



Implications of mating system and pollen dispersal indices for management and conservation of the semi-arid species *Prosopis flexuosa* (Leguminosae)



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ABSTRACT

Recruitment by fencing of semi-arid trees is a common woodland conservation strategy that may have both positive and negative implications. Domestic herbivores can act as efficient dispersal seed agents but they can also affect the survival and growth of the trees first stages. *Prosopis flexuosa* (algarrobo) woodlands represent the most important plant community in the Monte region in Argentina and as this resource is affected by deforestation, grazing, natural fires and replacement of natural ecosystems by croplands, in order to protect the “algarrobo” a reserve had been established. As efficient management and conservation program depends on the information on population structure and mating system, the objectives of this work were to estimate mating and pollen dispersal patterns in a wild *P. flexuosa* population from the Ñacuñan fenced to exclude domestic cattle for 40 years. This population is mainly outcrosser ($t_m = 0.996$), sensitive to inbreeding depression at seed stage, having relative reduced pollen mediated gene dispersal distance (4.56–20.35 m), which favors inbreeding as a consequence of mating between related individuals. Our results suggest that for *ex situ* conservation the distance that would be enough to avoid full sib sampling among harvested seed-trees is about 20 m. Collecting seeds from different pods reduce the proportion of full sibs within progeny arrays. For *in situ* conservation it is important to take into account that forest patches separated by more than 250 m are expected to be isolated, increasing the risk of genetic variability loss, inbreeding increase and population extinction. The results are discussed in reference to the “algarrobo” management program; while in some case excluding big mammals from the stands should be advisable, the interaction with long dispersal big herbivores might be considered as a strategy to promote increased outbreeding reducing the rate of mating between related individuals.

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1. Introduction

Forests cover near 47% of the terrestrial surface of Latin America and Caribe but each year two million forest ha are lost (FAO, 2017). The Monte eco-region (Fig. 1) occupies approximately 460,000 km² of the dry Western Argentina (Morello, 1958; Cabrera, 1976; Rundel et al., 2007) and deforestation, grazing and fires together with the replacement of natural ecosystems by croplands in irrigated areas are the most common disturbances that affect native woodlands in this region. Loss of woodlands are difficult to revert as tree species have low rate of regeneration and growth that can

be related with temporal variability of seed production, seed and seedling predation and low frequency of occurrence of the optimal climatic conditions promoting its establishment (Villagra et al., 2009).

Conservation efforts to promote the protection of environments against disturbance can include the change in the primary land-use by establishing protected areas, *in situ* conservation, and the conservation of germplasm *ex situ* (Ledig, 1986; Lindenmayer et al., 2006). From genetic and ecological standpoints, the creation of reserves implying the installation of fences can protect biodiversity by excluding process like grazing by domestic herbivores. Recruitment by fences can be affecting gene flow in the population that is trying to be conserved (Campos et al., 2016a; Aschero et al., 2016). The pattern of gene flow determine the dynamics of gene recombination from reproductive trees within population to a descendant

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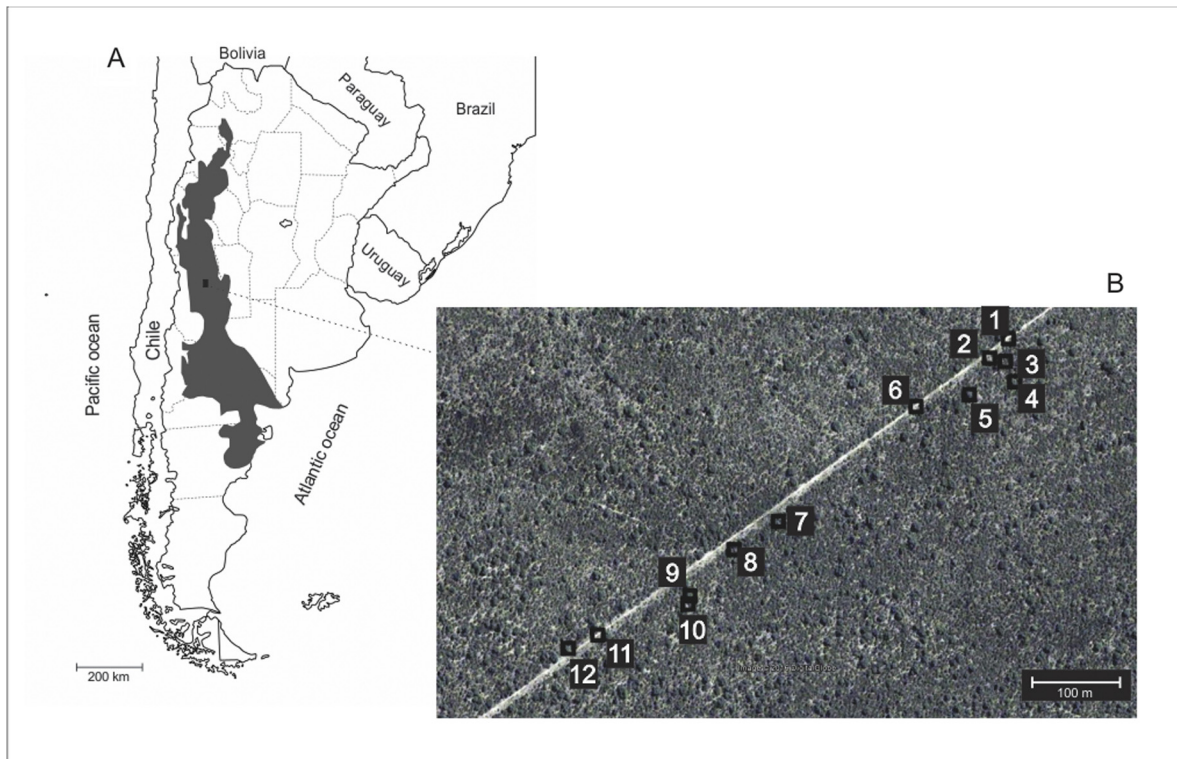


Fig. 1. Location of the Monte eco-region and the protected area of the Man and Biosphere Nacuñan Reserve sampled in this study (A). Spatial distribution of the sampled *Prosopis flexuosa* DC. trees labeled from 1 to 12 (B).

population, affecting the genetic diversity within families due correlated mating and mating among related individuals (Sebbenn, 2006; Moraes et al., 2012; Medina Macedo et al., 2016). Gene flow is one of the major factors in maintaining the high levels of genetic diversity found within forest tree populations and determining the population genetic structure (Biscaia de Lacerda et al., 2008).

Additionally, in natural trees populations, spatial genetic structure (SGS) can occur and the extent of this phenomenon is the result of the interaction between pollen and seed dispersal. When both are limited this will result in inbreeding and an increase of SGS (Chybicki et al., 2011; Forti et al., 2014). On the contrary when long-distance pollen movement occurs (as in wind pollen dispersal), SGS is determined by restricted seed dispersal, which might result in the grouping of siblings near to the maternal plant (Bittencourt and Sebbenn, 2007). Therefore, the knowledge of mating strategy, effective pollen movement, spatial genetic and family structure are indispensable for successful tree species conservation, management, breeding, and environmental reforestation.

Genetic markers have been employed efficiently to investigate mating system, spatial genetic structure and pollen dispersal events in natural population of tree species (Biscaia de Lacerda et al., 2008; Bessega et al., 2000, 2011, 2016; Marchelli et al., 2012; Duminil et al., 2016; Roser et al., 2017b). Microsatellite markers are ideal for quantifying inbreeding depression, outcrossing rates and pollen dispersal patterns (Dawson et al., 1997) due to their codominant inheritance and high polymorphism. Indirect approaches such as MLTR, KinDist and TwoGener analysis (Ritland, 1990; Austerlitz and Smouse, 2001; Smouse et al., 2001) have long been used for estimating the selfing rate and pollen dispersal distances. Particularly, molecular markers have been used to estimate effective pollen dispersal distance by comparing the segregation of genetic markers in the parental and offspring cohorts (Bittencourt and Sebbenn, 2008; Pometti et al., 2013; Duminil et al., 2016; Medina Macedo et al., 2016). They also allow the

detection of a higher genetic relatedness among near neighbors than among distant individual providing evidence of spatial genetic structure (SGS) (Vekeman and Hardy, 2004; Bittencourt and Sebbenn, 2008; Bessega et al., 2016; Duminil et al., 2016; Roser et al., 2017b). The distance of pollen movement is one of the main determinant process of the effective pollination neighborhood for a plant population (Austerlitz and Smouse, 2002), which is especially important in calculating the number of seed-trees for seed collection as part of management practices.

The most abundant tree species in the Monte (Argentina) are *Prosopis flexuosa* DC and *P. chilensis* (Mol.) Stuntz. (Roig, 1993). From an economic point of view *P. flexuosa* woodlands represent the most important plant community in Northern and Central Monte region. According to Roig (1993), the higher degradation of these woodlands occurred during the first decades of the 20th century associated to the railroad expansion, extraction of trees for firewood, charcoal, and the production of gas, as well as the major expansion of vineyards (1940–1960). As described in Villagra et al. (2009), in the Southern Monte, the history of use of the vegetation had a strong influence in changing disturbance regimes from ancient times when indigenous people frequently burned the shrub lands for hunting, communication or other purposes (Claraz, 1988), the introduction of domestic livestock at the end of the 19th century, and from the end of 1980's continuous grazing pressure begun to diminish as a consequence of the fall of wool price, coupled to rangeland degradation caused by overgrazing (Ares et al., 1990).

The UNESCO Man and Biosphere (MAB) Reserve of Nacuñan was established in Mendoza in 1972 to protect “algarrobo” (*Prosopis flexuosa*) woodlands to mitigate impact of tree cutting and extensive cattle grazing. Fencing is a common conservation strategy that relies on the idea that ecological communities require protection from some external influences, particularly anthropogenic activities (Aschero and García, 2012). Great herbivores like cattle are

expected to affect demography of *Prosopis* in multiple ways. Aschero and Vázquez (2009) suggested that herbivores may cause pre-dispersive losses of seeds due to decreased reproductive potential at seed set stage, based on the higher seed production found in trees inside the fenced reserve relative to that in the surrounding grazed ranches. Besides, herbivores may positively affect recruitment by facilitating seed dispersal (Campos and Ojeda, 1997; De Villalobos et al., 2005). And finally, negative effects on survival are also expected, as cattle forage on saplings and young trees when grass resources are scarce (Guevara et al., 1996).

The objectives of this work were to estimate mating system and pollen dispersal patterns in a wild *P. flexuosa* population located at the Ñacuñan Reserve in Mendoza fenced to exclude domestic cattle for 40 years. Our predictions were that (a) the protected population may be associated with a small neighborhood pollination area as a result of its high density, (b) both pollen and seed dispersal are limited as trees to be visited by pollinators are close each other and only small seed dispersal agents (small mammals) are available. As a consequence biparental inbreeding and significant SGS at short distances are expected. To evaluate our predictions the following questions were addressed: (1) what are the outcrossing, mating among relatives, and paternity correlation rates in the population? (2) What is the average distance of pollen dispersal inside the sampled area? (3) What are the effective number of pollen donors and the size of the effective pollination neighborhood area? (4) What is the number of seed-trees to collect seed, aiming for *ex situ* conservation of the effective number of 100? (5) Is there any evidence of inbreeding depression between seed and adult stages?

2. Material and methods

2.1. Study site and sample collection

Native woodlands currently are protected by national and provincial laws (Roma, 2001) (National Law 26331). Relict *Prosopis flexuosa* woodland is included in the Man and Biosphere Ñacuñan Reserve in the central Monte eco-region that is located in Mendoza Province (34° 02' S, 67° 58' W). The reserve encompasses 12,800 ha and is protected from livestock since 1972.

A population sample was collected in December 2014 (Fig. 1) following the sampling methodology described at Vilardi et al. (1988) and Saidman and Vilardi (1993). Seed pods were collected from 12 seed trees separated from each other by 50 m. As open pollinated seeds were collected from each mother tree, the progeny should potentially involve half-sibs, full-sibs, self-half-sibs, and self-full-sibs (Squillace, 1974). The density of adult trees in the collection area was estimated in $D = 11.25$ individuals/ha based on a detailed observation of a delimited area.

In the field, each mother tree was geo-referenced, leaf material was collected and preserved in silica gel bags and pods were conserved for future germination avoiding fungi and insects attacks. The corresponding *P. flexuosa* voucher specimens from all mother trees collected by CB and CC (ÑA001 to ÑA012) were deposited in BAFC. Germination was conducted according to the method described in Saidman (1985) and each seed was labeled according to plant mother (family) and pod origin. Germination was conducted up to the stage of green cotyledons, which was reached by nearly 100% of seeds. Fifteen green cotyledons from five different pods were randomly chosen from each family, yielding a total of 192 individuals including 180 progenies and 12 mother trees.

2.2. DNA extraction and genotyping

Total DNA was isolated from leaf material using DNA easy Plant mini kit (QIAGEN Inc., Valencia, CA, USA). We used 8 unlinked

microsatellites (SSR) developed by Mottura et al. (2005) and Bessega et al. (2013). The SSR's were amplified using forward primers labeled with a fluorescent dye (6-FAM and HEX, Invitrogen). The PCR amplifications were carried out in a 50 μ l reaction volume containing 10–30 ng DNA, 0.6 μ M each primer, 0.2 mM dNTPs, 0.3 U Taq DNA polymerase (Invitrogen, Foster City, CA, USA), and 1.5 mM MgCl₂. A Veriti Thermal Cycler (Applied Biosystems, Foster City, CA, USA) was used for amplifications with a cycling profile of: initial denaturation at 94 °C for 5 min followed by 35 cycles at 94 °C for 45 s denaturation, primer-specific annealing temperature (56°–59°) for 45 s and at 72 °C for 45 s extension, and a final extension step at 72° for 10 min. PCR products were electrophoresed in an ABI313XL (HITACHI) automated DNA sequencer and automatically sized using GENEMARKER ver 1.91 (SoftGenetics LLC™ www.softGenetics.com).

2.3. Data analyses

2.3.1. Genetic diversity and inbreeding in mother and offspring sampled groups

The presence of null alleles was tested using INEST 2.0 (Chybicki and Burczyk, 2009; Chybicki, 2015). As null alleles can affect population indices estimates which are based on heterozygosity, we estimate the frequency of null alleles (*f*_{null}) for mother and offspring groups, using the population inbreeding model (PIM) using INEST 2.0 (Chybicki and Burczyk, 2009).

Genetic diversity was quantified in mother and offspring cohorts by the mean of observed alleles per locus (A), effective number of alleles (N_{ae}), allelic richness (A_r), observed (H_o) and expected heterozygosity under Hardy–Weinberg equilibrium (H_e). Deviations from Hardy–Weinberg expectations were evaluated with the fixation coefficient (F). Significance of F estimates were calculated with the software Spagedi (Hardy and Vekemans, 2002) and their confidence intervals were obtained with the package *hierfstat* (Goudet, 2004) of the software R (R Development Core Team, 2017) with 1000 bootstraps.

The genetic diversity measured by H_o and H_e was compared between adult and offspring populations. H_o estimates were compared by paired Wilcoxon test, and H_e were compared by H_s test using the package *adegenet* (Jombart, 2008; Jombart and Ahmed, 2011) of R software (R Development Core Team, 2017) with 1000 permutations.

2.3.2. Mating system

Estimates of multilocus (tm) and mean single locus (ts) outcrossing rates, correlation of tm within progeny arrays (rt), and the correlation of outcrossed paternity (rp) were calculated using the MLTRwin software (Ritland, 2002). This program is based on the multilocus mixed-mating model and the estimation procedure of Ritland and Jain (1981) which assumes that progeny are derived from either random mating (outcrossing) or self-fertilization. The estimation of mating system indices was made by the expectation–maximization method to assure convergence and 10,000 bootstraps were used to get standard errors for each parameter.

Biparental inbreeding was estimated following Ritland (1990) as tm – ts . The effective number of pollen donors over all mothers trees were estimated under sibling pair model (Ritland, 1989) by the relation effective number of pollen donors $N_{ep} = 1/rp$. In a first analysis, all seeds within each family were considered a single group and all indices were estimated. As seeds within mother trees are grouped in pods, a second analysis was conducted considering the pod as an additional level within family.

The proportion of self-sibs (P_{ss}), half sibs (P_{hs}), full sibs (P_{fs}) and self-half-sibs (P_{shs}) was calculated for the population as $P_{ss} = s^2$, $P_{hs} = t^2_m(1 - r_{p(m)})$, $P_{fs} = t^2_m r_{p(m)}$ and $P_{shs} = 2st_m$ (Sebbenn, 2006).

2.3.3. Average coancestry coefficient (θ) and variance effective population size

Pairwise coancestry (θ_{xy}) multilocus coefficients were computed for all pairs of individuals within the mother and the offspring sample using the statistics of [Loiselle et al. \(1995\)](#) by the program Spagedi ([Hardy and Vekemans, 2002](#)). Within family coancestry coefficient (θ_{wf}) was calculated for each family as the average of θ_{xy} where X and Y are individuals belonging to the same family. The θ_{wf} were averaged over families to estimate an average population value (θ_{wfp}). Finally, θ_{xy} was compared among three groups involving individuals from (a) different mother plants (unrelated), (b) the same mother plant but different pod, and (c) the same pod. The comparison was conducted by the non-parametric two- and K -sample location test using the package *coin* ([Hothorn, 2015](#)) of the software R software ([R Development Core Team, 2017](#)).

The variance effective population size (N_{ev}) for each family was calculated following [Cockerham \(1969\)](#) as:

$$N_{ev} = \frac{0.5}{\theta_{wfp} \left(\frac{n-1}{n} \right) + \frac{1+F_o}{2n}}$$

where, n is the size family and F_o is the inbreeding coefficient in offspring. The number of seed trees (m) necessary for seed collection was calculated by the method of [Sebbenn \(2006\)](#) based on the relationship between the proposed effective population size of the conservation program ($N_{e(\text{reference})}$) and the average N_{ev} ([Sebbenn, 2006](#)):

$$m = \frac{N_{e(\text{reference})}}{N_{ev}}$$

2.3.4. Pollen dispersal distance

Based on the individual multilocus genotypes and the GPS coordinates from each mother tree, the pollen dispersal distances were inferred using KinDist ([Robledo-Arnuncio et al., 2006](#)) and TwoGener ([Smouse et al., 2001](#); [Austerlitz and Smouse, 2002](#)) using the POLDISP 1.0 software ([Robledo-Arnuncio et al., 2007](#)).

The TwoGener method is based on the relationships between physical distances among a sample of mother trees and pairwise intraclass genetic correlations (ϕ_{ft}) for the pollen pools sampled by these mothers. To fit pollen dispersal using the TwoGener approach, an independent estimate (or a joint estimation) of the effective density of pollen donors (de) is needed ([Austerlitz and Smouse, 2002](#)), which can complicate the accurate estimation of two indices. In addition, this method assumes uniformity in male fecundity and synchronous flowering. The KinDist method is based on a normalized measure of correlated paternity (ψ) between female pairs ([Robledo-Arnuncio et al., 2006](#)). The estimates of pollen dispersal indices obtained by KinDist are generally more accurate than those from TwoGener because KinDist does not rely on effective density, a parameter, which is very difficult to measure in the field. Joint estimation of de with TwoGener is associated with the usual statistical problems of increasing the dimensionality of the indices space. The KinDist method is also robust for the levels of male fecundity variation and asynchronous flowering commonly observed in forest tree populations ([Robledo-Arnuncio et al., 2006](#)).

The correlation of paternity within and among maternal families was first estimated by KinDist and, as indicated in POLDISP user's manual, the relationship between among-sibship correlated paternity and distance was tested for decreasing before running TwoGener. As a negative slope was obtained the TwoGener algorithm was run using the different dispersal distributions. The best dispersal distribution can be chosen by comparing least square residual. The mean pollen dispersal distances were obtained for

the same dispersal distribution in both algorithms. Different reference threshold distances were evaluated to define unrelated pollen pools in order to check stability of the results and the results here presented corresponds to threshold distance = 200.

The mean number of effective pollen donors (N_{ep}) under the pollen structure model was estimated under the relation

$$N_{ep} = \frac{1}{2\Phi_{ft}}$$

If we assume a bivariate normal distribution of pollen dispersal, the estimate of pollen structure ϕ_{ft} is inversely related the variance of pollen dispersal (σ^2) and reproductive adult density (d),

$$\Phi_{ft} = \frac{1}{8\pi\sigma^2d}$$

Given that ϕ_{ft} is known from genetic data and d from ecological observation (11.25 trees/ha), it follows that the variance in pollen dispersal can be computed from the expression above. Next, σ^2 can be used to estimate the effective number of pollen donors as:

$$N_{ep} = 4\pi\sigma^2d$$

The effective neighborhood pollination area (A_{ep}) was calculated following [Austerlitz and Smouse \(2001\)](#) as:

$$A_{ep} = 4\pi\sigma^2$$

2.3.5. Analysis of spatial genetic structure

Correlograms based on [Loiselle et al. \(1995\)](#) distances among individuals (θ_{xy}) against distance classes were evaluated either among adult and offspring populations using the function *eco.malecot* of package *EcoGenetics* ([Roser et al., 2017a](#)) of R ([R Development Core Team, 2017](#)). For the offspring generation 10 distance intervals were considered involving 1485 individual pairs each, and a minimal distance between individuals of 10 m. For the mother generation six distance intervals were considered involving 11 individual pairs each, and a minimal distance between individuals of 0 m.

3. Results

3.1. Genetic diversity and inbreeding in mother and offspring cohorts

The genotypes for eight loci were determined for each of the 12 sampled mother plants and 15 of their seeds (offspring). In the adult population f_{null} was close to zero in all loci, and in the offspring cohort only in two cases (GL16 and GL21) null allele frequencies were higher than 0.1 ([Table 1](#)). All eight loci were polymorphic showing from 4 to 13 alleles per locus with an average of 7.9 in the mother sample and 10.4 in offspring. The effective number of alleles varies from 1.4 to 9.2 with averages of 6.3 and 5.6 respectively for mothers and offspring and the allelic richness increases from 7.9 to 10.4 between the generations ([Table 1](#)). The mean observed heterozygosity (H_o) was significantly higher in the mother than in the offspring cohort (paired Wilcoxon $V = 3$, $P = 0.039$). By contrast, the mean expected heterozygosity (H_e) did not differ statistically among cohorts ($H_{test} \text{ obs} = 0.0016$, $P = 0.116$).

The fixation index (F) in the seed trees was positive but non significantly different from zero ($F = 0.07$, $P = 0.109$), however, but in the offspring cohort F was highly significant ($F = 0.13$, $P < 0.001$) indicating inbreeding.

Table 1
List of microsatellite loci and genetic diversity in both cohorts, mother (M) and offspring (O), of the studied population.

Locus ^a		N	A	N _{ae}	A _r	H _o	H _e	F	f null (SE)
GL12	M	12	8.0	7.1	8.0	0.75	0.86	0.14	0.03 (0.05)
	O	180	11.0	6.4	10.8	0.77	0.84	0.09**	0.04 (0.02)
GL15	M	12	8.0	7.5	8.0	0.75	0.87	0.14	0.04 (0.07)
	O	180	10.0	6.2	10.0	0.70	0.84	0.17**	0.09 (0.02)
GL9	M	12	7.0	6.6	7.0	1.00	0.85	-0.18	0.00 (0.00)
	O	180	9.0	5.9	9.0	0.76	0.83	0.08	0.05 (0.02)
GL16	M	12	8.0	6.6	8.0	0.67	0.85	0.23	0.08 (0.09)
	O	180	13.0	5.6	13.0	0.62	0.82	0.25**	0.14 (0.03)
Mo09	M	12	10.0	7.5	10.0	0.75	0.87	0.14	0.04 (0.06)
	O	180	13.0	6.9	12.9	0.69	0.85	0.19**	0.09 (0.02)
Mo08	M	12	11.0	9.2	11.0	0.92	0.89	-0.03	0.00 (0.00)
	O	180	13.0	8.4	13.0	0.85	0.88	0.04	0.01 (0.02)
GL21	M	12	7.0	3.8	7.0	0.58	0.74	0.22	0.02 (0.05)
	O	180	9.0	4.3	9.0	0.62	0.77	0.20**	0.12 (0.03)
Mo13	M	12	4.0	1.8	4.0	0.50	0.43	-0.17	0.00 (0.00)
	O	180	5.0	1.4	5.0	0.32	0.31	-0.04	0.09 (0.01)
MEAN	M	12	7.9	6.3	7.9	0.74	0.80	0.07	-
	O	180	10.4	5.6	10.3	0.67	0.77	0.13**	-

* and ** indicate respectively significant ($P < 0.05$) and highly significant ($P < 0.01$) F values.

^a name of locus as defined in by Mottura et al. (2005) and Bessega et al. (2013), M = mother trees, O = offspring, N = sample size, A = observed number of alleles, N_{ae} = effective number of alleles, A_r = allelic richness, H_o = observed heterozygosity, H_e = expected heterozygosity, F = fixation index (uncorrected for null alleles), fnull: frequency of null alleles.

3.2. Mating system

The analysis of the mating system (Table 2) showed an almost complete outcrossing rate ($tm = 0.996$). The difference $tm-ts$ was low but significant indicating a proportion of mating among relatives (~20%). The correlation of tm within progeny arrays (rt) was high, which might suggest that outcrossing rates differ among mother plants. However its standard error is too high (0.92) and tm was 1.000 in all mother plants but one in which tm was 0.94. Taken as a whole, these results indicate that the outcrossing rates are similar among mother plants. The correlation of outcrossed paternity was evaluated at three levels: global, among fruits and within fruits. In all cases rp was significantly greater than zero. The rp esti-

Table 2
Indirect estimations of mating system indices and pollen dispersal distances in *P. flexuosa* population.

Model	Indices	Estimate [CI]
Mixed mating	Multilocus outcrossing rate (tm)	0.996 ^c [0.812–1.180]
	Biparental inbreeding ($tm-ts$)	0.193 ^c [0.001–0.385]
	Correlated outcrossing rate (rt)	0.999 ^{NS} [-0.796–2.794]
	Correlated paternity (rp)	
	Global	0.312 ⁺ [0.194–0.430]
Among fruits		0.240 ⁺ [0.134–0.346]
	Within fruits	0.714 ⁺ [0.587–0.841]
Pollen pool structure	Φ_{it} (pollen structure parameter)	0.17
	Effective number of pollen donor (N_{ep})	2.93
	Kindist	
	Average distance of pollination (δ) Normal	4.56 m
	Least square estimation	2.03
	Average distance of pollination (δ) Exponential	5.38 m
	Least square estimation	2.04
	TwoGener	
	Average distance of pollination (δ) Normal	20.12 ^a /20.35 ^b m
	Average distance of pollination (δ) Exponential	17.83 ^a /18.04 ^b m
Effective neighborhood pollination area (A_{ep})	0.26 ha	

⁺ and ^{NS} indicate respectively significant ($P < 0.05$) and non-significant values.

^a Distance obtained from the global Φ_{it} value.

^b Pairwise Φ_{it} values assuming the density equals the census density (D). a and b estimates were obtained using TwoGener.

mate was maximum for sib sampled from the same fruit ($rp = 0.71$) but for different fruits on the same plants the value dropped in about 66% (0.24/0.71). These results indicates that the proportion of full sibs is about 71% for seeds proceeding from the same pod, whereas this proportion is only about 24% for seeds from different pods. The effective number of pollen donors estimated as ($1/rp$), indicated that about 4 pollen donors are siring each progeny array.

The progenies of open pollination in the population were constituted mainly for half sibs that represented 68.25% (P_{hs}) of the family array, whereas 30.95% of the progeny arrays were full sibs (P_{fs}). In contrast, a low proportion (0.8%) of self-half sibs (P_{shs}) was found and self-sibs (P_{ss}) were absent.

3.3. Average coancestry coefficient (θ) and variance effective population size

The effective number of pollen donors under sibling pair model (N_{ep}), paternity correlation (rp), coancestry coefficient θ_{wf} and the variance effective size of open pollinated families (N_e) were estimated for each of the 12 families (Table 3). The average paternity correlation was 0.4 ($CI_{95} = 0.03-0.65$), corresponding to numbers of pollen donors per mother plant ranging from about 2 to 8, with an average of 3.8. The number of pollen donors (N_{ep}) estimated for each family is not correlated with the geographic position as shown in Fig. 2. The average coancestry coefficient within families estimated according to Loiselle et al. (1995) ($\theta_{wfp} = 0.24$ $CI_{95} = 0.15-0.33$) was higher than the expected ($\theta = 0.125$) for panmictic populations where pollen donors are not related. The average variance effective population size estimated from the coancestry coefficients was 1.98 and the number of seed-trees necessary for seed collection aiming to retain an effective population size of 100 was estimated at the minimum of 52.

Coancestry was also analyzed at different levels. In the case of individuals from different families (unrelated) the average θ_{xy} were near 0, whereas for individuals from the same family (related) θ_{xy} were in average 0.23 and 0.27 for seeds from different and the same pod respectively (Fig. 3). The comparison among these groups yield highly significant results (Chi Sq = 5931.3, $P < 2 \cdot 10^{-16}$). The differences were also highly significant within families between individuals from different or the same pod ($Z = -3.63$, $P < 3 \cdot 10^{-4}$). The average θ_{xy} between individuals of the same family but from different pod is higher than the expected value

Table 3

Genetic structure of open pollinated families from *Prosopis flexuosa* DC. trees. N: sample size, N_{ep} : effective number of pollen donors under sibling pair model, rp : paternity correlation, θ_{wf} : coancestry coefficient within families, N_{ev} : variance effective size of open pollinated families, m : number of seed-trees necessary for seed collection aiming to retain the effective population size of $100 \pm 95\%$.

Family	N	rp	N_{ep}	θ_{wf}	N_{ev}	m
1	15	0.14	7.0	0.17	2.56	39.1
2	15	0.32	3.1	0.16	2.65	37.7
3	15	0.35	2.8	0.28	1.69	59.1
4	15	0.48	2.1	0.29	1.62	61.8
5	15	0.32	3.1	0.22	2.07	48.2
6	15	0.24	4.1	0.20	2.19	45.6
7	15	0.13	7.6	0.25	1.88	53.3
8	15	0.16	6.5	0.20	2.24	44.6
9	15	0.55	1.8	0.29	1.64	61.0
10	15	0.31	3.3	0.27	1.74	57.5
11	15	0.59	1.7	0.28	1.65	60.5
12	15	0.47	2.1	0.26	1.78	56.3
Mean	-	0.34	3.8	0.24	1.98	52.1
CI ₉₅	-	[0.03–0.65]	[0.34–7.88]	[0.15–0.33]	[1.26–2.69]	[35.03–69.10]

CI₉₅ = calculated by mean \pm 1.96 SE being SE the standard error.

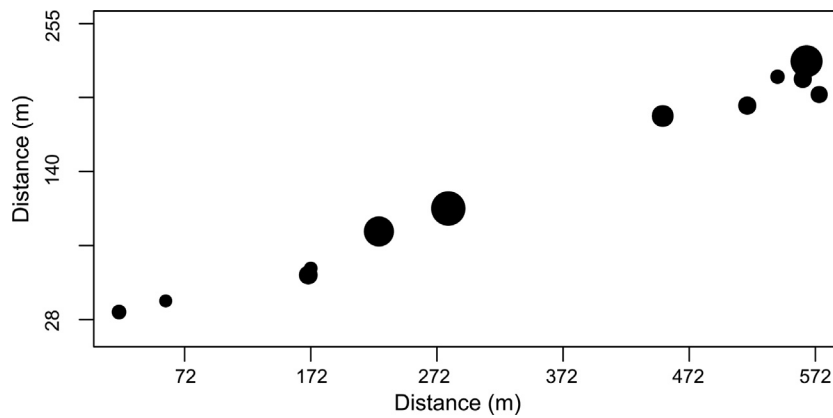


Fig. 2. Number of pollen donors (N_{ep}) under sibling pair model plotted considering geographic position. The size of the circle represents the number of pollen donors estimated for each family.

(0.125) for half sibs, and the corresponding average for individuals from the same pod are higher than the value expected for full sibs (0.25) (Fig. 3).

3.4. Pollen dispersal

Pollen pool structure analysis with POLDISP gave a Φ_{ft} estimate of about 17%, which translated into an effective number of pollen

parents (N_{ep}) represents about 3 pollen donors per mother plant (Table 2).

Applying KinDist, the average pollen dispersal distance was estimated in 4.56 m when assuming a normal and 5.38 when assuming exponential distribution of pollen dispersal distance curve. The model based on exponential distribution curve fits only slightly better than the normal as described by least square residuals of 2.03 and 2.04 respectively. Therefore both the normal and exponential curve was used for further analysis using TwoGener.

TwoGener yields estimated pollen distances higher than those obtained with KinDist. Considering a normal distribution, pollen dispersal distances varied depending on the estimation method between 20.12 and 20.35 m. When the same estimates were obtained using exponential curves; distance estimates varied between 17.83 and 18.04 m. No convergence was reached by TwoGener for the joint estimate of d_e and pollen dispersal variance.

Finally, the effective neighborhood area of pollination (A_{ep}) was estimated in 0.26 ha, that is, near a 29 m radius circle around each maternal tree.

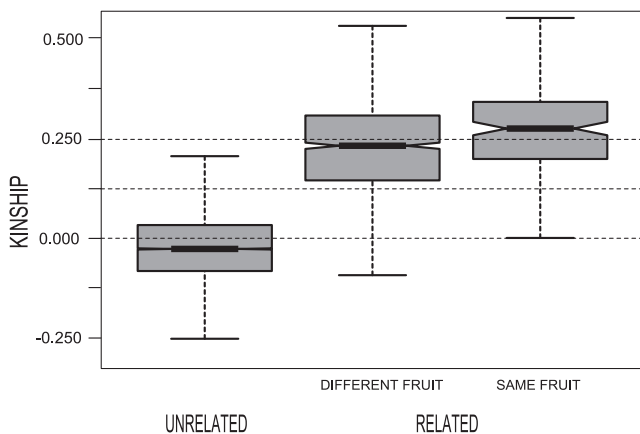


Fig. 3. Boxplots representing multilocus average coancestry estimated in three groups involving individuals from different mother plants (unrelated), the same mother plant but different pod, and the same pod.

3.5. Analysis of spatial genetic structure

Loiselle et al. (1995) kinship coefficient (θ_{xy}) decreases with the distance classes both in mother and offspring groups (Fig. 4). For mothers, the Mantel test (1967) for the association between θ_{xy} and $\ln(d_{xy})$ yielded significant results ($P = 0.01$). The average θ_{xy} was significant ($P = 0.02$) for the first distance class and decreases

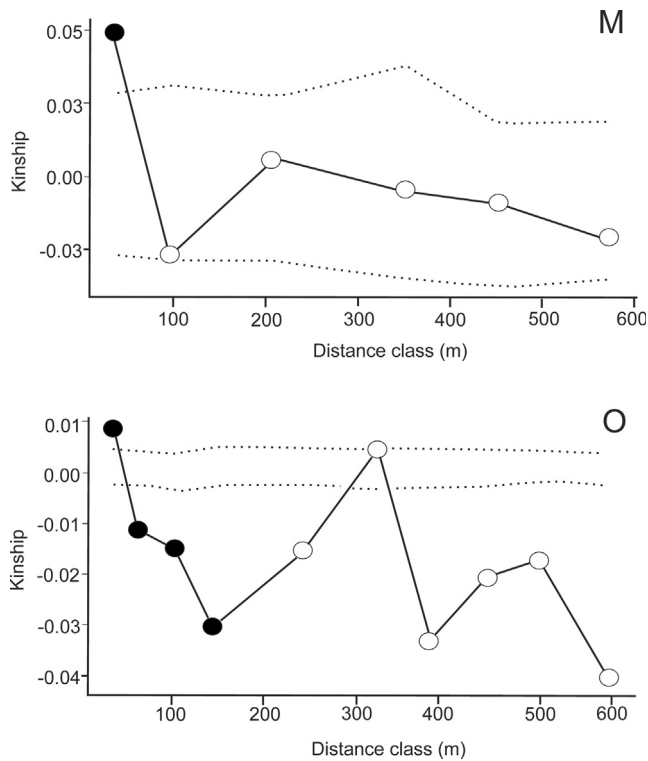


Fig. 4. Correlograms based on [Loiselle et al. \(1995\)](#) kinship coefficient for mothers (M) and offspring (O) populations. Significant correlation values ($P < 0.05$) are represented by black circles. Dotted lines represent bootstrap CI (95%) around 0.

abruptly becoming non-significant between 35.6 and 96.5 m. For offspring, the same trend is observed. The mantel test was also significant ($P = 0.01$) and average θ_{xy} was significant ($P < 0.04$) for the first 4 distance classes and significance is lost between 141.3 and 245.5 m, suggesting that trees separated by about 250 m are pollinated by non-related pollen clouds.

4. Discussion

Historically, the use of the native forests involves, in most cases, unsustainable extraction techniques and therefore represents a significant negative impact for the ecosystems. The need to reverse the process of environmental degradation in the Monte region has led to the development of programmes for the integrated management of multipurpose trees, especially for the two dominant *Prosopis* species in the Monte (*P. chilensis* and *P. flexuosa*). Woodland conservation based on fencing recruitment is a common strategy because it is assumed that herbivore megafauna can negatively affect tree regeneration through trampling and browsing on tree seedlings and saplings. However, some studies have found positive effects of large herbivores on tree recruitment as they can increase seed input into tree population by acting as seed dispersers and promoting seedling establishment by modifying micro environmental conditions ([Aschero and García, 2012](#); [Aschero et al., 2016](#)).

The establishment of a reserve like Ñacuñan to protect “algarrobo” gave rise to contrasting grazing regimes but, although differences in demographic process were detected such as lower seed production and higher probability of reversion to smaller size classes in young individuals when cattle were present, cattle grazing had no significant effect on the population growth rate ([Aschero et al., 2016](#)). This management practice as a protective method may affect the reproductive biology including mating system indices and spatial genetic structure. So, the incorporation of

genetic conservation criteria into forest and farm management practices may offer in many cases the best prospects for achieving management and conservation goals ([Hawkes et al., 1997](#)).

Our estimate of outcrossing rate from mixed-mating model was virtually one, demonstrating that *P. flexuosa* is mostly outcrossed. This result is similar to that obtained in *P. alba* Griseb, a related species inhabiting the Chaco Ecoregion, where selfing was shown to be absent ([Bessega et al., 2011](#)). High outcrossing rate may result from self-incompatibility systems or inbreeding depression at the embryo, seed, and seedling stages ([Hufford and Hamrick, 2003](#); [Ward et al., 2005](#); [Biscaia de Lacerda et al., 2008](#)). Despite the observed high outcrossing rate, some crosses in the population seem to involve related partners, suggesting that near 20% of the resulting seeds had been produced by biparental inbreeding in the population.

Additional evidence supporting the occurrence of biparental inbreeding was obtained from the analysis of pairwise kinship within family arrays. In consistency with the results from the MLTR global analysis, the coancestry coefficient within progenies ($\theta = 0.24$) was higher and the variance effective size ($N_{e(v)} = 1.98$) was lower than expected for true half-sib progenies ($\theta = 0.125$; $N_{e(v)} = 4$). Moreover, the average kinship estimate for seeds from the same fruit is higher than the expected for full sibs. As selfing rate is negligible, it cannot be the cause for increased coancestry within progenies, suggesting the correlated mating (rp).

The correlation of paternity can increase the relatedness and, consequently, the frequency of allele identity by descent within progenies ([Sebbenn, 2006](#)). Mating among relatives is indicative of spatial genetic structure and viceversa, here we detected significant spatial genetic structure for the population studied at relatively short distances. The spatial genetic structure observed in mother plant cohort may be criticized due to the low number of trees, but the same tendency can be shown in the offspring analysis, where the number of individual pair per distance class was much higher (1485). Consequently, nearest neighbor mating may explain the high rate of correlated paternity of outcrossed progenies ($rp = 0.13$ – 0.59) with a corresponding low mean effective number of pollen donors per mother tree ($N_{ep} = 3.8$).

Correlated mating may result from flowering desynchronicity, small population size and low numbers of reproductive trees, pollinators and seed disperser behavior. Desynchronicity has not been reported for this species, and is not to be considered as a plausible explanation for mating between relatives. In the present case effective population size does not seem to be small enough as to be accounted for biparental inbreeding as the Ñacuñan reserve encompasses 12.800 ha where populations has not been fragmented and density (near 11.25 trees/ha) of reproductive trees is relatively high for the species. *P. flexuosa* as all other species of the genus is pollinated by insects such as the bee species *Megachile* sp. and *Apis mellifera*, which tend to visit a few closely located trees and to forage the same inflorescence ([Simpson et al., 1977](#); [Genisse et al., 1990](#)). The calculated average distance among reproductive trees was short (from 4 to 20 m depending on the estimation method), favoring the interpretation of pollinator vectors visiting a few trees producing correlated mating. Thus, behavior of the pollinator could be a potential cause of the observed level of correlated matings in spite of the high population size and density. Finally seeds are dispersed by frugivorous mammals which tend to deposit together in dungs seeds from the same mother plant, favoring the developing of relative trees in the same neighborhood ([Keys, 1993](#); [Keys and Smith, 1994](#); [Bessega et al., 2000, 2011](#); [Campos et al., 2016a, 2016b](#)). The exclusion of megafauna by the reserve installation is expected to determine a reduction on the seed dispersal distances and an increase of the relatedness in the neighborhood trees. Within the reserve one of the main seed dispersal agents is the rodent *Ctenomys mendocinus* which is known

to be more abundant inside the reserve (Tabeni and Ojeda, 2003) than in the surroundings cattle ranches. The average dispersal distance promoted by this species are expectedly much shorter than that of cattles, however it was suggested that the negative effects on sapling growth could be qualitative similar. Consequently, seed disperser behavior may also be claimed to explain the occurrence of biparental inbreeding.

The family structure information is relevant to define the necessary number of seed trees to develop breeding, *ex situ* genetic conservation, and environmental reforestation programmes, as it provides the minimum effective population size for these purposes (Sebbenn, 2003, 2006). Here, estimated neighborhood pollination area is relatively small ($A_{ep} = 0.26$ ha), representing a ~ 20 m radius circle around each maternal tree. Sampled mother plants should be farther than this distance to insure the progeny arrays do not share the same pollen cloud. Indeed the correlation between kinship and geographical distances becomes non-significant after 250 m, so this distance is expected to avoid relationship due to both pollen and seed dispersal.

Considering that each progeny array represents 1.98 non inbred and relative individuals, the sample aiming to retain an effective population size of 100 should include at least 52 seed trees. An additional caution to be considered is that seeds collected from the same fruit are mostly constituted by full sibs, whereas the proportion of half sibs highly increases for seeds taken from different pods. Consequently, it is very important that sampled progeny arrays include seeds from different pods.

Biparental inbreeding might lead to a biparental inbreeding depression, a weaker type of inbreeding when compared to self-fertilization. However, it seems to be sufficiently strong to reduce the individual fitness in tree species (Stacy, 2001; Biscaia de Lacerda et al., 2008). Self-fertilization generates at least 50 percent of inbreeding in a descendent individual and mating among relatives produces inbreeding in offspring at an equal level than the value of the coancestry coefficient between the outcrossed individuals. Thus, since we find a very low proportion of self-fertilization in the studied population ($s = 0.004$), almost all inbreeding in the evaluated reproductive event can come from biparental inbreeding. The average fixation index obtained ($F = 0.133$) was significantly different from zero, indicating inbreeding in the progeny array. This estimate is not expected to be biased by viability differences between seeds, as roughly 100% of studied seeds reached the green cotyledon stage.

The low observed heterozygosity recorded in offspring population ($H_o = 0.67$) is compatible with inbreeding depression at seed stages as this coefficient is increased as seed goes to adult stage ($H_o = 0.74$) while F is reduced (0.07). Some seeds resulting in non-viable (inbred) genotypes would not reach mature stages supporting the idea that detrimental combinations in *P. flexuosa* are naturally purged as in many other trees (Duminil et al., 2016). Inbreeding depression should be taken into account for possible failure of seedlings in experimental fields and progeny trials.

Some tree species are extremely dependent on animals for pollination and seed dispersal, and these processes are strongly affected by anthropogenic effects on the landscape. Human-induced activities might produce habitat degradation and/or modification and promote large impacts on the SGS of forest populations (Paffetti et al., 2012; Rajendra et al., 2014; Sjölund and Jump, 2015). In particular, in *P. alba*, a close relative to *P. flexuosa*, SGS was evaluated and compared among a population highly disturbed and fragmented by agricultural activity and stock breeding and a not disturbed population. The latter showed a positive and significant SGS at short distances and consequently the neighborhood size and effective gene dispersal were reduced in comparison with the modified by anthropic activities. The differences in the SGS could be the result of differences in densities of individuals

and/or consequences of management practices (Bessega et al., 2016). Here, in *P. flexuosa* we also observed a limited pollen and seed dispersal that it is expected to give a spatial genetic structure at short distances and a small neighborhood area of pollination. The distance over which structure occurs is influenced primarily by seed dispersal and adult density (Dyer, 2007). Our results indicated a significant structure at short distances both for adult (<96 m) and offspring cohorts (<250 m) and may be explained by the fence installations and the lack of large herbivores. As discussed before, livestock tend to eat as many *Prosopis* fruits as possible from a single mother plant, depositing the scarified seeds in dung, which promotes the aggregation of full- and half-sib seeds in narrow whereas the dispersal may give rise to semi-isolated patches and tree groups.

5. Conclusions

Our results have important implications for the management of forest genetic resources of *P. flexuosa*. Mating system indices are valuable tools to be considered for both the conservation of the resource in its native range and its management in colonized areas. The population studied is mainly outcrosser, sensitive to inbreeding depression at seed stage, having relative reduced pollen mediated gene dispersal and important mating among related individuals. Our results suggest that for *ex situ* conservation the distance that would be enough to avoid full sib sampling among harvested seed-trees is 20 m. An additional caution is collecting seeds from different pods to reduce the proportion of full sibs within progeny arrays. For *in situ* conservation it is important to take into account that remaining forest patches separated by more than 250 m are expected to be isolated, increasing the risk of fragmentation consequences as loss of genetic variability, inbreeding increases and final loss of population.

Based on these results, the “algarrobo” management program has to consider the minimal distances needed for the establishment of future stands. Breeding programs may take advantage of the limited pollen dispersal to avoid contamination between selected stands and the surrounding population. In these case excluding big mammals from the stand should be advisable. While recruitment by fences seems to be an alternative to protect from external influences, it avoids long distance seed dispersal, and consequently gives place to a significant family structure. The interaction with long dispersal big mammals might be considered as a strategy to promote increased outbreeding reducing the rate of mating between related individuals. Silvo-pastoral practices may apply this criterion for a profitable use of resources.

Conflict of interest

The authors declare no conflicts of interest.

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