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Original article

Genetic and reproductive evidence of incomplete isolations barriers between *Pterodon emarginatus* and *P. pubescens* (Leguminosae, Papilionoideae)

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ABSTRACT

Pterodon emarginatus Vogel and *P. pubescens* (Benth.) Benth. are phylogenetically related trees that compose a clade of sister species abundant in the Brazilian Savanna. Despite their morphological differences, some individuals with intermediate morphological characteristics have been reported, indicating the formation of interspecific hybrids. This study proposed to evaluate the genetic structure and controlled pollination of individuals of *P. pubescens* and *P. emarginatus* in areas of sympatry with the presence of putative hybrids. For this purpose, we genotyped seven microsatellite loci from 61 individuals collected from four apparent contact zones between *P. pubescens* and *P. emarginatus*. Controlled pollination experiments were performed on 4,133 flowers from six trees of *P. emarginatus* and five of *P. pubescens*. We observed two genetic clusters (k= 2) that corroborate the divergence between *P. pubescens* and *P. emarginatus*. The individual genetic assignment showed evidence of natural hybridization between *P. pubescens* and *P. emarginatus*. The genetic assignment did not fully support the visual description of the diagnoses of individual morphological characteristics. Controlled interspecific pollination generated fruit and seed production, possibly indicating the absence of a reproductive barrier at the pollination and ovule fertilization levels between these species. Our results enlarge the understanding of the diversification process of *Pterodon* species.

Keywords: Controlled pollination; Microsatellite markers; Neotropical tree; Speciation; sucupira-branca.

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Introduction

The evolutionary consequences of hybridization range from the termination of reproductive isolation between species (Servedio & Noor 2003) to the homogenization of both species' gene pools (Currat *et al.* 2008; Marques *et al.* 2014). The outcome of these hybrids depends on their genetic combinations, demography, population structure, and reproductive viability (Yan *et al.* 2017). It is not clear which ecological and evolutionary factors drive one outcome of hybridization and not the other (Turchetto *et al.* 2022).

The presence of both biotic and abiotic factors suggests that the biomes of Savannas and grasslands might shelter a relatively abundant. Since Savannas are relatively young Neotropical biomes, it is likely that their hybrids reflect a minimal divergence among the endemic species that resulted from recent radiation (Schley *et al.* 2022; Turchetto *et al.* 2022). Moreover, these Neotropical biomes have widely distributed groups that present clear genetic evidence of hybridization (e.g., Ribeiro *et al.* 2018). However, there are only a few studies examining the impacts of hybridization on these biomes (Schley *et al.* 2022; Turchetto *et al.* 2022).

The Brazilian Savanna biome (also known as Cerrado) is considered one of the greatest biodiversity hotspots in the world (Myers *et al.* 2000), sheltering two non-endemic tree species belonging to the genus *Pterodon* (Leguminosae): *P. emarginatus* and *P. pubescens*. In addition to sharing the same popular name ("sucupira-branca"), the two species have resembling genetic variations (Lima 2019) and chromosomal structure similarities (Bandel 1974; Coleman & Demenezes 1980; Albernaz 2020), thus being phylogenetically recognized as closely related sibling species (Cardoso *et al.* 2013). In morphological terms, *P. emarginatus* (Fig. 1AD) has a glabrous leaf, glabrous or glabrescent rachis containing 4-10 leaflets, truncated to the strongly emarginate apex, and violaceous flowers (Rocha 2006). *Pterodon pubescens* (Fig. 1BE) has leaves containing 6-19 elliptic leaflets (usually 11-13), slightly ovate, pubescent on both sides, retuse to rounded apex, densely pubescent rachis, and pale pinkish almost white-to-dark pink flowers (Rocha 2006). Both mating systems are allogamous, and the flowers are pollinated by *Bombus atratus* and *Apis mellifera* bees, reaching flowering peaks in September, and the dispersing agent is the wind (da Silva Júnior 2012).

Our field observations revealed that both *Pterodon* species have a disjunct distribution. In addition to occurring in the Cerrado areas further South, the *Pterodon pubescens* is also present in the states of São Paulo, Mato Grosso do Sul, the south of the states of Mato Grosso, Minas Gerais, Goiás, and in the south of the Federal District. In turn, *P. emarginatus* occupies the following areas further North: north of the states of Mato Grosso, Federal District, Minas Gerais, and the north center of the states of Goiás, Tocantins, Maranhão, Bahia. Individuals showing intermediate characteristics appeared (SL Lima unpubl. res.) in the regions with overlapping distribution.

In a narrow range in the Distrito Federal (Brazil), Rocha (2006) detected individuals showing intermediate morphological features between both species (Fig. 1CF), in addition to some genetic hybrids (via RAPD). Such a finding reveals the possibility of interspecific permeability of species borders. In turn, a phylogeography study indicated that

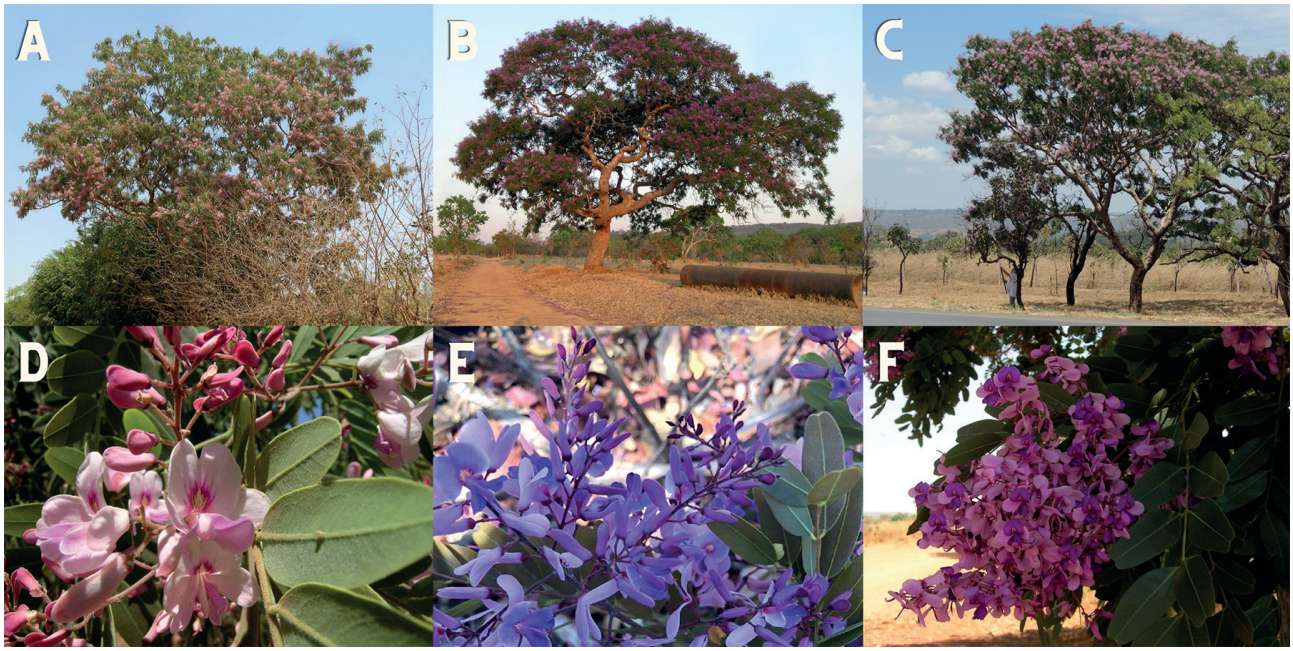

Figure 1. Trees of *Pterodon emarginatus* (A), *P. pubescens* (B), and potential interspecific hybrid (C) in the natural environment, followed by their respective flowers (D, E, and F). Elaborated by the authors, DMS Rocha & VFM Lima.

P. pubescens and *P. emarginatus* underwent recent divergence; therefore, the barriers of reproductive isolation between them might not be absolute (Lima 2019). Herein, our field observations detected four apparent contact zones between the two species, with flowering adults. Thus, this species pair represents a great study model for reproductive isolation, taxonomic cohesion, and natural hybridization. The reproductive isolation of related species (*e.g.*, Johnson *et al.* 2015; Kuligowska *et al.* 2015; Wang *et al.* 2015) is often associated with post-pollination mechanisms, and might occur in the following situations: if interspecific pollen grains do not germinate on the stigma; pollen tubes do not grow properly in the style, absence of fertilization, and if the embryo and/or endosperm does not develop well (Rieseberg & Carney 1998; Kuligowska *et al.* 2015). However, such barriers might have occurred if seeds are produced after interspecific cross-pollination. In this sense, controlled crossings might help understand the hybridization between these species.

Melo *et al.* (2022) used *P. pubescens* sequence data to develop a set of microsatellite markers (SSR) for studying the genetics of both populations. The author's proposal was unprecedented in this research line and indicated a high genetic diversity (higher in *P. pubescens*) with significant intrapopulation inbreeding.

More recently, methods of hybridization detection have estimated the degree of genetic variation shared between adjacent or co-occurring species, including calculating "hybrid indices" to assess the proportion of ancestry inherited from each parent (Schley *et al.* 2022). In this regard, population-level sampling strategies and molecular markers approaches (e.g., microsatellites, AFLPs, and SNPs from RADSeq) are used (Schley *et al.* 2022). Studies using molecular markers that can differentiate between closely related species and their hybrids help enlarge the knowledge on the speciation genetic basis and the effects of interspecific crossing on species integrity. Additionally, these studies provide further information on the evolutionary history of the species involved.

Despite the studies addressing these trees of wide economic and cultural potential (e.g., Mors *et al.* 1967; Bustamante *et al.* 2010; Basting *et al.* 2019; Kleinubing *et al.* 2022), the understanding of their intra- and interspecific genetic variations is yet to be investigated. Therefore, this study aimed to evaluate the genetic composition and controlled pollination of individuals from apparent contact zones of *P. pubescens* and *P. emarginatus* containing putative hybrids. Thereby, we sought to answer the three following main questions: (I) Are there genetic groups that support the divergence between *P. pubescens* and *P. emarginatus*? (II) Does the genetic composition of individuals characterized as putative hybrids in the field corroborate the hypothesis of natural hybridization between *P. pubescens* and *P. emarginatus*? and (III) Does interspecific crossing produce fruits with well-formed seeds?

Materials and methods

Material sampling and identification

We collected young leaves from 61 individuals in four natural sites presenting an apparent sympatry between *Pterodon pubescens* Benth. (Benth.) and *Pterodon emarginatus* Vogel (Fig. 2). Each individual collected was identified in the field (Table 1) following the visual description of diagnoses morphological characters (Rocha 2006): *P. pubescens* (pale pinkish flowers, pubescent leaves, and leaflets with retuse to rounded apex), *P. emarginatus* (violaceous flowers, glabrous leaves, and leaflets with truncate to strongly emarginate apex), or potentials hybrids (individuals with intermediate or discordant characteristics of Rocha's (2006) classification).

Vouchers were taken to record each phenotype/species and were deposited at Unidade de conservação/

Table 1. Description of the four natural sites in Brazil where apparent sympatry of *Pterodon pubescens* and *P. emarginatus* was reported, as well as the presence of potential hybrid (except in Itacaiú/GO). Field recording based on Rocha (2006).

Localization of natural sites	Geographical coordinates		Field record	N° of individuals
	Latitude	Longitude		
Itacaiú-GO	-15.020665	-51.309024	*Pterodon emarginatus*	1
			Pterodon pubescens	1
			Potential hybrid	0
Brasília-DF	-15.8906	-47.7502	*Pterodon emarginatus*	5
			Pterodon pubescens	6
			Potential hybrid	13
Corumbá de Goiás-GO	-15.847826	-48.766893	*Pterodon emarginatus*	2
			Pterodon pubescens	1
			Potential hybrid	3
Pirenópolis-GO	-15.80403	-48.8770	*Pterodon emarginatus*	2
			Pterodon pubescens	26
			Potential hybrid	1
All				61

PRPI – Herbário UFG (in Goiânia-GO, Brazil) –, as follows: *P. pubescens*, Herb Nº: 68.409 and 68.413; *P. emarginatus*, Herb Nº: 68.411, 68.414, 68.415, and 68.416, and the potential hybrid, Herb Nº: 68.410. The collected plant material was identified and packed in plastic bags containing spherical silica gel. Subsequently, these packages were transported to the Laboratório de Genética e Biodiversidade (LGBio) of the Universidade Federal de Goiás (Goiânia-GO, Brazil). After separating the material for DNA extraction, the remaining dried leaves were stored in a deep freezer (-80 ºC).

Molecular analysis

We have obtained total genomic DNA from leaf tissue using CTAB (cetyl-trimethyl ammonium bromide) extraction buffer, according to the extraction protocol by Doyle and Doyle (1987), modified by Ferreira and Grattapaglia (1996). DNA quality and concentration were assessed through electrophoresis on an agarose gel of 1%. The genotypes of the individuals were identified through seven nuclear microsatellite markers, developed for *P. pubescens* and transferred to *P. emarginatus* (Melo *et al.* 2022) (Table 2).

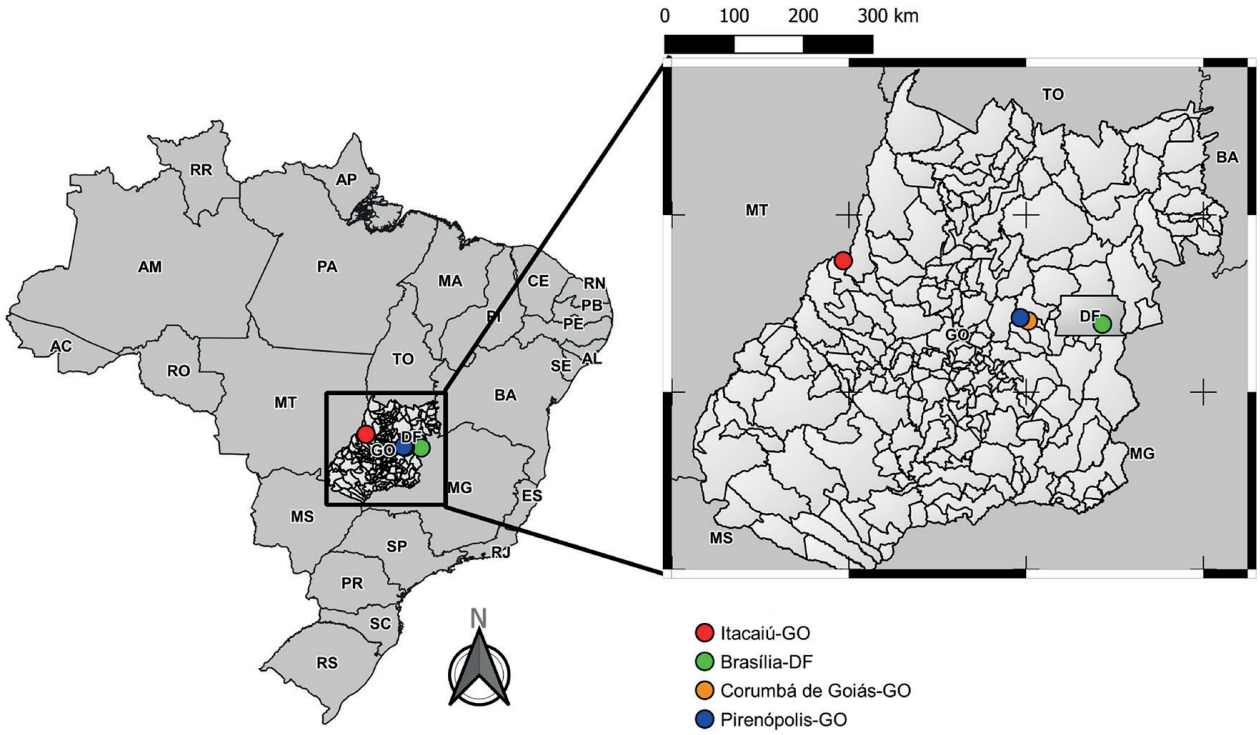

Figure 2. Representative map of the four natural sites presenting an apparent sympatry of *P. pubescens* and *P. emarginatus*. Table 1 shows the details of geographic coordinates and the number of individuals.

Table 2. List of application multiplexes used for genotyping of the studied individuals using seven microsatellite markers developed by Melo *et al.* (2022). Forward primers were labeled with specific DYE (fluorochromes) for detection in capillary electrophoresis (genotyping). Potential hybrids were tested at the annealing temperatures of *P. pubescens*.

Multiplexes	Locus	Repeat motif	Allele range (bp)	Annealing temperature	
				P. pubescens	*P. emarginatus*
1	PEM21	$(AG)_{20}$	254-306	56 ºC	56 ºC
	PEM24	$(AG)_{19}$	158-198	54 ºC	54 ºC
	PEM26	$(AG)_{17}$	342-408	56 ºC	56 ºC
2	PEM23	$(AG)_{19}$	308-342	54 ºC	54 ºC
	PEM18	$(AG)_{24}$	196-222	54 ºC	54 ºC
	PEM22	$(AC)_{19}$	194-226	54 ºC	54 ºC
	PEM25	$(AC)_{17}$	216-298	54 ºC	56 ºC

PCR reactions were performed in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems®) under the PCR protocol and the thermal cycling conditions described by Melo *et al.* (2022): one cycle at 94 °C for five min; 30 cycles of 94 °C for one min, annealing temperature for one min (depending on the locus, Tab. 2), and 72 °C for one min; and 72 °C for 30 min to enforce 3' Taq adenylation. The annealing temperature between *P. emarginatus* and *P. pubescens* presents a difference (of 2 °C) in only one marker (PEM25). Therefore, for the potential hybrid individuals, we tested the PCR reaction with the annealing temperature optimized to *P. pubescens*. The PCR products were multiplexed, denatured, and size-fractionated using capillary electrophoresis on an ABI 3500 DNA Analyzer (Thermo Fisher Scientific®) with a LIZ (600) molecular size standard (Thermo Fisher Scientific®). The molecular data obtained are available in Table S2 of the Supplementary Material.

Statistical analyses

We estimated the genetic diversity parameters on the FSTAT 2.9.3.2 (Goudet 2002) and GDA Genetic Data Analysis 1.0 (Lewis & Zaykin 2001) programs. The following parameters were estimated: the observed heterozygosity (Ho), the expected heterozygosity under Hardy-Weinberg equilibrium (*He*), the number of alleles (*A*), and the average allelic richness based on the minimum sample size (A_R). In addition, we also estimated the probabilities of identity (*PI*) and paternity exclusion (*Q*) on the Identity v.1.0 software (Wagner & Sefc 1999) to verify the quality of the microsatellite set in individual discrimination.

The genetic structure of the individuals was analyzed based on the Bayesian clustering method implemented on the STRUCTURE 2.3.4 software (Pritchard *et al.* 2000). We performed 30 independent runs for *K* values ranging from one (no genetic structure) to four (assuming four natural sites) genetic clusters. Each run covered 1,000.000 Monte Carlo simulations through Markov chains (MCMC), with a burn-in of 10%, assuming the mixture model with correlated alleles between individuals (admixture model). We then inferred the appropriate *K* values to explain the data, as proposed by Evanno *et al.* (2005) and Puechmaille (2016) in the STRUCTURE SELECTOR platform (Li & Liu 2018). The Structure Selector platform allowed us to obtain the coancestry values of each individual belonging to a given cluster and the assignment probability values were assigned through multiple replicates for the same *K* value (Kopelman *et al.* 2015) on the CLUMPAK package, provided by the same platform.

Through simulations on the NEWHYBRIDS 1.1 software (Anderson & Thompson 2002), we also evaluated the posterior probability (PP) of each individual belonging to one of the following probability classes of Mendel's law: Pure Parental A (*P. pubescens*), Pure Parental B (*P. emarginatus*), F1 hybrid, F2 hybrid, backcross A (with *P. pubescens*), and backcross B (with *P. emarginatus*). The assignment to different hybrid categories was systematized upon the individual belonging to any of the six classes considering a posterior probability ≥ 0.70. Individuals with PP ≥ 0.90 were considered to belong to pure parental lineages, whereas those without probability PP ≥ 0.70 for any of the six Mendel's laws classes were considered of an uncategorized hybrid origin. This test was performed with no prior information on allele frequencies, "Jeffery's like priors", and was based on 100,000 Monte Carlo simulations through Markov chains (MCMC) after a burn-in period of 100,000 to ensure the convergence of chains and homogeneity of runs.

Controlled pollination experiments

We conducted controlled pollination experiments (in 2019 and 2021) in six trees of *P. emarginatus* from a fragment of Cerrado, at the Planaltina Campus of the Universidade de Brasília (Planaltina-DF, Brazil). For *P. pubescens*, the experiments used five trees from a fragment of Cerrado at the Campus of Universidade de Brasília (Brasília-DF, Brazil). We performed hand pollinations during the co-flowering period to verify whether intra- and heterospecific pollen depositions produced fruits with well-formed seeds, in addition to comparing the seed set rates between intraspecific and interspecific cross-pollinations. We also performed manual self-pollination and checked the occurrence of self-pollination and natural production of fruit and seed (control). The following individuals were chosen for our analyses: a monospecific population of *P. pubescens* with previously genotyped trees (Rocha 2006) and a monospecific population of *P. emarginatus* with trees from a contiguous area to that studied by Rocha (2006), identified as *P. emarginatus* (both by morphological characteristics and RAPD).

The following four treatments were applied to the bagged flowers of each species (see Table 5 for sample size): (1) heterospecific pollination (hybridization): stigmas of several flowers of one of the species were pollinated with pollen from the other species; (2) conspecific cross-pollination: stigmas of several flowers were pollinated with pollen from flowers of another distant plant of the same species (at least 30 m); (3) manual self-pollination: stigmas of several flowers were pollinated with pollen from flowers of the same tree; (4) spontaneous self-pollination: flowers in pre-anthesis and bottom of several inflorescences were counted and the inflorescences were bagged to check whether self-pollination occurs spontaneously; (5) Control: counting of the flowers in several inflorescences of different plants of both species for tagging and fruit counting. From experiments 1 to 4, the inflorescences of the flowers examined were bagged before and after hand pollination. In experiments 1 and 2, the flowers were emasculated before anthers opened, manually pollinated, and bagged again until the fruits were set. Subsequently, the fruits were counted, and the reproductive success rates were compared between

treatments. The resulting well-formed fruits were collected and counted during the fruiting period.

We adopted general linear mixed models (GLMM) assuming a binomial error distribution (individual as a random factor) to test whether the fruiting rate differed between species and treatment; also in addition, the interaction between species and treatment was considered. Posthoc comparisons between treatment levels were established through the Tukey's method. All analyses were carried out in R version 4.1.2 (R Core Team 2021), using the packages lme4 v4.1-28 (Bates *et al.* 2014) and Emmeans v1.7.2 (Lenth 2022) for model fit and posthoc tests.

Results

Descriptive estimates of genetic variability

High estimate values of combined paternity exclusion probability ($Q \sim 0.99985$) were shown by the microsatellite set, thus demonstrating a strong ability of the markers to exclude potentially false paternity. We also found low values of combined probability of identity ($I \sim 8.441*10^{-11}$), indicating that the microsatellite set presents a strong power of individual discrimination since the estimated *PI* is almost zero.

The PEM25 locus is the only one that has a different annealing temperature between species. The first PCR test amplified the locus PEM25 in the hybrid individuals at the annealing temperature of *P. pubescens* (54 °C). Moreover, all loci were polymorphic, and 96 alleles (Table 3) were obtained, with an average allele richness per locus of 13.446. Higher values of allele number and heterozygosity were observed, thus indicating a greater content of marker information (Table 3).

Genetic structure

The Bayesian analysis using STRUCTURE suggested the following numbers of clusters: k= 2 by the Puechmaille (2016) method and k= 3 by the Evanno *et al.* (2005) method (Fig. 3). The k= 2 scenario summarized the information better than the k= 3 (Fig. 3). The k= 2 comprises the two parental species as contributors to the genetic structure of our samples, showing distinct cluster assignments between the *P. emarginatus* (PEM) and *P. pubescens* (PPU) individuals (coancestry values > 0.82). The k= 3 scenarios present a substructure of *P. pubescens* into two genetic clusters.

We identified the two parental species in k= 2 as contributors to the total gene pool sampled; therefore, individuals whose coancestry value was above or equal to 0.800 in any of the clusters were considered canonical species (for detailed coancestry values, see Table S1 in Supplementary Material). Thus, we assigned 40 individuals as *P. pubescens*, 15 as *P. emarginatus*, and six as hybrids.

The genetic assignment by STRUCTURE showed differences (compared with field record) in the number of individuals assigned, as follows: from 34 to 40 for *P. pubescens*, from 10 to 15 for *P. emarginatus*, and from 17 to 6 for the hybrids. Based on six individuals with a clear hybrid identity, this initial evaluation revealed a genetic admixture between the species.

Hybrid assignment

The Bayesian analysis by NEWHYBRIDS provided a clear genetic distinction of the individuals (for detailed posterior probability values, see Table S1 in Supplementary Material). In total, 87% of the individuals (n= 53) had significant values (≥ 0.700) of posterior probability (PP), thus belonging to either of the classes.

The genetic identification of the 61 individuals analyzed showed 13 *P. emarginatus* pure lineage (PP ≥ 0.930), 33 *P. pubescens* (PP ≥ 0.760), out of which only 28 were pure lineage (PP ≥ 0.900), and seven F2 hybrids (PP ≥ 0.773) (Fig. 4). The eight individuals wigth no significant probability (< 0.700) of belonging to any of the six classes were considered uncategorized hybrids, however with high genetic admixture values. Two of these uncategorized hybrids showed a higher posterior probability (PP) of being *P. pubescens* (0.452 < PP < 0.465), two *P. emarginatus* (0.520 < PP < 0.697), and the other four F2 hybrids (0.543 < PP < 0.633).

Table 3. The estimates of genetic variability parameters were analyzed from seven microsatellite loci for the sample of individuals registered as *P. pubescens*, *P. emarginatus*, and their potential hybrids. *A*: number of alleles; A_R: average allelic richness based on minimum sample size (n= 53); *He*: expected heterozygosity under HWE; *Ho*: observed heterozygosity; *PI*: identity probability; *Q*: paternity exclusion probability; *IC*: combined probability of genetic identity; and *QC*: combined probability of paternity exclusion. All values are rounded to three decimal places.

Locus	*A*	A_R	*He*	*Ho*	*PI*	*Q*
PEM21	20	19.383	0.918	0.845	0.015	0.819
PEM24	12	11.925	0.817	0.782	0.060	0.636
PEM26	12	11.834	0.840	0.679	0.049	0.668
PEM25	12	11.777	0.837	0.339	0.048	0.672
PEM23	15	15.000	0.905	0.698	0.020	0.792
PEM18	15	14.574	0.876	0.831	0.031	0.738
PEM22	10	9.650	0.809	0.433	0.067	0.614
Overall loci	96	13.449	0.857	0.658	IC = $8.441x10^{-11}$	QC= 0.99985

This fine genetic assignment provided 33 *P. pubescens* individuals, 13 *P. emarginatus,* and 15 hybrids. The NEWHYBRIDS results show the following differences in the number of individuals (compared with the field record): from 34 to 33 for *P. pubescens*, from 10 to 13 for *P. emarginatus*, and from 17 to 15 for the hybrids.

Agreement between hybrid assignment methods

We performed a combined evaluation of the field observation and genetic identification assignments (STRUCTURE and NEWHYBRIDS) (Table 4 and Table S1 in Supplementary Material). Approximately 67% of the

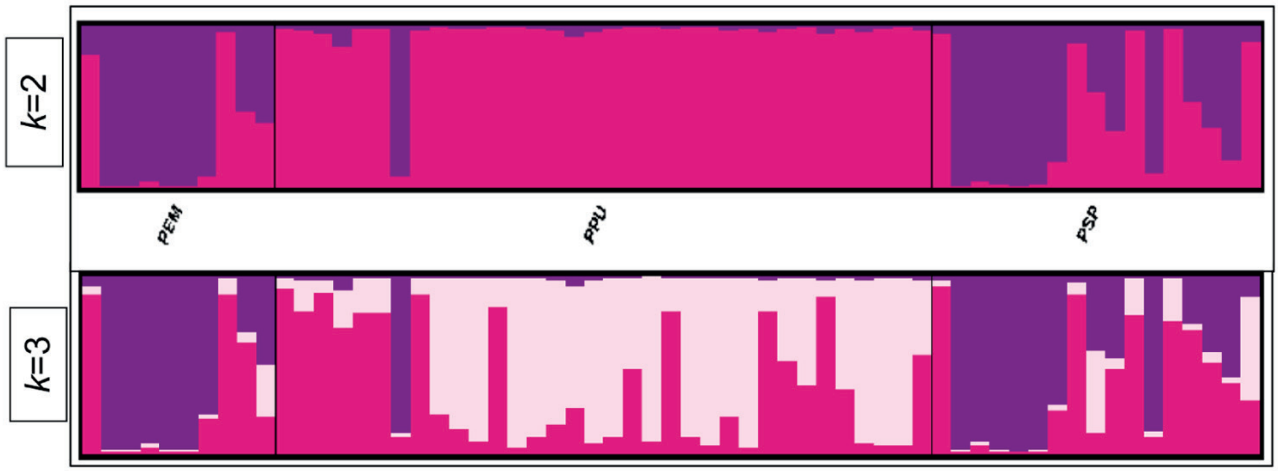

Figure 3. Bayesian clustering based on coancestry values, as follows: A) the formation of two genetic groups (*k*= 2) highlighted in dark pink and purple by the Puechmaille (2016) method and B) the formation of three genetic groups (*k*= 3) highlighted in dark pink, light pink, and purple by the Evanno *et al.* (2005) method. All data were grouped according to the field records, based on Rocha (2006), as follows: PEM - *P. emarginatus*, PPU - *P. pubescens,* and PSP - potential hybrids.

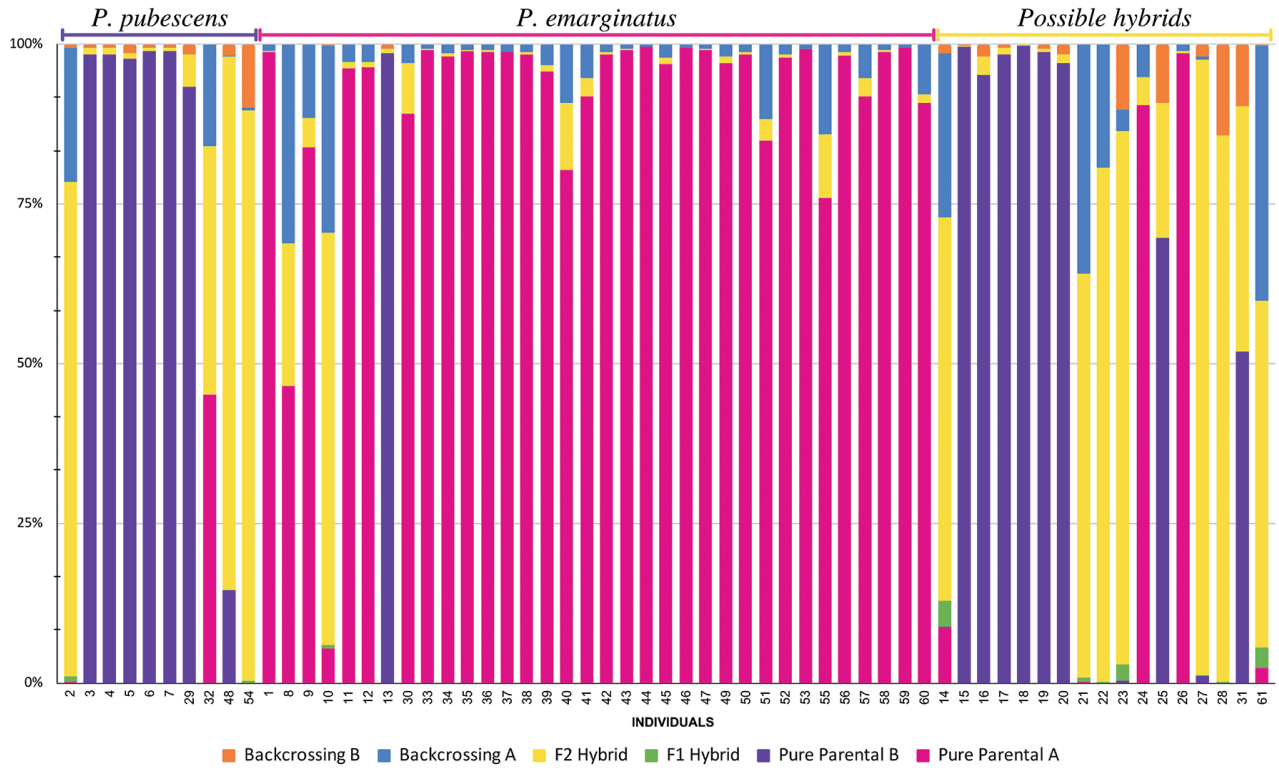

Figure 4. Posterior probability (PP) according to the category analyses on NEWHYBRIDS to verify hybridization between "sucupira-branca" species. Pure parent A (*P. pubescens*) is in pink; Pure parent B (*P. emarginatus*) is in purple. The size proportion of the categories in the individual bars indicates a higher assignment probability per class. All individuals were grouped according to the field records, based on Rocha (2006).

Table 4. The classification was compiled from the field record according to the morphological diagnosis by Rocha (2006) and the genetic identification obtained by STRUCTURE's coancestry and posterior probability (PP) in NEWHYBRIDS. Discrepancies in genetic identification between STRUCTURE and NEWHYBRIDS were classified as "Questionable hybrid" individuals. *Discrepancies between field records and genetic identification.

Localization	ID	Field record	Genetic identification
Brasília-DF	2	*P. emarginatus*	*P. emarginatus*
Brasília-DF	3	*P. emarginatus*	*P. emarginatus*
Brasília-DF	4	*P. emarginatus*	*P. emarginatus*
Brasília-DF	5	*P. emarginatus*	*P. emarginatus*
Brasília-DF	6	*P. emarginatus*	*P. emarginatus*
Brasília-DF	12	*P. pubescens*	Questionable hybrid*
Brasília-DF	13	*P. pubescens*	*P. pubescens*
Brasília-DF	14	*P. pubescens*	Questionable hybrid*
Brasília-DF	15	*P. pubescens*	*P. pubescens*
Brasília-DF	16	*P. pubescens*	*P. pubescens*
Brasília-DF	17	*P. pubescens*	*P. emarginatus**
Brasília-DF	45	Potential hybrid	Questionable hybrid
Brasília-DF	46	Potential hybrid	*P. emarginatus**
Brasília-DF	47	Potential hybrid	*P. emarginatus**
Brasília-DF	48	Potential hybrid	*P. emarginatus**
Brasília-DF	49	Potential hybrid	*P. emarginatus**
Brasília-DF	50	Potential hybrid	*P. emarginatus**
Brasília-DF	51	Potential hybrid	*P. emarginatus**
Brasília-DF	52	Potential hybrid	Questionable hybrid
Brasília-DF	53	Potential hybrid	Hybrid
Brasília-DF	54	Potential hybrid	Hybrid
Brasília-DF	55	Potential hybrid	*P. pubescens**
Brasília-DF	56	Potential hybrid	Questionable hybrid
Brasília-DF	57	Potential hybrid	*P. pubescens**
Corumbá de Goiás-GO	7	*P. emarginatus*	*P. emarginatus*
Corumbá de Goiás-GO	8	*P. emarginatus*	Questionable hybrid*
Corumbá de Goiás-GO	18	*P. pubescens*	*P. pubescens*
Corumbá de Goiás-GO	58	Potential hybrid	Hybrid
Corumbá de Goiás-GO	59	Potential hybrid	Hybrid
Corumbá de Goiás-GO	60	Potential hybrid	Questionable hybrid
Itacaiú-GO	1	*P. emarginatus*	Questionable hybrid*
Itacaiú-GO	11	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	9	*P. emarginatus*	Hybrid*
Pirenópolis-GO	10	*P. emarginatus*	Hybrid*
Pirenópolis-GO	19	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	20	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	21	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	22	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	23	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	24	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	25	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	26	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	27	*P. pubescens*	*P. pubescens*

Table 4. Cont.

Localization	ID	Field record	Genetic identification
Pirenópolis-GO	28	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	29	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	30	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	31	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	32	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	33	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	34	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	35	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	36	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	37	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	38	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	39	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	40	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	41	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	42	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	43	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	44	*P. pubescens*	*P. pubescens*
Pirenópolis-GO	61	Potential hybrid	Questionable hybrid

individuals (Six *P. emarginatus*, 31 *P. pubescens,* and four hybrids) were assigned to the same group in both the field and genetic identifications. About ~18% (n= 11) of the individuals showed agreement in the genetic identification but differing in the field records. These individuals presented the following behavior: six were genetically *P. emarginatus* and potentially hybrid according to the field records, two were genetically *P. pubescens* and potentially hybrid according to the field records, two were genetically hybrids and *P. emarginatus* according to the field records, and one was genetically *P. emarginatus* and *P. pubescens* according to the field record. The agreement between the field records and STRUCTURE reached ~70% of the individuals, while the field record and NEWHYBRIDS agreed in ~75% of the cases.

For both methods, only ~15% of the individuals (*n*= 9) showed different genetic assignments (STRUCTURE and NEWHYBRIDS), thus being considered "questionable hybrid" individuals (see Table 4 for detailed data). 10% (n= 6) of the total sampled individuals (n= 61) were genetically identified as hybrids by both software programs.

Controlled pollination experiment

All treatments of *P. emarginatus* generated ripe fruits (Table 5). The rate of fruit and seed set was higher for cross and interspecific pollinations comparing with self-pollination, which produced one manually self-pollinated fruit and one spontaneous fruit. Interspecific and cross-pollinations also produced fruits with seeds on *Pterodon pubescens* (Table 6). Self-pollination could not set any fruits, thus indicating that the species is completely allogamous and self-incompatible. The control treatment had very low rates of fruit set. The final fruiting rate was the same for both species (X^2= 3.42, p= 0.18) but different between the treatments (X^2= 44.44, $p < 0.0001$). Both species had the same outcome in the interspecific cross-pollination treatment (z= 1.33, p= 0.85) and the intraspecific cross-pollination treatment did not generate different results either for the *P. pubescens* (z= 2.12, p= 9.94) or *P. emarginatus* (z= 0.63, p= 0.99) hybrids.

Table 5. Results of controlled pollination experiments conducted during the flowering of *P. emarginatus* in 2019 and 2021.

Experiment	Number of Flowers	Number of fruits – 30 days	Number of fruits mature with developed seed	Fruit and seed set
Interspecific pollination	97	11	8	0.0825
Cross-pollination	74	9	7	0.0946
Manual self-pollination	118	1	1	0.0085
Spontaneous self-pollination	188	1	1	0.0053
Control	1790	42	29	0.0162

Table 6. Results of controlled pollination experiments conducted during the flowering of *P. pubescens* in 2019 and 2021.

Experiment	Number of Flowers	Number of fruits – 30 days	Number of fruits mature with developed seed	Fruit and seed set
Interspecific pollination	105	7	5	0.0476
Cross-pollination	38	2	2	0.0526
Manual self-pollination	80	1	0	0
Spontaneous self-pollination	393	0	0	0
Control	1250	8	2	0.0016

Discussion

Hybridization plays an important role in plant evolution (Abbott *et al.* 2016) and varied according to the taxa (Taylor & Larson 2019), presumably due to the biological differences between species pairs, such as timing and speciation mode (Payseur & Rieseberg 2016). Moreover, interspecific gene flow via natural hybrid zones might be a source of genetic variability for adaptation to environmental changes (Janes & Hamilton 2017; Turchetto *et al.* 2022).

Species that have diverged recently and occur in contact zones provide a unique opportunity to study the evolutionary process involved in speciation. Our study identified sympatric genetic individuals of *P. emarginatus* and *P. pubescens* and presenting potential hybrids. Our analysis was based on highly variable and informative microsatellite loci (Melo *et al.* 2022) with great potential for individual and species discrimination (paternity exclusion probability > 0.99 and almost no identity probability). Overall, the nuclear microsatellite loci demonstrated that the two "sucupira-branca" species are genetically distinct.

Based on our field observations and the literature, *P. emarginatus* seems to be distributed toward north and *P. pubescens* toward the south of the Cerrado, with a latitudinal contact range between them, thus forming contact zones (Rocha 2006). Rocha (2006) found individuals living in such a latitudinal range – our sampling site (Figure 1 and 2) – presenting morphological features and genetic composition that shown to be intermediary of the two species.

Contact zones opened space for the study of hybrid production and interspecific gene flow. Our results demonstrated that interspecific pollination experiments generated fruits and seeds, thus indicating that the two species have no reproductive barrier for pollen germination on the stigma, pollen tube development in the style, or ovule fertilization levels. Despite the absence of such barriers, the numbers of backcrosses and hybrids are relatively low; therefore, the barriers might be of an ecological nature. Our field observations revealed that some barriers might occur at the floral biology level since flowers of *P. pubescens* open earlier (around 6:00h) than those of *P. emarginatus* (around 8:00h), despite the time overlap between 8:00 to 12:00h. In addition, the two species might share pollinators, such as *Bombus* sp. Furthermore, we did not test whether there is a difference between the seed viability produced during interspecific and intraspecific cross-pollinations. There might be variations in seed germination, as well as seedling and plant development. In this sense, further studies should investigate their floral and pollination biology, seed germination, and seedling development to better understand how the two species avoid intercrossing.

The control test produced fewer fruits and seeds than manual cross-pollinations, which may be explained by a natural pollination deficit. The two cases of seed set after self-pollination of 206 flowers do not indicate the species are self-fertile because they can be a product of cross-pollen contamination. But further study might be conducted to better clarify these species' self-compatibility systems.

Our results corroborate the hypothesis of interspecific genetic mixing between *P. emarginatus* and *P. pubescens* at the contact zone, which is often reported in the literature for other phylogenetically closely related species (Lorenz-Lemke *et al.* 2006; Taylor *et al.* 2014; Ley & Hardy 2017). Additionally, our analyses confirm a hybrid genetic status in six individuals at the contact zones, out of which four also have intermediate morphology, according to the field records (Table 4: ID 53, 54, 58, and 59).

We also detected that the morphological and genetic records indicated different assignments, as reported in other studies involving phylogenetically close plants (Teixeira *et al.* 2019; Schnitzler *et al.* 2020). In fact, the unpredictable phenotype expression in hybrids hampers the morphological hybrid diagnosis (Rieseberg *et al.* 1993; Teixeira *et al.* 2019). Since several genetic and environmental factors influence the hybrid phenotypic expression, genetic analyses using molecular markers should provide more robust diagnoses for hybrids presenting the phenotypic expression of a parental species, as our results show (López-Caamal & Tovar-Sánchez 2014).

We found a uniform hybridization pattern at the four sampling sites, given that they were limited to F2 hybrids (see NEWHYBRIDS results), indicating a cross between F1 hybrids. However, NEWHYBRIDS analyses may underestimate backcrosses when evaluating parental species that have recently diverged, thus classifying individuals as belonging to the pure lineage of either parent (Vähä & Primmer 2006). Such a scenario could indicate a lower frequency of backcrosses in our observations.

The genetic composition of each studied individual, with clear genetic distinction, confirm that *P. emarginatus* and *P. pubescens* should be considered independent species. However, there are no definite reproductive barriers between the two species, thus promoting typical hybrid genetic mixing. We suggest that the small genetic admixture in the canonical species (see STRUCTURE results) might be associated with size homoplasy since the alleles have the same size but the sequence is different (Estoup *et al.* 2002). Nonetheless, such a hypothesis should be verified through genotyping by sequencing the microsatellites.

An easy and successful interspecific crossing depends directly on the phylogenetic relationship between the species involved in hybridization, in addition to chromosomal homology. Otherwise, there might be incongruities/ incompatibilities between the species' genomes. *Pterodon emarginatus* and *P. pubescens* have the same karyotypic number 2n= 16, with small and morphologically similar chromosomes (Bandel 1974; Coleman & Demenezes 1980; Albernaz 2020). More recently, Albernaz (2020) studied the genomes of *P. pubescens* and *P. emarginatus* and found highly similar repetitive fractions. Thus, there are several elements in *P. pubescens* and *P. emarginatus* that facilitate interspecific hybridization, such as phylogenetic proximity, recent divergence, and vast cytomolecular similarity (both structural and numerical).

It is difficult to detect the processes that lead to species hybridization, even more so for those with a history of introgression and ancestral polymorphisms persisting in the speciation process, such as "sucupira-branca" (Lima 2019). The function of these hybrids in diversifying the studied species are unknown, as well as whether they reinforce reproductive isolation (due to the low viability of hybrids). However, hybrid formation does not necessarily have a specific function, it might simply be result from incomplete species barriers, such as in genetic material exchange (e.g., Zhang *et al.* 2016). In this sense, further studies should detail the reproductive biology and viability of hybrids of *P. emarginatus* and *P. pubescens* to better understand the role of this phenomenon in their evolutionary history.

Our study enlarges the knowledge on the diversification process of the "sucupira-branca" species. The microsatellite set indicated genetic groups that distinguish the *P. pubescens* and *P. emarginatus* individuals. Although approximately 10% of the studied individuals were genetically identified as hybrids, the intermediate morphological features per se do not allow us to identify an individual as hybrid. These contrasts found between the genetic and morphological attributions of *P. pubescens*, *P. emarginatus,* and their potential hybrids, highlight that integrative studies must investigate the ecological and evolutionary scenarios of these species deeper.

Although Rocha (2006) and Sonsin-Oliveira *et al.* (2022) has suggested the existence of hybridization between *P. emarginatus* and *P. pubescens*, our study is the first to reveal the reproductive viability of hybrid formation, in addition to genetic evidence of natural hybrids between these species. On the other hand, considering their evolutionary proximity, studies adopting more specific approaches, including the genetic identification of hybrid seeds or broad genomic analyses, should differentiate recent hybridization from the retention of ancestral polymorphism/ ancient hybridization.

Supplementary Material

The following online material is available for this article:
Table S1 - Table with data compiled from the field record based on Rocha (2006) and the genetic assignment by coancestry of STRUCTURE and posterior probability (PP) in NEWHYBRIDS. For STRUCTURE (k= 2 obtained by Puechmaille (2016) method): Cluster 1 - *P. pubescens*, and Cluster 2 - *P. emarginatus*. For NEWHYBRIDS - the posterior probability of each individual belonging to one of the following probability classes of Mendel's law: PPU – Pure Parental A (*P. pubescens*), PEM – Pure Parental B (*P. emarginatus*), F1HYB – F1 Hybrid, F2HYB – F2 Hybrid, BPPU – Backcross with *P. pubescens*, and BPEM – Backcross with *P. emarginatus*.
Table S2 - Description of the molecular data used in the genetic analyses of the 61 individuals of *Pterodon* in this study. Field record based on Rocha (2006).

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