Genetic diversity of an endangered palm in forest and open grassland landscapes: informing seed collection and conservation of Butia eriospatha

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Abstract

The Atlantic Forest is a biodiversity hotspot facing severe habitat loss and fragmentation, with most of its remnants experiencing biomass and biodiversity declines. *Butia eriospatha*, a vulnerable palm species endemic to this biome, is threatened with extinction by habitat degradation, illegal poaching, and lack of regeneration. Despite its ecological and cultural importance, genetic studies have focused only on grassland populations, leaving the rare forest populations unexplored. Here, we compared genetic diversity aspects between a forest (FOR) and an open grassland (GRA) *B. eriospatha* populations, aiming to provide seed collection guidelines and further expand the knowledge on genetic conservation of this important species. We assessed genetic diversity, mating system, spatial genetic structure (SGS), and spatial pattern for GRA and FOR populations, by sampling mature and seedling individuals. Both populations exhibited moderate genetic diversity ($\widehat{H}_E$ = 0.429–0.445), with no significant fixation, but significant genetic differentiation ($\widehat{F}_{ST}$ = 2–3%) between them. Allogamy predominated in both populations, with outcrossing rates not different from unity, although FOR displayed a small proportion of mating among relatives. SGS was weak but extended over larger distances in FOR (up to 60 m) compared to GRA (up to 30 m), suggesting differences in mortality dynamics. For long-term conservation (N_e = 500) and to maximize genetic diversity, seeds should be collected from 132 up to 139 seed-trees, respecting SGS distances. Our findings highlight the need to conserve both grassland and forest populations to safeguard the *B. eriospatha* genetic diversity.

Introduction

The Atlantic Forest is one of the most diverse biomes on Earth with high levels of endemism, and yet, it has been one of the most fragmented and threatened ecosystems over the past centuries (Joly et al., 2014). Recent estimates suggest that only 28% of this biome remains in some preserved form (Project MapBiomas, 2025), within one of the most populated regions in South America (Lima et al., 2024). Habitat reduction drives biodiversity loss not only at the taxonomic level (Newbold et al., 2015), but also at the intraspecific level (Exposito-Alonso et al., 2022), further jeopardizing the maintenance of species and their ecological interactions, particularly under climate change scenarios (Calambás-Trochez et al., 2021; Marchioro et al., 2023). It is estimated that more than 80% of the remaining fragments in the Atlantic Forest suffer from biomass and biodiversity losses, what reduces their conservation value (Lima et al., 2020). Furthermore, 65% of all tree species in this biome were recently classified as threatened (Lima et al., 2024). Therefore, there is an urgent need and a congruent opportunity for better conserving and ecologically restoring the Atlantic Forest (Brancalion et al., 2019).

Despite the long-known importance of the maintenance of genetic diversity to safeguard the adaptive potential of endangered species in conservation and restoration programs (Jordan et al., 2019; Thomas et al., 2014), only recently this recognition has translated into practical recommendations and international policies in an attempt to safeguard minimum levels of genetic diversity within natural ecosystems (Hoban et al., 2021, 2023). Studies explicitly testing the effects of genetic diversity of source and sampled populations of trees on the success of restored tropical and subtropical forest ecosystems, more than just diversity of species, are still lacking but recent theoretical and empirical work has supported the

importance of adequate genetic diversity for maintaining the adaptive potential in other groups of organisms (Kardos et al., 2021). Better understanding the levels of intraspecific genetic variation is critical to inform management of endangered tree species (Hoban et al., 2021). This involves identifying unrelated individuals within and among genetically diverse populations for proper seed sourcing, and potentially taking into consideration different aspects of local adaptation and climate change for deployment of seedlings (Aitken et al., 2024; Jordan et al., 2024). Guidelines with recommended number and distance between sampled seed-trees, as well as number of seeds to be collected from each seed-tree, are crucial and may vary among species, but are still largely insufficient for endangered Atlantic Forest tree species (but see (Conceição et al., 2024; Costa et al., 2021; Montagna et al., 2019; Rodrigues et al., 2023).

One of the goals in conservation genetic studies is to investigate to what extent anthropogenic changes of natural ecosystems have affected the genetic composition and evolutionary trajectories of natural populations, and to provide genetic-based guidelines for restoration and conservation efforts, enhancing the chances of success in such actions. One way to approach this in trees is to compare populations from “less disturbed” sites with populations from “more disturbed” sites regarding multiple genetic parameters. Studies in this context have assessed the effects of habitat loss and fragmentation on the genetic composition of species by testing for genetic differences between populations within different levels of forest cover (e.g., Browne et al., 2015; Da Silva Carvalho et al., 2015; Santos et al., 2015; Zechini et al., 2018). However, for some palm species, after disturbance, individuals may occupy environments that do not correspond to a forest fragmentation gradient, with entire populations occurring in disturbed open grasslands (e.g., *Butia eriospatha*; Candido-Ribeiro et al., 2019). Comparisons regarding genetic diversity between forested and disturbed open grassland habitats are, therefore, critical to inform further conservation and restoration actions for endangered palm species in the Atlantic Forest.

Palm trees are key organisms, exceptionally relevant for maintaining their communities (Andreazzi et al., 2009; Galetti et al., 1999) and sustaining intimate relationships between human societies and their natural ecosystems (Johnson, 1988; Macía et al., 2011), especially in the Neotropics (Andrade et al., 2024; Muscarella et al., 2020). Restoration programs in tropical and subtropical regions should prioritize native palms species, given their capacity to facilitate the re-establishment of multiple ecological interactions and functions of depauperate communities on degraded environments (Reis et al., 1999). Given the ecological importance and unique cultural value of *B. eriospatha* in the highlands of southern Brazil (Sosinski et al., 2019; Wagner et al., 2025), and yet its worsening conservation status in the past decades, the need for restoration and conservation strategies for this species has become urgent. Illegal poaching of centenary individuals (Nazareno & Reis, 2014a), habitat loss and fragmentation, changes in seed morphology and reproductive patterns likely related to habitat changes (Candido-Ribeiro et al., 2019), and cattle grazing resulting in lack of regeneration by new recruitments (Nazareno & Reis, 2014b), are some of the major threats populations of *B. eriospatha* face. *Butia eriospatha* is currently listed as “Vulnerable” on the IUCN Red List (Noblick, 1998), but official regional lists classify this species as under high risk of extinction (CONSEMA-SC, 2014; SEMA-RS, 2014).

Despite being described as a grassland species (Reitz, 1974), *B. eriospatha* populations, formed almost exclusively of adult individuals, have also been observed in forested landscapes (Candido-Ribeiro et al.,

2019), and in current grassland areas that were covered by forest in the past (> 60 years ago) (Candido-Ribeiro, 2017). The current knowledge on the genetic conservation status of *B. eriospatha* is based solely on studies of grassland populations (Nazareno & Reis, 2014b; Reis et al., 2012), while forest populations have not been genetically characterized. Despite the higher number of adult *B. eriospatha* individuals observed in a forested landscape, a much lower proportion of individuals tends to reproduce within a season in this environment when compared to a nearby open grassland populations (Candido-Ribeiro et al., 2019). This raises questions of whether the effective population size of a forested population could be reduced, increasing potential genetic drift, reducing genetic diversity, and rising the risks associated with inbreeding depression in following generations. On the other hand, the forest environment could give more opportunities for more diverse interactions with pollinators than a disturbed open grassland landscape. This could increase outcrossing rates, optimize dispersal, and reduce risks associated with inbreeding in the population. Therefore, the potential differences in genetic diversity between these two contrasting habitats could be of key importance for the establishment of optimal conservation and seed sourcing strategies.

In this study, expanding on Candido-Ribeiro et al.'s (2019) work on *B. eriospatha*, we tested the following hypotheses: a) a given forested population should present higher outcrossing rates, and lower levels of fine scale spatial genetic structure among the reproductive individuals within the population when compared to an adjacent grassland population. b) No genetic differentiation in the cohort of adults should be expected between two populations occupying these two contrasting environments and separated by only 2 km, but a significant differentiation is expected in the cohort of seedlings. c) Lower genetic diversity would be observed in the seed cohort of both populations than in the adult populations. Finally, d) we tested the hypothesis of complete spatial randomness in the distribution of adult individuals in the grassland and forested populations. Here, we present a comprehensive description of the genetic diversity, mating system, and fine-scale spatial genetic structure in two populations of *B. eriospatha* occurring in two contrasting environments, open grassland and forest. We also provide seed collection guidelines to inform restoration and conservation efforts of this important palm species.

Methods

Study area and sampling design

In order to study two contrasting environments where populations of the palm *B. eriospatha* currently occur, we established two nearly squared plots of approximately five hectares each on a private property (−27°12'1"; −50°36'31") in the state of Santa Catarina, southern Brazil. One plot is in an open grassland environment (GRA), typical of open Araucaria Forest stands where the most economically valuable tree species were harvested for timber production and the area has been designated for cattle grazing likely for more than 60 years (Fig. 1). Adult individuals of *B. eriospatha* are the only arborescent plants left and maintained in this area, likely due to the economic importance of their fronds in the recent past, and the past and current appreciation for their fruits (amongst other reasons described in Candido-Ribeiro et al., 2019). Cattle is constantly grazing in this area and from time to time the area is also mowed or burned.

GRA has a total density of 19.1 individuals.ha^{-1}, with 92% being reproductive. The average height and diameter of individuals in GRA is 5.65 m and 31.92 cm respectively (Candido-Ribeiro et al., 2019).

The second plot (FOR) is located in a remnant of Araucaria Forest with *B. eriospatha* individuals co-occurring underneath the canopy of other remarkable tree species of this biome, such as *Araucaria angustifolia* and *Podocarpus lambertii*. The FOR area was systematically managed in the past for timber supply but was never transformed into an open grassland environment and only low-impact selective logging was practiced (Fig. 1). GRA and FOR are approximately 2 km apart. The density of individuals in FOR is 43.2 individuals.ha^{-1}, with only 28% reproductive (Candido-Ribeiro et al., 2019). The average height and diameter of individuals in FOR is 5.78 m and 33.37 cm respectively (Candido-Ribeiro et al., 2019).

Both plots were further subdivided into 400 m^2 squared subplots (20 x 20 m) to facilitate the identification of the relative position of individuals in a Cartesian plane (XY coordinates). All *B. eriospatha* individuals were identified, given a XY position within each plot, and classified according to the ontogenetic classes proposed by Nazareno & Reis (2014b). We sampled leaflets from all adult individuals within the demarcated plots for DNA extraction and genotyping (209 individuals in FOR and 109 in GRA). A more detailed description of the plots and the region can be found in Candido-Ribeiro et al., (2019).

As only a few regenerating seedlings of *B. eriospatha* were found in FOR and none was found in GRA, likely due to cattle herbivory (Nazareno & Reis, 2014b), we collected seeds from 18 individuals in FOR and 16 in GRA and assumed the seed cohort to represent the next generation of the two populations. We germinated the seeds in controlled conditions for subsequent genotyping and estimation of genetic parameters of the seed cohort and mating system.

DNA extraction and genotyping

We performed nuclear DNA extraction from fresh leaflets of sampled adults and seedlings following the protocol described by Doyle & Doyle (1990) with some modifications.

Genotyping was done with microsatellite markers (SSR) – seven SSR markers used were developed by Nazareno et al. (2011), and three were developed by the authors (Table S1. Supp. Mat. 1). In order to optimize the genotyping and assist the identification of alleles, we used two multiplex assays. One with five pairs of primers that amplified the microsatellite regions at an annealing temperature of 54° C, and the other with five pairs of primers with annealing temperature of 56° C. The forward sequence of each SSR primer was marked with a fluorochrome (FAM, PET, NED, and VIC). To avoid overlapping of bands marked with the same fluorescence pattern, the same fluorochromes were used only for distant markers based on the number of amplified base pairs.

The SSR regions were amplified via Polymerase Chain Reaction (PCR) with a reaction solution of 10 μl in each well of the 96-well plates. The reaction solution consisted of 5 μM of KAPA 2G Fast Multiplex PCR Kit (Kapa Biosystems), 0.04 μM to 0.11 μM of one of the multiplex assays, and 2 μM of the sample (pure DNA of an individual). The final volume was completed with Milli-Q water. For the PCR cycles, we used the

protocol described by Kapa Biosystem: 3 min at 95° C for the initial denaturation, 30 cycles of 15 s at 95° C for the denaturation, 30 s at the specified temperature of each multiplex for the annealing, 60 s at 72° C for the extension, and at the end of 30 cycles, 60 s at 72 ° C for the final extension. PCR amplifications were performed with a VERITI 96-Well thermocycler, 0.2 ml (Applied Biosystems).

In a new 96-well plate, we mixed 1 μM of diluted (1:20) amplified reaction solution, 8.75 μM of Formamide Hi-Di, and 0.25 μM of GS600 LIZ Size Standard (Thermo Fisher). The genotypes were identified by capillary electrophoresis in the ABI 3500xl – 24 capillary sequencer (Applied Biosystem) and the allele frequencies for each analyzed locus were verified and interpreted with GeneMapper v.4.1 (Applied Biosystem).

Due to genotyping errors, 12 adult samples from FOR and 5 from GRA were discarded to avoid inaccurate genetic estimates (Costa et al., 2019), totalizing 197 and 104 genotyped adult individuals, respectively. Due to failures in germination and genotyping, it was only possible to obtain the genotypes from 1 to 14 seedlings per reproductive individual. In total, 121 and 122 seedlings from FOR and GRA respectively were genotyped.

Analyses

Spatial distribution pattern

We tested the hypothesis of complete spatial randomness of all individuals and of only reproductive individuals in each plot (GRA and FOR), by applying the Ripley's K function (Ripley, 1977). For this analysis, we used the package "spatstat" v. 1.45-2 (Baddeley et al., 2016) in R – v. 4.0 (R Core Team, 2021), with a Ripley's isotropic function for edge effect correction (Ripley, 1977). To determine whether the observed spatial pattern differed significantly from complete spatial randomness, we obtained 95% confidence intervals after 1,000 Monte Carlo simulations. The maximum distance analyzed by the function corresponds to half the size of the smallest plot side (110 m). To improve the visual interpretation of the results, we transformed the Ripley's K function to the Ripley's L function. Direct comparisons were made to verify whether the two sampled plots differed regarding the spatial pattern distributions of individuals and at what distances such differences occurred.

Genetic diversity

First, we checked for the presence of null alleles with Microchecker - v.2.2.3 (Van Oosterhout et al., 2004), and linkage disequilibrium (LD) with FSTAT v.2.9.3.2 (Goudet, 1995). All loci were also tested for Hardy-Weinberg equilibrium by the hypothesis of heterozygous deficiency, applying the exact test (Guo & Thompson, 1992) implemented in Genepop v.4.6 (Rousset, 2008).

After performing pairwise tests among all ten loci for linkage disequilibrium (LD), all were found to segregate independently, with the exception of locus *B.eri07*, which showed significant LD in relation to locus *B.eri15* in adult populations ($p < 0.01$) and to locus *but23* in progeny populations ($p < 0.001$), after Bonferroni correction for multiple comparisons. Therefore, locus *B.eri07* was excluded from all subsequent

analyses, resulting in nine microsatellite loci used to determine genetic diversity, structure, mating system, and spatial genetic structure of both *B. eriospatha* populations.

From the allele frequencies obtained for all SSR loci in both populations and generations within populations, we estimated the following genetic parameters: total number of alleles, average number of alleles per locus ($\widehat{A}$), allelic richness ($\widehat{A}_R$), effective number of alleles per locus ($\widehat{A}_e$), number of exclusive alleles ($\widehat{A}_P$), number of rare alleles ($\widehat{R}$, allele frequency < 0.05), observed heterozygosity ($\widehat{H}_o$), and expected heterozygosity under Hardy-Weinberg Equilibrium ($\widehat{H}_e$). We also estimated the intrapopulation fixation index ($\widehat{f}$), and fixation index between populations ($\widehat{F}_{ST}$; Weir & Cockham, 1984). The effective population size ($\widehat{N}_e$) was estimated as proposed by Li (1976).

Total number of alleles, $\widehat{A}$, $\widehat{A}_e$, $\widehat{A}_P$, and $\widehat{R}$ were estimated in GenAlEx 6.503 (Peakall & Smouse, 2012). Before comparing the two populations for $\widehat{A}_R$, we standardized the number of alleles to the smallest sample size by applying the rarefaction method and bootstrapping (10.000) in R - v 4.0 (R Core Team, 2021). We used FSTAT 2.9.3.2 (Goudet, 1995) to estimate $\widehat{H}_o$ and $\widehat{H}_e$ and the PopGenKit R package (Paquette, 2012) to calculate 95% confidence intervals (CI) based on 1.000 bootstrap resamples among individuals for both parameters. We used FSTAT 2.9.3.2 (Goudet, 1995) to estimate $\widehat{F}_{IS}$, $\widehat{F}_{ST}$, and the 95% CIs. CI (95%) for $\widehat{f}$ was carried out in GDA (Lewis & Zaykin, 2002) using 1.000 bootstrap resamples over loci, excluding one population at a time. To test for differences between populations and generations within populations regarding their genetic diversity, we made direct comparisons between group confidence intervals (i.e., an overlap means that there is no significant difference).

Mating system

Only reproductive individuals with a minimum of six genotyped progenies were considered for the estimation of mating system parameters. Allele frequencies for six to 12 progenies from each of the 14 reproductive individuals (115 progenies) in FOR. and for seven to 14 progenies from each of the 12 reproductive individuals (116 progenies) in GRA were used.

We used the mixed mating model (Ritland & Jain, 1981) and the correlated mating model (Ritland, 1989) implemented in the software MLTR 3.4 (Ritland, 2009). The parameters estimated at the population level were multi-locus outcrossing rate ($\hat{t}_m$), single-locus outcrossing rate ($\hat{t}_s$), rate of selfing ($\hat{s} = 1 - \hat{t}_m$); selfing plus mating among relatives ($1 - \hat{t}_s$). mating among relatives ($\hat{t}_m - \hat{t}_s$), multi-locus correlation of paternity ($\hat{r}_{p(m)}$), inbreeding coefficient of maternal parents ($\widehat{F}_m$), proportion of half-sibs ($\widehat{P}_{hs}$), proportion of full-sibs ($\widehat{P}_{fs}$), proportion of self-sibs ($\widehat{P}_{ss}$), coancestry coefficient within progeny ($\widehat{\theta}_{xy}$; Sousa et al., 2005), effective size of the variance ($\widehat{N}_{e(v)}$; Cockerham, 1969), and estimated number of seed-trees necessary to form a population with an effective population size of 500 after seed collection ($\widehat{m}_{(500)}$) for medium to long-term conservation of genetic diversity within populations (i.e., >50 generations) (Alexandre Magno Sebbenn, 2002). We tested for significance and difference between populations for each parameter using confidence intervals (95% probability) constructed from 1.000

bootstrap resamples among families. From the results on paternity correlation, the effective number of pollen donors (N_{ep}) was also estimated ($N_{ep} = 1/\hat{r}_p$).

Fine-scale spatial genetic structure

To determine the fine-scale spatial genetic structure (SGS) within both FOR and GRA populations, we used all genotyped and mapped adult individuals described above. Multilocus pairwise comparisons among individuals within eight distance classes (K = 8. from 0 to 200 m) were performed to determine the average coancestry (i.e., Nason's kinship coefficient, $\widehat{F}_{ij}$) (Loiselle et al., 1995) within each class. The statistical significance calculated for each distance class was tested using confidence intervals, built from 10.000 permutations of individuals between different distance classes. This analysis was performed with the program SPAGeDi v. 1.4 (Hardy & Vekemans, 2002). Neighbourhood size ($\widehat{N}_b$; (Vekemans & Hardy, 2004) and deme size for reproductive individuals within each population were estimated as described in Montagna et al. (2018).

Results

Spatial distribution pattern

When considering all individuals present in the population (n = 209), FOR presented a predominantly random spatial pattern. Between 0 and 20 m, a random pattern was observed, becoming aggregated between 20 and 45 m, and random between 45 and 110 m (Fig. 2a). When looking only at reproductive individuals (n = 58), we observed that FOR maintained the predominant random pattern of spatial distribution, with a small exception between 9 m and 11 m (Fig. 2b). Therefore, individuals in the FOR population did not differ significantly from the hypothesized Complete Spatial Randomness (CSR) for most of the analyzed distances. The GRA population presented a predominantly aggregated spatial distribution pattern when all individuals were considered (n = 109). A random pattern was observed between 0 and 5 m, and between 90 and 110 m. Between 5 and 90 m, the distribution pattern was aggregated (Fig. 2c). When only reproductive individuals were considered (n = 101), the observed spatial distribution pattern was almost identical to the observed for the whole population (Fig. 2d). Therefore, the spatial distribution pattern observed in GRA differs significantly from the hypothesized CSR within most of the analyzed distances.

Genetic diversity and structure

In total, we sampled and genotyped 544 *B. eriospatha* individuals, 301 of which were adults and 243 were progenies. All the information regarding allele frequencies obtained for the nine microsatellite loci used, as well as the sample size obtained for each locus in both populations are shown in Table S1, Supp. Mat. 1. Across all adult individuals and progeny in FOR and GRA populations, 42 alleles were observed, and the *but07* locus showed the largest number of alleles, with nine in total (Table S1. Supp. Mat. 1). The loci *B.eri15*, *but17* and *but23* presented only two alleles each (Table S1. Supp. Mat. 1).

Both FOR and GRA populations showed deviations from Hardy Weinberg equilibrium (HWE) in the class of adults and progenies ($p < 0.001$). Specifically, within FOR, the loci *B.eri11*, *but07*, and *but09* for adults and *B.eri11*, *but07*, and *but23* for progenies showed deviations from HWE. Within GRA, the loci *B.eri11* and *but07* for adults, and *B.eri11, but07*, and *but08* for progenies were not in HWE.

Within the FOR population, 32 alleles were detected in the adults and 29 in the progeny. Within GRA, we detected 31 alleles in the adults and 32 alleles in the progeny. All loci were polymorphic in adults and progeny in both populations, with two to seven alleles per locus in FOR, and two to eight alleles per locus in GRA. On average, the number of alleles per locus ($\widehat{A}$) within each studied group ranged from 3.2 (FOR progenies) to 3.6 (FOR adults and GRA progenies). The average number of alleles per locus for the cohort of adults in the GRA population was 3.4 (Table 1).

The number of alleles found in the cohort of adults in FOR was not significantly different from GRA ($\widehat{A}_R$ = 3.3; *p-value* > 0.05). In the progeny, even after rarefaction correction, the allelic richness in GRA was higher than the observed in FOR ($\widehat{A}_R$ = 3.4 in GRA, and $\widehat{A}_R$ = 3.1 in FOR, *p-value* < 0.05) (Table 1). The progeny in GRA showed a number of effective alleles ($\widehat{A}_e$) of 2.25, and in FOR, 2.03. The adult cohort in GRA and FOR presented 2.15 and 2.08 effective alleles, respectively. Five exclusive alleles ($\widehat{A}_p$) were observed in adults within FOR, and four within GRA. Three exclusive alleles were observed in the progeny in FOR, and six in GRA. Eight rare alleles ($\widehat{R}$) were observed in the adults, and five in the progeny of both populations.

We did not detect a significant difference between GRA and FOR adult's observed heterozygosity ($\widehat{H}_o$, 0.447 and 0.440, respectively), nor expected heterozygosity ($\widehat{H}_e$, 0.445 and 0.441, respectively) (Table 1). In the progeny, no significant difference was observed between the two populations for $\widehat{H}_o$ and $\widehat{H}_e$, with 0.400 and 0.442 in GRA, and 0.357 and 0.429 in FOR respectively (Table 1).

The fixation index ($\widehat{f}$) in the adult and progeny cohorts did not differ significantly from zero in both populations (Table 1), indicating no deviation from Hardy-Weinberg equilibrium. Due to the low rate of fixation observed, the effective population size ($\widehat{N}_e$) in adults did not differ from the sample size (n) in both populations. For the progeny, $\widehat{N}_e$ was slightly smaller than n in both populations ($\widehat{N}_e$ = 104 in FOR and 111 in GRA, Table 1). A significant genetic differentiation between the two populations was observed, with an estimated $\widehat{F}_{ST}$ of ~ 2% for the adult population and ~ 3% for the progeny ($p < 0.05$, Table 1).

Table 1
Genetic diversity descriptors for adult and seedling cohorts in the two studied *Butia eriospatha* populations (FOR and GRA).

Adult												
FOR	197	32	3.6	3.3[a]	2.08	5	8	0.440 (0.417–0.465)	0.441 (0.423–0.455)	0.002[ns]	197	0.019*
GRA	104	31	3.4	3.3[a]	2.15	4	8	0.447 (0.422–0.472)	0.445 (0.422–0.458)	-0.004[ns]	104	
Progeny												
FOR	121	29	3.2	3.1[b]	2.03	3	5	0.357 (0.334–0.381)	0.429 (0.409–0.439)	0.167[ns]	104	0.029*
GRA	122	32	3.6	3.4[a]	2.25	6	5	0.400 (0.377–0.425)	0.442 (0.424–0.454)	0.097[ns]	111	

n: sample size; $\widehat{\varvec{A}}$: number of alleles per locus; $\widehat{\varvec{A}}_{\varvec{R}}$: allelic richness after rarefaction; $\widehat{\varvec{A}}_{\varvec{e}}$: effective number of alleles per locus; $\widehat{\varvec{A}}_{\varvec{p}}$: number of exclusive alleles; $\widehat{\varvec{R}}$: number of rare alleles; $\widehat{\varvec{H}}_{\varvec{o}}$ **(95% CI)**: observed heterozygosity (95% confidence interval); $\widehat{\varvec{H}}_{\varvec{e}}$ **(95% CI)**: expected heterozygosity (95% confidence interval); $\widehat{\varvec{f}}$: fixation index; $\widehat{\varvec{N}}_{\varvec{e}}$: effective population size; $\widehat{\varvec{F}}_{\varvec{ST}}$: fixation index due to population subdivision.

Mating system

The multi-locus outcrossing rate ($\hat{t}_m$) did not differ significantly from one (1.0) in both FOR and GRA populations ($\hat{t}_{m\ FOR}$ = 0.865; $\hat{t}_{m\ GRA}$ = 0.930) indicating that selfing is unlikely to happen within these two populations (Table 2). However, not all observed crossings in the FOR populations were among non-relatives, indicated by the $\hat{t}_s$ (single-locus crossing rate) significantly different from the unit (0.848). In the GRA population, $\hat{t}_s$ was not significantly different from one (Table 2). The proportion of the progeny generated by a single father ($\hat{r}_{p(m)}$) and the inbreeding coefficient of maternal parents ($\widehat{F}_m$), were not significantly different from zero in both populations. FOR showed a proportion 71% of half-siblings ($\widehat{P}_{hs}$), and GRA, 81% (Table 2). The coancestry observed within progeny ($\widehat{\theta}_{xy}$) in both populations, was not significantly different from 0.125, i.e., the expected coancestry among half-siblings. It would be necessary a number of 139 and 132 seed-trees in FOR and GRA populations respectively to create populations with an effective population size (N_e) of 500 (Table 2), and 28 and 26 seed-trees respectively if the goal were populations with an N_e of 100.

Table 2
Mating system estimates for both forest (FRO) and grassland (GRA) *Butia eriospatha* populations.

	Population [95% CI]*			
	FOR		**GRA**	
Number of sampled trees (number of progeny)		14 (115)		12 (116)
Multi-locus outcrossing rate: $\hat{t}_m$	0.865	[0.631–1.009]	0.930	[0.858–1.010]
Sigle-locus outcrossing rate: $\hat{t}_s$	0.848	[0.568–0.996]	0.935	[0.838–1.014]
Selfing rate: $1-\hat{t}_m$	0.135	[0.000-0.369]	0.070	[0.000-0.142]
Selfing + mating among relatives: $1-\hat{t}_s$	0.152	[0.004–0.432]	0.065	[0.000-0.162]
Mating among relatives: $\hat{t}_m-\hat{t}_s$	0.017	[0.000-0.097]	-0.005	[0.000-0.045]
Multi-locus correlation of paternity: $\hat{r}_{p(m)}$	0.052	[0.000-0.124]	0.059	[0.000-0.104]
Inbreeding coefficient of maternal parents: $\widehat{F}_m$	0.060	[-0.200-0.158]	-0.036	[-0.200-0.084]
Proportion of half-siblings (%): $\widehat{P}_{hs}$	70.93	[39.44–97.82]	81.39	[71.81–100.0]
Proportion of full-siblings (%): $\widehat{P}_{fs}$	3.890	[0.000-9.187]	5.103	[0.000-9.431]
Proportion of self-siblings (%): $\widehat{P}_{ss}$	1.800	[0.000-13.60]	0.500	[0.000-2.100]
Coancestry coefficient within progeny: $\widehat{\theta}_{xy}$	0.139	[0.117–0.150]	0.132	[0.118–0.141]
Variance effective size: $\widehat{N}_{e(v)}$	3.592	[3.204-4.000]	3.783	[3.543-4.000]
Number of seed-trees for a $\widehat{N}_e$ of 500: $\widehat{m}_{(500)}$	139	[116–156]	132	[118–141]

	Population [95% CI]*			
	FOR		GRA	
Number of seed-trees for a $\widehat{N}_e$ of 100: $\widehat{m}_{(100)}$	28	[25–31]	26	[25–28]

*Confidence interval estimated from 1.000 bootstrap resampling.

Fine-scale spatial genetic structure

We observed significant levels of coancestry among individuals within a radius of up to 60 m in FOR and up to 30 m in GRA (Fig. 3). The maximum level of coancestry was observed for individuals up to 15 m apart ($\widehat{F}_{ij}$= 0.038 in FOR; $\widehat{F}_{ij}$= 0.051 in GRA). No coancestry was observed when only reproductive individuals were considered in FOR. In GRA, the spatial genetic structure remained unchanged with only reproductive individuals. The neighborhood size ($\widehat{N}_b$) estimated for reproductive individuals in GRA was 237, and in FOR, 260. Deme sizes based on the density of reproductive individuals in each population were 13 ha and 21.6 ha in GRA and FOR, respectively.

Discussion

Establishing seed collection criteria for endangered tree species aimed at restoration and conservation programs (e.g., minimum number of seed trees and the distances among them) is critical to safeguard the long-term maintenance of genetic diversity in these species. Nevertheless, the lack of basic information about the genetic status of populations for many tree species in the tropics and subtropics is still a barrier for such actions. Here we provided a detailed description of the genetic diversity, mating system, and fine-scale spatial genetic structure in two populations of *B. eriospatha* occurring in open grassland and forest habitats in order to inform seed collection efforts for the genetic conservation of this remarkable endangered palm species.

To the best of our knowledge, our study represents the first attempt in describing the levels of genetic diversity in a *B. eriospatha* population occurring in a forest environment. Overall, we detected important differences between the two studied populations despite their proximity, high levels of outcrossing rate, and their similar moderate levels of genetic diversity. In fact, significant levels of genetic differentiation were observed between the two populations. A more marked spatial genetic structure, with related individuals spanning larger areas, was observed within the forest environment, but when only reproductive individuals were considered, no internal structure was observed. On the other hand, reproductive individuals in the open grassland population showed significant levels of spatial genetic structure. Allogamy was predominant within both populations, but a small proportion of crossings among relatives was detected in the forest environment.

Differences between populations

The open grassland population (GRA) was spatially more aggregated than the forest population (FOR) for the analyzed scale range. The site where GRA is located has been subjected to more intense management (e.g., cattle presence, mowing, fire, and more accessible to fruit collectors) in the past decades despite the maintenance of the adult *B. eriospatha* individuals. These more stressful conditions can increase mortality rates, maintaining groups in more favorable micro-environments. When mortality occurs due to abiotic factors, there is a tendency for individuals to occur in more suitable habitat patches where resources are less limited (Hutchings, 1997). This can promote a negative density-dependence relationship, increasing the aggregation of individuals. Other species-specific factors, such as reproduction and dispersal regime (Silva et al., 2012), can contribute to the spatial distribution of trees (Willson, 1993). No inferences about the spatial distribution pattern of lower age classes in both populations was possible given that no seedlings or saplings were observed in GRA, and only a handful of seedlings were found in FOR. The lack of continuity of generations in the two sites, makes it difficult to fully understand the potential causes and effects of the spatial pattern observed among adults, and also suggests an imminent collapse of these two populations.

In general, the assessed microsatellite loci presented low polymorphism, ranging between 2 and 8 alleles per locus, with allelic richness varying between 3.2 and 3.6. An average of 4.4 alleles per locus was previously observed among four *B. eriospatha* grassland populations (Nazareno & Reis, 2014b). Low allelic richness has also been reported for other palm species studied with microsatellites (Rodríguez-Peña et al., 2014), however, some species may exhibit moderate, e.g., *Oenocarpus batua* (6.5 alleles per locus) (Ottewell et al., 2012), or high allelic richness, e.g., *Euterpe edulis* (9.2 alleles per locus) (Carvalho et al., 2015). When comparing the two populations, allelic richness, and the number of effective alleles in GRA was higher than in FOR (Table 1), and the number of exclusive alleles in the progeny of GRA was double ($\widehat{A}_p$ = 6) the number observed in the progeny of FOR ($\widehat{A}_p$ = 3). These differences suggest that open grassland areas are more likely to promote gene flow than forested areas and are also more prone to gene flow with pollen originating from outside the population.

In both populations, observed heterozygosity was close to the expected heterozygosity in adult individuals. This resulted in non-significantly different from zero fixation indices ($\widehat{f}$), indicating that the probability of two alleles being identical by descent at a locus within the same individual is close to zero (Allendorf et al., 2022) in both populations. Despite the slight increase in $\widehat{f}$ for the progeny in both populations, these values were still not significantly different from zero, indicating that genetic diversity could be maintained in these two populations, if a new generation was able to get established. However, if we consider the eight natural populations of *B. eriospatha* studied by Nazareno & Reis (2014a), only two did not show significant $\widehat{f}$ values, indicating that many populations have already lost considerable genetic diversity. Another study with 14 *B. eriospatha* populations found substantial variation for this parameter, with six populations presenting high and significant $\widehat{f}$ (Reis et al., 2012).

We observed a significant genetic differentiation ($\widehat{F}_{ST}$) between the GRA and FOR for both adult and progeny cohorts, suggesting that even short distances (e.g., 2 km) can produce a certain degree of reproductive isolation between *B. eriospatha* populations. Furthermore, the observed differentiation in both cohorts indicates a lack of historical and current gene flow between populations. This may be associated with the pollination and dispersal syndrome of the species, carried out by small insects and animals that travel short distances. Long-distance pollen dispersal could prevent genetic isolation, allowing the cross-pollinating population to extend beyond its geographic limits (Cottrell et al., 2009). Nevertheless, the increased structuring in the progeny observed in this study may indicate a deficiency of these vectors in the landscapes where *B. eriospatha* occurs. Strong and significant $\widehat{F}_{ST}$ has been previously observed among more distant *B. eriospatha* populations in the state of Santa Catarina (Nazareno & Reis, 2014a; Reis et al., 2012). This indicates that a large proportion of the genetic variation in the species is likely maintained among populations. Over time, genetic structuring can lead to the fixation and loss of alleles, increasing homozygosity and reducing genetic diversity within populations (Hartl & Clark, 1997). In fact, GRA and FOR presented alleles that were not present in the two populations used in the primer note to describe seven of the nine loci used here (Nazareno et al., 2011), nor in other nine populations analyzed by Nazareno & Reis (2014a), which also used these same seven microsatellite loci.

We confirmed the predominance of allogamy in *B. eriospatha* for the assessed reproductive event in both GRA and FOR population. Similar outcrossing rates have been observed in other palm species, such as *Euterpe edulis* (Seoane et al., 2005), *Astrocaryum aculeatum* (Ramos et al., 2011), *Oenocarpus batua* (Ottewell et al., 2012), and *Acrocomia aculeata* (Lanes et al., 2016). Likewise, a similar result was observed by Nazareno & Reis (2012) in another grassland *B. eriospatha* population in Santa Catarina state, albeit a small significant deviation from unity ($\hat{t}_m$= 0.961). Many tropical tree species have mechanisms to prevent self-fertilization and mating among related individuals (Degen & Sebbenn, 2016). *Butia eriospatha*, for example, despite being monoecious, exhibits protandrous inflorescences (Nazareno & Reis, 2012), what prevents pollen from male flowers fertilizing female flowers on the same individual. Despite this, Nazareno & Reis (2012) results suggest that self-fertilization can also be present in the species. The wide confidence interval observed in FOR (Table 2), may be associated with a large variation in $\hat{t}_m$ among seed-trees.

The single-locus outcrossing rate ($\hat{t}_s$) was not significantly different from the unity in GRA, indicating that all sampled seeds in the population derived from crosses between unrelated individuals, and reinforcing that gene flow is likely more efficient in GRA. In FOR, on the other hand, the estimated $\hat{t}_s$ suggests that although there were no signs of self-fertilization, a small portion of the sampled seeds were generated from inbreed crosses. Although this level of $\hat{t}_s$ in FOR was not enough to make the fixation index in the progeny significant (Table 1), it indicates a certain deficiency in gene flow and contradicts our initial expectation of higher outcrossing rates in the forested population. In addition, the estimated number of fathers per mother plant indicates that about 9% of the reproductive population in FOR contributed to the formation of the seeds of each mother plant, while this proportion reached 15% in GRA. Outcrossing levels should be further investigated in other forested populations, and the main pollinators in forested and grassland populations should be described in order to confirm and potentially explain these patterns.

Spatial genetic structure (SGS), formed due to clustering of related individuals within populations of trees, can lead to inbred crosses (Sebbenn, 2006). Some differences were observed between GRA and FOR regarding SGS among adult individuals. The maximum coancestry in FOR was observed at a distance scale of up to 15 m ($\widehat{F}_{ij}$= 0.038). This is less than the expected between first-degree cousins (0.0625). Therefore, up to a distance scale of 15 m, the probability of two randomly sampled alleles in two adult individuals being identical by descent in the population is less than 4%. At the same distance scale (15 m), coancestry in GRA reached a higher value ($\widehat{F}_{ij}$ = 0.051). When considering all individuals present in FOR, family structure was detected up to a scale of approximately 60 m, despite being of low intensity. In GRA, coancestry was detected up to 30 m of distance between individuals. However, when only the reproductive individuals were considered, coancestry ceased to be significantly different from zero at any distance in FOR, but not in GRA. This suggests that a possible alternation of reproductive individuals at each reproductive event in forest *B. eriospatha* populations (Candido-Ribeiro et al., 2019) could help reduce inbred crosses, but the reduced number of individuals participating in one reproductive event still produces inbred offspring, as suggested by the single-locus outcrossing rate observed in this population (Table 2). On the other hand, a more effective gene flow and the larger number of reproductive individuals in GRA seems to offset the stronger coancestry observed at shorter distance scales in this population, maintaining diversity in the progeny.

A potential explanation for the overall weak SGS observed within both GRA and FOR is the described demographic pattern of both populations, currently composed of only adult senescent individuals (Candido-Ribeiro et al., 2019). Mortality of senescent individuals, despite representing a loss of alleles due to genetic drift, tends to decrease family structure within populations. Studies that analyzed SGS at different ontogenetic stages within tropical palm populations detected a reduction in family structure as individuals advanced through demographic classes, precisely due to mortality in the populations (Browne et al., 2015; Choo et al., 2012). Therefore, it is likely that in *B. eriospatha* populations like these, seed dispersal, so important in determining the family structure of palm populations (Choo et al., 2012), gradually lost its importance as a determining factor in the distribution of genetic variation, and mortality of adult individuals has become the main factor shaping SGS in both GRA and FOR populations.

Implications for conservation and recommendations for seed collection

GRA and FOR populations exhibited considerable levels of genetic diversity with non-significant fixation indexes, making them suitable for seed collection for conservation, restoration, or the establishment of breeding populations. The absence of differences in genetic diversity levels indicates that collections could be conducted in either population. However, the presence of exclusive alleles in the progenies ($\widehat{A}_p$ = 3 and 6 for FOR and GRA, respectively), along with detectable genetic divergence ($\widehat{F}_{ST}$ = 3%), suggests that greater genetic diversity would be contemplated if seed collections were conducted in both populations. Selfing is unlikely to be happening in these two populations, which is important for avoiding the rise of inbreeding in the progeny. However, inbreeding can also arise from crosses among related individuals. This was observed in FOR but it was not sufficient to generate a significant fixation index.

In general, gene flow has been efficient in promoting random crosses within each population. Yet, in order to generate a new population with an effective population size ($\widehat{N}_e$) of 500, seeds would have to be collected from 132 and 139 seed trees in GRA and FOR, respectively. If the two studied populations were designated for germplasm collection and conservation, special attention should be given to the minimum area required for seed collection. Considering the density of reproductive individuals in the FOR population (12 individuals.ha^{-1}), for example, a minimum area of c. 11.6 ha would be necessary. This would allow the collection of seeds in a way that maintains the long-term genetic diversity of the population without any considerations for minimum distances between seed trees given the lack coancestry observed among reproductive individuals. For GRA, the minimum necessary collection area, considering only the number of reproductive individuals in the population (17.7 individuals.ha^{-1}), would be c. 7.5 ha. However, distances of less than 30 meters between seed trees should be avoided given the significant coancestry observed among reproductive individuals in this population. Bearing in mind the strong aggregation of individuals observed in this population (i.e., non-random pattern in the spatial distribution of individuals), a substantially larger area for seed collection should be considered. In order to form populations with smaller effective population sizes such as $\widehat{N}_e$ of 50, 13 and 14 seed trees should be used for collection in GRA and FOR, respectively.

The main limitation of our study is the lack of replications within each habitat (i.e., forest and open grassland), which precludes the possibility of general inferences about the environments where these two populations occur. Therefore, extrapolations of our results to other forest and open grassland *B. eriospatha* populations should be made with extreme caution. Furthermore, the diversity and genetic structure data related to the progeny should also be taken with some caution. Since we did not find sufficient seedlings to be sampled directly from the populations in their natural environments, seedlings were germinated under laboratory conditions, from seeds taken at random in each population, and therefore, seeds were not exposed to selection in their natural environmental conditions. However, the effort to estimate such parameters in this ontogenetic class was considered pertinent as it gave us some understanding about the genetic variation that is transmitted through generations in both *B. eriospatha* populations.

Final remarks

The extinction risk populations of *B. eriospatha* currently face is undeniable. Illegal poaching of centenary individuals (Nazareno & Reis, 2014a), habitat loss and fragmentation (Candido-Ribeiro, 2017), changes in seed morphology and reproductive patterns likely related to habitat changes (Candido-Ribeiro et al., 2019), cattle grazing, fruit consumption, senescence of adult individuals with lack of regeneration by new recruits (cattle), and the fast spread of exotic tree plantations pressure the already depauperate populations. To reverse this scenario, it is crucial that new populations of the species be founded, in addition to the conservation and enrichment of existing populations. Thus, establishing appropriate criteria for collecting seeds with high genetic diversity allows new populations to have adaptive potential.

Considering the conservation status of *B. eriospatha* in both assessed habitat types, open grassland populations are likely under higher anthropogenic pressure due to cattle grazing and land conversion to silviculture, combined with a lack of legal protection, as these populations occur within productive

agricultural sites. Forest *B. eriospatha* populations, on the other hand, are under a relatively lower threat due to the existence of forest protection laws (Atlantic Forest Law), but these populations are much rarer to find. In any case, conservation strategies should consider these two habitats. Besides proper seed collection and restoration actions, efforts for *in situ* and *ex situ* conservation must be prioritized, with a particular focus on promoting the natural establishment and survival of new seedlings in current populations. The general lack of regeneration in *B. eriospatha* populations, and therefore, lack of generation turnover, in addition to the mortality and poaching of senescent individuals (Candido-Ribeiro, 2017; Nazareno & Reis, 2014a), are likely the most important threats to the genetic conservation of the species. Finally, as exotic tree plantations, such as *Pinus* sp., expand across southern Brazilian landscapes, the effects of commercial forest plantations on the maintenance of genetic diversity within and among *B. eriospatha* populations should be investigated.

Declarations

Competing Interest

The authors declare no competing interests.

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Author Contribution

RCR and MSR conceived the study and the sampling design. RCR, TM, and NCFC collected the data, sampled the seeds, and collected tissue for DNA extractions. RCR performed the lab work and analyzed the data. RCR wrote the manuscript with inputs from TM, NCFC, and MSR. All authors have approved the final version of the manuscript.

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Data Availability

The data used and analysed during this study are available from the corresponding author on reasonable request.

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Figures

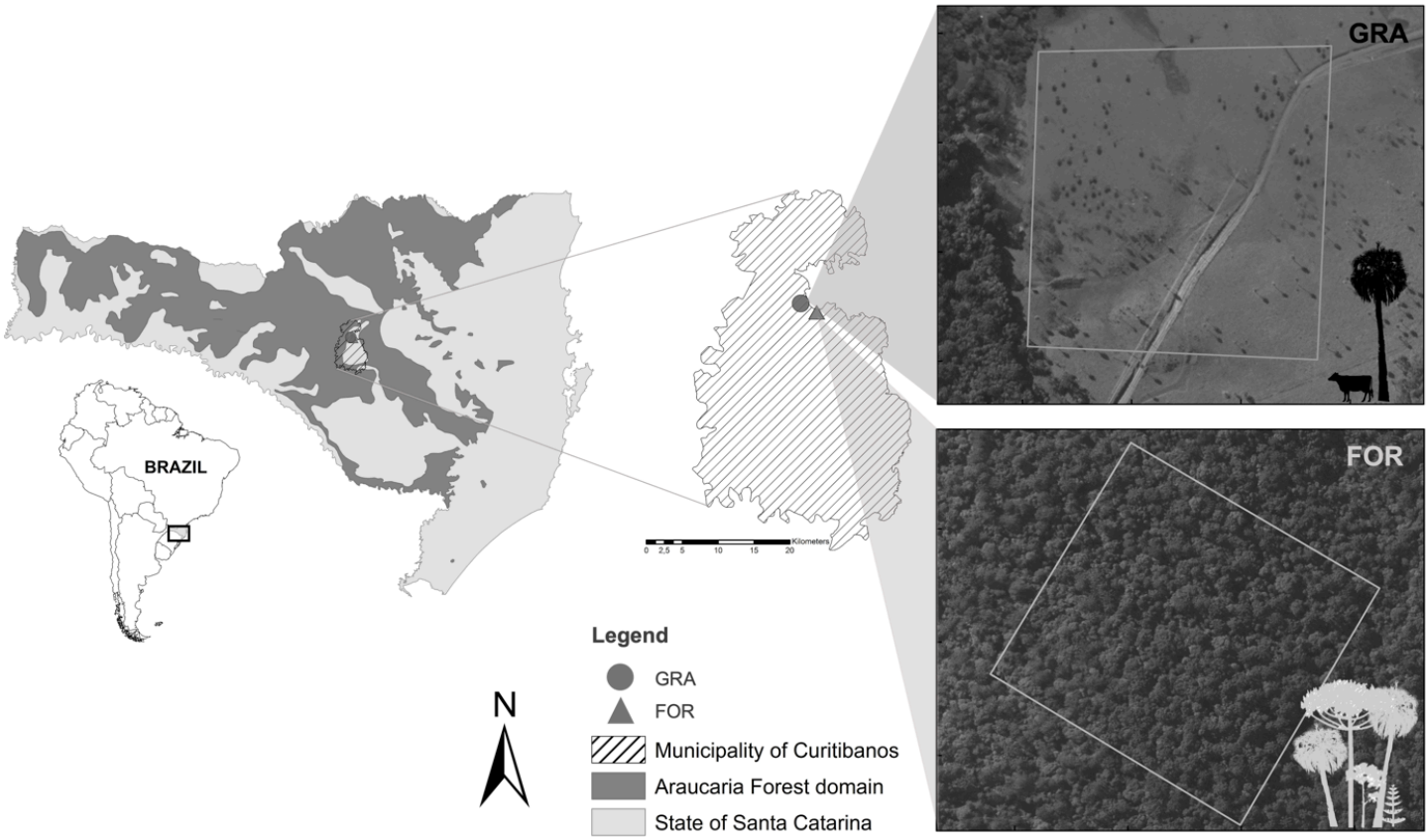

Figure 1

Studied *Butia eriospatha* populations, GRA (open grassland) and FOR (forest), where the nearly square plots of c. 5 ha each were established (grey squares within each population). The two populations are located in Curitibanos, SC, Brazil. Aerial photos WMS were obtained from the Santa Catarina's geographic information system at http://sigsc.sc.gov.br/sigserver/SIGSC/wms.

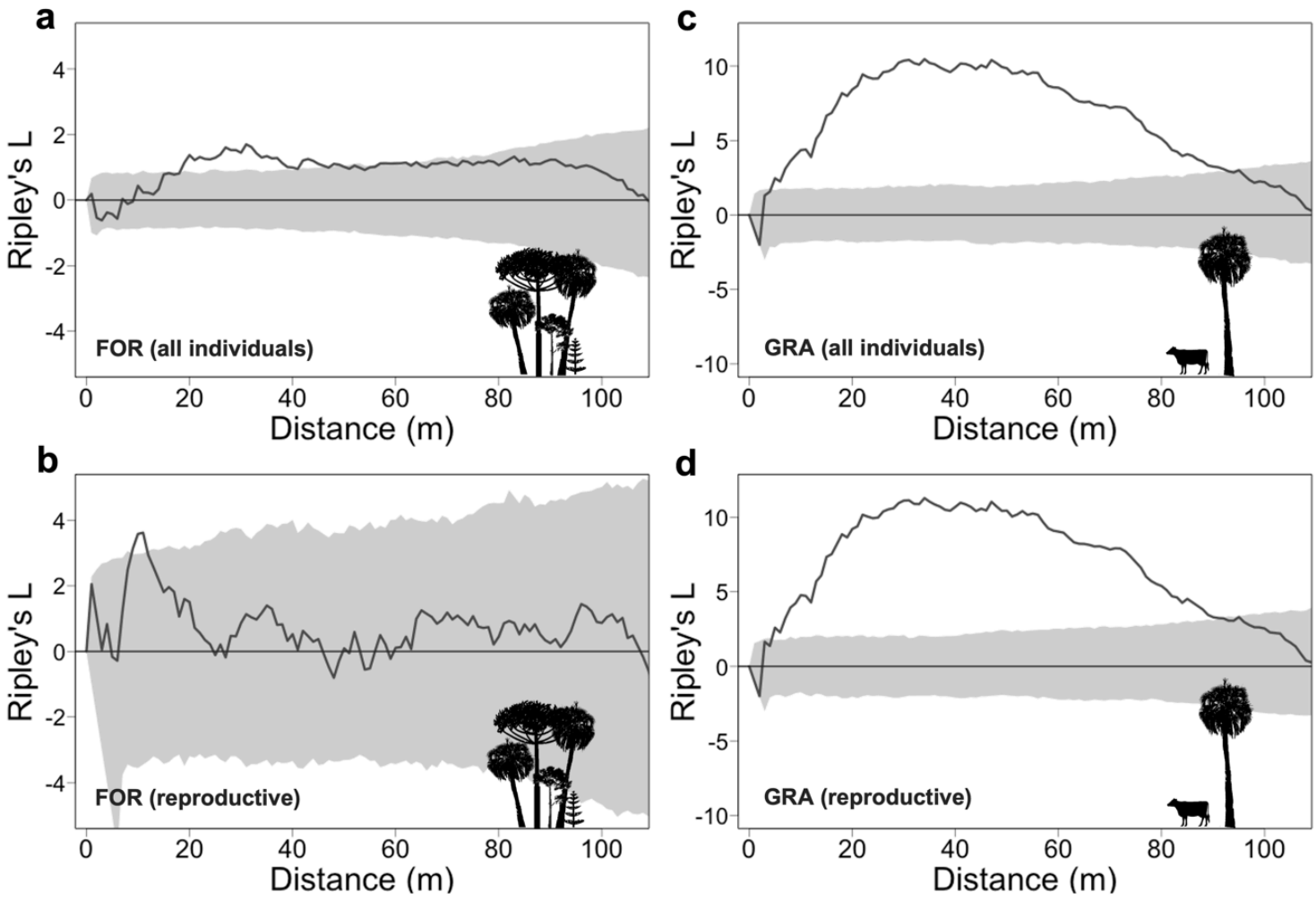

Figure 2

Spatial distribution pattern in the two *Butia eriospatha* populations represented by the Ripley's K transformed (L). Spatial distribution pattern of all individuals in the forest population (FOR) (a), and in the open grass land population (GRA) (c), and only of reproductive individuals in FOR (b), and GRA (d). Gray ribbons depict the 95% confidence intervals after 1,000 Monte Carlo simulations. Complete randomness is given by an overlap between the black line the confidence interval.

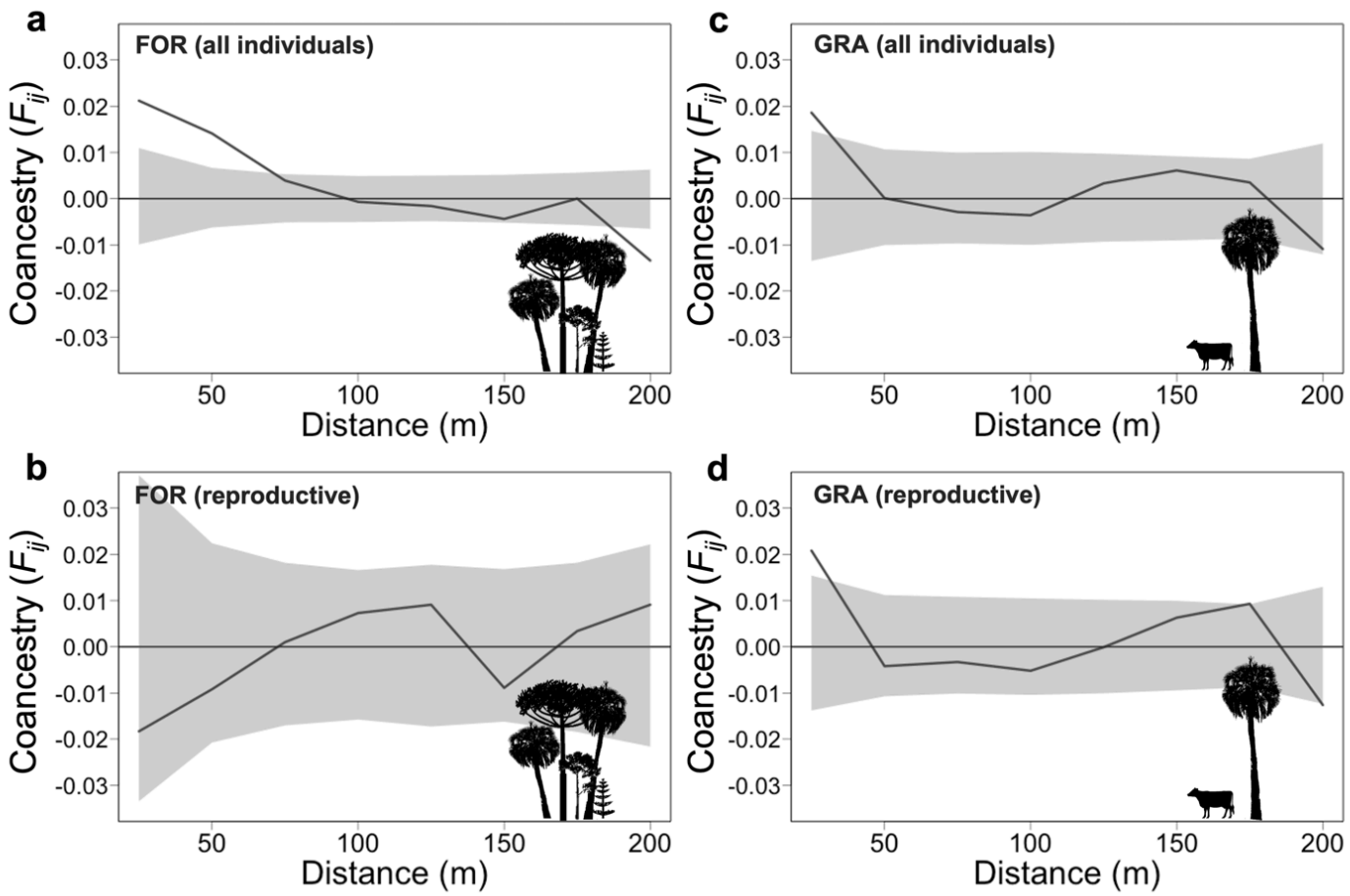

Figure 3

Correlograms of coancestry (F_{ij}) for pairs of *Butia eriospatha* individuals. (a) All individuals in the forest population (FOR). (b) Reproductive individuals only in FOR. (c) All individuals in the open grass land population (GRA). (d) Reproductive individuals only in GRA. Ribbons depict 95% confidence intervals around zero (no kinship).

Supplementary Files

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