

Reproductive success of the hybrid tree *Dimorphandra wilsonii*: implications for conservation

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- Atlantic Forest
- conservation
- endangered species
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- stingless bees

Palavras-chave:

- Mata Atlântica
- conservação
- espécies em perigo
- aptidão reprodutiva
- abelhas sem ferrão

Abstract

Dimorphandra wilsonii is a rare and endangered tree taxon that was once considered a species, but is now believed to be a hybrid. However, there is no available information on the reproductive status of its remaining populations. Therefore, we aimed to assess the reproductive fitness of *D. wilsonii* and its implications for the conservation of this endangered tree. We analysed the reproductive system, phenology, seed germination, and seedling development and survival of *D. wilsonii*, as well as the abundance and behaviour of its floral visitors. Manually self-pollinated and cross-pollinated flowers produced more fruit (0.04% and 0.042%, respectively) than spontaneously self-pollinated flowers (0.01%). Seed germination rates were highest for those pollinated naturally (53.3%) and through cross-pollination (44.2%). However, seedlings resulting from manual self-pollination had the lowest survival rate among treatments (only 5% survived after 180 days). Stingless bees, which generally facilitate self-pollination, were the most likely biotic pollinators. Recent evidence suggests that individuals of *D. wilsonii* are hybrids resulting from the crossing of two other *Dimorphandra* species. Our results indicate that existing populations can successfully interbreed and produce offspring. These findings provide insight into the taxonomic and conservation status of *D. wilsonii* and its parental species.

Resumo

Dimorphandra wilsonii é uma árvore rara e ameaçada de extinção que já foi considerado uma espécie, mas agora acredita-se que seja um híbrido. Entretanto, não há informações disponíveis sobre o status reprodutivo de suas populações restantes. Portanto, nosso objetivo foi avaliar a aptidão reprodutiva de *D. wilsonii* e suas implicações para a conservação desta árvore ameaçada de extinção. Analisamos o sistema reprodutivo, fenologia, germinação de sementes e desenvolvimento e sobrevivência de plântulas de *D. wilsonii*, bem como a abundância e o comportamento de seus visitantes florais. Flores autopolinizadas manualmente e de forma cruzada produziram mais frutos (0,04% e 0,042%, respectivamente) do que flores autopolinizadas espontaneamente (0,01%). As taxas de germinação de sementes foram mais altas para aquelas polinizadas naturalmente (53,3%) e por polinização cruzada (44,2%). Entretanto, mudas resultantes de autopolinização manual tiveram a menor taxa de sobrevivência entre os tratamentos (apenas 5% sobreviveram após 180 dias). Os polinizadores bióticos mais prováveis foram abelhas sem ferrão, que geralmente facilitam a autopolinização. Evidências recentes sugerem que indivíduos de *D. wilsonii* são híbridos resultantes do cruzamento de duas outras espécies de *Dimorphandra*. Nossos resultados indicam que populações existentes podem cruzar-se com sucesso e produzir descendentes. Essas descobertas fornecem insights sobre o status taxonômico e de conservação de *D. wilsonii* e suas espécies parentais.

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Introduction

Dimorphandra wilsonii Rizzini (1969: 239; Leguminosae, Caesalpinioideae) is an endemic tree restricted to a few remaining areas of *Cerrado* / Atlantic Forest ecotone in the central region of the state of Minas Gerais, Brazil (Fernandes & Rego 2014). Since its description in 1969, *D. wilsonii* has been considered a rare species (Rizzini 1969). In 2016, only around 420 adult individuals were found in nature (F.M. Fernandes 2018, personal communication). Moreover, these individuals were found in highly anthropised areas, with none observed in fully protected areas. For this reason, it was thought that the vast majority of the population had been eliminated due to environmental alterations (Martins *et al.* 2014). The rapid destruction of the habitat of *D. wilsonii*, and its restricted occurrence as isolated individuals far from remaining protected areas, led to it being listed as Critically Endangered in the IUCN Red List of Threatened Species (IUCN 2011), the List of Threatened Species of Brazil (MMA 2013) and the List of Threatened Species of the state of Minas Gerais (Drumond *et al.* 2008).

Although the last decade has seen efforts to implement strategies for the conservation of *D. wilsonii*, the recent study of Muniz *et al.* (2020) proposed that individuals of the species are in fact hybrids from the crossing of two other species: *Dimorphandra exaltata* Schott and *Dimorphandra mollis* Benth (Muniz *et al.* 2020). *Dimorphandra mollis* is a common and economically important species from the *Cerrado* (Ratter *et al.* 2003), classified as facultatively allogamous (Mendes *et al.* 2013), while *D. exaltata* is a rare and threatened species from the Atlantic Forest (Muniz *et al.* 2019) whose reproductive biology is currently unknown. This could imply changes to some of the goals of the conservation programme of *D. wilsonii*, since hybrids are not protected by most legislation on threatened species (vonHoldt *et al.* 2018).

Events of hybridisation among plants can modify their mating systems, phenology and floral morphology, which has direct effects on their reproductive fitness and ecological interactions with pollinators (Whitney & Glover 2007; Rezende *et al.* 2020). However, it is necessary to understand the reproductive conditions that populations of *D. wilsonii* are subjected to in order to contribute to determining the taxonomic and conservation status of the nothotaxon, as well as conservation actions to be implemented. Therefore, we aimed to evaluate the reproductive fitness of *D. wilsonii* and its consequences for the conservation of the nothotaxon. For this, we analysed the breeding system and reproductive success, floral morphology, phenology, seed germination, and

seedling development and survival of *D. wilsonii*, as well as the species richness and behaviour of its floral visitors.

Materials and Methods

Study area

The study was carried out on individual trees located in the municipalities of Sete Lagoas, Paraopeba and Caetanópolis in the central region of the state of Minas Gerais, Brazil. The occurrence and distribution of individuals of *D. wilsonii* is described in Fernandes & Rego (2014). The region's climate is characterised as humid subtropical, with rainy summers and a dry season from April to September. The average temperature is 22 °C, and the annual rainfall varies between 1,300 mm and 1,500 mm (Silva *et al.* 2008).

The region is in the *Cerrado* Domain (Ab'Saber 1977), which is characterised by physiognomies that vary from grasslands (*campo limpo*) to savannas (typical *cerrado* or *cerrado sensu stricto*) and *cerrado* woodlands (*cerradão*). The individuals of *D. wilsonii* were found in areas of *Brachiaria* sp. (Poaceae) pasture on private properties and near native vegetation generally characterised as transitional between woodland and semideciduous forest.

Floral visitors

Fieldwork was carried out from October 2008 to December 2010 for a total of 70 h of field observations, including observations of the behaviour of floral visitors and the number and frequency of visits throughout the day (between 7 am and 7 pm). The species richness and abundance of floral visitors were measured and foraging behaviour was observed in the inflorescences of *D. wilsonii*. The presence of pollen on visitors' bodies was noted. Whenever possible, floral visitors were filmed or photographed and collected for identification to the lowest taxonomic level possible. Exemplars were deposited in the entomological collection of the Instituto de Ciencias Biológicas of the Federal University of Minas Gerais.

Floral biology

From November to February in 2008 and 2009, we observed the phenological stage of 10 labelled individual trees once a month to determine the flowering period and peak of *D. wilsonii*. During the peak of the flowering season, we recorded the number of open flowers per ear, the number of ears per bunch and the number of bunches of flowers in two inflorescences of 10 labelled tree individuals of *D. wilsonii* every two weeks. We also measured the duration of flower opening for 10 marked flowers.

Breeding system and reproductive fitness

Each inflorescence of *D. wilsonii* consisted of up to 15 dense ears, with each ear containing 80–100 flowers, resulting in approximately 1,000 flowers per inflorescence. The inflorescence was chosen as the sampling unit for the pollination treatments due to the small size of the flowers and the difficulty of handling them individually without causing them to detach. Inflorescences with flowers at the bud stage were labelled and isolated with organza bags. After opening, the flowers underwent the following treatments: (1) spontaneous self-pollination ($N = 158$ inflorescences), where bagged flowers were left intact; (2) hand self-pollination/geitonogamy ($N = 35$ inflorescences), where pollen from previously bagged flowers or from the same flower (as the flowers were not previously emasculated) was manually transferred to the stigma of the same inflorescence, followed by subsequent re-bagging; and (3) hand cross-pollination ($N = 61$ inflorescences), where pollen was manually transferred between flowers of different tree individuals, or transferred to the stigma of the same flower, as flowers were not emasculated beforehand. Flowers in treatments 2 (hand self-pollination) and 3 (hand cross-pollination) were not emasculated prior to controlled pollen transfer. Thus, treatment 2 (hand self-pollination) could reflect both manual self-pollination (as *D. wilsonii* is autocompatible) and geitonogamy. Similarly, treatment 3 (hand cross-pollination) involved pollen transfer between flowers of different plants without emasculation of the treated flowers, which could result in a combination of cross-pollination and self-pollination. We chose not to emasculate the flowers to maintain the natural state of the flower during the experiment, as emasculation can accelerate ovule degeneration, reduce fruit set and seed production, and shorten flower life by inducing wound-induced senescence (O'Neill 1997; Hedhly *et al.* 2009; Guerra *et al.* 2010). Inflorescences from all treatments were then bagged until fruit maturation to calculate “fruit set”, here considered as the number of fruit formed per treated inflorescence. Other inflorescences were labelled but not bagged to observe the number of fruit formed through natural pollination (control, $N = 66$ inflorescences).

Reproductive fitness was assessed by germinating the seeds set from the pollination experiments described above, and following the growth of the seedlings. For each pollination treatment, 120 seeds were selected; only seeds with intact seed coats, and of regular shape and uniform size, were included in the experiment. The fruit were collected and processed to remove the seeds, which were then cleaned and mechanically scarified on the side opposite to the hilum. Scarification

was performed because it increases germinability by about 75% in relation to intact seeds (Matheus *et al.* 2017). The seeds were then incubated in Styrofoam trays in a germination chamber (347/CDG, FANEM) at 28 °C and 12 h photoperiod for about 30 days. The number of germinated seeds was determined daily from day 3 to day 14, and again on day 30. Seeds were considered to have germinated when they developed roots and cotyledons. After this 30-day period of the germination experiment, all surviving seedlings were transplanted into plastic bags (18 × 24 cm) and kept in the same greenhouse and under the same conditions (temperature, photoperiod and irrigation). The number of surviving seedlings differed between the pollination treatments as follows: spontaneous self-pollination: $N = 29$; hand self-pollination/geitonogamy: $N = 8$; hand cross-pollination: $N = 23$; natural pollination: $N = 21$. After 30 days in the greenhouse (60 days from the start of the germination experiment), the number of surviving seedlings was counted and transferred to an open field with 80% shade. Sixty days later (120 days after the start of the germination experiment) the seedlings were transplanted into larger plastic bags (25 × 40 cm) and kept under full sun. The number of seedlings reflected the cumulative survival during each observation period - 30, 90 and 180 days - after the start of the germination experiment. Soil from the region where the nothotaxon occurs was collected for use as a substrate for seed germination and seedling growth.

Statistical analyses

The normality of each data set was first tested using QQ-Plots and a Shapiro-Wilk test. As none of our fixed effects were normally distributed, we conducted nonparametric tests.

To analyse whether the number of viable seeds per fruit of *D. wilsonii* (count response variable) was influenced by pollination treatment (fixed effect), we used a generalised linear mixed model (GLMM) with a gaussian distribution using the `glmmTMB` function in the `glmmTMB` package (Magnusson *et al.* 2019). The individual plant was included as a random effect. Another GLMM model (truncated Poisson distribution) was used to analyse the effects of pollination treatment and number of incubation days (fixed effects) on *D. wilsonii* seed germination (number of germinated seeds, count response variable). The area of seed origin was included as a random effect in this model. A general linear model (GLM) with a binomial distribution was used to analyse the effects of pollination treatment and the number of days since the start of the germination experiment (fixed effects) on the survival of *D. wilsonii* seedlings (presence or absence of seedlings, response variable). Estimated marginal means

for each pollination treatment and the number of days since the start of the germination experiment were tabulated using the R package “emmeans” (Lenth 2019). Pairwise differences were tested for significance using the Tukey method. Type II Wald chi-square (χ^2) tests were specified via the Anova() function in the “car” package (Fox *et al.* 2018). The fit of all models was assessed using diagnostic plots from the “DHARMA” package (Hartig 2020). All statistical analyses were performed using R software version 4.1.2 (R Core Team 2022).

Results

Floral biology

The racemose inflorescence of *D. wilsonii* (20–30 cm in length; $N = 20$) is composed of up to 20 bunches, each comprising up to 15 dense ears (6–12 cm long, Fig. 1). Ears possess 80–100 flowers ($N = 20$). The production of flower buds started in November and extended until February in 2008 and 2009. The flowers ($n = 20$) are yellow; the calyx is 2–3 mm long, with 5 lobes at the apex, and the corolla has 5 bowl-shaped petals 3–4 mm long. The androecium has 5 stamens (3.5–5 mm long) which are antepetalous, epipetalous and glabrous - the anthers are almost the same size as the filaments - and 5 staminodes, which are antesealous and linear, with a widened apex and a clubbed inner surface. The ovary is fusiform (2–2.5 mm long), angular, glabrous and multiovulate (20–30 ovules); the style is thick (0.5 mm long) and generally curved; the stigma is punctate, and is adaxial with respect to the anthers. Flowers start to open gradually about a month after budding, reaching the greatest intensity in December and January. Flowers open asynchronously in the ear and inflorescence. Pollen is released gradually through rimose anthers over a period of 1–2 days, and the stigma remains receptive for the same period. The receptive stigmatic surface consists of a crater, usually at the apex of the style, which is full of clear liquid in fresh flowers. After 2–3 days, the petals, stamens and staminodes fall, leaving the sepals and pistil.

Floral visitors

Insects - mainly bees - visited the flowers of *D. wilsonii*. About 82% of all floral visitors were stingless bees (Meliponini, Apidae, Figs. 1–2). *Tetragona clavipes* (Fabricius 1804) was the most frequent visitor, followed by *Trigona spinipes* (Fabricius 1793), *Cephalotrigona capitata* (Smith 1854) and *Trigonisca intermedia* Moure, 1990 (Fig. 2). When they neared a flower, they landed on the petals, walked on the stamens, actively collected pollen by rubbing against it with their bodies and with their collected nectar, and frequently contacted

the stigma. In the process, the bees' entire bodies became covered with pollen, after which they generally visited several other flowers nearby. Other insects (butterflies, wasps, ants, beetles and flies) were also seen on flowers of *D. wilsonii*; however, they were less common than the stingless bees, collected only nectar and generally did not touch the stigma or anthers during visits.

Reproductive fitness

Fruit set (fruit/inflorescence) for *D. wilsonii* was very low, with only 0.02% of the inflorescences setting fruit under natural treatment (control). Inflorescences subjected to self-, cross- and natural pollination set a similar number of fruit, and more than those subjected to spontaneous self-pollination (Tab. 1). Moreover, spontaneously self-pollinated inflorescences had about 1.8 times fewer chances to set fruit than those that were naturally pollinated, and 3.9 times fewer than cross-pollinated inflorescences (Tab. 1). About 0.01% of spontaneously self-pollinated inflorescences set fruit, while hand self- and hand cross-pollinated inflorescences set 0.04% and 0.042% fruit, respectively (Tab. 1). Moreover, the fruit set from cross-pollinated inflorescences produced a higher proportion of viable seeds (~79% of all seeds), which was greater than the other pollination treatments; fruit from naturally pollinated inflorescences set more viable seeds than were set by hand-pollinated inflorescences (Tab. 1; Fig. 3).

Germination started with radicle emission on the 4th day and cotyledon appearance on the 13th day after sowing, and gradually decreased until the 28th day. The proportion of germinated seeds varied between pollination treatments (Tab. 2), being higher in seeds set by natural pollination (53.3%) and hand cross-pollination (44.2%), followed by spontaneous self-pollination (38.3%) and hand self-pollination (27.5%; Fig. 3). There was no significant difference in the number of germinated seeds between hand cross-pollination and spontaneous self-pollination treatments (Tab. 2), whereas the other pairwise comparisons were significant (Tab. 2). The number of incubation days (3–14 days) had no effect on the number of germinated seeds (truncated Poisson distribution, GLMM: $\chi^2 = 0.39$, $df = 1$, $p = 0.5298$).

Seedling survival decreased throughout the 180 days from germination (Tab. 3; Fig. 4). From 14 to 30 days (since the start of the germination experiment), a strong decrease in the number of living seedlings was observed; however, from 30 to 120 days, the number of living seedlings decreased more gradually, with no significant difference being detected between days of experiment (Tab. 3; Fig. 4). Seedling survival was also related to pollination



Figure 1 – a-c. The floral architecture of the inflorescence of *Dimorphandra wilsonii*, and its floral visitors – a. Racemose inflorescence of *D. wilsonii* (20–30 cm long), consisting of up to 20 bunches, each with up to 15 dense ears (6–12 cm long) - each ear has 80–100 flowers; b. a stingless bee visiting *D. wilsonii* flowers; c. a wasp visiting the flowers.

treatment (Tab. 3). Hand self-pollination had the lowest seedling survival among the treatments after 180 days of incubation, with only 5% survival (Tab. 3; Fig. 4). No significant differences were found among the other treatments, although the 180-day survival of seedlings was 20% from spontaneous self-pollination, 17.5% from hand cross-pollination and 15.8% from natural pollination (Fig. 4). The interaction between pollination treatment and the number of days of incubation did not affect seedling survival (pollination treatment*days: $\chi^2 = 11.47$, $p = 0.245$, AIC = 1850.3).

Discussion

Our results indicate that *D. wilsonii* can successfully reproduce and produce offspring, providing new evidence for the hybridisation process that produces individuals of *D. wilsonii* (Muniz *et al.* 2020). Therefore, it is necessary to update the taxonomic and conservation status of *D. wilsonii* and consider new perspectives for the conservation of its parental species.

Stingless bees enhance self-pollination

The flower / fruit ratio for *D. wilsonii* was low: about two in 10,000 flowers, or 0.02%, set fruit under natural conditions, and our results suggest that the reproductive fitness of *D. wilsonii* would be severely reduced in the absence of a biotic pollen vector. Hand cross-pollination increased fruit and seed set and germination rates, causing more seed to reach the seedling stage. Although cross-pollination is related to higher reproductive fitness for *D. wilsonii*, individual plants seem to be subject to deficient or unstable cross-pollination services.

The most frequent floral visitors of *D.*

wilsonii were stingless bees, mainly *Trigona* spp. All stingless bees are eusocial, and many species tend to forage in large groups of up to 20,000 workers (Chinh *et al.* 2005). These bees tend to specialise on a single floral species at a time (Waser 1986; Ramalho *et al.* 1994; Slaa *et al.* 2003), which is a good strategy to reduce the use of energy that would be redundantly spent choosing other plants (Gruter & Ratnieks 2011). As a consequence, a greater number of nestmates can be recruited to forage the same tree, dominating it numerically (high local abundance) and behaviourally (controlling feeders) (Lichtenberg *et al.* 2010). This strategy is especially advantageous when foraging on plants with mass flowering, such as *D. wilsonii*, which has millions of small flowers open on a single tree per day. Moreover, the flight distances of these bees range from 621 to 1,800 m (Van Nieuwstadt & Iraheta 1996; Araújo *et al.* 2004; Nunes-Silva *et al.* 2020 and references therein), which is shorter than those of the honeybee (Visscher & Seeley 1982; Kakutani *et al.* 1993). Therefore, the most frequent visiting bees of *D. wilsonii* seem not to be cross-pollination promoters due to their tendency to forage intensively on the same tree instead of moving among plants. However, this does not mean that *Trigona* spp. and other stingless bees are poor pollinators of *D. wilsonii*.

In contrast to studies showing that *Trigona* species are floral visitors with a negative impact on reproductive success due to their damaging and cutting behaviour - sometimes destroying entire flowers (Renner 1983; Rego *et al.* 2018) - there are some cases where they can be effective pollinators (Slaa *et al.* 2006; Silberbauer-Gottsberger *et al.* 2013; Giannini *et al.* 2014; Pinto & Schlindwein 2014) or secondary pollinators (Stein & Hensen 2011). In fact, the damaging and cutting behaviour commonly associated with *Trigona* spp. was not observed during their visits to *D. wilsonii* flowers. This could be explained by flower size in relation to bee body size, where bees smaller than the stigma-anther distance may have suboptimal or low pollination efficiency (Willmer & Finlayson 2014; Jauker *et al.* 2016; Stanley *et al.* 2016; Baronio *et al.* 2020; Morais *et al.* 2020). The tiny flowers of *D. wilsonii* are close to or even smaller in size than the bodies of *Trigona* spp., which may explain why *Trigona* bees can act as pollinators without having to destroy the flowers to access the nectar. Indeed, the label “rescue pollinator” was recently proposed for the very common *T. spinipes* due to its capacity to colonise degraded habitats and its persistence in heavily altered landscapes (Jaffé *et al.* 2016). However, due to the numerous reports of negative impacts that bee species of *Trigona* have on pollination and fruit set for many plant species they visit, the term appears to be misapplied. Therefore, stingless

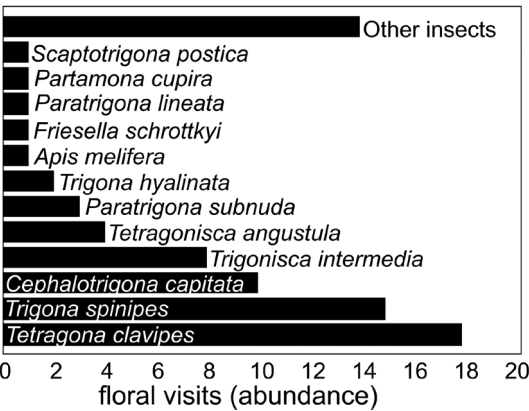


Figure 2 – Total number of visits per insect species to flowers of *Dimorphandra wilsonii*. Note that 82.3% of all visitors were stingless bees. “Other insects” comprises unidentified species of butterflies, wasps, ants, beetles and flies.

Table 1 – Fruit and seed set per pollination treatment and contrast of the number of viable seeds/fruit set among pollination treatments for *Dimorphandra wilsonii*. Generalised linear mixed model (GLMM negative binomial distribution) analysis: (1) number of fruit set per inflorescence as the fixed effect - “individual plant” was included as a random effect; (2) the number of viable seeds per fruit as the fixed effect - “individual plant” and “area of seed origin” were included as random effects. The “odds ratio” represents how the odds of a particular outcome change when the predictor variable (pollination treatments) increases by one unit, controlling for random effects in the model; “SE” is the standard error of the odds ratio; “t-ratio” is a test of the significance of the predictor variable (pollination treatments), calculated as the ratio of the estimated coefficient to its standard error; “p-value” is the significance of the fixed effects. *P*-values in bold indicate significant effects according to post-hoc Tukey’s test.

	Treatment	N-flowers	Fruit set (%)	Fruits/10 ³ flowers Mean (min–max)	Viable seeds/fruit Mean (min–max)
Fruit and seed set	Spontaneous self-pollination	158,000	0.011	2.35 (1–5)	8.20 (1–14)
	Hand self-pollination	35,000	0.040	3.57 (1–11)	7.43 (0–16)
	Cross-pollination	61,000	0.043	5.3 (1–15)	12.42 (3–22)
	Natural pollination (control)	66,000	0.020	3.69 (1–9)	9.87 (1–16)
	Contrasts	Odds ratio	±SE	t-ratio	P-value
Fixed effect: Fruit set $\chi^2 = 26.35$, $p < 0.0001$	Hand self- vs Natural	0.70	0.48	-0.51	0.986
	Hand self- vs Cross-	0.41	0.25	-1.45	0.597
	Hand self- vs Spontaneous	8.95	5.73	3.43	0.005
	Natural vs Cross-	0.58	0.27	-1.18	0.763
	Natural vs Spontaneous	12.79	9.28	3.51	0.004
	Cross- vs Spontaneous	22.10	15.12	4.52	< .001
	Contrasts	Odds ratio	±SE	t-ratio	P-value
Fixed effect: Viable seeds/fruit $\chi^2 = 81.78$, $p < 0.001$	Hand self- vs Natural	-2.53	0.89	-2.85	0.0244
	Hand self- vs Cross-	-5.85	0.76	-7.67	< .0001
	Hand self- vs Spontaneous	-1.23	0.89	-1.39	0.5078
	Natural vs Cross-	-3.32	0.72	-4.57	< .0001
	Natural vs Spontaneous	1.30	0.85	1.52	0.4254
	Cross- vs Spontaneous	4.61	0.71	6.49	< .0001

bees (*Trigona* spp.) should especially promote self-pollination, and eventually also cross-pollination, of *D. wilsonii*.

In fact, self-pollinated flowers had reasonable fruit and seed set, while inbred seeds had moderate germination rates and seedling survival. Additionally, individual *D. wilsonii* plants are typically isolated in fragmented landscapes, and their main visitors are bees that tend to forage intensively on the same individual plant instead of moving between individuals. Conveniently, self-pollination is favourable for individuals of *D. wilsonii* due to the fact that the plants are subject to conditions that could limit pollen dispersal distance

(Bai *et al.* 2007; Mohsenipoor *et al.* 2010; Bartoš *et al.* 2020), and asexual reproduction is absent or very rare (Muniz *et al.* 2020). Therefore, despite cross-pollination resulting in higher reproductive fitness, self-pollination (autonomous and pollinator-mediated) and geitonogamy are the most probable mating systems of *D. wilsonii*.

Consequences of self-pollination for reproductive fitness of D. wilsonii

The high number of flowers produced per inflorescence combined with low fruit production led to low fruit/flower ratios in *D. wilsonii*. The relationship between high numbers of flowers

and low fruit production is a typical characteristic of many woody species (Bawa & Webb 1984) and has also been reported for other species of Leguminosae (Bawa & Buckley 1989). These studies mainly attributed this characteristic to limited resources and sexual selection. Other studies have hypothesised that the low fruit/flower ratio may be adaptive, including some evidence for the subfamily Caesalpinioideae, to which *D. wilsonii* belongs (Gibbs *et al.* 1999). One hypothesis is that the “excess” flowers optimise seed number or quality by allowing the plant to discard young fruit with unfertilised ovules or defective embryos. Another hypothesis is that non-fruiting flowers help to increase male reproductive success by improving the distribution of pollen within the population. Therefore, based on these hypotheses, the high flower production of *D. wilsonii* may have the function of attracting floral visitors so that

the high frequency of pollinators can compensate for the negative effects of geitonogamy in this nothospecies, while sometimes also promoting cross-fertilisation and thus maintaining the genetic quality of its seeds.

The reproductive flexibility made possible by autogamy assures the reproductive maintenance of the studied populations of *D. wilsonii*. These characteristics prevent flaws in the reproductive cycle that could increase the risk of extinction of *D. wilsonii*. Autogamy can be advantageous for fixing important genotypes in populations (Harder & Wilson 1998; Barringer 2007; Williams 2007), and gives autogamous species other ecological advantages, such as “reproductive safety” (Richards 1986; Lloyd 1992). This would be especially relevant in environments with small populations of plants, periods of pollinator absence, or low pollinator frequency, making cross-pollination unlikely. However, the costs of autogamy include pollen discounting (Lloyd 1992; Harder & Wilson 1998), where pollen that could be used in cross-fertilisation is spent on self-fertilisation. The high frequency of self-pollination, whether by autogamy or geitonogamy, can also result in inbreeding depression if there are deleterious genes in the population (Richards 1986; Lloyd 1992; Barringer 2007; Williams 2007). Since asexual reproduction is absent or very rare in *D. wilsonii* (Muniz *et al.* 2020), the establishment of new individuals in the population depends especially on successful pollination and dispersion of diaspores to suitable habitats (as discussed in the following section).

Reproductive success, hybridisation and conservation

The survival of a population over time depends on the degree of plants' dependence on pollinators for pollen exchange and sexual reproduction, and the implications of hybridisation for this process (Miller & Venable 2000; Whitney & Glover 2007; Glick *et al.* 2016). This can include important consequences for the mating system, flower morphology and phenology, which has direct implications for reproductive fitness (Whitney & Glover 2007; Rezende *et al.* 2020). The most extreme consequence for plant reproduction is complete hybrid sterility, which represents an evolutionary dead-end (Arnold & Hodges 1995; Arnold *et al.* 2001; Husband 2004; Vallejo-Marín *et al.* 2017; Meeus *et al.* 2020). However, fertile hybrids are rare because hybrids with intermediate phenotypes may be selected against in the parental habitats, leading to ecological hybrid inviability (Baack *et al.* 2015). Nevertheless, our findings indicate that hybrids of *D. wilsonii* are capable of interbreeding successfully and producing viable seeds and seedlings.

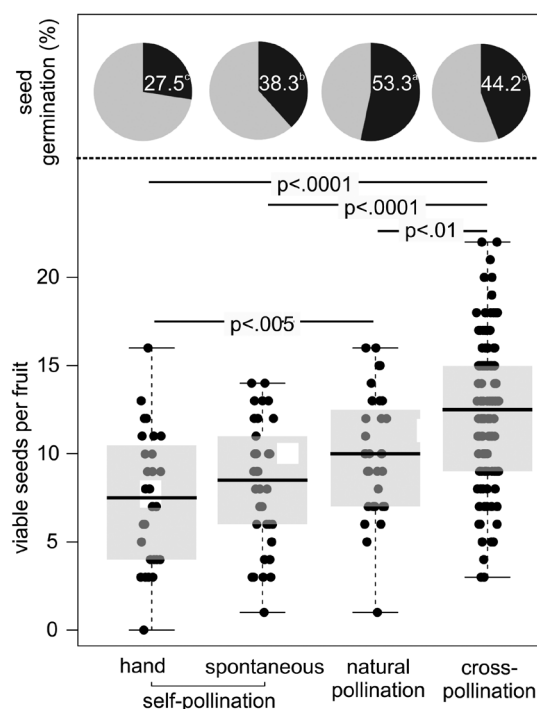


Figure 3 – Number of viable seeds per fruit and seeds germinated (%) per pollination treatment of *Dimorphandra wilsonii*. Statistical analysis: (1) viable seeds per fruit, GLMM: $\chi^2 = 81.78$, $df = 3$, $p \leq 0.001$, AIC = 1149.1, gaussian distribution. *P*-values indicate statistical significance according to Tukey's method; (2) N of seeds germinated, GLMM: $\chi^2 = 102.3$, $df = 3$, $p \leq 0.001$, AIC = 751.2, truncated Poisson distribution. Percentages of seeds germinated with different superscript letters are significantly different from each other as indicated by post-hoc Tukey's test.

Table 2 – Number of *Dimorphandra wilsonii* seeds germinated per number of incubation days (3–14 days) and per pollination treatment. Generalised linear mixed model (GLMM, truncated Poisson distribution) analysis. Number of seeds germinated was the fixed effect (N = 120 seeds tested per treatment); area of seed origin was included as a random effect. *P*-values in bold indicate significant effects according to Tukey’s test. The number of incubation days had no significant effect on the number of germinated seeds ($\chi^2 = 0.39$, df = 1, $p = 0.5298$). The “odds ratio” represents how the odds of a particular outcome change when the predictor variable (pollination treatments) increases by one unit, controlling for random effects in the model; “SE” is the standard error of the odds ratio; “t-ratio” is a test of the significance of the predictor variable (pollination treatments), calculated as the ratio of the estimated coefficient to its standard error; “p-value” is the significance of the fixed effects.

Contrasts		Odds ratio	±SE	t-ratio	P-value
seed germination $\chi^2 = 102.36$, $p < 0.0001$	Spontaneous vs Hand self-	1.38	0.10	4.76	< .0001
	Spontaneous vs Natural	0.73	0.04	-5.31	< .0001
	Spontaneous vs Cross-	0.87	0.05	-2.25	0.1161
	Hand self- vs Natural	0.53	0.03	-9.86	< .0001
	Hand self- vs Cross-	0.63	0.04	-6.95	< .0001
	Natural vs Cross-	1.19	0.07	3.09	0.0130

The germinability of *D. wilsonii* (53–79%) appears to be intermediate between the germinability of its parental species *D. exaltata* (32%) and *D. mollis* (89–97%) (Matheus *et al.* 2017; Borghetti *et al.* 2019; Silva *et al.* 2020). Seedlings of *D. mollis* have higher survival probabilities (55–75%, Borghetti *et al.* 2019) than those of *D. wilsonii* (5–20%). There are no specific studies on the dispersal of *D. wilsonii*, but comparisons with the parental *D. mollis*, which has similar seed and fruit characteristics (Lopes & Matheus 2008), suggest that they may share dispersers. The tapir (*Tapirus terrestris*), which is critically endangered and no longer found in the areas where *D. mollis* occurs, was once an important seed disperser of *D. mollis* (Bizerril *et al.* 2005). Currently, cattle may act as seed dispersers for *D. wilsonii* in disturbed areas, as seeds have been found in their faeces (Fernandes & Rego 2014). We therefore suggest that the absence of native seed dispersers and the presence of a putative exotic disperser may reduce seed dispersal and recruitment of new individuals of *D. wilsonii*. In fact, species classified as rare with respect to their distribution ranges, such as *D. wilsonii*, tend to have low dispersal capacity (Kunin & Gaston 1997).

Furthermore, the intermediate morphology of hybrids is expected to make them poorly suited for establishment in the parental environments (Rice & Hostert 1993; Rundle & Whitlock 2001), which may explain why *D. wilsonii* is rare and restricted to the ecotone of its parental species’ biomes (Rocha *et al.* 2024). While one of the parental species (*D. mollis*) is native to the *Cerrado*, the other parental species (*D. exaltata*) is the only known

species within the genus *Dimorphandra* endemic to the Atlantic Forest, and *D. wilsonii* occurs in the *Cerrado*/Atlantic Forest ecotone (Rocha *et al.* 2024). Indeed, colonisation of different habitats has been identified as a key factor shaping the late evolutionary history of the genus *Dimorphandra* (Rocha *et al.* 2024). The Amazon Forest is likely to have given rise to the *Dimorphandra* lineage, which in turn gave rise to the savannah lineage, which in turn gave rise to a more recent lineage that returned to a forest habitat, the Atlantic Forest (Rocha *et al.* 2024). These habitat changes, combined with the presumed breakdown of prezygotic and postzygotic barriers, may have favoured the diversification of the genus *Dimorphandra*, but also the emergence of hybrids such as *D. wilsonii*. Trees of *D. wilsonii* are self-compatible, and stingless bees - generally self-pollination promoters - are the most probable biotic pollinators. However, cross-pollination would increase fruit and seed set, which would result in more seedlings. Although *D. wilsonii* individuals are the result of hybridisation, our results indicate that these individuals are capable of sexual reproduction and can produce offspring. The conservation programme for *D. wilsonii*, originally for biodiversity purposes, aimed to produce F2 saplings in nurseries, with some of them being reintroduced into the field (Martins *et al.* 2014). The first reintroduced individuals are now about nine years old and 7 m tall, and some have flowered, although fruit or seeds have not yet been observed (F.M. Fernandes 2018, personal communication). Congruent with this, *D. wilsonii* has a low dispersal capacity and

Table 3 – Results of a general linear model (GLM, binomial distribution) analysis for the effects of pollination treatment and number of days from germination on the survival of *Dimorphandra wilsonii* seedlings. The presence or absence of seedlings was the response variable. *P*-values in bold indicate significant effects based on Tukey’s test. AIC: Akaike information criterion. T-ratio: test of the significance of the predictor variable (pollination treatments), calculated as the ratio of the estimated coefficient to its standard error.

Fixed effects	AIC	Contrast	t-ratio	p-value
Pollination treatment $\chi^2 = 38.95$, $p < .0001$	1969.5	Hand self- x Natural	-5.22	< .0001
		Hand self- x Hand cross-	-5.50	< .0001
		Hand self- x Spontaneous self-	-5.89	< .0001
		Natural x Hand cross-	-0.26	0.994
		Natural x Spontaneous self-	-0.69	0.899
		Hand cross- x Spontaneous self-	-0.44	0.971
Days $\chi^2 = 129.17$, $p < .0001$	1886.8	14 x 30 days	7.92	< .0001
		14 x 90 days	8.47	< .0001
		14 x 180 days	8.76	< .0001
		30 x 90 days	0.71	0.891
		180 x 30 days	-1.19	0.628
		180 x 90 days	-0.49	0.961

a low recruitment of new individuals in nature, with a low proportion of young individuals. To understand more generally the changes in sexual reproduction of *D. wilsonii* caused by hybridisation, it is crucial to understand the mating system, flower morphology, phenology, possible pollinators and ploidy level not only of *D. wilsonii* but also of its parental species. In the same way, experimental crosses between the three species and successive

evaluations of F1 reproductive success could clarify the hybridisation pathways of the genus *Dimorphandra*.

In summary, contrary to the possibility of complete sterility (Baack *et al.* 2015), *D. wilsonii* hybrids have reproductive autonomy and can produce viable offspring, which could make them a functionally distinct biological entity from their parental species. However, our results also provide evidence of inbreeding depression in *D. wilsonii*, as evidenced by the difference in fruit production and seed quality between the cross-pollination and self-pollination treatments. Thus, the protection of the habitat where *Dimorphandra* species co-occur, specifically the Cerrado/Atlantic Forest ecotone, is essential for the continuity of the eco-evolutionary processes of habitat shifts to which the *Dimorphandra* lineage is naturally subjected (Rocha *et al.* 2024). This would mean conserving not only *D. wilsonii* but also its parental species, *D. exaltata* and *D. mollis*, thus preserving the genetic diversity of the three species and reducing their risk of extinction. However, our results raise new a question: are F3 individuals able to reproduce? If the answer is yes, then hybrids of *D. wilsonii* would be able to produce fertile offspring. Furthermore, an assessment of the genetic mechanisms that allow *D. wilsonii* to be reproductively fertile is needed to help to determine the taxonomic and conservation status of *D. wilsonii*.

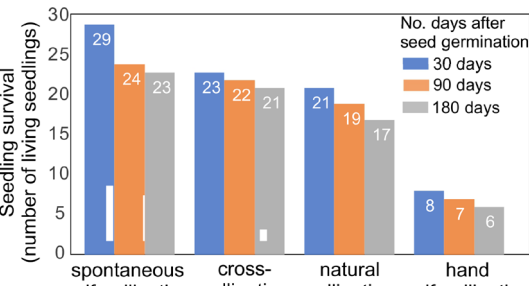


Figure 4 – Survival of *Dimorphandra wilsonii* seedlings from different pollination treatments (spontaneous self-pollination, manual self-pollination/geitonogamy, manual cross-pollination, natural pollination). The bars represent the cumulative number of living seedlings during each observation period: 30, 90 and 180 days after the start of the germination experiment.

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Data availability statement

In accordance with Open Science communication practices, the authors inform that all data are available within the manuscript.

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