

Linking Phenology to Mating System: Exploring the Reproductive Biology of the Threatened Palm Species *Butia eriospatha*

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Abstract

The reproductive biology of the vulnerable palm species *Butia eriospatha* was studied to provide important information that contributes to our understanding and conservation of the species. In order to determine when and how *B. eriospatha* reproduces, we combined data from 7 nuclear microsatellite loci with ecological data on flowering and fruiting phenology collected between 2009 and 2011 from a population ($N = 515$) in the Atlantic Rainforest, Southern Brazil. Periods of flowering and fruit production were seasonal and variable across reproductive events. Mating system analyses indicate that *B. eriospatha* is a predominantly outcrossing species, ($\hat{f}_m = 0.961$), since a certain degree of biparental inbreeding does occur. The species is self-compatible and reproduction may also occur by geitonogamy, indicating the ability of isolated populations to survive and persist. Open-pollinated seeds varied in relatedness, including mainly half-sibs and full-sibs. The effective population size was lower than that expected for panmictic populations. Hence, seeds for conservation programs must be collected from a large number of seed-trees to ensure an adequate effective population in the sample. The collection of germplasm is a high-priority strategy that should be employed to maintain the genetic variability that remains.

Key words: circular statistics, conservation genetics, mating system estimation, microsatellites, outcrossing

The palm family (Arecaceae) has about 2450 species distributed in many of the world's tropical forests and it is one of the better studied tropical families of angiosperms (Barfod et al. 2011). Although many important advances have been made in palm evolutionary biology concerning life history (Henderson 2002; Rodrigues-Buriticá et al. 2005; Eiserhardt et al. 2011), molecular evolution (Chase 2004; Smith and Donoghue 2008), population genetics (Luna et al. 2005; Shapcott et al. 2009), and pollination mechanisms (Barfod et al. 2011), our knowledge is still somewhat limited with respect to reproductive biology, specifically relating to the mating system (Bovi et al. 2003; Conte et al. 2008; Ramos et al. 2011; Abreu et al. 2012).

In plants, mating systems that employ cross- and/or self-fertilization control the patterns of genetic transmission within and among populations. Although self-fertilization (i.e., selfing) restricts gene migration through pollen flow, outcrossing promotes pollen flow and reduces the

likelihood of microgeographic differentiation and population substructuring. However, even in the presence of long-distance pollen flow, population substructuring can occur due to localized seed dispersal. Mating patterns are determined by reproductive and environmental features such as self-incompatibility mechanisms (Goodwillie et al. 2005), gender and degree of dichogamy in flowering plants, foraging behavior of pollinators (Hirao et al. 2006), flowering phenology (Oddou-Muratorio et al. 2006), spatial isolation of trees (Fuchs et al. 2003), and plant density (Murawski and Hamrick 1991).

Tropical trees are mainly outcrossing (Ward et al. 2005); however, mixed mating systems—which include both selfing and outcrossing—frequently occur in a wide variety of plants (Goodwillie et al. 2005), including palms (Conte et al. 2008; Ramos et al. 2011; Abreu et al. 2012). In addition, reproductive biology studies have shown that self-compatibility in plant species is also frequent (Goodwillie 1999, 2001). Although it is generally

accepted that palm families are predominantly self-compatible (Read 1975; Scariot et al. 1991; Ashburner et al. 2001; Barfod et al. 2003; Bovi et al. 2003; Chan et al. 2011), few studies have tested the self-compatibility of specific palm species.

Self-compatibility within a species is advantageous when no pollinator vectors are available and could improve the chances of reproductive success when mates are scarce or when the population is small. As reductions in population sizes become common due to anthropogenic disturbances, such as land conversion, self-compatible species would be able to survive and persist more than a few generations in fragmented habitats or at small population sizes. Hence, understanding the reproductive biology of a plant species is a key element in conservation programs and is particularly important for those species that are at risk of extinction.

Natural populations of the neotropical palm species *Butia eriospatha* (Martius ex Drude) Beccari are suffering the consequences of human intervention, especially through loss of habitat due to exotic tree species reforestation, illegal sale of specimens in both local and international trade, overexploitation of fruit, and cattle farming. Furthermore, the remaining populations of *B. eriospatha* mainly consist of mature individuals aged 100 years or older (Nazareno et al. 2011). This vulnerable species (IUCN 2010) is a long-lived palm (subfamily Arecoideae, subtribe Buttinae; Dransfield et al. 2005) locally known as *butiá-da-serra*. It is native to the Atlantic Forest, which ranges from Southern Brazil to Uruguay (Reitz 1974) and grows in high-altitude grasslands (a subtype of the Atlantic Forest Domain). Previous observations indicate that *B. eriospatha* ($2n = 32$; Correa et al. 2009) is monoecious (Reitz 1974). *Butia eriospatha* is a palm species with numerous male and female yellow flowers arranged in distinct parts of the same inflorescence (i.e., androgynous). The inflorescences are protandrous in which male flowers come into anthesis before female flowers. Since there may be several inflorescences on a single individual, selfing (i.e., geitonogamy) can occur due to pollen flow from one inflorescence to another one with female receptive flowers. The fleshy fruit of the species is approximately 2.0 cm in diameter and is eaten by both local human populations and frugivores, such as birds and squirrels. No published study has examined the details of the reproductive biology of *B. eriospatha* and therefore the goal of this study is to shed light on this important aspect of the species.

In order to elucidate when and how *B. eriospatha* reproduces, we coupled genetic data with ecological data on individual size, flowering, and fruiting. Specifically, we address the following questions: 1) Is there a pattern of seasonality in flowering and fruiting? 2) Is the species strictly outcrossing? 3) Is there some mechanism to avoid self-fertilization? Our results will influence long-term decisions for in situ and ex situ conservation and breeding of this threatened palm species.

Materials and Methods

Study Site

We sampled one population of *B. eriospatha* located near the municipality of Curitiba (27°16' S, 50°34' W), in Santa

Catarina State, Western Plateau, Southern Brazil. This population covers an area of about 16 hectares with individuals in a clustered distribution (Figure 1). In order to reduce the risk of illegal poaching and trade of mature individuals from this population, we do not provide the exact location of the study site. Historically, the study region was the first area on the plateau of Santa Catarina State colonized by Europeans and the area has consistently been occupied by mainly live-stock herders. Cattle have been present for at least 90 years in this *B. eriospatha* population's habitat. Based on previous observations, this population has a bimodal age structure made up of adult plants and seedlings with no evidence of recruitment to later stages of maturity (Nazareno and Reis, unpublished data). The Western Plateau of Santa Catarina has a humid subtropical climate according to the Köppen (1948) system. In the study area, the annual rainfall from 2009 to 2010 was approximately 1500 mm (Figure 2). The average total monthly rainfall is 150 mm from October to March (wet season) and 80 mm from April to September (dry season). The mean annual temperature is 17.1°C, with average highs in February of 21.4°C and lows in June of 12.6°C. Climate data for the study site was provided by the National Institute of Meteorology (INMET – Instituto Nacional de Meteorologia) that operate a station located approximately 10 km from *B. eriospatha* population.

Flowering and Fruiting Phenology

Field Measurements

Phenological observations were carried out during 2 consecutive reproductive events (October 2009–May 2010 and October 2010–May 2011). In the studied population (Figure 1), all individuals ($N = 515$) were tagged with a numbered aluminium plate, and the diameter at breast height (DBH) was recorded. Due to mortality, the number of monitored palm trees in 2010–2011 was 504 individuals. The following phenological stages (i.e., phenophases) were recorded and data collected at 15-day intervals: 1) inflorescence with male flowers, 2) inflorescence with female flowers, 3) infructescence with unripe fruit, and (4) infructescence with ripe fruit. For every palm, we recorded the number of reproductive structures (i.e., inflorescence and/or infructescence) and their specific phenophase during each phenological observation. In order to detect evidence of geitonogamy, flowering overlap was also assessed (i.e., the presence of both male and female flowers during inflorescence in a same plant).

Phenological Statistical Analyses

The analyses were based on the period of open flowers (male and female) and (un)ripe fruits, hereafter called flowering and fruiting. The size of the reproductive *B. eriospatha* population was calculated as the number of individuals that reproduced during the 2009–2011 period. We analyzed differences in reproductive effort (number of inflorescence per palm tree) with Kolmogorov–Smirnov test between the reproductive events. In addition, the effect of DBH on flowering (yes/no) was inferred by an unpaired *t*-test (Zar 1996) under the H_0

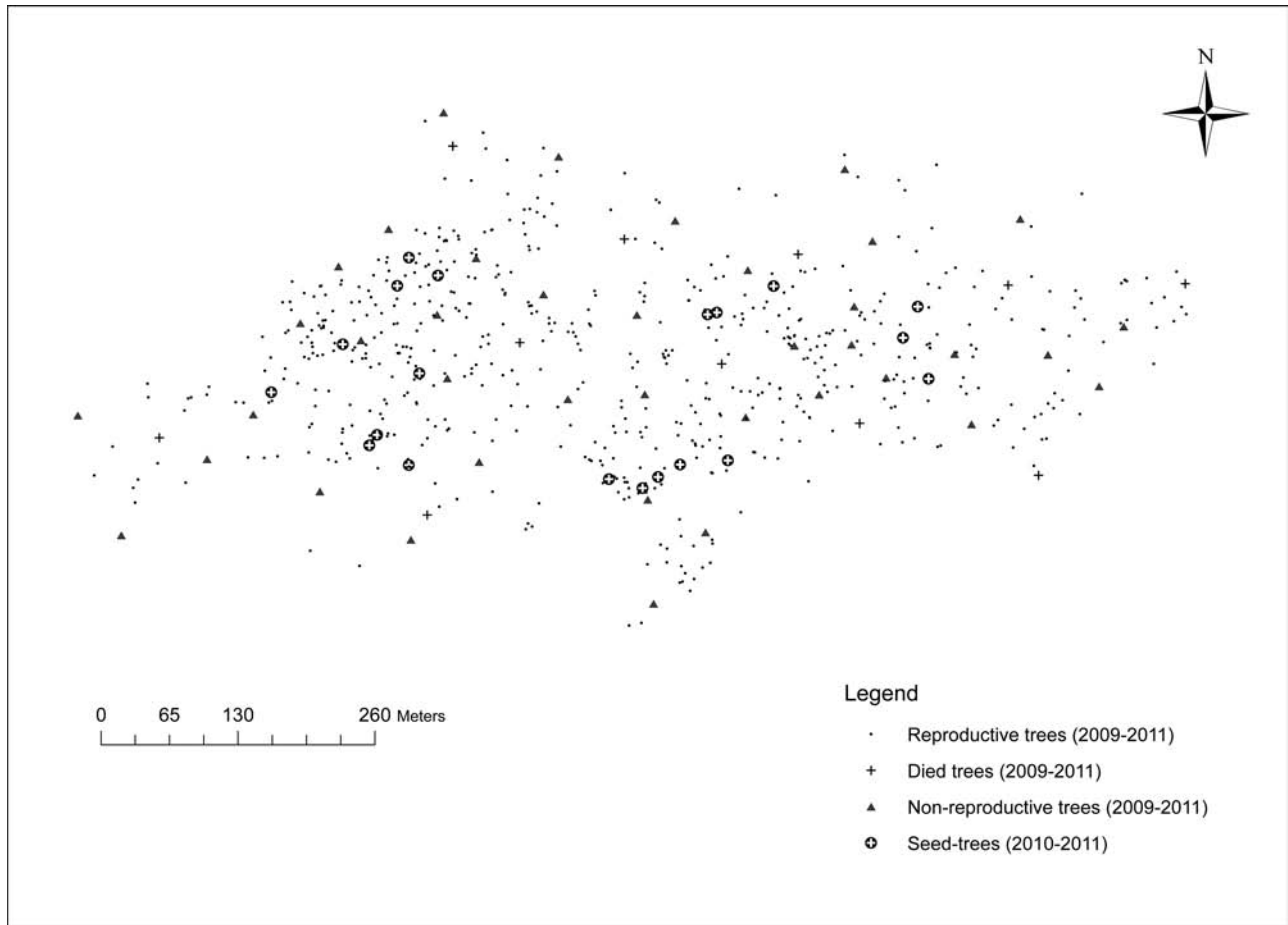


Figure 1. Map of the study population showing the spatial distribution of all studied *Butia eriospatha* (Martius ex Drude) Beccari individuals in the Atlantic Rainforest, Santa Catarina State, Southern Brazil. Reproductive and nonreproductive trees, died trees, and seed-trees are shown.

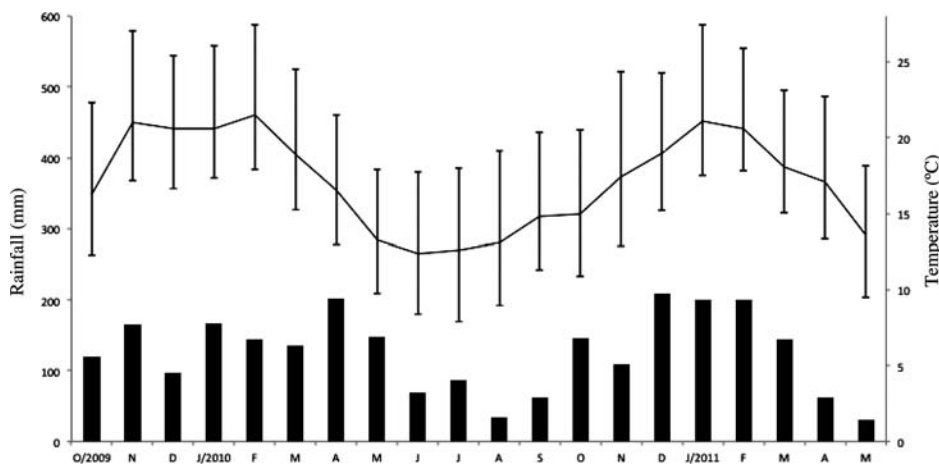


Figure 2. Annual distribution of average monthly temperatures (°C) including maximum and minimum temperatures, and rainfall distribution (mm) for the municipality of Curitiba, in the Santa Catarina State Western Plateau, Southern Brazil. Bars represent rainfall data and line represents temperature data.

hypothesis: where the mean DBH of reproductive palm trees is the same as the mean DBH of nonreproductive palm trees.

Circular statistics were used to determine seasonality, as proposed by Morellato et al. (2000). To calculate the circular statistic parameters, months were converted to angles, from 0° January (no. 1) to 330° December (no. 12) at intervals of 30°. The frequency of reproductive structure in a specific phenophase within each angle was calculated and the following parameters estimated for each reproductive event: mean angle a , and the length of vector r , a measure of concentration around the mean angle that ranges from 0, when an equal number of phenological records occur at each angle, to 1, when all records occur at one single angle or month. The significance of the mean angle was estimated using the Rayleigh(z) test (Zar 1996). The mean date for each phenophase was determined by converting the mean angle to corresponding mean dates.

The seasonality hypothesis also followed Morellato et al. (2000) by defining H_0 as the condition where phenological variables are distributed uniformly throughout the year, thus suggesting no seasonality. If H_0 is rejected, the phenological variables are not uniformly distributed, and there is a significant mean angle or date for the phenological variable or seasonal pattern. The intensity of concentration around the mean angle denoted by r can be considered a measure of the degree of seasonality (Morellato et al. 2000). When the mean angle was significantly different from 0, a two-sample Watson-Williams test (F) was performed to compare the mean dates between the reproductive events. Possible climatic conditions such as temperature and rainfall were correlated with the phenological events (Spearman's correlation). All analyses followed Zar (1996) and were performed with ORIANA software (Kovach 2004).

Genetic Analysis

Sampling, DNA Extraction, and Microsatellite Marker Amplification

In order to determine the mating system, we analysed 20 open-pollinated progeny from 20 seed-trees sampled randomly in the final reproductive event (2010–2011; Figure 1). Genomic DNA extraction from leaves of seed-trees followed the standard cetyltrimethylammonium bromide procedure (Alzate-Marin et al. 2009). For the progeny arrays, DNA was extracted directly from the embryo due to the low germination potential of dormant seeds. For DNA extraction from embryos, we used the NucleoSpin kit (MACHEREY-NAGEL GmbH & Co. KG), according to manufacturers' instructions. The PCR and profile used to amplify the 7 microsatellite loci (But06, But07, But08, But09, But11, But16, and But23) are described in Nazareno et al. (2011). PCR products were denatured and separated using 10% denaturing polyacrylamide gels (39:1 acrylamide to bisacrylamide) stained with silver nitrate. Gels were run with 1X TBE buffer (90 mM Tris, 92 mM boric acid, and 2.5 mM EDTA) on a vertical electrophoresis at a constant electric current (21 mA for each gel). Allele sizes were estimated by

comparison with a 10 bp DNA ladder standard (Invitrogen, Carlsbad, California, USA).

Prior Genetic Analysis

We tested for Mendelian inheritance of alleles and gametic disequilibrium before including the microsatellite set in our study. Loci that are included in analyses despite gross violations of these assumptions or high rates of error could lead to inaccurate and biased genetic estimates (Selkoe and Toonen 2006). Since the mating system analysis presupposes that alleles at different loci segregate independently, we undertook Mendelian inheritance analyses for each locus, based on the mother tree and their open-pollinated family as proposed by Gillet and Hattemer (1989). As gametic disequilibrium creates pseudoreplication for analyses in which loci are assumed to be independent samples of the genome, we used the FSTAT software (Goudet 2002) to test all loci for linkage disequilibrium, applying the Bonferroni correction for multiple comparisons.

Determination of the Mating System

The mating system of *B. eriospatha* was analyzed under the mixed-mating and correlated mating models, using the Multilocus mating system program MLTR version 3.2 (Ritland 2008). Since the null alleles can reduce the power to estimate mating systems, the analysis was performed taking into account that all loci may contain null alleles even if there are none. The parameters estimated were multilocus outcrossing rate (\hat{t}_m); single-locus outcrossing rate (\hat{t}_s); selfing correlation (\hat{r}_s); biparental inbreeding rate ($\hat{t}_m - \hat{t}_s$); and multilocus paternity correlation ($\hat{r}_{p(m)}$) or proportion of full sibs among outcrossed progeny. The inbreeding coefficient of maternal parents (\hat{F}_m) was also calculated. Analyses at the population level were carried out using the probabilities of expectation maximization numerical method and at the individual level using the method of moments according to Ritland (2004). The standard error for each parameter was calculated from 1000 bootstrap replicates with resampling among families. To determine whether the values were significantly lower than 1 ($\hat{t}_m - \hat{t}_s$) or greater than 0 (\hat{F}_m , $\hat{t}_m - \hat{t}_s$, and $\hat{r}_{p(m)}$), a 95% confidence interval was calculated.

From the mating system parameters, other demographic and genetic parameters were assessed. The neighborhood size \hat{N}_{ep} , that is, the number of pollen donors contributing to each family, was estimated as $1/\hat{r}_{p(m)}$ (Ritland 1989). The average proportion of self-sibs (\hat{P}_{ss}), half-sibs (\hat{P}_{hs}), full-sibs (\hat{P}_{fs}), and self-half-sibs (\hat{P}_{shs}) within families was estimated as $\hat{P}_{ss} = \hat{s}^2$; $\hat{P}_{hs} = \hat{t}_m \hat{r}_s (1 - \hat{r}_{p(m)})$; $\hat{P}_{fs} = \hat{t}_m^2 \hat{r}_{p(m)}$ and $\hat{P}_{shs} = 2\hat{s} \hat{t}_m$ where \hat{s} ($= 1 - \hat{t}_m$) is the selfing rate. The confidence intervals for these parameters were calculated based on both upper and lower confidence limits estimated from mating system parameters using the MLTR program.

In addition, we calculated the coancestry coefficient among plants within progenies ($\hat{\theta}_{xy}$) from the correlation coefficient of relatedness among plants within progenies (\hat{r}_{xy}), as proposed by Ritland (1989): $\hat{r}_{xy} = 0.25 (1 + \hat{F}_m) [4\hat{s} + (\hat{t}_m^2 +$

$\hat{f}_m \hat{f}_s \hat{f}_y (1 + \hat{f}_{p(m)})$. Based on the coancestry coefficient, we estimated the effective size of variance $\hat{N}_{sp} = 0.5/\hat{\theta}_{sy}$ (Cockerham 1969), considering that for diploid species the coefficient $\hat{\theta}_{sy}$ is half the coefficient \hat{f}_{sy} (Lynch and Walsh 1998). It is important to note that the minimum coancestry coefficient expected is 0.125 for half-sibs. Further, this parameter is expected to accommodate different levels of relatedness within families: self-sibs, half-sibs, full-sibs, and self-half-sibs. Thus, in open-pollinated offspring incorporating different kinds of relatives, $\hat{\theta}$ is expected to have values between 0.125 and 1 (the coancestry expected between two self-sib individuals from an autogamous species). In addition, the Nason's estimator of kinship coefficient (f_{ij} or coefficient of coancestry) described in Loiselle et al. (1995) was performed for maternal trees using the software SPAGeDi (Hardy and Vekemans 2002).

The coefficient of inbreeding in embryos (\hat{F}_o) was inferred by calculating the fixation index using the FSTAT program (Goudet 2002). To test if \hat{F}_o was significantly different from 0, 10 000 permutations of alleles among individuals were performed. The total coefficient of inbreeding $\hat{F}_o (= \hat{F}_s + \hat{F}_{m-s})$ in embryos was split into its components resulting from self-fertilization $\hat{F}_s = 0.5\hat{f}(1 + \hat{F}_m)$ (Barrett and Kohn 1991) and mating among relatives, \hat{F}_{m-s} .

We also estimated the number of seed-trees \hat{m} from which it would be necessary to collect seeds in order to retain the reference effective population size ($N_{e(reference)}$) of 500 (Nunney and Campbell 1993). This was calculated following the method of Sebbenn (2006), based on the relationship between the desired effective population size of the conservation program ($N_{e(reference)}$) and the average variance effective population size estimated for plants within progenies $\hat{N}_{e(i)}$.

In order to determine if the mating events of the *B. eriostatha* population occurred due to random mating, we estimated the coefficient of pollen pool structure Φ_{β} from 2-generation analyses (TWOGENER, Smouse et al. 2001). It is an estimator of genetic differentiation among pollen pools (ranges from 0 to 1), which is an analogue of Wright's F_{ST} . The standard error for Φ_{β} was calculated using the jackknife procedure over loci. Calculations were performed using the R language for TWOGENER analysis as was written by A. S. Hirao (available from <http://hosho.ees.hokudai.ac.jp/~hirao/TWOGENER/TwoGener.html>).

Results

Flowering and Fruiting Phenology

The reproductive population of the sampled *B. eriostatha* population ranged from 474 ($N_{2009-2010} = 515$) to 459 individuals ($N_{2010-2011} = 504$). Approximately 8% of the total population did not exhibit reproductive activity over the 2009–2011 study periods (Figure 1). The flowering intensity was significantly different between reproductive events ($D = 0.69$, $P < 0.05$). In the 2010–2011 study period, the number of inflorescence ($N = 868$, mean per plant = 2.93, minimum = 1, maximum = 6) was 34% lower than during the

2009–2010 period ($N = 1316$, mean per plant = 3.15, minimum = 1, maximum = 7). The smallest individual that flowered had a DBH of 6.68 cm. Furthermore, some palm trees with a large diameter did not flower during the study period. In agreement, there is no significant difference in the mean DBH for reproductive ($DBH_{2009-2010} = 10.88$, $DBH_{2010-2011} = 10.76$) and nonreproductive ($DBH_{2009-2010} = 10.38$, $DBH_{2010-2011} = 10.87$) palm trees ($t_{2009-2010} = 2.311$, $P > 0.05$; $t_{2010-2011} = 1.961$, $P > 0.05$). In both reproductive events, there was an overlap in flowering for 18% ($N_{2009-2010} = 85$) to 19% ($N_{2010-2011} = 87$) of individuals, indicating that geitonogamy can occur in *B. eriostatha*.

We also found that both the periods of flowering and fruit production were variable between reproductive events. The peak in reproduction during the 2010–2011 study period occurred about 1 month later than that observed during the 2009–2010 reproductive event. Flowering (male and female) was concentrated in the wet season, between October and March (Figure 1). The *B. eriostatha* population produced fruit for a long period of time, from November through July, with a peak of unripe fruit 1 month later than that reported in 2009–2010 period (Table 1). The period of ripe fruiting activity for animal dispersal occurred from February to July with the peak amount of ripe fruit variable between reproductive events (Table 1).

For each reproductive event, the mean date of flowering and fruiting was significant (Table 1), indicating that phenological variables are not uniformly distributed throughout the year. The r values, ranging from 0.76 (male and female flowers at 2010–2011) to 0.96 (ripe fruits at 2010–2011; Table 1), denote a high degree of seasonality. When phenophases were compared between reproductive events, the mean angle or mean date was significantly different between male flowers ($F = 303.5$, $P < 0.001$), female flowers ($F = 30.8$, $P < 0.001$), unripe fruit ($F = 559.9$, $P < 0.001$) and ripe fruit ($F = 737.6$, $P < 0.001$). Therefore, these variables seem to exhibit uneven seasonal patterns.

We found significant correlations between climate variables and flowering and fruiting in both study periods. Flowering (female) was both positively correlated with mean temperature ($r_s = 0.76$, $P = 0.0001$) and precipitation ($r_s = 0.54$, $P = 0.014$). However, the same correlation was not found for male flowers ($r_s = 0.42$, $P = 0.063$), which was minimally but significantly correlated with mean temperature ($r_s = 0.58$, $P = 0.007$). For fruiting, significant correlations were found only for unripe fruit: mean temperature ($r_s = 0.91$, $P < 0.0001$) and precipitation ($r_s = 0.54$, $P = 0.013$).

Mating System

The microsatellite set for *B. eriostatha* had a Mendelian inheritance (see Supplementary Material online) and no significant linkage disequilibrium ($P > 0.001$) was found between loci for seed-trees and progenies. Therefore, the microsatellite markers used are appropriate for investigating the mating system of this palm tree species.

Table 1 Results of circular statistical analyses testing for seasonality in phenological behavior for *Butia eriospatha* (Martius ex Drude) Beccari in the Atlantic Rainforest, Santa Catarina State, Southern Brazil

	Phenological variables							
	Male flowers		Female flowers		Unripe fruits		Ripe fruits	
	2009–2010	2010–2011	2009–2010	2010–2011	2009–2010	2010–2011	2009–2010	2010–2011
Observations (N)	1316	868	1295	849	3406	3207	1202	845
Mean angle (\hat{a})	318.11 ^{oa}	342.45 ^{ob}	350.54 ^{oa}	0.73 ^{ob}	24.99 ^{oa}	46.74 ^{ob}	61.43 ^{oa}	86.45 ^{ob}
Mean date	21/Nov	14/Dec	22/Dec	01/Jan	25/Jan	16/Feb	03/Mar	29/Mar
Length of mean vector (\hat{r})	0.95	0.76	0.79	0.76	0.83	0.81	0.96	0.94
Rayleigh(z) test of uniformity (\hat{p})	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Significant values $P < 0.05$. Mean angle for each phenophase does not differ by the Watson-Williams test ($P < 0.001$) if it is followed by the same letter (a or b).

Table 2 Mating system parameters, estimates of inbreeding, and relatedness of a *Butia eriospatha* (Martius ex Drude) Beccari population in the Atlantic Rainforest, Santa Catarina State, Southern Brazil

Mating system	95% CI	
Multilocus outcrossing rate: \hat{t}_m	0.961	0.956–0.966
Single-locus outcrossing rate: \hat{t}_s	0.909	0.906–0.912
Selfing rate: $\hat{s} = 1 - \hat{t}_m$	0.039	0.034–0.044
Mating among relatives: $\hat{t}_m - \hat{t}_s$	0.052	0.048–0.056
Correlation of selfing: \hat{r}_s	0.104	0.103–0.105
Multilocus paternity correlation: $\hat{r}_{p(m)}$	0.442	0.439–0.445
Effective number of pollen donors: \hat{N}_{ep}	2.260	2.241–2.283
Inbreeding and genetic structure		
Inbreeding coefficient of maternal parents: \hat{F}_m	0.000	0.000–0.000
Inbreeding coefficient of progeny: \hat{F}_o	0.050	0.019–0.073
Inbreeding in progeny from selfing: \hat{F}_s	0.020	0.008–0.054
Inbreeding in progeny from mating among relatives: \hat{F}_{m-t_s}	0.030	0.017–0.069
Proportion (%) of self-sibs pairs: \hat{P}_{ss}	0.160	0.120–0.190
Proportion (%) of half-sibs pairs: \hat{P}_{bs}	51.54	50.55–55.21
Proportion (%) of full-sibs pairs: \hat{P}_{fs}	40.80	40.02–44.06
Proportion (%) of self-half-sibs pairs: \hat{P}_{shs}	7.500	6.531–8.460
Coancestry within offspring: $\hat{\theta}_{sy}$	0.187	0.173–0.192
Effective size of variance: $\hat{N}_{e(v)}$	2.670	2.632–2.793
Number of seed-trees for seed collection: \hat{m}	187.0	179.0–189.0
Sample size		
Number of seed-trees	20.0	
Average number of offspring for seed-tree	19.5	

CI is the confidence interval calculated by 1000 bootstraps.

As the multilocus ($\hat{t}_m = 0.961$) and single-locus ($\hat{t}_s = 0.909$) outcrossing rates (Table 2) differ statistically from unity, the *B. eriospatha* is a predominantly outcrossed species. The individual multilocus and single-locus outcrossing rates were variable, ranging from 0.746 to 1.136 and from 0.629 to 1.044, respectively (Table 3). Estimates of outcrossing rates greater than 1 are an artifact of the algorithm used in the computer analysis; however, the individual estimates for (\hat{t}_m) and (\hat{t}_s) did not differ statistically from unity (Table 3). The difference between the multilocus and single-locus outcrossing rate was

low but significantly different from 0 ($\hat{t}_m - \hat{t}_s = 0.052$), suggesting a low proportion of mating among relatives (Table 2). However, individual differences in ($\hat{t}_m - \hat{t}_s$) show significant and high values among the seed-trees, ranging from 0.155 to 0.328 (Table 3). Also, the individual estimates of selfing were high and significantly different from 0 for 2 families (7 and 19, Table 3). Estimates of selfing and the presence of overlap during flowering episodes (Table 3) indicate that *B. eriospatha* is a self-compatible palm species. The estimate of the correlation of selfing (\hat{r}_s) was low and significantly different from 0 ($\hat{r}_s = 0.104$, $P < 0.05$) indicating low variation in the individual self-fertilization rate in this population.

The multilocus paternity correlation was significantly different from zero ($\hat{r}_{p(m)} = 0.442$, Table 2). This $\hat{r}_{p(m)}$ value indicates that less than 3 fathers ($\hat{N}_{ep} = 2.26$) contributed to individual progeny arrays in the *B. eriospatha* population. Furthermore, the coefficient of pollen pool structure of maternal trees was high and statically different from 0 ($\Phi_{ji} = 0.225$, $P < 0.05$), indicating a nonrandom mating pattern probably due to the restriction of pollen distribution in the population. In addition, combining the estimate of the paternity correlation with the estimated multilocus outcrossing rate, it is possible to determine the proportion of different kinship types within families (Table 2). In *B. eriospatha* populations, the offspring within families were composed predominantly of half-sibs (51.54%), followed by full-sibs (40.80%).

The fixation index of mother plants (\hat{F}_m) estimated for the progenies was nil (Table 2), indicating an absence of inbreeding in the sampled mother plants. Although inbreeding was low in progenies as result of the kinship coefficient of their parental trees ($\hat{f}_{ij} = 0.04$, 95% confidence interval after 10 000 permutations over loci ranging from -0.16 to 0.14), biparental inbreeding was the main cause of the observed inbreeding (Table 2). The mean coefficient of coancestry within progenies $\hat{\theta}_{sy}$ was 0.187. This value was lower than expected for full-sib families ($=0.25$). The estimate of effective population size ($\hat{N}_{e(v)} = 2.67$) was also lower than that expected for half-sib families ($=4$). The number of seed-trees \hat{m} from which it is necessary to collect seeds, aiming to retain an effective population size of 500, is 187 palm trees (Table 2).

Table 3 Estimates of the mating system and flowering overlap for each *Butia eriopatha* (Martius ex Drude) Beccari seed-trees in the Atlantic Rainforest, Santa Catarina State, Southern Brazil

Family/[n]	\hat{t}_m	CI 95%	\hat{t}_s	CI 95%	\hat{s}	CI 95%	$\hat{t}_m - \hat{t}_s$	CI 95%	Flowing overlap
01 [20]	1.001	0.950–1.051	0.997	0.756–1.238	0.000	–0.052–0.049	0.004	–0.186–0.194	–
02 [20]	0.957	0.894–1.019	<u>0.629</u>	0.413–0.845	0.043	–0.019–0.105	<u>0.328</u>	0.175–0.480	–
03 [20]	0.866	0.691–1.040	0.857	0.672–1.041	0.134	–0.040–0.308	0.009	–0.001–0.018	+
04 [20]	1.069	0.931–1.206	1.011	0.805–1.216	0.000	–0.206–0.068	0.058	–0.010–0.127	–
05 [20]	0.992	0.896–1.088	0.923	0.750–1.095	0.008	–0.088–0.104	0.069	–0.007–0.145	–
06 [20]	0.983	0.891–1.075	0.877	0.614–1.139	0.017	–0.075–0.109	0.106	–0.064–0.276	–
07 [20]	<u>0.746</u>	0.514–0.977	<u>0.582</u>	0.229–0.934	<u>0.254</u>	0.022–0.485	<u>0.164</u>	0.042–0.285	+
08 [20]	1.063	0.967–1.159	1.044	0.732–1.356	0.000	–0.159–0.033	0.019	–0.197–0.234	–
09 [20]	1.057	0.912–1.202	1.002	0.759–1.245	0.000	–0.202–0.088	0.055	–0.043–0.153	–
10 [19]	1.136	0.961–1.131	0.976	0.567–1.495	0.000	–0.310–0.038	0.160	–0.185–0.504	–
11 [20]	1.028	0.985–1.071	1.010	0.859–1.161	0.000	–0.071–0.015	0.018	–0.089–0.126	–
12 [16]	1.034	0.856–1.212	1.002	0.774–1.229	0.000	–0.212–0.144	0.032	–0.017–0.081	–
13 [20]	0.997	0.875–1.118	0.993	0.798–1.187	0.003	–0.118–0.124	0.004	–0.068–0.076	–
14 [20]	1.059	0.919–1.198	0.970	0.674–1.266	0.000	–0.198–0.080	0.089	–0.067–0.246	–
15 [19]	1.086	0.917–1.254	0.963	0.669–1.257	0.000	–0.254–0.082	0.123	–0.002–0.248	–
16 [20]	1.019	0.868–1.169	1.005	0.783–1.226	0.000	–0.169–0.132	0.014	–0.056–0.084	–
17 [20]	1.048	0.912–1.183	1.023	0.682–1.360	0.000	–0.183–0.087	0.025	–0.180–0.230	–
18 [20]	1.008	0.847–1.168	0.999	0.774–1.224	0.000	–0.168–0.152	0.009	–0.055–0.073	–
19 [19]	<u>0.873</u>	0.767–0.978	<u>0.718</u>	0.477–0.959	<u>0.127</u>	0.021–0.232	<u>0.155</u>	0.019–0.290	+
20 [20]	1.041	0.862–1.219	1.003	0.650–1.356	0.000	–0.219–0.137	0.038	–0.136–0.212	–

CI is the confidence interval calculated by 1000 bootstraps. n , number of progenies; \hat{t}_m , multilocus outcrossing rate; \hat{t}_s , single-locus outcrossing rate; \hat{s} , selfing rate; $\hat{t}_m - \hat{t}_s$, outcrossing rate among relatives; (+) indicates flowering overlap; confidence intervals that fall within 1 (for \hat{t}_m and \hat{t}_s estimates) or 0 (for \hat{s} and $\hat{t}_m - \hat{t}_s$ estimates) are not significant. Underlying values were significant at $P < 0.05$.

Discussion

Flowering and Fruiting Phenology

We found considerable variation in flowering and fruiting between reproductive events in the studied *B. eriopatha* population. The number of flowering trees, flowering intensity, and fruiting were all greater during the 2009–2010 study period than during the 2010–2011 period. Further, we did not find any impact of DBH, a common measurement of tree size, on reproductive success. The strategy of converting growth into fitness through the correlation of size and reproductive success is a documented strategy for plants which has been reported for several trees species (Weiner et al. 2009), including palms (e.g., *B. capitata*, Castellani et al. 1998). In addition, the breeding population of *B. eriopatha* seems to be diminishing, probably because the population consists of mature individuals aged 100 years or older. Furthermore, it is known that the location of individuals in the landscape and local conditions affects flowering intensity (Oddou-Muratorio et al. 2005); for example, the availability of light promotes flowering. As the *B. eriopatha* inhabits open environments, the availability of light is likely not a factor. Nevertheless, the spatial distribution of individuals might contribute to reproductive success because plants located in proximity might compete for resources (e.g., availability of soil nutrients). In Southern Brazil, isolated *B. capitata* individuals are more successful in reproduction than those located in clusters (Azambuja AC, personal communication).

We also found that flowering was more synchronous among individuals during the 2009–2010 reproductive event in comparison to the 2010–2011 period (see r values for male and female flowers in Table 1). However, *B. eriopatha*

individuals displayed a significant degree of overlap in sexual phases. Hence, sexual reproduction may then occur by geitonogamy. A similar pattern was also documented for the congeneric palm species *B. capitata* in the Atlantic Rainforest (L. Rosa, personal communication) and for other palms species (Borchsenius 1997; Martén and Quesada 2001).

Butia eriopatha also shows seasonality of flowering and fruiting with significant correlations for flowering and unripe fruit production with the wettest and warmest periods. Unlike the data reported in Reitz (1974) for the same region, flowering of *B. eriopatha* occurred from October to March and fruits ripened from February to July. Several studies have shown that reproductive phenological behavior in palms is extremely variable (Henderson 2002); flowering can be restricted to a particular season, and fruiting is frequently nonseasonal (De Steven et al. 1987; Peres 1994; Henderson et al. 2000; Genini et al. 2009).

Although *B. eriopatha* does not provide sufficient resources for all vertebrate frugivores throughout the year, their importance as a major resource in this habit is supported by the production of ripe fruit for about 6 months of the year. Terborgh (1986) stressed that tropical forest plants, such as palms and figs, sustain a large proportion of the vertebrate animal community during annual seasons of food scarcity. Further studies at the community level are essential in order to determine if *B. eriopatha* is a key plant resource for frugivores in the high-altitude grasslands.

Mating System

This study represents the first analysis of mating system parameters of the *Butia* genus. We found that

B. eriospatha is a predominately outcrossing species and seems to be self-compatible. The self-compatibility of this palm species can be supported by three lines of evidence: 1) significant and high selfing rates observed in individual families, although the number of progeny analyzed per maternal tree was low; 2) flowering overlap between inflorescence of individual palms; and 3) the production of viable seeds from an isolated individual, which were germinated and grown into seedlings at the Botanical Garden of São Paulo, São Paulo State, Brazil (personal observation). This evidence indicates the potential of *B. eriospatha* to reproduce in the absence of pollinators or in isolation.

Despite the advantages of this reproductive strategy, selfing can incur negative consequences because through autogamy a recessive allele can increase its incidence by up to 50% in the population (Fisher 1941). Furthermore, self-fertilization is detrimental when selfed offspring suffer reduced viability due to inbreeding depression (Herlihy and Eckert 2002; Goodwillie et al. 2005) brought on by increases in the frequency of homozygotes. Like *B. eriospatha*, most palms studied to date are self-compatible, including *Thrinax parviflora* (Read 1975), *Acanthococos emensis* (Silberbauer-Gottsberger 1990), *Bactris gasipaes* (Clement and Arkcoll 1991), *Acrocomia aculeata* (Scariot et al. 1991), *Butia capitata* (L. Rosa, personal communication), *Cocos nucifera* (Ashburner et al. 2001), *Licuala spinosa* (Barfod et al. 2003), *Archontophoenix* spp. (Bovi et al. 2003), *Syagrus coronata* (KMR Rocha, personal communication), *Johannesteijsmannia lanceolata* (Chan et al. 2011), *J. magnifica* (Chan and Saw 2011); however, the compatibility among many major groups of palms is still unknown (Henderson 2002).

Even though self-compatibility seems to be a prevailing breeding strategy in palms, different mechanisms to promote outcrossing have developed. In species with hermaphrodite flowers, self-pollination is avoided by a temporal separation of female and male phases, for instance, by protandry (*Thrinax*) or protogyny (*Cryosophila*) (Silberbauer-Gottsberger 1990). Palms with androgynous inflorescence likewise may be protandrous (e.g., *Butia* and *Syagrus*) or protogynous such as the genera *Bactris* and *Acrocomia* (Silberbauer-Gottsberger 1990). For *B. eriospatha*, outcrossing is predominantly promoted by protandry. The high multilocus outcrossing rate in *B. eriospatha*, which confirms its allogamy, is comparable with rates found for other palm species, including *Astrocaryum mexicanum* (Eguiarte et al. 1992), *Euterpe edulis* (Reis et al. 2000; Gaiotto et al. 2003; Conte et al. 2008), *Astrocaryum aculeatum* (Ramos et al. 2011), and other mainly outcrossing tropical tree species such as *Symphonia globulifera* (Degen et al. 2004), *Solanum lycocarpum* (Martins et al. 2006), *Carapa guianensis* (Cloutier et al. 2007), and *Ficus arpaçusa* (Nazareno and Carvalho 2009).

Nevertheless, *B. eriospatha* shows a certain level of biparental inbreeding, indicating that outcrossing occurs between relatives. Further, the paternity correlation, which indicates the proportion of plants generated by biparental crosses, was high, suggesting a nonrandom process of cross-pollination. Similarly, the high value of the coefficient of pollen pool structure indicates that assortative mating may have occurred

in the *B. eriospatha* population studied. High differentiation in pollen gene pool implicates a restriction in the effective number of pollen donors as was observed for *B. eriospatha* (Table 2) and also reported for other tree species (Lacerda et al. 2008; Llorens et al. 2012). One assumption of the mixed-mating model is that the allele frequencies in the pollen and ovule pools are homogeneous due to random mating (Ritland and Jain 1981). Several factors could account for the departure from this model, including unequal male and female contributions among adult trees within the population, pollen coming from outside of the population, selection between the time of pollination and progeny sampling, and/or nonrandom mating of genotypes during outcrossing events (Murawski and Hamrick 1992; Doligez and Joly 1997; Lee et al. 2000). In *B. eriospatha*, assortative mating may have occurred through nonrandom mating and/or due to the limited flight range of pollinators.

Although there is currently no information regarding the behavior of pollinators for this species, we predict that *B. eriospatha* is likely pollinated by beetles. This is based on the high predation of seeds (25%, $N = 4367$; unpublished data) and by Silberbauer-Gottsberger's (1990) argument that the palm-beetle relationship has evolved to a balance between parasitism and successful pollination. In addition, for the nearest relative of *B. eriospatha*, the palm species *B. leiospatha* (Barb. Rodr.) Becc, two beetle species (Curculionidae and Nitidulidae) breed in different parts of the inflorescence and flowers. Some of them are effective pollinators (e.g., *Anchylorhynchus* sp.), and their females oviposit on the gynoecium after having passed over the stigmas of various flowers (Silberbauer-Gottsberger 1990). And in both *B. eriospatha* and *B. leiospatha*, larvae of beetles break out from the fruit (Silberbauer-Gottsberger 1990). Future studies on the pollination biology of *B. eriospatha* are necessary and can help clarify the reproductive biology of the species.

The coancestry in the progenies of the study population means there is an 18.7% probability that 2 alleles sampled in 2 plants of the same progeny are identical by descent. The coancestry coefficient is also important in estimating the effective size of variance, which measures the genetic representativeness of progenies compared to the reference population. For *B. eriospatha*, the effective size of variance was estimated at 2.67, indicating that part of progenies are full-sibs. Mating among relatives was the main cause of the observed inbreeding in offspring. On the other hand, the remaining inbreeding detected in offspring could be attributed to selfing. Although we did not observe inbreeding among maternal parents, a previous study indicated that there is significant inbreeding among adult plants in this population (unpublished data), which may be an artifact of the sampled seed-trees.

Although this study provides important information regarding aspects of *B. eriospatha* reproductive biology, the sample is only one population with data from a limited time period. As mating patterns may vary substantially among populations and from one flowering event to another (Reis 1996; Hoebee et al. 2007), our study cannot be replicated at the population level. As well as providing preliminary data on *B. eriospatha* biology,

the conclusions of this study indicate that the studied population is a suitable source from which to sample seeds for *B. eriostpatha* conservation programs. Clearly, more long-term studies of this palm are needed, particularly those that monitor individual palms and their reproductive structures. A detailed picture of contemporary pollination processes and investigations of the floral biology will strengthen the guidelines for effective conservation of the *B. eriostpatha* species.

Implications for Conservation

Recently, the studied *B. eriostpatha* population was reduced to 490 mature individuals due to natural mortality. According to our observations, about 8% of all individuals are non-reproductive, thus suggesting a current breeding population of 450. Considering a mortality rate of 2% (mean calculated across the 2009–2011 period), this population could be at risk of local extinction in the short term, and the number of seed-trees (=187, estimated through the mating system) necessary for seed collection to retain the effective population size of 500 may be scarce in the near future. Beyond the size of the reproductive population, about 19% of all individuals are capable of selfing by geitonogamy. Although selfing would be advantageous to *B. eriostpatha* when no pollinator vectors are available, the effects of inbreeding can lead to a decrease in genotype diversity. Further, if the population is small, the inbred plants may be in danger when there is an adverse change in habitat or environment due to low levels of genetic diversity or if deleterious genes persist in progenies in the long-term. Hence, ex situ conservation based on estimates of effective size of variance for *B. eriostpatha* is a high-priority strategy that will help ensure the conservation of the genetic variability that remains.

Although ex situ conservation programs and monitoring of the remaining populations are essential strategies in protecting *B. eriostpatha*, combating illegal trade is necessary to improve the current state of this species. Given the amount of trade of *B. eriostpatha* abroad, it is also appropriate to include this species in CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) Appendix I, which prohibits the international trade of threatened species. Another possible step in preserving the species is related to cattle herding. Placing enclosures around some reproductive plants could encourage seedling survival. This relatively simple technique has been used effectively for similarly threatened palms in other parts of the world (e.g., *Rhopalostylis baueri*, Walls 2000; *Ptychosperma macarthurii*, Liddle et al. 2006). However, such a strategy requires effective management as restricting the area can lead to genetic structuring within populations.

Supplementary Material

Supplementary material can be found at <http://www.jhered.oxfordjournals.org/>.

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