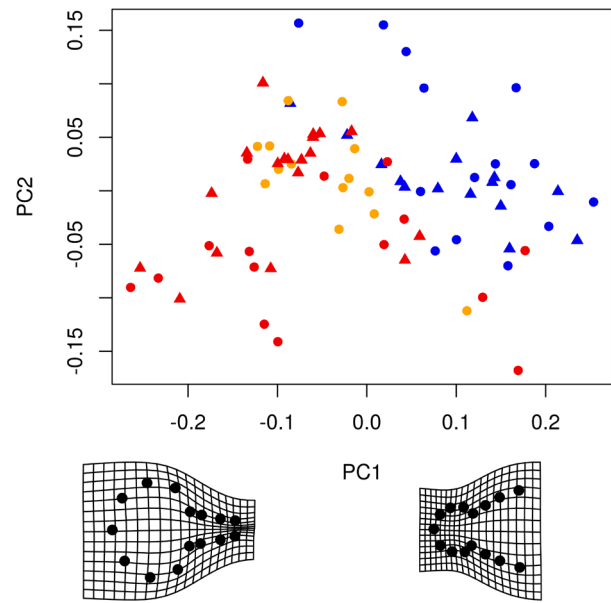
	Residual	DF	F	p
Number of stamens	69	2	0.6220	0.5398
Stigma area	69	2	6.4097	0.00279
Anther size	69	2	12.1346	0.00003
Gynaecium length	69	2	6.1636	0.00344
Androecia length	69	2	1.6270	0.2039

Table 4. Nested Procrustes ANOVA for shape of floral and leaf traits of *C. americana* in the three geographical regions studied (Southern Cerrado, Santarém, and Northern Cerrado). DF: Degree of freedom, MS: Mean Square Sum, p: p value.

		DF	MS	R2	p			DF	MS	R2	p
Leaf	Region	2	0.605	0.13	<0.001	Petal	Region	2	0.0103259	0.07225	0.007
	Population	3	0.178	0.06	<0.001		Population	2	0.0106042	0.07420	0.0027
	Individual	6	0.024	0.02	0.006		Individual	20	0.0048138	0.33682	0.001
	Residual	731	0.010	--	--		Residual	49	0.0030143	--	--
Ovary	Region	2	0.297805	0.31901	<0.001	Sepal	Region	2	0.0116036	0.080207	0.006
	Population	2	0.038002	0.04071	0.03		Population	2	0.0074344	0.051388	0.051
	Individual	21	0.024181	0.27198	<0.001		Individual	20	0.0045225	0.312605	0.02
	Residual	52	0.013224	--	--		Residual	49	0.0032820	--	--

Figure 5. Principal component analysis (PCA) using geometric morphometric coordinates for ovary shape of *Curatella americana*. Each dot is an individual measure projected according to the first two axes of the PCA. Colours indicate regions: orange is Santarém, red is Northern Cerrado and blue is Southern Cerrado. Different shapes indicate different populations within each region.

Discussion

Pollinator availability varies greatly for the Neotropical tree *C. americana* throughout its wide distribution range. The availability of pollinators (represented by bees larger than *A. mellifera,* such as species of *Bombus*, *Centris*, *Epicharis* and *Xylocopa*) had a direct effect on the level of natural pollination, and a more complex relationship with autogamy. We also found that *C. americana* populations at edge areas (Northern region and populations 9, 10 at the Southern limit of Cerrado) had higher levels of autogamy. We expected smaller flowers and lower herkogamy in more self-pollinated populations and, as predicted, these populations had larger stigma area, smaller anthers, and

Figure 4. Comparison (ANOVA) of floral traits of *Curatella americana* from different regions in the Brazilian Cerrado (Neotropical savanna).

less herkogamy. Meanwhile, other structures such as petals and sepals did not vary in size according to the regions studied. Interpretations of these results are discussed in the following sections.

Floral biology, pollination, and pollen tubes

The mass-flowering pattern of *C. americana* was already reported for another Neotropical Dilleniaceae species – *Davilla kunthii* (Rech *et al.* 2011). Apart from flower colour (white in *C. americana* and yellow in *D. kunthii*) and the closing sepals of *D. kunthii,* both species have similar floral biology, pollination, and mixed mating systems (Rech *et al.* 2011). The open flower morphology with exposed anthers makes it easy for visitors to contact anthers and stigmas in a single visit, however, many flower visitors hardly moved between individuals, making them unlikely to be significant pollen vectors (Ollerton *et al.* 2007). Pollen as the only reward and the white UV absorbing pattern found in the petals, indicates a flower that is attractive to bees (Lunau *et al.* 2011), which was clearly confirmed by visitation frequency (AR Rech *et al.* unpubl. res.).

Some populations presented very low levels of natural fruit production, as well as low availability of effective pollinators. We interpreted the negative relationship between fruit set under natural pollination and autogamy as an assurance strategy selecting autogamy in areas with low pollinator availability, herein, the Northern region and populations at the Southern edge of the Brazilian Cerrado (Baker 1967; Cheptou 2012). Two populations at the Southern edge of the *C. americana* distribution (Jatai and Caldas Novas) presented natural fruit sets that were equal to the populations in Central Brazil, but the levels of autogamy were similar to the populations in the North. Palynological evidence shows that the Southern edge of Brazilian savannas presented very dry, treeless environments from 18,000 to 6,000 bp (Salgado-Labouriau *et al.* 1997). Only from 5,000 bp onwards did trees, such as *C. americana,* colonize the area. Thus, the level of autogamy in the area may still be a consequence of the colonization process. Indeed, genetic data suggests a recent range expansion for *C. americana* in Brazilian savannas (Canuto 2011). Also, changes in mating systems may be flexible, as seen in artificial selection experiments (Levin 2012). Furthermore, two generations of strong induced directional selection were enough to elevate the autogamy level from 4 % to 56 % in *Phlox drummondii* (Bixby & Levin 1996). Therefore, past climate and colonization events related to expansion of Neotropical Savannas could underpin the distribution and consequently the contemporary autogamy rate found in *C. americana* (Canuto 2011; AR Rech *et al.* unpublished res.).

Curatella americana has a lifespan of over 200 years (Costa 2013), with a slower process of population replacement when compared to short-lived herbs such as *P. drummondii*. Therefore, if there is an ecological filter preventing colonization by animal-pollination dependent individuals, the establishment of a predominantly autogamous population could be very quick, as is the case for some invasive plant species (Ollerton *et al.* 2012). Nonetheless, recovery of higher rates of self-incompatibility when population size increases and pollinators come back into the environment can be slower, specially for long lived species like *C. americana*. It is important to consider that populations are normally not exclusively self-pollinated, so the unfavourable effects of a pure strategy may be reduced in mixed mating systems (Goodwillie *et al.* 2005). We also cannot rule out the possible effect of inbreeding depression caused by selfing at edge populations, which leads to less visitation and should be the focus of future genetic studies.

Morphology

Most of the traits studied here had a latitudinal gradient of variation. Moreover, individuals from Santarém frequently presented intermediate shape and size compared to Northern and Southern populations and were more similar to one region or another depending on the structure considered. This seems to be related to a similar palaeoecological history as the Northern region, and a pollinator availability similar to the one found in the Southern region. All the savannas studied herein, currently within isolated regions, were probably connected in the past and the Northern region was probably the first to disconnect from the others (Mayle & Power 2008).

At the regional level, floral and vegetative traits varied similarly but with different intensities. The synchronized variation in floral and vegetative traits in plants with generalized pollination systems (open flower morphology and many unrelated species of pollinators) is one of the expectations to "Berg's rule" (Berg 1960; Fenster 1991; Conner & Sterling 1995; Armbruster *et al.* 1999). As floral traits derive ultimately from the vegetative ones, decoupled patterns of variation are not expected unless clearly different regimes of selection drive the evolution of different groups of traits in different ways (Berg 1960; Armbruster *et al.* 1999). However, the variation pattern found in the ovary structure is much clearer than in the other structures. Therefore, we interpreted the progressive segregation of stigmas from north to south as a strategy selected to increase genetic diversity by touching different pollinators or pollinators' body parts during visits.

However, contrary to the expectation, not all traits varied in the same direction as an integrated unit or "pleiades" (Berg 1960). Stigma area was larger and there was less herkogamy in the North. These two traits may be related to female reproductive insurance, as both increase the chance a flower will be self-pollinated (see review in Levin 2012). The same pattern was also reported in *Nicotiana glauca* after it was introduced to parts of the world where there

were no suitable pollinators present, reinforcing the role pollinators have in mediating the phenotypic variation of flowers (Ollerton *et al.* 2012). On the other hand, anthers were larger in the Southern populations. Considering that anther size and pollen production are positively correlated (Harder & Thompson 1989; Philipp *et al.* 1990), and pollen removal can be related to seed set (Broyles & Wyatt 1990), there seems to be a pressure on the male component (pollen production) of fitness in areas with higher levels of cross-pollination (Lloyd 1984).

Finally, we showed that floral structures did not have an integrated pattern of variation across regions and populations. Conversely, different flower traits may respond independently to the pressures imposed by pollinator availability and the importance of autogamy in the different populations (Rosas-Guerrero *et al.* 2010; Opedal *et al.* 2016). These divergent responses fulfil the function of the flower by maximizing reproduction, with flexibility to adapt to different scenarios.

Conclusions

Trees have longer lives, and, therefore, slower individual substitution rates than most herbs and smaller woody plants. However, herein we found a similar pattern of variation in the mating systems and flower morphology of a tropical tree, as previously demonstrated for herb and a vine species, indicating colonization filters and pollinator availability as important drivers of flower morphology and mating system (Opedal *et al.* 2016). The results discussed here re-emphasize mating systems as a property of populations and not of species (Whitehead *et al.* 2018). Even though, in general, longer-lived organisms seem to have higher outcrossing rates, pollinator availability may also help explain local reproductive strategies and interpopulation variations in mating system.

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