CSIRO PUBLISHING

Australian Journal of Botany, 2018, **66**, 360–368
https://doi.org/10.1071/BT17195

A high level of outcrossing in the vulnerable species *Prosopis rubriflora* in a Chaco remnant

Fábio M. Alves[A,B], *Ângela L. B. Sartori*[C], *Maria I. Zucchi*[D], *Ana M. G. Azevedo-Tozzi*[A], *Evandro V. Tambarussi*[E] *and Anete P. de Souza*[A,B,F]

[A]Department of Plant Biology, Institute of Biology, PO Box: 6109, University of Campinas – UNICAMP, 13083-970, Campinas, SP, Brazil.
[B]Center for Molecular Biology and Genetic Engineering (CBMEG), University of Campinas – UNICAMP, CEP 13083-875, Campinas, SP, Brazil.
[C]Center for Biological and Health Sciences (CCBS), Federal University of Mato Grosso do Sul – UFMS, Cidade Universitária s/n, PO Box 549, 79070-900, Campo Grande, MS, Brazil.
[D]São Paulo Agency of Technology and Agro-Business, Pólo Apta Centro Sul, SP 127, km 30, 13400-970, Piracicaba, SP, Brazil.
[E]Department of Forestry Engineering, State University of the Central-West, PR 153, km 7, Irati, PR, 84500-000, Brazil.
[F]Corresponding author. Email: anete@unicamp.br

Abstract. *Prosopis rubriflora* Hassl. is a tree species typically found in chaquenian areas, mainly with an arborised phytophysiognomy in the southern region of the Pantanal wetland. This species has become vulnerable in recent decades as a result of considerable increases in anthropogenic activities such as cattle breeding, and this vulnerability has also been observed in several other native species. The goal of this study was to estimate the mating system of *P. rubriflora* in a Chaco remnant by analysing 10 microsatellite markers. Samples were collected over 2 years (2010–213 seedlings and 2011–180 seedlings), and the results suggest that the mating system of *P. rubriflora* is preferably allogamous. A progeny array was predominantly composed of half-sibs (from 76 to 79%), full-sibs (from 15%) and self-half-sibs (from 6 to 9%). The outcrossing rate between related individuals was significant in 2011 but not in 2010. The average co-ancestry coefficient ($\bar{\theta}$) ranged from 0.158 to 0.162, and the variance effective size (N_e) ranged from 3.05 to 3.13. The number of seed trees required for seed collection (m) to retain an effective size of 150 in progeny array samples was 48–49. The high levels of outcrossing of *P. rubriflora* appear to be related to several mechanisms that avoid selfing and due to the behaviour of native pollinators, which clearly contribute to the gene flow of the species.

Additional keywords: conservation, mating system, neotropics, pantanal, stepic savanna.

Received 11 October 2017, accepted 5 July 2018, published online 6 August 2018

Introduction

The genus *Prosopis* L., belonging to the subfamily Mimosoideae DC. and the family Leguminosae Adans., includes 45 species that are distributed primarily on the American continent but also occur in Africa and South-east Asia. These species are important components of arid or semiarid areas (Lewis *et al*. 2005; Catalano *et al*. 2008). In South America, certain species of *Prosopis* are characteristic of arid and semiarid regions, such as Monte, Patagonia, Puna, and Chaco (Catalano *et al*. 2008). Chaco or Gran Chaco, which is described as the largest South American dry forest, covers Argentina, Paraguay, Bolivia, and Brazil (Hueck 1972). According to the Brazilian Institute of Geography and Statistics (IBGE 2012), these areas are classified as stepic savanna. The genus is pollinated by insects, which are reported as having a short-range pollen-dispersion behaviour (Burkart 1976; Bessega *et al*. 2000; Bessega *et al*. 2012). The seed dispersion is described as authocoric (Solbrig and Cantino 1975; Freitas *et al*. 2013), but it is mainly registered as zoochoric through domesticated animals such as, cattle, goats and horses and also through wild animals such as rodents, armadillos, foxes and skunks (Burkart 1976; Campos *et al*. 2016).

Prosopis rubriflora Hassl. (Fig. 1) is a tree species with branches that display prickles, red inflorescences and reduced linear leaflets with hermaphrodite flowers (Burkart 1976). The species occurs primarily in Chaquenian areas with an arborised stepic savanna phytophysiognomy, whereas other *Prosopis* sp. are commonly observed in areas with a ‘forest stepic savanna’

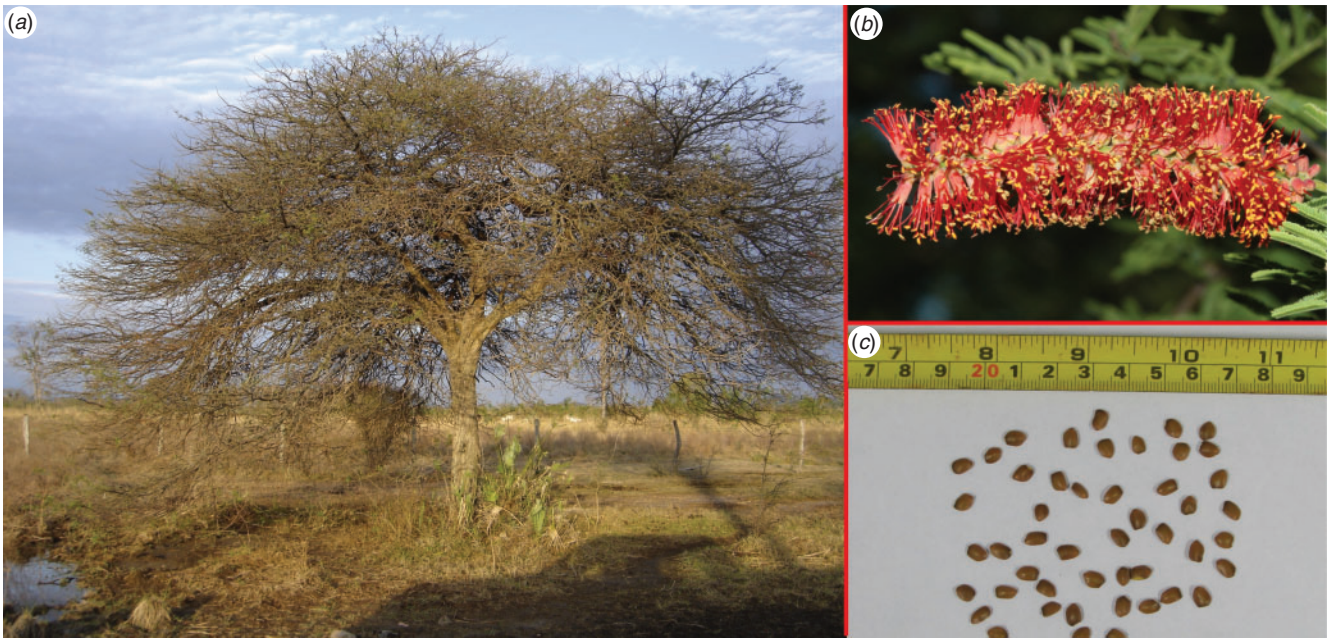

Fig. 1. *Prosopis rubriflora* registered in Porto Murtinho: (*a*) habit; (*b*) red inflorescence in raceme (characteristic of the species); (*c*) sample of the seeds used in the study. Photographs (*a*) and (*c*) by FM Alves, (*b*) by ALB Satori.

physiognomy (Pott and Pott 2003; Alves *et al*. 2018) distributed in the south-west of the state of Mato Grosso do Sul (MS), Brazil and Paraguay (Burkart 1976). The 'arborised stepic savanna' (ASS) consists of sparse, prickly nanophanerophytes (IBGE 2012), and *P. rubriflora* is often found in clusters interspersed with other species in this region. *Prosopis rubriflora* is a dominant taxon in conserved areas (Lima 2012), thus it is a good indicator of arborised stepic savanna.

The species has two flowering peaks, which occur in February and August. Although maximum fruiting occurs from October to January, the species continuously flowers throughout the year at a lower intensity and is mainly pollinated by insects (Sigrist *et al*. 2018). *Prosopis rubriflora* wood can be used for firewood, coal, agricultural implements and other uses in lightweight joinery (Lorenzi 2002). In addition, symbiotic associations with nodulating nitrogen-fixing soil bacteria in their roots is characteristic of Leguminosae and is reported for representatives of the genus, such as *Prosopis farcta* (Sol. ex Russell) J.F.Macbr. (Fterich *et al*. 2011). *Prosopis rubriflora* is also highly important ecologically, providing food resources such as pollen and nectar to local fauna through the year (Freitas *et al*. 2013; Sigrist *et al*. 2018).

Prosopis rubriflora was once considered to be an endangered species according to the International Union for Conservation of Nature (IUCN) 1997 list for Paraguay (Walter and Gillett 1998). Nonetheless, the species may be considerable vulnerable at a global scale due to the small-range distribution in north-east Paraguay, with an unknown exact distribution for the country, and in Brazil (Burkart 1976), with a predominant localisation in Porto Murtinho city, MS (Souza-Lima *et al*. 2017). A considerable decrease in Chaquenian areas (more than 35%) was reported for this region in 2008 (MMA-IBAMA 2010). The consequences of habitat fragmentation as a reduction in the number of individuals decreases the population genetic diversity and the effective population size, followed by genetic drift and a loss of rare alleles (Young *et al*. 1996; Furlan *et al*. 2012; Szczecińska *et al*. 2016). Furthermore, habitat fragmentation might result in inbreeding in the long term because of the increased probability of pollination between related and inbred individuals (Kageyama and Gandara 1998).

To understand the mating system of this poorly studied but ecologically important taxon, we performed a mating system analysis for *P. rubriflora* using polymorphic microsatellite markers based on 2 years of sampling. For this study, we tested the hypotheses that: (i) *P. rubriflora* presents a mixed mating system, as evidenced by the dispersion of pollinators and the distribution of this taxon in the sampled remnant; and (ii) both years should have similar results, providing stronger support for the findings. With this study, we expect to provide additional data for genetic conservation and environmental restoration programs.

Materials and methods

Fruit collection occurred in November 2010 and November 2011 at Fazenda Retiro Conceição (57°46′40″W, 21°41′03″S; Figs 2 and 3). Fifteen *P. rubriflora* seed trees were sampled (spaced 50 m apart); seeds extracted from pods sampled from each tree were grouped in Petri dishes, with 16 seeds sampled randomly from each progeny array for germination and subsequent genotyping.

To break the dormancy of the *P. rubriflora* seeds, we adopted the mechanical scarification method of lightly abrading selected seeds using sandpaper (Rocha *et al*. 2009). This method has been reported to be efficient for breaking dormancy in *Prosopis*

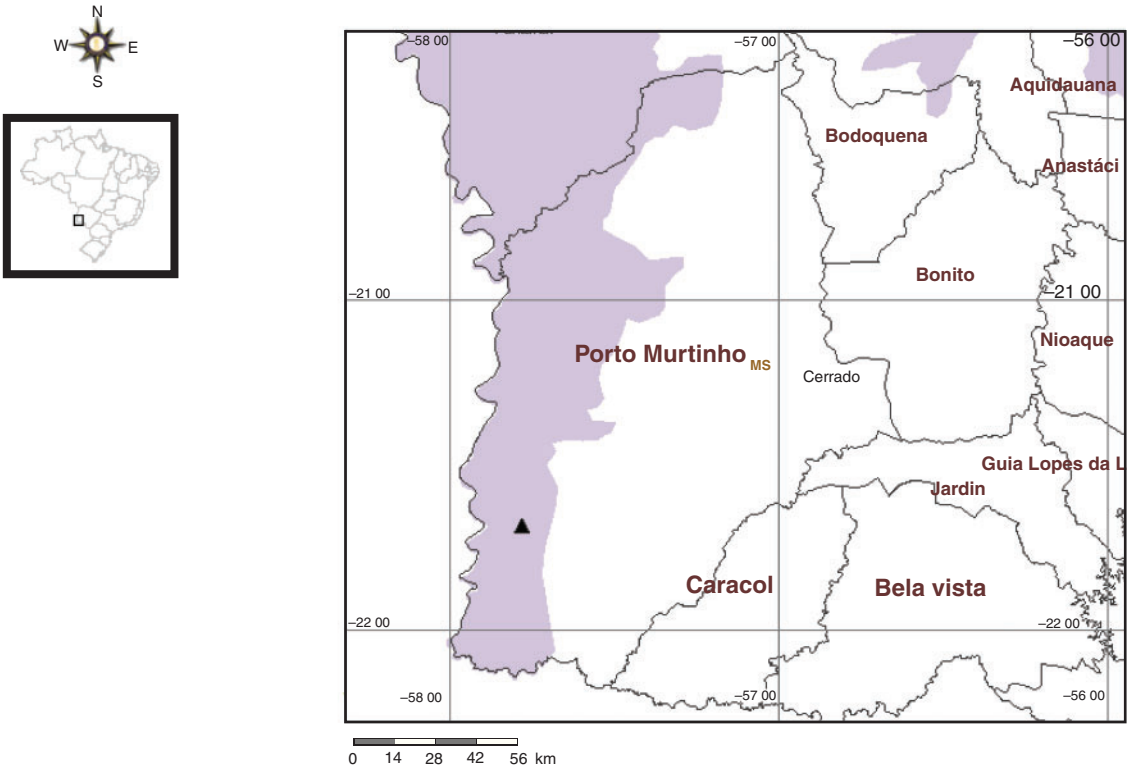

Fig. 2. Location of the studied Chaco remnant Fazenda Retiro Conceição in Porto Murtinho, Mato Grosso do Sul, Brazil. The map was created using the speciesMapper tool available from the speciesLink project (http://splink.cria.org.br/tools, accessed 10 November 2015).

Fig. 3. Arborised stepic savanna area (Savana Estépica Arbórea) in Fazenda Retiro Conceição (photograph by ALB Sartori).

species (Vilela and Ravetta 2001; Rocha *et al*. 2009; Miranda *et al*. 2011). The seeds were placed in Petri dishes lined with filter paper soaked in distilled water. Germination began with the emergence of rootlets after an average of two days at room temperature (~25°C).

The germinated seeds were transferred to plastic cups (with 2–3 seedlings per cup) containing a sterilised substrate composed of sand and vermiculite (1 : 1). Irrigation was conducted 2–3 times per week. We obtained 393 seedlings, which were collected following leaf emergence and stored in a freezer (−80°C).

Genomic DNA was extracted with a BIOPUR Extraction Kit Mini Spin Planta (SR Produtos para Laboratórios, Curitiba, PR, Brazil) according to the manufacturer's protocol. The quantity and quality of the DNA were assessed by 1% agarose gel electrophoresis with ethidium bromide staining, and the DNA was adjusted to 8 ng μL^{-1} by comparison with a Lambda DNA standard (Invitrogen, Carlsbad, CA, USA). The fragments were polymerase chain reaction (PCR) amplified using 8 ng of template DNA, 2 mM of $MgCl_2$, 50 mM of KCl, 20 mM of Tris–HCl (pH 8.4), 0.2 mM of deoxynucleiotides (dNTPs), 0.19 mg mL^{-1} of bovine serum albumin (BSA), 0.15 mM of each primer and 1 U of Taq DNA polymerase; a final reaction volume of 20 µL was achieved with ultrapure water. Forward primers were synthesised with the following fluorophores: 6-carboxyfluorescein (6-FAM; Sigma-Aldrich, St. Louis, MO, USA), Vic., NED and PET (Applied Biosystems, Foster City, CA, USA). The PCR temperatures were selected according to the guidelines described by Mottura *et al*. (2005), and the annealing temperatures were set using the protocol by Alves *et al*. (2014). For population genotyping, we used 10 simple sequence repeat (SSR) markers, eight specific markers and two markers from *Prosopis ruscifolia* Griseb. developed by Alves *et al*. (2014).

The amplified products were visualised on a 3% agarose gel with ethidium bromide staining to determine the quality before genotyping. For genotyping, we used 0.03–0.17 µL of the PCR product, depending on the observed intensity, and 0.2 µL of LIZ-600 label (Applied Biosystems) with formaldehyde Hi-Di in a total volume of 11 µL. The mixture was denatured at 95°C

for 5 min and subjected to capillary electrophoresis using an automatic sequencer ABI 3500 Genetic Analyzer (Applied Biosystems). The results were examined using the software GeneMarker ver. 6.0 (SoftGenetics LLC, State College, PA, USA), and the data were transferred to a spreadsheet for use with data analysis tools. The genotyping procedure was performed using 10 microsatellites markers with a total of 15 progeny (213 seedlings) in 2010 and 12 (180 seedlings) in 2011 for the statistical analysis (Table 1).

The mating system analyses, the including multilocus outcrossing rate (t_m), single-locus outcrossing rate (t_s), outcrossing rate between two related individuals ($t_m - t_s$), multilocus correlation of selfing (r_s) and multilocus paternity correlation ($r_{p(m)}$), were performed using MLTR ver. 3.4 software (Ritland 2002), which employs a multilocus mixed-mating model (Ritland and Jain 1981) that assumes progeny are generated in part from self-fertilisation events and in part from random mating (Ritland 1989). Family-level analyses were carried out using the expectation-maximisation (EM) method, with standard deviation (s.d.) calculated by 1000 bootstrap replicates (using family as a resampling unit). The inbreeding coefficient (F_m), the observed heterozygosity (H_o) and linkage disequilibrium (LD) were estimated using FSTAT (Goudet 1995), the expected heterozygosity (H_e) and inbreeding coefficient of the progeny (F_p) were estimated according to the methodology proposed by Manoel *et al*. (2015), and null alleles were assessed with FreeNA (Chapuis and Estoup 2007).

Other analyses, including the selfing fraction due to biparental inbreeding, $1 - r_s$ (Ritland 2002), the degree of selfing (s), where $s = 1 - t_m$, and the effective number of pollen donors (N_{ep}), where $N_{eP} = 1/r_{p(m)}$ (Ritland 1989), were manually estimated. The degree of kinship among progeny, such as full-sibs (P_{FS}), half-sibs (P_{HS}), self-half-sibs (P_{SHS}) and self-sibs (P_{SS}), which are sibs from two distinct ancestral trees without selfing, as proposed by Squillace (1974), were estimated according to Sebbenn (2006), where $P_{FS} = t_m^2\, r_{p(m)}^2$; $P_{HS} = t_m^2(1 - r_{p(m)}^2)$; $P_{SHS} = 2st_m$; and $P_{SS} = s^2$.

Progeny coancestry was estimated according to the equation

$$\bar{\theta} = 0.125(1 + \hat{F}_p)[4\hat{s} + (\hat{t}_m + \hat{s}\hat{t}_m\hat{r}_s)(1 + \hat{r}_{p(m)})], \quad (1)$$

(Sebbenn 2002), where $\hat{F}_p$ is the parental inbreeding coefficient, s is the degree of selfing, $\hat{t}_m$ is the multilocus outcrossing rate, and r_p is the multilocus paternity correlation. The variance effective size was estimated as

$$N_e = \frac{0.5}{\bar{\theta}\left(\frac{n-1}{n}\right) + \frac{1+\hat{F}_o}{2n}}, \quad (2)$$

where n is the number of samples and $\hat{F}_o$ is the progeny fixation index (Sebbenn 2002; Tambarussi *et al*. 2016).

The number of trees required to harvest seeds for conservation and management programs based on the progeny examined in this study was calculated according to the following expression proposed by Sebbenn (2002):

$$\hat{m} = \frac{N_{e(reference)}}{N_e}, \quad (3)$$

where $N_{e(reference)}$ is 150 (Eduardo *et al*. 2008) and N_e is the variance effective size.

Results

Seventy-five alleles were identified across all of the loci characterised. With respect to each individual locus, the number of alleles ranged from 2 to 14 across both years or 2 to 12 within each year (Tables S1 and S2, available as Supplementary Material to this paper). The number of rare alleles (frequency <0.05) was 25 in 2010 and 31 in 2011, and considering the standard deviations, these amounts ranged from 19 to 41 (2010) and 25 to 44 (2011). Eight alleles were exclusively observed in 2011 and six were detected only in 2010 (Table S2). The pollen and ovules exhibited heterogeneous allele frequencies for most of the evaluated loci, which the exception of Prb3 and Prb5 in 2010 and Prb7 in 2011, with 34.3 and 39.13% differences in allele frequencies in 2010 and 2011 respectively (Table S2). The H_e ranged from 0.118 to 0.818 (2010) and 0.221 to 0.799 (2011), and the H_o also ranged from 0.061 to 0.849 (2010) and 0.194 to 0.729 (2011) for the characterisation of SSR markers (Table S1). No evidence of null alleles was observed (Table S3, supplementary material), and no significant LD was observed for any of the markers after Bonferroni correction (P-value for 1% = 0.000222–Table S3). A few markers, including Prb2 for M2 in 2010 and Prb4 for M1 in 2011, presented HW departure after Bonferroni correction (P-value for 1% = 0.001 – Table S4, supplementary material).

The t_m values were 0.952 (s.d. = 0.935–0.969) and 0.968 (s.d. = 0.952–0.984) in 2010 and 2011, respectively, and the t_s values were 0.939 (s.d. = 0.923–0.955) and 0.908 (s.d. = 0.894 to 0.922), respectively. The estimates of t_s in both years and t_m

Table 1. Estimates of mating system parameters for *Prosopis rubriflora* based on 2 years of sampling in Fazenda Retiro Conceição

The values in parentheses correspond to standard deviations (s.d.) estimated using 1000 bootstraps

Statistical parameters	Estimate, mean (s.d.)	
	2010	2011
Number of seed trees – number of seeds	15–213	12–180
Single-locus outcrossing rate (t_s)	0.939 (0.923–0.955)	0.908 (0.894–0.922)
Multilocus crossing rate (t_m)	0.952 (0.935–0.969)	0.968 (0.952–0.984)
Mating among relatives ($t_m - t_s$)	0.013 (-0.003–0.029)	0.060 (0.047–0.073)
Selfing correlation (r_s)	0.069 (0.055–0.083)	0.055 (–0.022–0.132)
Self-pollination (s)	0.048 (0.031–0.065)	0.032 (0.016–0.048)
Multilocus paternity correlation ($r_{p(m)}$)	0.163 (0.140–0.186)	0.162 (0.135–0.189)

for 2010 were significantly different than unity (1.0), suggesting a low level of selfing for *P. rubriflora* in this population. The lower t_s value in 2011 reflects a significantly higher level of mating between relatives in that year, and the outcrossing rate between related individuals ($t_m - t_s$) ranged from 0.013 (2010) to 0.060 (2011) and differed significantly between 2010 and 2011. As result, this analysis presented a significant difference for the year 2011 according to the SDs (Table 1).

The estimated proportion of self-pollination (s) suggests that there was ~5% selfing in 2010, a value that decreased to 3% in 2011. The correlation of selfing (r_s) ranged from 0.069 (2010) to 0.055 (2011) and differed from zero, which suggests individual variation in outcrossing rates in the sampled population. The selfing fraction due to biparental inbreeding ranged from 0.941 (2010) to 0.945 (2011), suggesting that the low rate of selfing is related to two mates that are more genetically similar, as opposed to self-pollination. The assessment of multilocus correlated paternity ($r_{p(m)}$) presented values that significantly differed from zero in both years, which suggests correlated mating and indicates that the progeny arrays are composed of both half-sibs and full-sibs. The effective number of pollen donors (N_{ep}) per seed tree was approximately six in both years (6.1 and 6.2 respectively). The P_{SHS} proportion was 9% in 2010 and 6% in 2011, values that are insignificant proportions of P_{SS}. The average P_{FS} was 15% in 2010 and 2011, and the approximate P_{HS} proportion was 76% in 2010 and 79% in 2011.

The progeny fixation index (F_{IS}) was 0.101 (s.d. = –0.261 to 0.463) in 2010 and 0.131 (s.d. = –0.208 to 0.470) in 2011, and the inbreeding coefficient for the maternal genotype F_{IS} was 0.04. The coancestry coefficient ($\bar{\theta}$) was estimated as 0.162 in 2010 and 0.158 in 2011. The variance effective size (N_e) ranged from 3.05 (2010) to 3.13 (2011). Based on the estimated N_e, 48–49 trees are needed to obtain an effective number of progeny of 150.

For the sampled progeny arrays in 2010 and 2011, t_s varied from 0.753 to 1.000 and 0.765 to 1.000, respectively, whereas t_m ranged from 0.792 to 1.000 and 0.938 to 1.000, respectively. Mating among relatives ($t_m - t_s$) ranged from –0.111 to 0.247 in 2010 and from 0.000 to 0.235 in 2011. The multilocus paternity correlation ($r_{p(m)}$) was 0.000 to 0.046 in 2010 and 0.000 to 0.079 in 2011 (Table 2).

Analysis of family groups between 2010 and 2011 showed significant differences for the single-locus outcrossing rate and mating among relatives, but similar values for t_m, s, r_s and ($r_{p(m)}$) were found. Individual differences in the progeny arrays, which showed varying significance, were observed in both years. The most obvious differences were obtained for $t_m - t_s$ (progeny M1, M2, M3, M4, M5, M6, M7, M10, M11, M13, M14 and M15), $r_{p(m)}$ (progeny M1, M2, M3, M4, M6, M7, M10, M11, M13 and M14), t_s (progeny M1, M2, M3, M10, M11, M13 and M15), and t_m (progeny M3, M7, M10 and M13) (Table 2). In addition, mating estimates for *P. rubriflora* varied within an individual among years.

Table 2. Single-locus and multi-locus crossing rates for *Prosopis rubriflora* organised by progeny arrays over 2 years of sampling in the remnant Fazenda Retiro Conceição

Abbreviations: N, number of progeny; t_m, multilocus outcrossing rate; t_s, single-locus outcrossing rate; $r_{p(m)}$, multilocus paternity correlation; $t_m - t_s$, mating among relatives; s.d., standard deviation reported by MLTR estimated using 1000 bootstraps. Values in bold indicate significant differences between the 2 sampling years

Family	N	$t_s \pm$ s.d.	$t_m \pm$ s.d.	$r_{p(m)} \pm$ s.d.	$t_m - t_s \pm$ s.d.
M1 (2010)	16	**0.996 ± 0.000**	1.000 ± 0.000	**0.015 ± 0.000**	**0.004 ± 0.000**
M2 (2010)	16	**0.999 ± 0.000**	1.000 ± 0.000	**0.035 ± 0.000**	**0.001 ± 0.000**
M3 (2010)	14	**0.921 ± 0.000**	**0.929 ± 0.005**	**0.000 ± 0.000**	**0.008 ± 0.000**
M4 (2010)	16	0.999 ± 0.005	1.000 ± 0.000	**0.022 ± 0.000**	**0.001 ± 0.000**
M5 (2010)	16	0.998 ± 0.002	1.000 ± 0.000	0.000 ± 0.000	**0.002 ± 0.000**
M6 (2010)	16	0.999 ± 0.005	1.000 ± 0.000	**0.046 ± 0.001**	**0.001 ± 0.000**
M7 (2010)	16	0.916 ± 0.004	**1.000 ± 0.000**	**0.026 ± 0.000**	**0.084 ± 0.000**
M8 (2010)	15	0.913 ± 0.004	0.933 ± 0.000	0.006 ± 0.000	0.021 ± 0.000
M9 (2010)	9	0.901 ± 0.004	0.889 ± 0.000	0.000 ± 0.000	-0.012 ± 0.000
M10 (2010)	14	**0.902 ± 0.004**	**0.792 ± 0.002**	**0.000 ± 0.000**	**-0.111 ± 0.000**
M11 (2010)	14	**0.929 ± 0.003**	1.000 ± 0.000	**0.022 ± 0.000**	**0.071 ± 0.000**
M12 (2010)	12	1.000 ± 0.000	1.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
M13 (2010)	11	**0.753 ± 0.004**	**1.000 ± 0.000**	**0.000 ± 0.000**	**0.247 ± 0.000**
M14 (2010)	15	0.997 ± 0.005	1.000 ± 0.000	**0.033 ± 0.000**	**0.003 ± 0.000**
M15 (2010)	13	**0.997 ± 0.004**	1.000 ± 0.000	0.000 ± 0.000	**0.003 ± 0.000**
M1 (2011)	16	**1.000 ± 0.000**	1.000 ± 0.000	**0.000 ± 0.000**	**0.000 ± 0.000**
M2 (2011)	15	**0.992 ± 0.001**	1.000 ± 0.000	**0.006 ± 0.000**	**0.008 ± 0.000**
M3 (2011)	16	**0.994 ± 0.000**	**1.000 ± 0.000**	**0.029 ± 0.000**	**0.006 ± 0.000**
M4 (2011)	16	0.996 ± 0.000	1.000 ± 0.000	**0.035 ± 0.000**	**0.004 ± 0.000**
M5 (2011)	15	0.996 ± 0.005	1.000 ± 0.000	0.000 ± 0.000	**0.004 ± 0.000**
M6 (2011)	16	0.996 ± 0.005	1.000 ± 0.000	**0.003 ± 0.000**	**0.004 ± 0.000**
M7 (2011)	16	0.911 ± 0.002	**0.938 ± 0.000**	**0.029 ± 0.000**	**0.027 ± 0.000**
M10 (2011)	15	**0.993 ± 0.006**	**1.000 ± 0.000**	**0.044 ± 0.000**	**0.007 ± 0.000**
M11 (2011)	15	**0.947 ± 0.000**	1.000 ± 0.000	**0.000 ± 0.000**	**0.053 ± 0.000**
M13 (2011)	16	**0.948 ± 0.004**	**0.948 ± 0.004**	**0.011 ± 0.000**	0.000 ± 0.000
M14 (2011)	14	0.999 ± 0.005	1.000 ± 0.000	**0.079 ± 0.000**	**0.001 ± 0.000**
M15 (2011)	10	**0.765 ± 0.000**	1.000 ± 0.000	0.000 ± 0.000	**0.235 ± 0.000**

Discussion

Two markers presented departure from the Hardy–Weinberg equilibrium (HWE) in two progeny arrays, with a different marker for each year; such a low departure rate may indicate that mating was not completely random, likely due to pollinator behaviour. This adherence to HW for most loci reflected a low level of F_{IS} for both the maternal genotypes and progeny, indicating a low or absent selfing rate for the population. This was confirmed for the recorded multilocus outcrossing rate for both years in this study, suggesting an allogamous tendency of mating for *P. rubriflora*. Other *Prosopis* species, such as *Prosopis alba* (Bessega *et al*. 2012; Carreras *et al*. 2017), *Prosopis flexuosa* (Mottura 2006), *Prosopis glandulosa* and *Prosopis nigra* (Bessega *et al*. 2000), are also allogamous. Conversely, species such as *P. ruscifolia* and *Prosopis velutina* (Bessega *et al*. 2000) present a mixed mating system.

The *Prosopis* genus was originally reported as containing only protogynous species (Burkart 1976; Solbrig and Cantino 1975), which prevents or significantly hinders self-fertilisation. Although protogyny is not supported according to the findings of Genise (1990), suggesting another self-incompatibility mechanism for *Prosopis* spp., Sigrist *et al*. (2018) discuss that *P. rubriflora* actually presents protogyny before anther opening and also herkogamy (partial or temporal), which might prevent selfing. Solbrig and Cantino (1975) proposed a few additional factors that might explain the high outcrossing rate observed in this study, as follows: the trees appear to grow close to one another, enabling cross-pollination at a large scale; large numbers of pollinators visit the trees; and a hypothesised inhibitory compound might act in combination with fertilisation to prevent the development of nearby flowers. Aizen and Feinsinger (1994) studied *P. nigra* and observed that compared with pollen tubes formed by outcrossing, pollen tubes formed by manual selfing required 3.5-times longer to reach the stylus after 36 h, which discourages selfing. *Prosopis rubriflora* appears to effectively employ these mechanisms, as reflected in the low proportion of selfing (s), with only 3–5% recorded for this study.

The significant correlation of selfing (r_s) indicates individual variation in the outcrossing rate of this species, resulting in progeny with different levels of inbreeding and coancestry. Similar to the observed differences in allele frequencies in pollen and ovules, the correlation of selfing may be attributed to pollinator behaviour and asynchrony in flowering, which is responsible for the contribution of correlated mating, selfing and mating between related individuals (Mori *et al*. 2013) to the different levels of inbreeding observed in this study.

The outcrossing rate between related individuals in 2011 suggests an intrapopulation spatial genetic structure and indicates that related individuals might surround the tree matrix and mate with one another (Sobierajski *et al*. 2006; Mori *et al*. 2013). As previously discussed, the *Prosopis* genus presents zoochoric (Solbrig and Cantino 1975; Burkart 1976; Campos *et al*. 2016) and autochoric (Solbrig and Cantino 1975; Freitas *et al*. 2013) seed dispersal. Although the fruits that do not disperse are subject to severe attacks by bruchid insects (also observed in several sampled fruits in the present study), a proportion of seeds can overcome these obstacles and germinate near the matrix trees (Solbrig and Cantino 1975), which might explain the possible structure of *P. rubriflora* in this remnant. Other *Prosopis* species, such as *P. alba* (Bessega *et al*. 2012), *Prosopis chilensis*, *P. flexuosa*, (Mottura 2006), *P. glandulosa* and *P. nigra* (Bessega *et al*. 2000), exhibit a similar pattern, indicating that such a genetic structure also occurs in other representatives of the genus, regardless of whether the presumed reproduction system is allogamous or mixed.

Nonetheless, it is most likely that the main factors derived from the biparental inbreeding analysis of 2010–2011 were the non-peak flowering of *P. rubriflora* and the reduced synchrony in 2011, which limited pollen distribution. In addition, interference might have occurred during visits by native pollinators (typically bees) in 2011. According to Sigrist *et al*. (2018), hummingbirds and native insects such as bees. butterflies, flies and wasps visit *P. rubriflora* trees searching for food resources with a relatively high frequency; they collect pollen and nectar from a few inflorescences per plant and quickly move to another tree, which reduces the likelihood of inbreeding. The higher visiting frequency and also this behaviour of the native fauna ensures effective gene flow. However, native fauna are less common than *Apis mellifera*, which visits the same inflorescence several times, thereby increasing the likelihood of mating between related individuals. This is supported by the biparental inbreeding fraction analysis ($1–r_s$), possibly promoting the observed heterogeneity in pollen and ovule allele frequencies presented in Table S2.

Other factors that can contribute to differences in allele frequencies between pollen and ovules include non-equivalent contributions of pollen and ovules in adult trees within the population, pollen migration from another population, selection between the time of pollination and sampling of progeny, and non-random mating (Ribeiro and Lovato 2004; Pometti *et al*. 2011).

The correlation of pollen $r_{p(m)}$ value estimated in the present study was significant for both years, and to the best of our knowledge, such correlations have been reported to varying degrees for all *Prosopis* species, with similar values obtained for *P. alba* ($r_{p(m)} = 0.166$) (Bessega *et al*. 2012) and *P. flexuosa* ($r_{p(m)} = 0.154$) (Mottura 2006). The factors driving this correlation might be related to the behaviour and short distance covered by pollinators (Bessega *et al*. 2012), low synchronisation and the period during which the sampling was conducted. The correlation of pollen results suggests that the majority of the progeny must be half-sibs with a proportion of full-sibs and with a lower degree of self-half-sibs, as based on the lower rate of selfing; this is supported according to the degree of kinship analysed for both years.

The approximate number of pollen donors registered for the sampled area was six for both years, similar to the results reported for *P. alba* ($N_{eP} = 5.9$) (Bessega *et al*. 2012) and *P. flexuosa* ($N_{eP} = 6.4$) (Mottura 2006). The number of pollen donors might vary depending on the density of the area and the family structure and might be underestimated when nearby trees are highly related because of the difficulty in distinguishing between the paternal genotypes of trees and related trees (Bessega *et al*. 2012). A very high family structure might be responsible for the results reported by Bessega *et al*. (2000), who found that *P. alba*, *P. chilensis*,

P. flexuosa, *P. ruscifolia*, *P. velutina* and *P. glandulosa* individuals share one paternal tree that acted as the pollen donor; this might be attributed to the large number of related individuals in the sampling areas.

The values of the co-ancestry coefficient within progeny were higher than expected for half-sib progeny ($\bar{\theta} = 0.125$) but lower than the expected value for full-sibs ($\bar{\theta} = 0.250$) (Doolittle 1987), reinforcing the finding that the observed progeny were primarily composed of half-sibs, with a small proportion of full-sibs. The coancestry coefficient in this remnant was similar to that determined for *P. alba* ($\bar{\theta} = 0.165$) (Bessega *et al*. 2012), which suggests a similar mating system for both species.

Lastly, due to the small degree of selfing and correlated mating in both evaluations, the average coancestry coefficient within families ($\bar{\theta}$) was higher and the variance effective size (N_e) was lower than expected for panmictic populations ($\bar{\theta} = 0.125$, $N_e \approx 4$) (Sebbenn 2002; Vencovsky and Crossa 2003). As such, a greater sampling effort is required to achieve better genetic diversity for use in conservation plans, reforestation or genetic improvement. Based on the effective population size, seed collection must be carried out in 48–49 seed trees to obtain samples with a reference effective size of 150 *P. rubriflora* for short-term genetic conservation (10 generations).

Prosopis rubriflora exhibited variation in progeny over 2 years, and most of the progeny were generated by crossing pollination, resulting in an allogamic pattern with a low degree of overall selfing. The most notable levels of selfing, presented in Table 2, for the progenies of M13 (2010) and M15 (2011) could be related to the frequent visits by *A. mellifera*, as suggested by Sigrist *et al*. (2018). Other reasons for the observed differences in the results for the same seed trees in two years might have been related to a lower synchrony of flowering in a given year, as discussed above.

Field observations in other areas that present cattle breeding activities suggest that *P. rubriflora* might be among the most highly suppressed tree species. This suppression might be related to characteristics of this species, such as prickle-covered branches and low height, which tend to damage cattle hides. Regardless, strong evidence of anthropomorphic disturbances was not observed in the study area, with the exception of cattle breeding activities, which typically require the suppression of most trees. Evidence of fragmentation was also not observed in the surrounding area. Thus, similar results should be observed in other conserved areas or even in remnants with a low incidence of anthropogenic disturbances.

A primary problem in the Chaco areas of Porto Murtinho, Brazil, is the lack of conservation areas for the protection of native species (Pott and Pott 2003), which might be due to the high level of cattle breeding activity in the county. A similar lack of protected areas is also a reality in Paraguay, where created protected areas cover less than 2% of land area (Yanosky 2013). If human activities result in a loss of native fauna, such as pollinators and seed dispersers, we would expect to observe a significant increase in selfing due to frequent visits from the exotic bee *A. mellifera*. In addition, reductions in native seed dispersers, their extinction or their replacement with livestock can affect the landscape structure and alter ecosystem functioning (Campos *et al*. 2016). Fences, which limit the movement of livestock, can lead to a higher family structure of trees, leading to genetic diversity decline and decreasing the number of the pollen donors. Other consequences of current anthropogenic activities can lead to inbreeding depression, which tends to reduce fitness and affects the fertility and survival of individuals (Charlesworth and Willis 2009), particularly those of non-inbred species (Ellstrand and Elam 1993) such as *P. rubriflora*.

We provide three recommendations for future studies pertaining to conservation of the genetic diversity of *P. rubriflora*.

(1) When collecting fruits and seeds, a higher number of seeds per tree should be collected because bruchid insects lay eggs in developing fruits, and after the eggs hatch inside the fruit, the larvae will depredate the seeds (Solbrig and Cantino 1975). After the fruits have been sampled and the seeds have been removed, storage at 4 °C is desirable to prevent larval development and maintain viable seeds for subsequent use.
(2) The mortality rate of progeny should be considered a factor that affects germination and growth. Although mortality was not the focus of this study, a maximum survival rate of 93% was observed in *P. glandulosa* germinated in the laboratory; in contrast, a survival rate of only 19% was observed under field conditions without the presence of competing plants, and a survival rate of 2% was observed when other herbaceous plants were present during the initial germination stage (Bush and van Auken 1990). A similar mortality rate can be anticipated for *P. rubriflora*.
(3) During seed sampling, a certain distance should be maintained between parent trees to prevent the accidental collection of related individuals. A mean of 50 m between individuals appeared to be sufficient; however, a greater distance, such as 100 m (Bessega *et al*. 2012), is recommended for areas that contain barriers that might reduce the mobility of zoochoric seed dispersers.

Conclusions and conservation recommendations

Prosopis rubriflora presents a preferably allogamous mating system in which the progeny are predominantly half-sibs. The majority of seeds originate from pollen from different paternal trees, which might be related to the mechanisms presented by Solbrig and Cantino (1975), Aizen and Feinsinger (1994), and Sigrist *et al*. (2018). Additionally, frequent visits by native pollinators might contribute to the low degree of selfing, despite constant visits by *A. mellifera*. Analysis of the mating system in 2 years (2010 and 2011) yielded similar results, though the small difference in the outcrossing rate between related plants might reflect a unique event in 2011, such as a low synchronisation of flowering during the sampling period that resulted in pollinators visiting and distributing pollen from nearby trees. To conserve the genetic diversity of *P. rubriflora* in the short term (10 generations), following the recommendation of retaining an effective number of 150 individuals, it is necessary to collect 48–49 progeny arrays.

The data presented herein provide important information for the conservation of genetic resources in the near future. Conservation measures will not only benefit *P. rubriflora* but

also other animal and plant species that depend on this vegetation type.

Conflicts of interest

The authors have declared that they have no competing interests in the publication of this study.

Acknowledgements

The authors are grateful to Miklos Maximiliano Bajay and Mariana Araújo Barreto for their support in the statistical analysis. We also thank the participants of the field excursions, including Zildamara dos Reis Holsback-Menegucci, Fábio Junior Kochanovski, Thayane Ely Lima, Thiago Henrique Stefanello, Laise Cristina Souza Magalhães, Elidiene Priscila Seleme Rocha and João Roberto Fabri. A PhD fellowship was granted to Fábio de Matos Alves from FAPESP (proc. 2010/51242-6) and a PD fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Computational Biology Program and CAPES – EMBRAPA Program). A research grant was awarded to Anete Pereira de Souza from FAPESP (proc. 2008/52197-4) and CAPES – Computational Biology Program. Additional support was provided by CNPq – Conselho Nacional de Desenvolvimento Científico and Tecnológico – CNPq (Casadinho/Procad # 552352/2011-0), which funded the field excursions to collect the materials for the study. Anete Pereira de Souza was the recipient of a research fellowship from CNPq.

References

Aizen MA, Feinsinger P (1994) Forest fragmentation, pollination, and plant reproduction in a Chaco dry forest, Argentina. *Ecology* **75**, 330–351. doi:10.2307/1939538

Alves FM, Zucchi MI, Azevedo-Tozzi AMG, Sartori ALB, Souza AP (2014) Characterization of microsatellite markers developed from *Prosopis rubriflora* and *Prosopis ruscifolia* (Leguminosae – Mimosoideae), legume species that are used as models for genetic diversity studies in Chaquenian areas under anthropization in South America. *BMC Research Notes* **7**, 375. doi:10.1186/1756-0500-7-375

Alves FM, Sartori ALB, Zucchi MI, Azevedo-Tozzi AMG, Tambarussi EV, Alves-Pereira A, Souza AP (2018) Genetic structure of two *Prosopis* species in Chaco areas: a lack of allelic diversity diagnosis and insights into the allelic conservation of the affected species. *Ecology and Evolution* doi:10.1002/ece3.4137

Bessega C, Ferreyra L, Julio N, Montoya S, Saidman B, Vilardi J (2000) Mating system parameters in species of genus *Prosopis* (Leguminosae). *Hereditas* **132**, 19–27. doi:10.1111/j.1601-5223.2000.00019.x

Bessega C, Pometti CL, Ewens M, Saidman BO, Vilardi JC (2012) Strategies for conservation for disturbed *Prosopis alba* (Leguminosae, Mimosoideae) forests based on mating system and pollen dispersal parameters. *Tree Genetics & Genomes* **8**, 277–288. doi:10.1007/s11295-011-0439-6

Burkart A (1976) A monograph of the genus *Prosopis* (Leguminosae subfam. Mimosoideae) (Part 2.). Catalogue of the recognized species of *Prosopis*. *Journal of the Arnold Arboretum* **57**, 450–525.

Bush JK, van Auken OW (1990) Growth and survival of *Prosopis glandulosa* seedlings associated with shade and herbaceous competition. *Botanical Gazette* **151**, 234–239. doi:10.1086/337822

Campos CM, Campos VE, Miguel F, Cona MI (2016) Management of protected areas and its effect as ecosystem function: removal of *Prosopis flexuosa* seeds by mammals in Argentinian drylands. *PLoS One* **11**(9), doi:10.1371/journal.pone.0162551

Carreras R, Bessega C, López CR, Saidman BO, Vilardi JC (2017) Developing a breeding strategy for multiple trait selection in *Prosopis alba* Griseb., a native forest species of the Chaco Region in Argentina. *Forestry: An International Journal of Forest Research* **90**(2), 199–210.

Catalano SA, Vilardi JC, Tosto D, Saidman BO (2008) Molecular phylogeny and diversification history of *Prosopis* (Fabaceae: Mimosoideae). *Biological Journal of the Linnean Society. Linnean Society of London* **93**, 621–640. doi:10.1111/j.1095-8312.2007.00907.x

Chapuis MP, Estoup A (2007) Microsatellite null alleles and estimation of population differentiation. *Molecular Biology and Evolution* **24**(3), 621–631. doi:10.1093/molbev/msl191

Charlesworth D, Willis JH (2009) The genetics of inbreeding depression. *Nature Reviews. Genetics* **10**, 783–796. doi:10.1038/nrg2664

Doolittle DP (1987) 'Population genetics: basic principles.' (Springer-Verlag: New York)

Eduardo A, De Lacerda B, Kanashiro M, Sebbenn AM (2008) Long-pollen movement and deviation of random mating in a low-density continuous population of a tropical tree *Hymenaea courbaril* in the Brazilian Amazon. *Biotropica* **40**(4), 462–470. doi:10.1111/j.1744-7429.2008.00402.x

Ellstrand NC, Elam DR (1993) Population genetic consequences of small population size: implications for plant conservation. *Annual Review of Ecology and Systematics* **24**, 217–242. doi:10.1146/annurev.es.24.110193.001245

Freitas TG, Souza CS, Aoki C, Arakaki LMM, Stefanello TH, Sartori ALB, Sigrist MR (2013) Flora of Brazilian humid Chaco: composition and reproductive phenology. *Check List* **9**, 973–979. doi:10.15560/9.5.973

Fterich A, Mahdhi M, Caviedes MA, Pajuelo E, Rivas R, Rodriguez-Llorente ID, Mars M (2011) Characterization of root-nodulating bacteria associated to *Prosopis farcta* growing in the arid regions of Tunisia. *Archives of Microbiology* **93**, 385–397. doi:10.1007/s00203-011-0683-z

Furlan E, Stoklosa J, Griffiths J, Gust N, Ellis R, Huggins RM, Weeks AR (2012) Small population size and extremely low levels of genetic diversity in island populations of the platypus, *Ornithorhynchus anatinus*. *Ecology and Evolution* **2**(4), 844–857. doi:10.1002/ece3.195

Genise J (1990) Observaciones sobre la biologia floral de *Prosopis* (Leguminosae, Mimosoideae). II. Fases florales y visitantes en el distrito chaqueño serrano. *Darwiniana* **30**, 71–85.

Goudet J (1995) Fstat (version 2.9.3.2.): a computer program to calculate F-statistics. *Journal of Heredity* **86**, 485–486. doi:10.1093/oxfordjournals.jhered.a111627

Hueck K (1972) 'As florestas da América do Sul: ecologia, composição e importância econômica.' (Polígono: São Paulo, Brazil)

IBGE (2012) 'Manual técnico da vegetação Brasileira.' (IBGE: Rio de Janeiro)

Kageyama PY, Gandara FB (1998) Consequências genéticas da fragmentação sobre populações de espécies arbóreas. *Série Técnica IPEF* **32**, 65–70.

Lewis GP, Schrire B, Mackinder B, Lock M (2005) 'Legumes of the world.' (Royal Botanic Gardens, Kew, UK)

Lima TE (2012) Perturbação ambiental em remanescentes de Chaco e mecanismos de defesa em leguminosas. MSc thesis. Universidade Federal de Mato Grosso do Sul, Brazil.

Lorenzi H (2002) 'Árvores brasileiras: manual de identificação e cultivo de plantas arbóreas do Brasil. Vol. 2.' (2nd edn) (Instituto Plantarum: Nova Odessa-SP, Brazil)

Manoel RO, Freitas MLM, Furlani-Júniro E, Alves PF, Morales MLT, Sebbenn AM (2015) Individual, fruit, and annual variation in correlated mating in *Genipa americana* population. *Silvae Genetica* **64**(3), 108–116.

Miranda RQ, Oliveira MTP, Correia RM, Almeida-Cortez JS, Pompelli MF (2011) Germination of *Prosopis juliflora* (Sw) DC seeds after scarification treatments. *Plant Species Biology* **26**, 186–192. doi:10.1111/j.1442-1984.2011.00324.x

MMA-IBAMA (2010) 'Projeto de monitoramento do desmatamento dos biomas brasileiros por satélite. Monitoramento do bioma Pantanal 2002 a 2008.' (MMA: Brasília)

Mori ES, Sebbenn AM, Tambarussi EV, Guries RP (2013) Sistema de reprodução em populações naturais de *Peltophorum dubium*. *Scientia Forestalis* **41**, 307–317.

Mottura MC (2006) Development of microsatellites in *Prosopis* spp. and their application to study the reproduction system. PhD thesis. TheUniversity of Göttingen, Göttingen, Germany.

Mottura MC, Finkeldey R, Verga AR, Gailing O (2005) Development and characterization of microsatellite markers for *Prosopis chilensis* and *Prosopis flexuosa* and cross-species amplification. *Molecular Ecology Resources* **5**, 487–489.

Pometti CL, Vilardi JC, Saidman BO (2011) Mating system parameters and genetic structure in Argentinean populations of *Acacia caven* (Leguminosae, Mimosoideae). *Plant Systematics and Evolution* **292**, 25–32. doi:10.1007/s00606-010-0389-8

Pott A, Pott VJ (2003) 'Espécies de Fragmentos Florestais em Mato Grosso do Sul. In 'Fragmentação florestal e Alternativas de Desenvolvimento rural na Região Centro-Oeste.' (Ed. RB Costa). pp. 26–52. (UCDB: Campo Grande, Brazil)

Ribeiro RA, Lovato MB (2004) Mating system in a Neotropical tree species, *Senna multijuga* (Fabaceae). *Genetics and Molecular Biology* **27**, 418–424. doi:10.1590/S1415-47572004000300018

Ritland K (1989) Correlated matings in the partial selfer *Mimulus guttatus*. *Evolution* **43**, 848–859. doi:10.1111/j.1558-5646.1989.tb05182.x

Ritland K (2002) Extensions of models for the estimation of mating systems using *n* independent loci. *Heredity* **88**, 221–228. doi:10.1038/sj.hdy.6800029

Ritland K, Jain S (1981) A model for the estimation of outcrossing rate and gene frequencies using *n* independent loci. *Heredity* **47**, 35–52. doi:10.1038/hdy.1981.57

Rocha BN, Martins CR, Missio EL (2009) Superação de dormência e germinação de sementes de Inhanduvá (*Prosopis affinis*) Sprenger. *Revista da FZVA* **16**, 278–287.

Sebbenn AM (2002) Número de árvores matrizes e conceitos genéticos na coleta de sementes para reflorestamento com espécies nativas. *Revista do Instituto Florestal* **14**, 115–132.

Sebbenn AM (2006) Sistema de reprodução em espécies arbóreas tropicais e suas implicações para a seleção de árvores matrizes para reflorestamentos ambientais. In 'Pomares de sementes de espécies nativas'. (Eds AR Higa, LD Silva) pp. 193–198. (FUPEF: Curitiba, Brazil)

Sigrist MR, Stefanello TH, Souza CS, Vargas W, Almeida KSM, Laroca S, Mansano VF (2018) Phenology and pollination ecology of *Prosopis rubriflora* (Leguminosae, Mimosoideae), a species from the semi-arid Brazilian Chaco. *Brazilian Journal of Botany* **41**(1), 103–115. doi:10.1007/s40415-017-0433-9

Sobierajski GR, Kageyama PY, Sebbenn AM (2006) Sistema de reprodução em nove populações de *Mimosa scabrella* Bentham (Leguminosae). *Scientia Forestalis* **71**, 37–49.

Solbrig OT, Cantino PD (1975) Reproductive adaptations in *Prosopis* (Leguminosae, Mimosoideae). *Journal of the Arnold Arboretum* **56**, 185–210.

Souza-Lima ES, Sinani RT, Pott A, Sartori ALB (2017) Mimosoideae (Leguminosae) in the Brazilian Chaco of Porto Murtinho, Mato Grosso do Sul. *Rodriguésia* **68**, 263–290. doi:10.1590/2175-7860201768131

Squillace AE (1974) Average correlations among offspring from open-pollinated forest trees. *Silvae Genetica* **23**, 149–156.

Szczecińska M, Sramko G, Wołosz K (2016) Genetic diversity and population structure of the rare and endangered plant species *Pulsatilla patens* (L.) Mill in east central Europe. *PLoS One* **11**(3), doi:10.1371/journal.pone.0151730

Tambarussi EV, Boshier DH, Vencovsky R, Freitas MLM, Di-Dio OJ, Sebbenn AM (2016) Several small: how inbreeding affects conservation of *Cariniana legalis* Mart. Kuntze (Lecythidaceae) the Brazilian Atlantic Forest's largest tree. *International Forestry Review* **18**(4), 502–510. doi:10.1505/146554816820127550

Vencovsky R, Crossa J (2003) Measurements of representativeness used in genetic resources conservation and plant breeding. *Crop Science* **43**, 1912–1921. doi:10.2135/cropsci2003.1912

Vilela AE, Ravetta DA (2001) The effect of seed scarification and soil-media on germination, growth, storage, and survival of seedlings of five species of *Prosopis* L. (Mimosaceae). *Journal of Arid Environments* **48**, 171–184. doi:10.1006/jare.2000.0735

Walter KS, Gillett HJ (1998) '1997 IUCN Red list of threatened plants.' (IUCN – The World Conservation Union: Gland, Switzerland)

Yanosky A (2013) Paraguay's challenge of conserving natural habitats and biodiversity with global markets demanding for products. In 'Conservation biology: voices from the tropics'. (Eds PH Raven, NS Sodhi, L Gibson), pp. 113–119. (John Wiley & Sons Ltd: Oxford, UK)

Young A, Boyle T, Brown T (1996) The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology & Evolution* **11**, 413–418. doi:10.1016/0169-5347(96)10045-8

Handling Editor: Margaret Byrne