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REPRODUCTIVE BIOLOGY OF THE NARROW ENDEMIC *Dianthus morisianus*

BIO/03 Botanica Ambientale e Applicata

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Contents

General introduction

The Mediterranean basin (Fig. 1A) is one of the 34 most important biodiversity hotspots of the planet (Mittermeier *et al.* 2004), with around 25,000 species of which 13,000 are endemic. In the Mediterranean, there are nearly 5,000 islands, and these are characterized by a high rate of endemism due to their high diversity of environments and their isolation (Médail and Quézel 1999) and the species usually are confined in reduced areas and populations (de Montmollin and Strahm 2005; Thompson 2005; Rosselló *et al.* 2009). These characteristics make island species extremely vulnerable to habitat destruction, overgrazing, urban expansion and the presence of invasive alien species (Cowling 1996; Baillie *et al.* 2004; de Montmollin and Strahm 2005; Cuttelod *et al.* 2009). Caujapé-Castells *et al.* (2010) regard island species as one of the most critically threatened of the world.

Sardinia is the second largest island of the Mediterranean basin after Sicily and it has around 2,494 plant species (Conti *et al.* 2007), 347 of which are endemic. The 45.8% of them are exclusive Sardinian endemisms and several are threatened (Fenu *et al.* 2014).

During the last decades, the loss of biodiversity has been increasing rapidly as a consequence of human action, to the extent that Leakey and Lewin (1996) suggested that we are undergoing a "sixth extinction". There are numerous causes for the loss of biodiversity, but habitat destruction via change land uses is considered the main factor (Baillie *et al.* 2004).

The reproductive biology of endangered and threatened species is one of the most important research topics, after habitat conservation, in order to identify conservation priorities (Bernardello *et al.* 2001; Neal and Anderson 2005; Dudash and Murren 2008; Anderson *et al.* 2012; Stuessy *et al.* 2014). The data of the reproductive biology provide information to analyse the population viability of the species (Menges 1991; Metz *et al.* 2010, Dudash and Murren 2008), to plan appropriate conservation strategies (Schemske 1994; Bernardello *et al.* 2001; Neel 2002; Albert *et al.* 2001), and help us to determine what causes the species' vulnerability (Schemske 1994). Conservation biology is a multidisciplinary science which has two key tasks, namely, identifying the factors that affect the abundance of the species and evaluating their level of endangerment (Mejías *et al.* 2002). Although the importance of obtaining these information is widely accepted, today there are still many island species whose reproductive system remains unknown (Bernardello *et al.* 2001). In this thesis, we investigate some of these aspects in order to better understand the vulnerability of the narrow endemic *Dianthus morisianus* Vals. (Caryophyllaceae).

The Caryphyllaceae family is represented by more than 80 genera (comprising about 300 species), it is distributed around the world and its highest presence is in the temperate regions of the Northern hemisphere. The genus *Dianthus* is one of the best represented in this family and is one of the most difficult genera from a taxonomic point of view (Valente *et al.* 2010). *D. morisianus*, our study species, belongs to the *Dianthus sylvestris* Wulfen group, which is also one of the most complex within this genus (Bacchetta *et al.* 2010). In Europe, there are more than 100 *Dianthus* species, of which more than 70 are endemic. These endemic species are usually characterized by a limited distribution, which could indicate a recent diversification (Valente *et al.* 2010). In Sardinia, there are other 7 species belonging to this group: *D. cyathophorus* Moris; *D. sardous* Bacchetta, Brullo, Casti & Giusso; *D. genargenteus*, Bacch., Brullo, Casti & Giusso; *D. ichnusae* subsp. *ichnusae*, Bacch., Brullo, Casti & Giusso; *D. ichnusae* subsp. *toddei*, Bacch., Brullo, Casti & Giusso, *D. oliastrae*, Bacch., Brullo, Casti & Giusso, *D. insularis* Bacch., Brullo, Casti & Giusso.

Dianthus L. species are usually perennial, but there are also some annual and biennial species. Plants are herbaceous or small shrubs, with linear and parallel-veined leaves. Flowers are solitary or in heads, surrounded by bracts. The epicalyx has 4 (6) scales, usually appressed to calyx. The calyx is tubular, with 5 teeth and without scarious commisures. Flowers are composed by 5 petals, long-clawed, entire, dentate or laciniate but not deeply bifid; and do not present coronal scales, with 10 stamens and 2 styles. The dehiscing capsule is characterized by 4 teeth and the carpophore is often present. Male-sterile plants occur sporadically and add some difficulties to their identification, since such plants are smaller. Most of the species are intercrossable but, since they are usually geographically or ecologically isolated, hybrids are rather local (Tutin 1964).

In this study, we focused on *D. morisianus*, the only psammophilous species of *Dianthus sylvestris* Wulfen group in the Mediterranean (Bacchetta *et al*. 2010). *D. morisianus* is a threatened narrow endemic species, which grows in only one natural population on a stabilized dune system at 10-55 m a.s.l. in Portixeddu (south-western Sardinia; Figure 1B and 1C; Bacchetta *et al.* 2010) in the *Cisto-Lavanduletalia* coastal habitat (Fig. 2A-2C; Fenu *et al.* 2016a). During the last decades, this population has been affected by grazing, farming, reforestation (Fig. 2A), and urbanization, which reduced and fragmented the population. *D. morisianus* is classified as endangered in the National Red List (Conti *et al.* 1992) and as Critically Endangered in the International Union for Conservation Nature (IUCN) (Fenu *et al.* 2010, 2011). In previous studies were studied the life history and demographic features of the natural population observing that the natural population presents a stable size and the adult plants present high survival rate (Cogoni 2011), and also the germination ability of the species which indicate that seedlings emergence is the most critical phase

and the lack of persistent soil seed bank (Cogoni *et al.* 2012). Also, due to the high level of endangerment of the species, two reintroductions in areas located near the natural population were conducted in previous years (Fig. 1C; Cogoni *et al.* 2013, Fenu *et al.* 2016b), but the reproductive biology of the species had never been investigated.

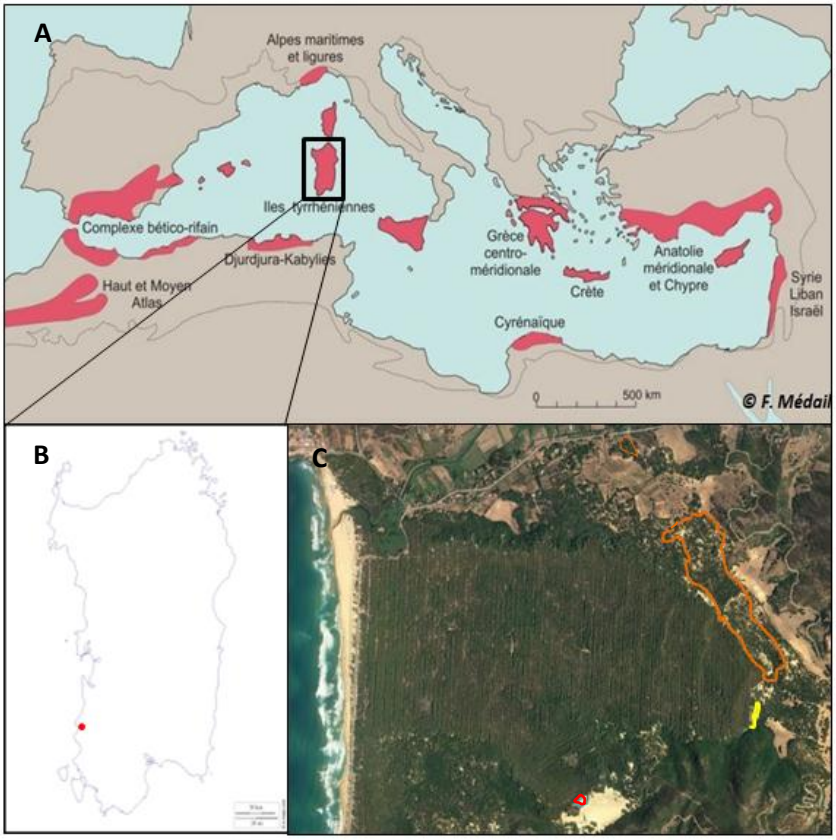

Figure 1. A) Mediterranean hotspots; B) location of the natural population of *Dianthus morisianus*, Sardinia (Italy); and C) area of the natural population (orange) and the two reintroductions (the yellow one is the studied population, and the red the other one).

D. morisianus is a suffrutex of 30-50 (60) cm tall; with woody stocks loosely branched with branches of 0.5-3.0 cm long. The basal leaves are inserted along the woody stock branches, they are flat, measure 2.5-15.0 cm long × 1.5- 2.0 mm wide, and are acute at the apex. It has 2-10 (1-18) stems of 20-45 cm long, with 4-6 internodes. The calyx measures 25-30 mm long × 4.5-5.5 mm diameter, with lanceolate teeth, margins membranaceous, acute and overlapping below, 5.5-7.0 mm long. Petals measure 35-40 mm long and the claw 23-26 mm. The limb is pink, cuneate-rounded, 10-15 × 8-11 mm with 6-8 teeth and 0.3-1.0 mm long. Anthers are 4.5 mm long and the ovary is 7.5 mm long. The style and stigma are 14 mm long and the fruit is a cylindrical capsule included in the calyx (Fig. 2D; Bacchetta *et al.* 2010).

Figure 2. The pictures A) and B) show the habitat of *D. morisianus*. In A), we can observe the afforested area, C) an individual with numerous open flowers growing protected by *Cistus salvifolius* and *Pistacia lentiscus*, D) three flowers of the studied species in three different stages: (A) first of anthesis, (B) male phase and (C) female phase.

Objectives

The aims of this PhD studies were: 1) to study the reproductive biology of the narrow endemic species *Dianthus morisianus*, 2) to analyse if there are factors that contribute to variations in the inbreeding and mating system estimates in the natural and in one reintroduced population and 3) to provide information about the plant-pollinators relationships.

This thesis is divided in four chapters, the first study (chapter 1) was conducted on *ex situ* plants and the other three were carried out in the natural and in one reintroduced population.

Chapter I: The aim of this study was to investigate, at a general level: 1) the floral biology; 2) the flowering phenology; 3) the breeding system; and 4) the inbreeding depression of the study species *ex situ*, in order to know the main characteristics of the reproductive system without disrupting the natural population (Nebot *et al.* 2016).

Chapter II: Once we obtained information on the self-compatibility of the species we deepened the knowledge of the reproductive system and mating system in order to identify the possible biological factors that threaten this plant. The aims of this study were: 1) to investigate the flower phenology; 2) to gain information on the breeding system and related indexes; 3) to evaluate

the reproductive success; 4) to assess the germination capacity and survivorship of the seedlings; and 5) to estimate the level of inbreeding depression in the natural population.

Chapter III: In this chapter, we compared the reproductive biology of the offsprings of the reintroduced population with the results obtained in the natural population (chapter 2), with the aim to evaluate the success of the first reintroduction. In particular, the aims were 1) to deepen the knowledge of the success of the first reintroduction; 2) to analyse the female reproductive success of both the natural and the reintroduced populations, 3) to investigate the flowering phenology of the offsprings; and 4) to obtain information on the breeding system of offsprings of the reintroduced population, in order to determine the presence of inbreeding depression, pollen limitation and the pollinator dependence of the population.

Chapter IV: In the second and third chapter, we observed that the species is highly dependent on pollinators. Identifying the set of pollinators of an endangered species is fundamental for the development of conservation plans and they should protect also these mutualisms to maintain sustainable populations of *D. morisianus*. The aims of this study were: 1) to investigate the presence of diurnal and nocturnal pollinators and their effectiveness to produce fruits and seeds; 2) to verify the presence of a generalist or a specialist pollination system; 3) to assess the nectar production of *D. morisianus*; and 4) to verify the presence of nursery pollination (insects that lay their eggs on the same flowers that they pollinate to rear offspring on the seeds and it potentially impose large fitness costs on their plant hosts).

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CHAPTER 1

FLORAL BIOLOGY AND BREEDING SYSTEM OF THE NARROW ENDEMIC *Dianthus morisianus* Vals. (CARYOPHYLLACEAE)

1 Chapter I - Floral biology and breeding system of the narrow endemic *Dianthus morisianus* Vals. (Caryophyllaceae)

1.1 Introduction

The reproductive biology of a plant affects, at least to some extent, its reproductive success (Rymer *et al.* 2005) and can have important consequences for the viability of its populations (Evans *et al.* 2003). Specifically for threatened plants, the reproductive biology is of special importance because of its direct effect on populations' growth, dispersal and colonization (Saunders and Sipes 2006). It can also provide indirect information related to conservation efforts such as patterns of genetic diversity, threats of diversity loss, risk of inbreeding depression, and risks associated with changes in pollinator abundance or effectiveness (Menges 1991; Nell 2002; Evans *et al.* 2003).

Mating patterns are prime determinants of the levels of inbreeding (Barrett and Kohn 1991). Inbreeding depression and selfing rates play important roles in studies describing the evolution of plant mating systems (Charlesworth and Charlesworth 1978; Collin *et al.* 2009). Inbreeding depression is not a fixed state; it can vary depending on which life history stage is most negatively affected (Kittelson and Maron 2000). Negative effects of inbreeding on reproductive traits and offspring establishment have been documented in several plant species (Fishman 2001; Sedlacek *et al*. 2012; Bellanger *et al.* 2015).

In general, outcrossed progeny have higher levels of genetic diversity than those produced by self-fertilization, in which the effects of inbreeding depression are observed (Teixera *et al.* 2009; Cursach and Rita 2012). However, selfed progeny offers reproductive assurance when pollinators are scarce, or limited pollen transfer reduces the reproductive output (Barret 1998; Knight *et al.* 2005; Cursach and Rita 2012). Several studies highlight that threatened plants exhibit slightly higher levels of self-compatibility than common plants (Saunders and Sipes 2006).

The genus *Dianthus* L. (Caryophyllaceae) is one of the most diverse plant genera in Europe and is characterized by several endemic *taxa* in the Mediterranean region (Valente *et al.* 2010). In Sardinia, eight endemic *taxa* of the *Dianthus sylvestris* Wulf. group have been recorded, and four of them are narrowly distributed (Bacchetta *et al.* 2010). *Dianthus morisianus* Vals. is the only psammophilous plant of this group in the Mediterranean Basin and grows with only one natural population on the Portixeddu coastal dune system (Buggerru, South–West Sardinia; Bacchetta *et al.* 2010). The natural habitat of *D. morisianus* has been strongly modified by human activities, causing

habitat loss and fragmentation (Cogoni *et al.* 2013). The small size of the population and the limited seedling recruitment make *D. morisianus* potentially prone to extinction and, for this reason, this plant is considered as one of the most threatened plants on the island (Bacchetta *et al.* 2012) and it is categorized as Critically Endangered on the IUCN Global Red Lists (Fenu *et al.* 2013). In order to reduce the extinction risk, two experimental translocation programs were realized. The first reintroduction was in a protected and fenced site, while the second one was in an open and unprotected site. Both these sites are located in areas near to the natural population (Cogoni *et al.* 2013; Fenu *et al.* 2016).

Despite its status and past conservation efforts, several traits of the reproductive biology of this species remain unknown.

Classical greenhouse experiments are excellent for implementing detailed studies of mating systems and inbreeding depression at successive reproductive stages (Alonso and Garcia-Sevilla 2013). This is required to understand the potential advantages and disadvantages of selfing in those species whose extensive manipulation in the field may be problematic or difficult (Alonso and Garcia-Sevilla 2013). In this context, this work aims to provide new information about the reproductive biology of *D. morisianus* in an *ex situ* experiment; in particular, the following aspects were investigated: (1) floral biology; (2) flowering phenology; (3) breeding system; and (4) inbreeding depression.

1.2 Material and methods

1.2.1 Study species and plant materials

Dianthus morisianus Vals. (Caryophyllaceae) is a perennial characterized by numerous woody stocks, erect stems and a basal rosette with thin and linear leaves (Fig. 1A). It is a gynomonoecious plant that presents hermaphroditic and, in low frequency, female flowers. The stems bear terminal multi-flowered heads; the calyx is characterized by lanceolate teeth and the color of the corolla is pink. Petals present 6–8 teeth, rounded and irregularly lobed. Anthers measure 4.5 mm long, the ovary 7.5 mm long and style and stigma 14 mm long (Bacchetta *et al.* 2010; Fig. 1B–C). The flowering season is from early May to late June, and ripe fruits can be found during June–July (Bacchetta *et al.* 2010). Seedling emergence represents the most critical stage for the long-term persistence of *D. morisianus*, while the lack of a persistent soil seed bank represents a hazard to the persistence of the natural population (Cogoni *et al.* 2012).

Figure 1. Cultivated plant of *Dianthus morisianus* (A), flower at male stage (B) and flower at female stage (C).

Ripe fruits of *D. morisianus* were collected in July 2010 from 50 plants growing in the natural population. The seeds were cleaned and stored according to the mother at the Sardinian Germplasm Bank (BG-SAR) under controlled conditions in a dry room (15°C and 15% relative humidity [RH]). In October 2010, 100 seeds from 20 different plants (five seeds per plant) were sown in 100 square pots (7 l; five pots per plant) filled with universal substrate and sand (70:30). The plants were cultivated in the open space, under natural conditions (ambient temperature, humidity and natural light), at the Botanical Garden of the Cagliari University, but watered three times a week. The third year (2013), the 86 surviving plants (belonging to 18 mother plants) were used to carry out this experiment.

1.2.2 *Ex situ* phenology and floral biology

During the flowering season, observations of the open flowers of each plant were carried out once a day, during the flower's lifespan. These observations allowed us to detect the different stages during the flower's lifespan. Each flower was marked with a univocal code on the calyx using adhesive tape and a permanent marker. The following floral parameters were obtained: maximum flowering (considered to be the maximum number of open flowers per plant) and time to reach maximum flowering (number of days from the first open flower in the population to the day of the maximum flower count on each plant (Bishop and Schemske 1998); flowering duration per plant and group of plants studied (in days); flowering phenology, and flowering synchrony. The last

parameter is described as the number of days that the flowering of an individual overlaps with the flowering of every other plant (Augspurger 1983) and calculated following the formula:

$$X_i = \left[\frac{1}{(n-1)}\right]\left(\frac{1}{f_i}\right)\sum e_{j\neq i}$$

where n is the number of plants in the population, ej the number of days during which both individuals i and j flower synchronously, fi is the number of days when individual i is in flower (Augspurger 1983). The flowering synchrony ranges from 0 to 1, where 1 indicates that plants present a complete flowering overlap, whereas0 indicates that plants flower in different moments (Augspurger 1983).

1.2.3 Breeding system

The Cruden's index (Cruden 1977) was calculated in order to estimate the average pollen–ovule ratio (P/O) value. The number of pollen grains and ovules was calculated following the protocol in Dafni *et al.* (2005), as modified by Cursach and Rita (2012). For the determination of pollen grain and ovule numbers, six mature buds belonging to six different plants were collected just before flower opening and preserved in 70% ethanol. To determine the pollen grain number, dehiscing anthers were placed in Eppendorf tubes filled with 1 ml ethanol and shaken with a vortex mixer (V-1 plus Biosan Ltd.) to release the pollen; for each Eppendorf, five subsamples of 10 µl of solution were scored, and the number of pollen grains was counted at 8x magnification by a dissecting magnifying glass (Zeiss Discovery V8). The number of pollen grains per Eppendorf was extrapolated to obtain the number of pollen grains available per anther. The number of pollen grains per anther was multiplied by 10 (number of anthers per flower) in order to obtain the number of pollen grains per flower. The ovaries were dissected with a scalpel and placed in a drop of water on a microscope slide. Ovules were counted at 1.5× magnification under a dissecting magnifying glass (Zeiss Discovery V8).

The breeding system of *D. morisianus* was experimentally assessed considering all flowering plants (82 of a total of 86 cultivated plants). Two types of pollination treatments were conducted: xenogamous hand pollination, with pollen taken from another plant of the same population (133 hand pollinations; 1–13 flowers per plant), and geitonogamous hand-pollinations (33 hand pollinations; 1–3 flowers per plant), with pollen derived from a flower of the same floral stem. The flowers were emasculated, then bagged with fine-mesh polyester bags before they became receptive in order to exclude insects. The bags were removed during hand pollinations, and then the flowers were re-bagged. Pollen was applied once by removing one dehiscent anther from the stamens with

forceps and applying the pollen to the mature stigmas. The bags were removed when the flower had withered in order to minimize the effects of bagging on fruit formation. At fruit maturity, tea bags were placed on the immature fruits to ensure the collection of all mature seeds. The fruits were collected when ripe, and the seeds were stored in a dry room at 15°C and 15% RH.

The reproductive success of each pollination treatment was compared in terms of fruit set, assessed as the proportion of treated flowers that eventually produced fruits; seed set, calculated as the number of seeds per fruit/number of ovules per flower; number of seeds per fruit (total mature seeds per fruit); seed weight (mg); germination rate (%) and seed germination speed (T50: indicates the number of days until 50% of the seeds germinate; Cogoni *et al.* 2012). To obtain data on the germination rate and T50, 20 seeds from five capsules were sown per treatment on the surface of 1%agar water in 60 mm plastic Petri dishes, incubated at a constant temperature (15◦C), irradiated 12 h per day (test chamber SanyoMLR-351 equipped with white fluorescent lamps—FL40SS.W/3770–10 μmol $m^{-2}s^{-1}$), according to the germination requirements for this species (Cogoni *et al.* 2012). Germination was scored daily for 30 days, and the germinated seeds were removed.

1.2.4 Seedlings' emergence and growth

From the geitonogamy treatments we obtained 145 seeds from15 plants and 16 different fruits and from xenogamy treatments 150 seeds from 25 plants and 29 different fruits. According to Cogoni *et al.* (2012) they were sown in square pots of 7l filled with universal substrate and sand (70:30) at a maximum burial depth of 3 cm. The seedlings obtained, 57 from geitonogamy (from 15 fruits of 14 plants) and 53 from xenogamy (from 26 fruits of 20 plants) treatments were monitored over 30 days, and the following morphometric parameters were measured daily with digital calipers (Top Cal 150 PW): stem height, number of leaves and length of the longest leaf. All these parameters were considered in order to calculate the relative growth rate at day 30 (RGR30) following the formula in Mustajärvi *et al.* (2005), modified by Gargano *et al.* (2011):

$$\mathrm{RGR} = \frac{\left[\Sigma\left(\frac{Sh+nL+lL}{t_i}\right)\right]}{N}$$

where Sh = stem height, nL = number of leaves, lL = length of longest leave, ti= time (in days) from the beginning to the end of the experiment, and N = number of measurements.

1.2.5 Inbreeding depression

The inbreeding depression value was calculated using eight different plant parameters, from fruit production to seedling growth: fruit set, seeds per fruit, seed set, seed weight, germination rate,

T50, number of days of first leaf appearance and RGR30 of seedlings. The magnitude of inbreeding depression (δ) for the different traits was calculated according to Charlesworth and Charlesworth (1987), following the formula:

$$\delta = 1 - \left(\frac{W_s}{W_o}\right)$$

where Ws and Wo are mean trait values of geitonogamous hand-pollinations and xenogamous hand pollination, respectively. The values of inbreeding depression range from −1 to 1, whereas zero indicates the absence of inbreeding depression, positive values that the outcrossed offspring outperform the selfed offspring (inbreeding depression) and negative values an opposite trend (Charlesworth and Charlesworth 1987).

Finally, the cumulative value of inbreeding depression with the relationship among the data of the eight parameters was calculated according to Husband and Schemske (1996) following this formula adapted to our work:

$$\delta = 1 - \left[\left(\frac{W_{sf}}{W_{of}}\right) \times \left(\frac{W_{ssf}}{W_{osf}}\right) \times \left(\frac{W_{ss}}{W_{os}}\right) \times \left(\frac{W_{ssw}}{W_{osw}}\right) \times \left(\frac{W_{ss\%}}{W_{os\%}}\right) \times \left(\frac{W_{sT50}}{W_{oT50}}\right) \times \left(\frac{W_{sRGR}}{W_{oRGR}}\right) \times \left(\frac{W_{sla}}{W_{ola}}\right)\right]$$

where *f* = fruit set values, *sf* = seeds per fruit, *s* = seed set, *sw* = seed weight, *s%* = germination percentage, *T50* = value of T50, *RGR* = value of RGR30 and *la* = number days of first leave appearance.

1.2.6 Statistical analysis

The effect of pollination experiments (geitonogamy/xenogamy) on our response variables was tested by fitting generalized linear mixed models (GLMMs). GLMMs provides a flexible way to model traits which do not satisfy the assumptions of a standard linear model, allowing the distinction between fixed and random factors in the model. Their use is justified by the non-normal distribution of dependent variables under consideration and for the inclusion of random sources of variation (Littell *et al.* 1996). Accordingly, to test the effects of the pollination experiments and mother effect (natural plant precedence) we used GLMMs (Bolker *et al.* 2009). In the analysis, treatment and mother was considered as the fixed and random factors, respectively. All analyses were conducted using R3.2.2 (R Core Team 2015) by using the *glmm* package (Knudson 2015) and binomial or Poisson error distributions with logit or log link functions.

1.3 Results

1.3.1 *Ex situ* phenology and floral biology

The flowering period started on the 8th of May and finished on19thof July (lasting 72 days; N = 82 flowered plants). The flowers were clustered on flowering stems, and an average (±S.E.) of 11.49 ± 6.13 (ranging from 1 to 32) stems and 2.72 ± 0.84 (ranging from 1 to 11) flowers on each stem were observed. The *ex situ* cultivated plants presented high values of synchrony (mean of 0.81 ± 0.16).

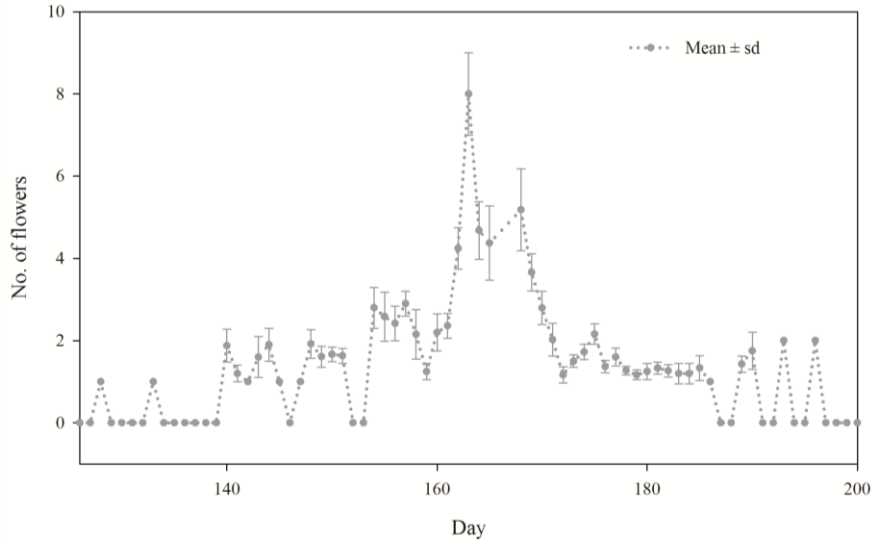

Figure 2. Flowering phenology (number of open flowers per day) of *D. morisianus* plants cultivated at the Botanical Gardens. The X-axis indicates the day of the year from 8 May (day number 128) to 19 July (day number 200).

The mean flowering duration per plant was 38.47 ± 8.76 days (mean ± S.E.), ranging from 18 to 62. A plant produced 32.59 ± 21.97 flowers, ranging from 5 to 94. The maximum flowering peak occurred between the 11th (162th day of the year) and 20th of June (171th day of the year), 34–43 days after the beginning of flowering (Fig. 2). On these days, 60% of the flowers bloomed. The mean flower lifespan of hermaphroditic flowers was 7.5 days (from stage c to l; Fig. 3). On the following days, the petals were withered, covering the stamens and the stigma, and then floral parts began to fall, with the exception of the ovary and calyx. Each hermaphroditic flower had first a male and then a female phase. Nine different phenological stages, from bud development to fruiting, were differentiated (Fig. 3). The male phase, which lasted three days, was divided into three different stages. When 10 stamens were no longer upright, the female phase (three to six days) started. Stage h in Fig. 3 is the moment at which the flower presented maximum receptivity to pollen. When the flowers were pollinated, petals closed wrapping styles; the last stage was fruiting

and lasted 45.17 ± 5.21 days from flower withering to ripe fruits. Plants produced few female flowers, the lifespan of which was not studied.

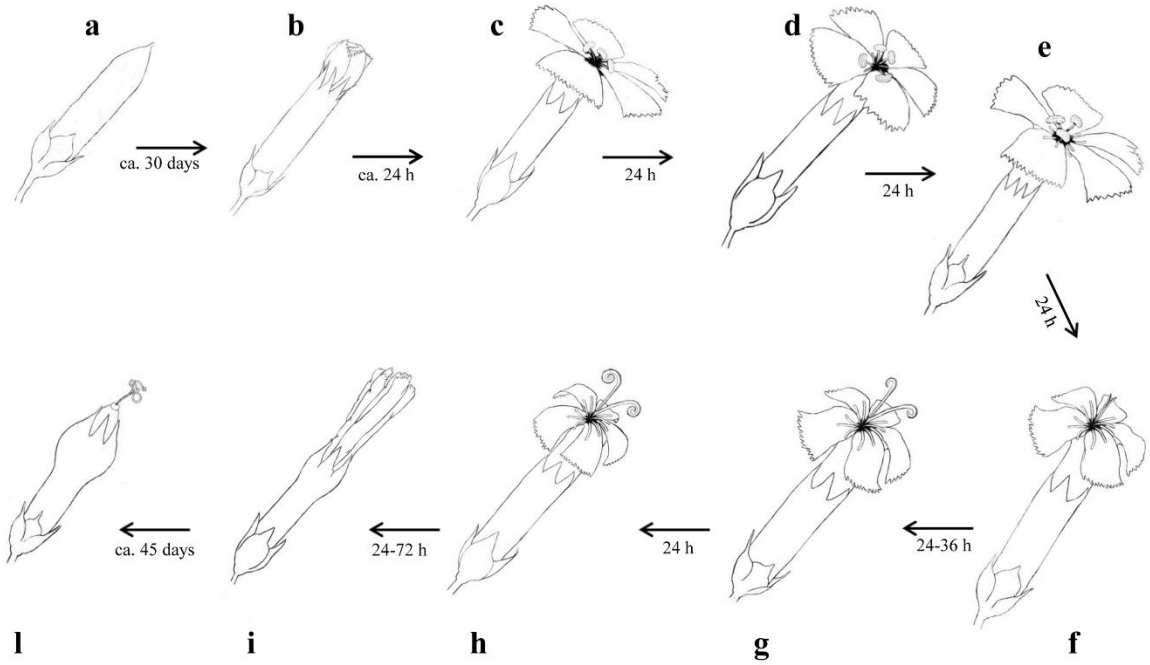

Figure 3- Different stages during the lifespan of a hermaphroditic flower of *D. morisianus*, from bud formation to fruit production. Stage a: bud development; stage b: bud; stages c–e: male stages, when the stamens grow in three different whorl, each stage takes around 24 h; stages f–h: female stages, when the anthers have already fallen and the styles begin to elongate, stage f sometimes takes 36 h and g and h around 24 h; stage i: after pollination of the flower, the time depends on the moment of pollination

1.3.2 Breeding system

The average number of pollen grains and ovule per flower was 27,920 ± 4,916 and 97.14 ± 16.67, respectively. The P/O ratio was 287.42, and, according to Cruden's category (Cruden 1977), the breeding system of this species belongs to the facultative xenogamy category.

Both pollination treatments formed ripe fruits. Only fruit set and first leaf appearance differed ($P < 0.05$) between the treatments applied (Table 1). The mother effect was significant for number of seeds and first leaf appearance (Table 1).

Table 1. Effects of different pollination treatments (geitonogamous/xenogamous) on several traits of *Dianthus morisianus* measured on 82 flowered plants cultivated under common garden conditions. The effects of pollination treatment (fixed factor) and mother (random factor) were tested by implementing GLMMs.

	Geitonogamy	**Xenogamy**					
	Mean ± SD	*Mean ± SD*		*Estimate ± SE*	*Z value*	*P*	*d.f.*
Fruit set (%)	81.25	97.84	Mother	0.116 ± 0.071	1.624	> 0.05	167
			Treatment	-1.960 ± 0.051	-2.828	**< 0.05**	
Seed/fruit (No.)	42.12±3.67	48.07±1.69	Mother	1.649 ± 0.549	3	**< 0.05**	136
			Treatment	-0.034 ± 0.034	-1.004	> 0.05	
First leaf appearance	40.59±5.63	37.13±3.48	Mother	1.341 ± 0.489	2.739	**< 0.05**	81
(days)			Treatment	-0.245 ± 0.051	-4.758	**< 0.05**	
Seed germination speed	5.83±0.88	3.95±0.40	Mother	0.016 ± 0.013	1.249	> 0.05	9
(T50, days)			Treatment	0.421 ± 0.296	1.419	> 0.05	
Seed set (%)	44.13±3.48	48.66±1.79	Mother	1.306 ± 0.435	3	> 0.05	146
			Treatment	-0.367 ± 0.030	-10.830	> 0.05	
Seed weight (mg)	11.50±3.6	12.60±3.80	Mother	0.555 ± 0.185	3	> 0.05	138
			Treatment	0.291 ± 0.663	4.392	> 0.05	
Germination rate (%)	93.89±4.84	95.00±3.16	Mother	2.349 ± 1.356	1.732	> 0.05	9
			Treatment	-0.021 ± 0.065	-0.325	> 0.05	
Relative Growth Rate	0.47±0.11	0.46±0.11	Mother	1.300 ± 0.475	2.739	> 0.05	85
(RGR30; mm × $days^{-1}$)			Treatment	0.321 ± 0.031	10.156	> 0.05	

1.3.3 Inbreeding depression

The level of inbreeding depression was low for fruit set, seeds per fruit, seed set, seed weight, germination rate, relative growth rate and first leaf appearance, but quite high for seed germination speed (Table 2). The cumulative level of inbreeding depression (δ_c) was 0.002 in the stages of the reproductive cycle analyzed.

Table 2. Coefficients of inbreeding depression (δ) in different traits of *Dianthus morisianus*.

Plant traits	**δ**
Fruit set (%)	-0.17
Seeds/fruit (No.)	0.12
Seed set (%)	0.09
Seed weight (mg)	0.08
Germination rate (%)	0.01
Seed germination speed (T_{50}; days)	-0.50
Relative Growth Rate (RGR; mm×$days^{-1}$)	-0.02
First leaf appearance (days)	-0.09

1.4 Discussion

The study carried out on *ex situ* cultivated plants highlighted that *D. morisianus* present inbreeding depression only on the seed germination speed (T50) parameter. Inbreeding depression is a strong evolutionary force that is able to modulate changes in angiosperms' mating system to effectively attract pollinators while reducing selfing (Barret 1998, 2003). It may negatively affect plant demography and thus poses a threat to the conservation of endangered species (Menges 1991; Oostermeijer 2000). However, in our case this did not seem to have a strong impact, as the levels of inbreeding depression were low in the studied plants, at least in the stages evaluated. Although a recent meta-analysis links the magnitude of inbreeding depression more to plant population size than to species-specific traits (Angeloni *et al.* 2011), previous studies have suggested that plant species which typically outcross tend to have greater inbreeding depression than species that typically self (Husband and Schemske 1996). The magnitude of inbreeding depression may also vary across a plant's life-cycle stages (Husband and Schemske 1996; Angeloni *et al.* 2011). In the plants of *D. morisianus* cultivated *ex situ*, the average level of inbreeding depression for all stages studied was lower than the average level found in predominantly out-crossing species (Husband and Schemske 1996). Inbreeding depression in *D. morisianus* was detected only in seed germination speed, a stage of the early life cycle. This result differs from the results reported by Husband and Schemske (1996), which indicate that predominantly outcrossing species present inbreeding depression early and late in the life cycle.

Husband and Schemske (1996) indicate that while inbreeding depression occurs more often during growth and seed set in angiosperms, inbreeding depression is not uncommon at the germination level, as presented by our studied plants. Our plants presented a low value of the cumulative inbreeding depression, which benefits the presence of a high selfing rate.

Phenology is affected by the environment in which plants grow (e.g., Dart and Eckert 2013; Cogoni *et al.* 2015). Thus, our data should be treated with caution due to the fact that environmental conditions and temperatures in natural populations could be different. In *D. morisianus*, the plants present a high degree of flowering synchrony and asynchronous proterandry within individuals; hence, this plant presents several flowering stages at the same time, and the proterandry itself unlikely guarantees outcrossing (de Jong *et al.* 1993; Perglová *et al.* 2006). Due to the flowering synchrony, each plant can exchange genes with most plants of the population, increasing the genetic diversity of the same population (Augspurger 1981; Ollerton and Lack 1998; Martínez-Sánchez *et al.* 2011). The high degree of flowering synchrony may be related to attracting pollinators or simply because the plants live in a very homogeneous habitat in terms of ecological conditions (Thompson

1980). In exogamous species with asynchronous flowering phenology, those individuals that are highly asynchronous with respect to the population mode show a remarkable reduction in reproductive fitness (Augspurger 1981; Ollerton and Lack 1998). Furthermore, an asynchronous flowering can cause reproductive isolation among individuals or groups of individuals that flower differently within the population's flowering time (Tarayre *et al.* 2007), because cross pollination is only possible between synchronously flowering individuals (Hendry and Day 2005).

Flower longevity is a key characteristic for plant reproduction, and it is determined through a trade-off between the cost to maintain the flower open and the pollen dispersal and receipt rates (i.e., male and female functions; Ashman and Schoen 1994). Flowers of *D. morisianus* were open for approximately eight days, and thus can be considered long-lived (Primack 1985; Mejías *et al.* 2002). This could be an advantage for the species because a long lifespan enhances the probability of receiving a visit by pollinators (Ashman and Schoen 1994). However, in the natural conditions, the longevity of flowers may be lower if abundance of pollinators is high, since flower senescence occurs quickly after pollination.

Our results provide the first description of the reproductive sys-tem of *D. morisianus*. The species shows high reproductive output for both geitonogamous and xenogamous treatments. Although *D. morisianus* produces only around half of its potential seeds, it has a high fruit set, seeds present a high rate of germination and germinate quickly for the two types of pollination treatments. These results demonstrate that *D. morisianus* is a self-compatible species. Nevertheless, negative effects of geitonogamy were found for fruit set and the appearance of the first leaf according to the origin of the pollen. While the lower production of fruits could be problematic because it reduces the reproductive assurance of the species, the number of days seedlings take for the appearance of the first leaf does not directly affect reproductive assurance, and thus should not be a problem for the rareness of our plant species.

Since *D. morisianus* is self-compatible and an individual presents numerous open flowers of different flower stages at the same time, it is likely that geitonogamy frequently occurs in natural situations (Carrió *et al.* 2008; Jorge *et al.* 2015). Self-compatibility is an adaptive strategy of reproduction for many narrow endemic species that might otherwise be particularly vulnerable to inconsistent availability of pollinators (Karron 1989). Other *Dianthus* are also proterandrous and self-compatible; however, the level of inbreeding depression and the time of occurrence during the life cycle vary from species to species (Collin and Shykoff 2003; Gargano *et al.* 2009).

Comparing our data on P/O with other data of related plants (Jürgens *et al.* 2002), *D. morisianus* presents similar values of pollen grains and ovules as *Dianthus deltoides* L. Conversely, taxonomically related *D. sylvestris* presents a higher value of P/O (450 ± 100). Following the conservative indicator of Cruden (1977), *D. morisianus* presents a facultative xenogamous breeding system. The most recent review on mixed mating systems estimated that 42% of flowering plants have this type of breeding system (Goodwillie *et al.* 2005). Plants with this type of mating are considered to have an advantage over plants with pure mating strategies, as they com-bine the possibility of outcrossing while guaranteeing reproduction in the absence of pollinators or potential mates (Goodwillie *et al.* 2005). The mechanisms that drive the mixed-mating strategy are still poorly understood (Jorge *et al.* 2015).

1.5 Conclusions

From a conservation viewpoint, threats to narrow endemic species, such as *D. morisianus*, could generally arise from their genetic background (Ellstrand and Elam 1993; Angeloni *et al.* 2011), limited reproduction (Alonso *et al.* 2010) or a combination of both factors. Further studies on the natural population of *D. morisianus* (*in situ* experiments) focusing on ecological requirements, mating system, inbreeding depression in later stages, pollinators and pollen limitation are required to better understand the status of the species and to plan conservation strategies.

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CHAPTER 2

IN SITU PHENOLOGY STUDIES AND BREEDING SYSTEM OF *Dianthus morisianus* Vals. (CARYOPHYLLACEAE)

2 Chapter II. *In situ* phenology studies and breeding system of *Dianthus morisianus* Vals. (Caryophyllaceae)

2.1 Introduction

Narrow endemic plant populations are often small and fragmented and are therefore subjected to a higher extinction risk (Cowling *et al.* 1996) due to habitat destruction, inbreeding depression (Karron 1997; Keller and Waller 2002) and/or pollinator decline (Kearns *et al.* 1993; Aguilar *et al.* 2006).

The phenological characteristics (the date of flowering initiation, flowering duration, synchrony and intensity) of a species determine the periods of flower availability to pollinators and thus the reproductive success of the studied species. High flower intensity and synchrony can attract more pollinators, therefore increasing the pollination rate (Albert *et al.* 2001; Buide *et al.* 2002). At the level of the individual plant, high intensity of flowering in self-compatible species can increase the geitonogamy pollination (Harder and Barrett 1995; Eckert 2000). These phenologic traits are influenced by both biotic and abiotic factors (Sola and Ehrlén 2007), and in Mediterranean environments, where climatic conditions are seasonal, species are highly dependent on these conditions (Aizen 2003). The most important climatic factors are temperature, precipitation and photoperiod (Arroyo 1990). These factors are particularly important for pollinator-dependent plant species (Rathcke and Lacey 1985). The study of the mating system is fundamental in order to understand these effects, especially on endemic and threatened plants. The knowledge of the mating system of a species can also contribute to understand the biotic threats (Schemske *et al.* 1994), determine to what extent it depends on the pollinators and which is the survivorship capacity of the species in specific environments (Schemscke *et al.* 1994; Fisogni *et al.* 2011). As indicated above narrow endemic plant populations are often affected by pollinator decline and this could cause pollen limitation. The consequences of pollen limitation (poor quality or quantity of pollen) have been largely investigated in the last years (e.g. Ashman *et al.* 2004; Lázaro and Traveset 2006; Andersson *et al.* 2016). Pollen limitation is often associated to low pollinator abundance (Gómez *et al.* 2010): this can be an important cause of reduced reproductive success in fragmented and small populations of pollinator-dependent plants (Aguilar *et al.* 2006). Pollen limitation can reduce population viability (Ashman *et al.* 2004; Knight *et al.* 2005; Fernández *et al.* 2012) and lead to selection on plant mating system and floral traits (Ashman and Morgan 2004). The effect of pollen

limitation may be reduced by traits enabling selfing (Larson and Barret 2000) being more easily detected in highly outcrossed or self-incompatible species (Neel 2002).

The inbreeding depression is another of the threats for narrow endemic species (Karron 1997; Keller and Waller 2002). Inbreeding depression is the decrease of the fitness of the inbred offspring relative to the outbred one (Charlesworth and Charlesworth 1987; Husband and Schemske 1996). The mating system, the degree of fragmentation, the size of the population and the environmental conditions are some of the factors that can influence the level of inbreeding depression (Angeloni *et al.* 2011). Small population size is usually correlated with high values of inbreeding depression and can also vary at the different stages of the life cycle, making it interesting to study both early and late stages of a particular plant (Husband and Schemske 1996). Husband and Schemske (1996) predicted that there are different levels of purging among early and late stages and that they vary depending on the breeding system. Outcrosses usually present inbreeding depression during both early and late stages of the species' life cycle, while selfers only in late stages. The level of inbreeding depression affects the efficiency of selfing on mixed mating systems (Lande and Schemscke 1985; Keller and Waller 2002). Species that have historically been outcrossed usually present higher values of inbreeding depression than selfing species, which present high level of selfing (Lande and Schemske 1985) and can reduce the level of inbreeding depression by purging deleterious alleles (Charlesworth *et al.* 1990).

D. morisianus is a psamophilous narrow endemic species which grows only in one natural population on established sand dunes in Portixeddu (south-western Sardinia; Bacchetta *et al.* 2010). Its natural habitat has been strongly modified by human activities, causing habitat loss and fragmentation (Cogoni *et al.* 2013). The small population size and the limited seedling recruitment make *D. morisianus* potentially prone to extinction and, for this reason, this plant is considered as one of the most threatened of the island (Bacchetta *et al.* 2012) and it has been categorized as Critically Endangered on the IUCN Global Red Lists (Fenu *et al.* 2013). In order to reduce the extinction risk, two experimental translocation programs were realized. The first reintroduction was carried out in a protected and fenced site, while the second one was performed in an open and unprotected site. Both these sites are located near the natural population (Cogoni *et al.* 2013; Fenu *et al.* 2016).

Different characteristics of *D. morisianus* were investigated in previous studies; namely, the life history and demographic features of the population (Cogoni 2011), the germination ability in natural and controlled conditions (Cogoni *et al.* 2012) and some characteristics of the breeding

system of the species in cultivated plants (Nebot *et al.* 2016 [Chapter 1]). However, the degree of dependence from pollination vectors and ability to spontaneous self-pollination, the degree of limitation of reproductive success due to pollinators resources and the ability and degree of abiotic pollination have never been investigated. As explained above, the phenology, and thus the reproductive success of plant species, is influenced by biotic and abiotic factors. The aim of this study was to deepen the knowledge of the reproductive system and mating system of *D. morisianus* in order to identify the possible biological factors that threaten the species. In particular the aims of this study were: 1) to investigate the flower phenology; 2) to gain information on the breeding system and related indexes; 3) to evaluate the reproductive success; 4) to assess the germination capacity and survivorship of the seedlings; and 5) to estimate the level of inbreeding depression in the natural population.

2.2 Material and methods

2.2.1 Study species

Dianthus morisianus Vals. (Caryophyllaceae) is a perennial plant characterized by numerous woody stocks, erect stems and a basal rosette with thin and linear leaves. The stems bear terminal multi-flowered heads; lanceolate teeth characterize the calyx and the colour of the corolla is pink. Petals present 6–8 teeth, rounded and irregularly lobed. Anthers measure 4.5 mm long, the ovary 7.5 mm long and style and stigma 14 mm long (Bacchetta *et al.* 2010). The flowering season is from early May to late June and ripe fruits can be found during June–July (Bacchetta *et al.* 2010). The species is characterized by proterandry and mixed mating system and is a gynomonoecious plant (Nebot *et al.* 2016 [Chapter 1]) with both hermaphroditic and, female flowers, the latter being found at a low frequency (9.19% of the cultivated plants presented some female flowers). Seedling emergence represents the most critical stage for the long-term persistence of *D. morisianus*, while the lack of a persistent soil seed bank constitutes a hazard to the persistence of the natural population (Cogoni *et al.* 2012).

2.2.2 Experimental design

This study was conducted during two consecutive flowering seasons (spring 2014 and 2015) on the only natural population of *D. morisianus*. The reproductive experiments detailed in this manuscript were performed on individual plants that were separate more than five meters apart from one another. In each experiment, plants were randomly selected (20 in 2014 and 40 in 2015) and tagged prior to flowering. In 2014, some more plants were selected in the first week of study because animals had eaten numerous flowering stems belonging to tagged plants. In 2015, selected

plants were protected from cattle and natural herbivores immediately after the pollination treatments.

2.2.3 Phenology

Plants were monitored daily during the flowering season in 2014 (from the 7th of May to the 23th of June) and 2015 (from the 15th of May to the 10th of June) by recording the number of open flowers. In order to gain information on the flowering phenology, the calyxes of freshly opened flowers were labelled each day with a univocal code. For each plant, the following parameters were calculated: intensity (maximum number of simultaneously open flowers; Albert *et al.* 2001), flowering duration (individual and total), maximum flowering moment (*i.e.*, the number of days between the day when the first flower in the population opens and the day when the maximum peak is recorded; Bishop and Schemske 1998), and flowering synchrony (*i.e.*, the number of days during which the flowering of an individual overlaps with the flowering of every other plant) following the formula proposed by Augspurger (1981):

$$S_i = [1/(n-1)](1/f_i)\sum e_{j \neq i}$$

where *n*: number of study plants, *ej*: number of days during which both individuals *i* and *j* flower synchronously, *fi*: number if days during which individual *i* is in flower. The synchrony index values vary from 0 to 1: 0 means that there is no synchrony, 1 means that the plants flower at the same time. The level of synchrony in the population (Z) was analysed using the following formula:

$$Z = \frac{1}{n}\sum_{i}^{n} S_i$$

2.2.4 Breeding system

To better understand the breeding system of the species, six types of pollination treatments were carried out (Table 1). To analyse the ability of autonomous self-pollination, unmanipulated flowers were bagged with a fine mesh bag prior to anthesis in order to prevent pollination. To test the geitonogamy and xenogamy treatments, flowers were bagged with a fine mesh bags prior to anthesis, emasculated and, when stigma were receptive, they were hand-pollinated. The flowers used to verify the geitonogamy ability were pollinated with pollen coming from a flower of the same plant; while those used to test the xenogamy were pollinated with pollen coming from a flower which was located more than five meters away from the pollinated plant in order to avoid parental relationship. To analyse the presence of pollen limitation in the population, supplemental

pollination experiments were conducted, which consisted of carrying out supplemental manual pollination on flowers which were open pollinated. In addition, the capacity of wind pollination was tested by the anemophily test: these flowers were bagged with a 0.5 mm mesh bags, thus excluding floral visitors, allowing the passage of airborne pollen and the wind which could deposit the own pollen on the stigma. These flowers were not emasculate. Finally, the control treatment allowed us to compare the different pollination treatments with the reproductive success in nature. We initially tried to conduct all the pollination treatments on all the studied plants; however, it was not always possible. During the 2015 study, 20 plants located more than five meters away from the plants subjected to a pollinator exclusion treatment were selected to verify if the presence of bags was biasing the result of the control fruits. During the fruiting period, bags were left on all the treated fruits in order to make sure that seeds were not dispersed when fruits ripened.

Table 1. Number of flowers and plants used to test the different pollination treatments in each year of study.

	2014		2015	
	Replicates	N° plants	Replicates	N° plants
Autonomous self-pollination	18	11	35	35
Geitonogamy	21	16	37	36
Xenogamy	27	16	43	38
Supplementary pollination	6	6	37	25
Anemophily	19	12	17	16
Control	9	9	46	32

All treated capsules were harvested when ripe and individually conserved in paper envelopes to analyse the reproductive success of each treatment. Fruits were stored in a dry room at 15°C and 15% relative humidity (RH) until they were analysed. In the laboratory, the number of unfertilized ovules, aborted seeds and mature seeds were counted. After that, ripe seeds were weighted (mg).

The reproductive success was analysed by measuring the fruit set (percentage fruits / treated flowers), the seed/ovule ratio (mature seeds per fruit / number of ovule per flower), and mean weight of 10 seeds. The obtained data allowed us to calculate different indexes related to the breeding system, *i.e.*, the Self Fertility Index (SFI) and the Self-compatibility Index (SCI) (Lloyd and Schoen 1992); the seed/ovule ratio was used instead of the fruit set to carry out the calculations. The SFI index, which indicates the ability of plants to produce seeds in the absence of pollinators, was calculated using the seed/ovule ratio of the autonomous self-pollination and the seed/ovule ratio of the xenogamy treatments. The SCI index, which indicates the level of self–compatibility of the species, was intended as the ratio between the seed/ovule ratio of the geitonogamy treatment and that of the xenogamy treatment. Values under 0.5 indicate partial self-fertility or self-

incompatibility, 0.5 indicates complete self-fertility or self-compatibility and values above 0.5 indicate preferentially self-fertile or self-compatible individuals (Wani *et al.* 2015). The Selfing rate at the population level (S) was also calculated following Charlesworth and Charlesworth (1987) using the following formula:

$$S = \frac{(P_x - P_0)}{(P_x - P_s)}$$

where P_x is the reproductive performance after xenogamy, P_s after geitonogamy (Aizen and Basilio 1995), and Po after the control treatment (Charlesworth and Charlesworth 1987).

The supplemental and the control treatments were used to analyse the effect of increased pollen quantity on the styles by calculating the pollen limitation index (PL) at the population level:

$$PL = 1 - \left(\frac{P_0}{P_s}\right)$$

where P_o is the seed/ovule ratio of the control treatment and P_s is the seed/ovule ratio of the supplemental hand pollinations (Larson and Barret 2000). 0 or negative values indicate absence of pollen limitation and 1 pollen limitation (Becker *et al.* 2011).

2.2.5 Germination experiments in the laboratory

In the laboratory were tested the seeds obtained from the breeding system experiment carried out in 2014. Seeds were sown on 60 mm Petri dishes and placed inside a germinating chamber (Sanyo MLR-351 equipped with white fluorescent lamps—FL40SS.W/3770–10 μmol $m^{-2}s^{-1}$) at the optimal germination temperature of 15°C, 12h light/12h dark (Cogoni *et al.* 2012). Four replicates from each pollination treatment were tested; however, the number of seeds per replicate varied depending on the treatment due to the different number of resulting seeds: 25 seeds per replicate were used in the xenogamy and the geitonogamy treatment, 10 seeds were used in the anemophily and supplemental treatments and 16 in the control. The autonomous self-pollination treatment produced a low number of seeds, which was not enough to perform germination tests. Petri dishes were observed daily for a month to analyse the germination rate and the T50 (time to 50% of the seeds have germinated). After the germination rate analysis, some seedlings were sown in small pots with universal substrate in order to calculate the relative growth rate at the 30th day (RGR30) and the rate of survivorship. Seedlings were measured weekly for one month following Nebot *et al.* (2016) [Chapter 1]. 84 days after the first germination, seedlings were removed from the substrate to calculate the total seedling weight and the shoot and root weight. The root and shoot seedlings

were then transferred in an oven at 80°C during 48 hours (Cordazzo 2002), after which they were weighted again to calculate the dry weight. The data of the supplemental, the anemophily and the autonomous self-pollination treatments were not analysed in this experiment due to the absence of a sufficient number of survived seedlings.

2.2.6 Germination experiments in the field

Seeds obtained in the breeding system experiment during 2015 were brought back to the natural population and sown in the field. For each pollination treatment, three replicates of 10 seeds were sown by placing them in biodegradable peat pots, which were buried in the sand; each seed was planted at a depth of around 0.5-1 cm, as detailed in Cogoni *et al.* (2012). Treatments were distributed equally on each replicate. The sand used for the experiment was previously sieved in order to avoid the presence of other seeds that could alter the results. This experiment was conducted in the study area but around 10 meters away from the natural plants of *D. morisianus* in order to prevent the arrival of seeds from other individuals. The replicates were positioned in different places to include the environmental variability. This experiment started the 8th of September and was monitored monthly during 7 months from the beginning, the last day corresponded to six months after the first germination had been recorded.

2.2.7 Inbreeding depression

The mean value of inbreeding depression (δ) was analysed considering the following parameters: fruit set, seed/ovule ratio, seed weight, germination rate in the field and survivorship in the field. To analyse the level of inbreeding depression we used the formula proposed by Charlesworth and Charlesworth (1987) $\delta = 1- (Ws/Wo)$, where *Ws* is the mean production resulting from the geitonogamy pollination and *Wo* that from the xenogamy pollination. We also calculated the cumulative value of inbreeding depression on the fitness ratio of these five reproductive traits. The formula was modified by incorporating the analyses of the germination rate and the survivorship in the field using the formula $\delta_c = [(w_{sa}/w_{oa})\times(w_{sb}/w_{ob})\times(w_{sc}/w_{oc})\times(w_{sd}/w_{od})\times(w_{se}/w_{oe})]$, as proposed by Husband and Schemsche (1996). Values of inbreeding depression vary from -1 to +1, where 0 indicates absence of inbreeding depression (the outcrossed progeny is fitter than the selfed one), +1 high inbreeding depression and -1 high outbreeding depression (the selfed progeny is fitter than the crossed one).

2.2.8 Statistical analyses

Phenology (number of flowering stems, number of flowers per stem and the duration of flowering) and germination data (germination rate, T50, and survivorship) were compared by

General Linear Models (GLM) using the function *glm()*. We used the Gaussian family for normal distributed data, the Poisson family for counts with non-normal distributed data, and binomial for proportions. We then analysed the correlation among the different phenology parameters and among the germination results using Pearson correlations for normal distributed data. We used General Linear Mixed Models (GLMMs) in order to compare the results of the reproductive success in the different pollination treatments (fixed factor) between the two years of study, considering fruit set, seed/ovule ratio, aborted seeds, and seed weight as response variables and year and individual as random factors. In order compare the results of germination rate in the natural population and in optimal conditions, we conducted another GLMM analysis using "place" as random factor, while treatment was the fixed factor. The package lme4 version 1.1-12 (Bates *et al.* 2015) with the function *lmer()* was used to carry out these analyses. Since the current version of *lmer* does not provide F-tests for fixed effect, we compared our model with the null model to test the significance of the fixed effect (Faraway 2005). If the ANOVA test indicates significant differences ($P < 0.05$), this means that the pollination treatment influences the analysed parameter of reproductive success. As for the *post hoc* contrasts, we used the package *lsmeans* version 2.24 (Lenth 2016) with the function *lsmeans()* to verify if there were differences among treatments. All analyses were conducted using R3.3.1 (R Core Team 2016).

2.3 Results

2.3.1 Phenology

In 2014, wild plants of *D. morisianus* started to flower in early May (7th) and finished the 23th of June, while the 2015 flowering season started the 13th of May and lasted until the 10th of June (Fig. 1). The studied plants produced 5.8 ± 4.95 (mean ± sd) flowering stems, ranging from 1 to 29; and flowering stems bore a mean of 2.59 ± 0.85 flowers. There were not significant differences between years neither in the number of stems nor in the number of flowers per stem ($P > 0.05$). The flower intensity showed a mean of 4.76 ± 3.6 (from 1 to 15) simultaneous open flowers during 2014 and 4.95 ± 2.7 (from 2 to 13) during 2015. The maximum flowering moment occurred between the 24th of May and the 7th of June (18 and 32 days after flowering started, respectively) in 2014 and between the 25th of May and the 2nd of June (13 and 21 days after flowering started) in 2015. In 2014, the mean duration of flowering per plant varied from 7 to 36 days, with a mean of 24.54 ± 7.13 days, while in 2015 it varied from 9 to 27 with a mean of 18.29 ± 4.72 days, with significant differences ($P < 0.05$). The studied plants flowered for 48 days in 2014 and 29 days in 2015. Even if the flowering duration was reduced on 19 days, the maximum flowering moment occurred during the same period (Fig. 1). Flowers had a lifespan of around 7-8 days. The studied plants in the

natural population of *D. morisianus* showed a synchrony of 0.63 ± 0.13 per plant and 0.62 at population level during 2014, and 0.81 ± 0.08 per plant and 0.83 at population level during 2015. The correlation analyses between the flowering starting date and the duration of flowering indicated that plants that flowered earlier in 2015 had a longer flowering period than those that flowered later ($P < 0.05$), but the same was not observed in the plants studied in 2014 ($P = 0.05$). In addition, plants with long flowering period had more flowers than plants with short flowering period ($P < 0.05$, Table 2). In 2014 8.33% of plants presented some female flowers and five plants were partial or completed eaten and lost between the 14.28% to 100% of the floral stems. In 2015 no one of the studied plants presented female flowers and we were able to protect our plants and no one was eaten.

Table 2. Pearson rank correlation coefficients between the flowering starting date and the flowering duration and between the flower number and the flowering duration. Results were studied separately for each year. Values in bold indicate significant differences.

	Start/duration		Flowers/duration	
Year	2014	2015	2014	2015
Pearson rho	-0.41	-0.73	0.43	0.49
P	0.05	**<0.01**	**<0.05**	**<0.01**

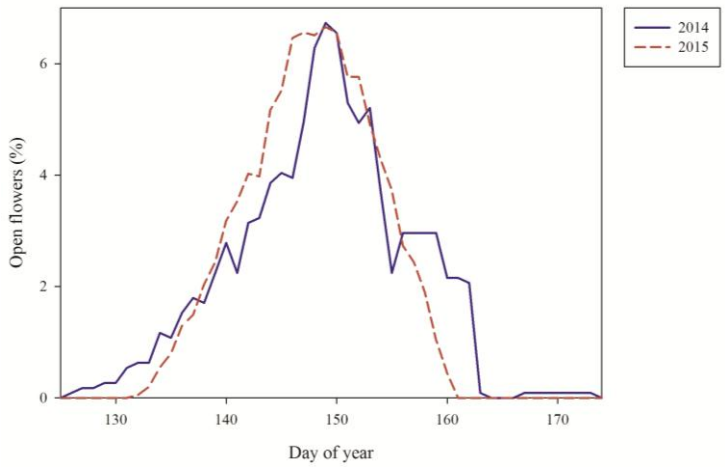

Figure 1. Flowering phenology (number of open flowers per day) of *D. morisianus*. The x-axis indicates the day of the year from 5 May (day number 125) to 23 June (day number 174).

2.3.2 Breeding system

All pollination treatments produced fruits but the reproductive success varied among pollination treatments. The GLMMs showed that there were not significant differences neither between years nor between individuals (Table 3). Anemophily and autonomous self-pollination showed significant differences on seed/ovule ratio with respect to the supplemental, xenogamy,

geitonogamy and control treatments (Table 4). As for the weight parameter, the geitonogamy treatment produced the lightest seeds and the anemophily treatment the heaviest (Table 4). The anemophily seeds showed significant differences with respect to the supplemental, control, xenogamy, and geitonogamy seeds, while the autonomous self-pollination seeds were significantly different from the xenogamy and geitonogamy ones (Table 4). The control fruits from untreated plants had a higher seed/ovule ratio (59.20 ± 13.70) than control fruits from treated plants, while weight did not show significant differences (10.396 ± 2.588) ($P > 0.05$).

Table 3. Effect of the different pollination treatments on the female reproductive success. Data were analysed by GLMMs. Significant effects were showed in bold.

	Factor	d.f.	Chi-square	*P*>chi-square	Random
Fruit set	Treatment	6	68.966	<**0.01**	Residual>year Residual>individual
Seed/ovule ratio	Treatment	6	52.221	<**0.01**	Residual>year Residual>individual
Aborted seeds	Treatment	6	36.993	<**0.01**	Residual>year Residual>individual
Weight	Treatment	6	24.548	<**0.01**	Residual>year Residual>individual

Table 4. Reproductive success of the studied plants during 2014 and 2015, germination rate (%), T50 and survivorship calculated in controlled conditions during 2014 and in field conditions during 2015. Different letters indicate significant differences at $P < 0.05$.

					Controlled conditions (2014)			Field experiments (2015)	
Treatment	Fruit set	Seed/ovule ratio	Aborted seeds	Seed weight (mg)	Germination rate (%)	T50	Survivorship (%)	Germination rate (%)	Survivorship
Control	100.00^{b}	50.73 ± 21.88^{cd}	19.00 ± 15.37^{ab}	10.339 ± 2.796^{b}	87.39^{a}	6.17 ± 1.77^{b}	80.00^{c}	50.00^{a}	30.77^{a}
Xenogamy	91.66^{b}	49.97 ± 22.03^{cd}	23.80 ± 15.67^{b}	9.890 ± 2.748^{ab}	97.00^{a}	4.01 ± 0.53^{a}	63.13^{bc}	80.79^{a}	57.14^{a}
Geitonogamy	91.23^{b}	44.54 ± 19.73^{bc}	25.30 ± 14.44^{b}	8.912 ± 2.568^{a}	99.00^{a}	3.87 ± 0.09^{a}	36.36^{ab}	57.69^{a}	60.00^{a}
Autonomous self-pollination	51.11^{a}	10.51 ± 8.64^{a}	15.47 ± 18.27^{a}	12.031 ± 3.112^{b}	-	-	-	50.00^{a}	38.46^{a}
Anemophily	57.14^{a}	27.25 ± 21.75^{ab}	16.15 ± 6.61^{a}	12.630 ± 3.663^{ab}	95.00^{a}	3.98 ± 0.41^{a}	21.05^{a}	76.92^{a}	35.00^{a}
Supplemental pollination	82.35^{b}	48.78 ± 21.61^{cd}	19.97 ± 11.32^{b}	10.686 ± 2.316^{ab}	90.00^{a}	5.01 ± 0.52^{ab}	39.40^{ab}	69.23^{a}	50.00^{a}
Total					94.77			64.10	46.00

In the control, xenogamy, geitonogamy and supplemental treatments, we observed that around 30% of the ovules remained always without seed formation or abortion, could be due to that ovules had not been fertilized. Our results showed that the species is self-compatible (SCI = 0.94) and self-fertile (SFI = 0.21), and the selfing rate value was -0.13. Hand pollinations on open pollinated flowers did not significantly increase the reproductive success; the PL index supported this result with a value of 0.24.

2.3.3 Germination tests

The autonomous self-pollination treatment was not analysed in 2014 due to the low number of obtained seeds. There were not significant differences among treatments in the germination rate in the laboratory (Table 4 and 5). The germination rate was not correlated with the weight of the seeds (r_s: 0.017, $P > 0.05$). The T50 varied from 3.87 to 6.17 days, being the control the treatment with the highest value (Table 4), this treatment showed significant differences with respect to geitonogamy, xenogamy and anemophily (Table 4 and 5). The T50 was not correlated neither with the survivorship rate (r_s: 0.14, $P > 0.05$) nor with the seed weight (r_s: 0.117, $P > 0.05$). The seedlings of the control treatment showed significant differences among treatments in the survival rate (Table 4 and 5). The survival rate was not correlated with the weight of the seeds (r_s: 0.016, $P > 0.05$). Since the germination ability depends on the environment, the data obtained in laboratory experiments have to be treated with caution due to the fact that the tests are carried out under optimal conditions (χ^2= 6.753, d.f. = 4, $P > 0.05$) and the random factor presented significant effect on germination (residual<place).

In the field experiment, xenogamy was the treatment with the highest germination rate (Table 4 and Fig. 3A) and geitonogamy the one with the highest survival rate (Table 4). There were not significant differences neither in the germination rate nor in the survivorship values (Table 4 and 5) among the different pollination treatments. The weight of the seeds was correlated neither with the germination rate nor with the survival time of the seedlings (r_s: 0.12, $P > 0.05$). The survivorship rate was the only parameter which was correlated with the weight of the seeds (r_s: -0.013, $P < 0.01$). Seeds germinated during October, November and December and showed the highest germination rate in November (Fig. 3A), when 55% of the seeds had germinated. December was the month with the highest mortality rate, with a value of 56.25% (Fig. 3B).

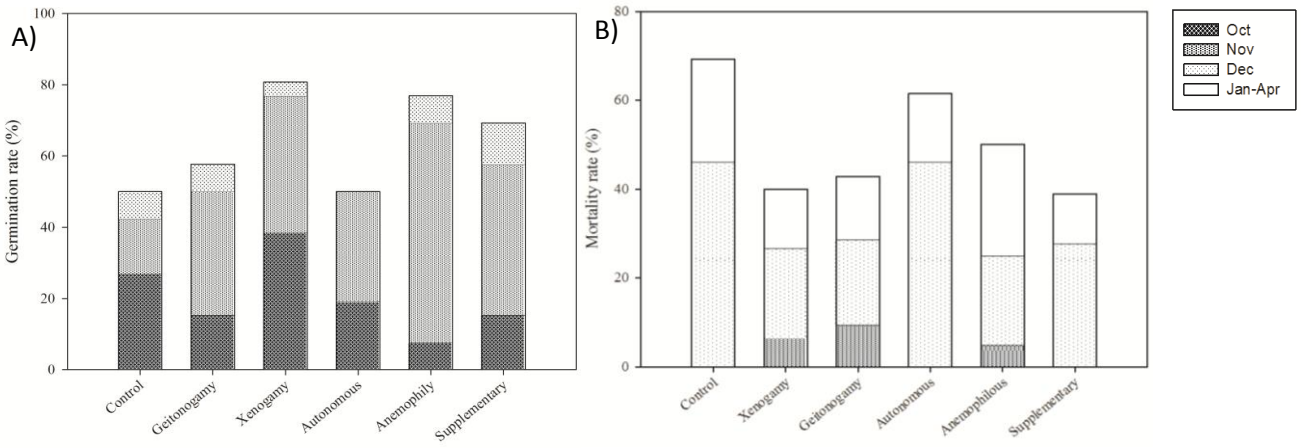

Figure 3. Results of the germination experiment in the field. A) Germination rate (%) and B) mortality rate (%) of seeds from pollination treatments. Each pattern indicate different month.

Table 5. Effect of the different pollination treatments on germination in laboratory and field conditions comparing the results with the null model. Data were analysed by GLM. Values are significant at $P < 0.05$.

		Estimate	Std. Error	Z value	*P*
Lab germination rate	Intercept	3.476	2.931	1.186	0.236
	Control	-1.666	3.265	-0.510	0.610
	Geitonogamy	0.416	4.620	0.090	0.928
	Supplementary	-1.279	3.372	-0.379	0.704
	Anemophily	-0.532	3.722	-0.143	0.886
	Autonomous	-	-	-	-
T50	Intercept	1.387	0.249	5.554	**<0.010**
	Control	0.432	0.321	1.347	0.178
	Geitonogamy	-0.034	0.356	-0.096	0.923
	Supplementary	0.224	0.335	0.670	0.503
	Anemophily	-0.007	0.354	-0.021	0.983
	Autonomous	-	-	-	-
Survival rate in the lab	Intercept	-1.322	0.398	-3.322	**<0.010**
	Control	2.708	0.605	4.472	**<0.010**
	Geitonogamy	0.762	0.506	1.505	0.132
	Xenogamy	1.861	0.521	3.572	**<0.010**
	Supplementary	0.891	0.534	1.668	0.095
	Autonomous	-	-	-	-
Field germination rate	Intercept	1.362^{-15}-	3.922^{-1}	0.000	1.000
	Control	1.528^{-15}	5.547^{-1}	0.000	1.000
	Geitonogamy	2.819^{-1}	5.575^{-1}	0.506	0.613
	Supplementary	8.001^{-1}	5.776^{-1}	1.385	0.166
	Anemophily	1.208	6.091^{-1}	1.984	**0.047**
	Xenogamy	1.450	6.354^{-1}	2.282	**0.022**
Survival rate in the field	Intercept	-0.470	0.570	-0.824	0.410
	Control	-0.341	0.828	-0.412	0.681
	Geitonogamy	0.875	0.776	1.128	0.259
	Supplementary	1.163	0.758	1.534	0.125
	Anemophily	0.470	0.725	0.649	0.517
	Xenogamy	0.758	0.721	1.051	0.293

2.3.4 Seedlings produced in the laboratory experiments

This experiment produced results only for the xenogamy, the geitonogamy and the control treatments (Table 6), while the other treatments did not produce enough seedlings. The control treatment was characterized by significant differences with respect to the geitonogamy one in RGR (Table 6 and 7), while the shoot dry weight and root dry weight did not show significant differences among treatments (Table 6 and 7). The RGR was positively correlated with the T50 (r_s: 0.38, $P < 0.01$).

Table 6. Results of the laboratory experiments (mean ± sd). Different letters indicate significant differences at $P < 0.05$.

	Shoot dry weight (mg)	Root dry weight (mg)	RGR
Control	0.036 ± 0.011^{a}	0.012 ± 0.009^{a}	0.861^{ac}
Geitonogamy	0.017 ± 0.006^{a}	0.023 ± 0.017^{a}	0.611^{b}
Xenogamy	0.026 ± 0.009^{a}	0.015 ± 0.009^{a}	0.714^{bc}
Autonomous self-pollination	-	-	-
Anemophily	-	-	-
Supplemental pollination	-	-	-

Table 7. Effect of the different pollination treatments on seedling growth in controlled conditions comparing the results with the null model. Data were analysed by GLM.

		Estimate	Std. Error	Z value	*P*
Shoot dry weight	Intercept	-3.272	1.685	-1.941	0.052
	Geitonogamy	-0.783	2.968	-0.264	0.792
	Xenogamy	-0.336	2.595	-0.130	0.897
Root dry weight	Intercept	-3.822	2.185	-1.750	0.080
	Geitonogamy	0.061	3.045	0.020	0.984
	Xenogamy	-0.367	3.402	-0.108	0.914
RGR	Intercept	0.962	0.138	6.963	**<0.010**
	Geitonogamy	-1.467	0.371	-3.958	**<0.010**
	Xenogamy	-1.303	0.305	-4.270	**<0.010**

2.3.5 Inbreeding depression

The level of inbreeding depression was 0.01 for the fruit set, 0.11 for the seed/ovule ratio, 0.12 for the seed weight, 0.29 for the germination rate in field and -0.19 for the survivorship in field. The level of cumulative inbreeding depression was 0.35, thus indicating the presence of some degree of inbreeding depression in the investigated phases of the reproductive cycle.

2.4 Discussion

In situ plants of *D. morisianus* produced around half the flowering stems with respect to the cultivated plants, but the number of flowers per stem was very similar (2.59 versus 2.72 on *ex situ* plants) (Nebot *et al.* 2016 [Chapter 1]). Nowadays there are a lot of studies about the effect of the temperatures on the flower phenology. The effects varies among studies, some of them indicate an earlier flowering with the increase of the temperatures (Anderson *et al.* 2012; Ellwood *et al.* 2013), while others demonstrate that some species delay the starting of the flowering when the temperatures are higher (Fitter and Fitter 2002; Cook *et al.* 2012). Actually, in May 2014 the flowering started when the mean temperature was lower than in 2015, flowering started before and, at the same time, the duration and the maximum flowering moment were longer than that recorded in 2015. The population also presented lower synchrony and intensity: individuals with early flowering had a longer flowering period and produced more flowers than late flowering plants; this is in accordance with Ollerton and Lack (1998) and Albert *et al.* (2008). The lifespan of flowers lasted around 7-8 days, like in *ex situ* experiments (Nebot *et al.* 2016 [Chapter 1]). In most species, flower senescence can be accelerated by pollination (Rogers 2006; Marshall *et al.* 2010) but, in our case, the lifespan required six days until the styles presented the maximum receptivity (Nebot *et al.* 2016 [chapter 1]) due to proterandry. After that, the flowers of *D. morisianus* closed rapidly (in less than 24 h) after pollination. This is a normal characteristic in carnations (O'Neill 1997). As indicated in the introduction, high levels of flower synchrony could allow the exchange of genes

among more than half of the studied plants. High synchrony and high floral display can attract more pollinators (Méndez and Díaz 2001), this is an important result due to the fact that around 80% of the pollination in this species depends on insects. *D. morisianus* presented a large floral display at the same time and, usually, each one was in a different phenologic stage. The first characteristic promotes the geitonogamous pollination, while the second one, depending on the stages might reduce the levels of geitonogamy. At the same time, the heterogeneity of stages is positive for the population, since during the days with low synchrony pollinators can carry pollen of plants which are located further away (Rathcke and Lacey 1985) and therefore promote genetic diversity.

The results of the breeding system confirmed those obtained by Nebot *et al.* (2016) [Chapter 1], who found that *D. morisianus* is a self-compatible species with a mixed mating system. According to the autonomous self-pollination test and the self-compatibility index (SCI = 0.94) *D. morisianus* is self-compatible and is able to reproduce by pollen vectors. The significant differences in the fruit set and seed/ovule ratio between the geitonogamy treatment and the autonomous self-pollination could be due to the proterandry of the species or by the age of the pollen, due to for the manual pollination was applied young pollen while the autonomous self-pollination applied old pollen due to the proterandry. The results were not caused by genetic incompatibility, as demonstrated by the geitonogamy treatment, but could be due to the low quantity and quality of pollen, as reported by Buide and Guitián (2002) for other species, namely [*Silene acutifolia*]. In *D. morisianus,* pollen was exposed for three to five days until the styles reached their maturity. In this case, dichogamy reduces the capacity but cannot avoid the self-pollination, as also demonstrated by Collin and Shykoff (2003) for *D. sylvestris*. Some self-fertility is an advantageous characteristic to maintain the reproductive assurance in the absence or reduction of pollinators (Kalisz and Vogler 2003). The SFI indicated that the species is characterized by partial self-fertility (Lloyd and Schoen 1992). The results of the anemophily treatment also produced a low number of mature seeds; in this case, this could be a result of the low amount of pollen. The low values of anemophily and the low value of SFI confirmed the need for pollinators to obtain high reproductive success. It seems that, in nature, the production of seeds in *D. morisianus* is highly dependent on pollinators, since the control flowers produced a significantly higher number of seeds than flowers subjected to treatments involving pollination exclusion without manual pollination (*i.e.*, autonomous self-pollination and anemophily). This was supported by the Selfing rate (S = -0.13), which indicated a low rate of selfing at the population level. The results of the supplemental and the control treatments were not significantly different, whereas the pollen limitation index supported the finding that the addition of pollen to natural pollinated flowers does not result in more seeds being produced. Some reviews

indicate that pollen limitation is widespread in plants (Larson and Barrett 2000; Ashman *et al.* 2004, Knight *et al.* 2005); in contrast with these results, our species did not show pollen limitation. Rather, this is in accordance with Fenster and Martén-Rodríguez (2007), who indicated that many self-compatible species have an ability to autonomously self-pollinate and thus are usually characterized by low levels of pollen limitation (Alonso *et al.* 2010). Due to the low level of self-fertility, *D. morisianus* might minimize pollen limitation but cannot eliminate it. The significant differences in weight observed in autonomous self-pollination and anemophily with respect to the other treatments may be triggered by the different resource allocation (Guo *et al.* 2010). The lower seed/ovule ratio in the control treatment compared to control plants outside study indicated that the bags affected the behaviour of pollinators, and the higher weight could be also due to distribution of resources.

The germination rate declined between 17% and 30% in the field experiments as compared to the laboratory experiments, this may be due to the laboratory experiments were conducted in optimal conditions and in field the conditions are very variable. The temperature, the lack of nutrients and water and/or the burial of seeds can reduce the germination and survivorship of seedlings. As indicated by Fenner and Thompson (2005), the burial of seeds can be responsible for a high death rate of seedlings prior to reaching the sand surface.

Our experiments revealed that, in *D. morisianus*, seed weight is not correlated with the germination capacity. This is in accordance with Fernández *et al.* (2015), who reported that the seed weight of *Erysimum popovii* was not correlated with the germination success and time. Seed mass has long been regarded as an important aspect of plant reproductive biology (Cordazzo 2002; Navarro and Guitián 2003). Larger seeds are usually characterized by a greater germination rate (Navarro and Guitián 2003), they can allocate more reserves to getting established in areas with active sand burial (Cordazzo 2002), they can cope better with the hazards (Westoby 2002) and their seedlings grow more slowly (Meyer and Carlson 2001) in the early stages than lighter seeds. In our case, weight was positively correlated only with the number of obtained seedlings in the laboratory experiments, in accordance with the results obtained by Cordazzo (2002) on tree species growing on southern Brazilian coastal dunes, and negatively correlated with the survivorship of the seedlings in the field. This is in contrast with the results reported by Shaukat *et al.* (1999) and Khurana and Singh (2000) where larger seeds showed greater germination rate. T50 is another important characteristic for the species' subsequent survival (Fenner 2012). Usually, small seeds germinate more quickly than large ones, presenting competitive advantage (Howell 1981); nevertheless, in *D. morisianus* we did not observe any correlation between the weight and the T50.

In the field, seeds germinated from October until December and the highest germination rate was recorded in November. This result indicates an earlier germination with respect to previously obtained results; more specifically, Cogoni *et al.* (2012) indicated that *D. morisianus* usually starts to germinate in November, when the temperatures are lower than 20 ºC and the water availability achieves its maximum. This earlier germination could be due to lower temperatures and increased rainfall in October, since the 2015 rainfall pattern was different from the historical data relative to the same months when our experiments were carried out. Likewise, the high mortality recorded in December could be due to the low rainfall recorded in this month.

The species showed low values of inbreeding depression, being 0.29 the highest value relative to the germination rate in the field. Gargano *et al.* (2009) observed the same level of inbreeding depression for this parameter in *D. guliae*. Our results are also in accordance with Husband and Schemske (1996), who wrote that inbreeding depression is not uncommon at the germination stage. The current low values could be due to the purge of lethal and deleterious alleles (Husband and Schemske 1996). In addition, they support the results obtained in the *ex situ* experiment by Nebot *et al.* (2016) [Chapter 1] where *D. morisianus* presented inbreeding depression in seed germination speed (T50). The results of inbreeding depression could indicate that *D. morisianus* could be protected of the inbreeding depression generated by selfing. In the last decades, there has been a lot of discussion about the effects of environmental conditions on inbreeding depression. For example, some studies carried out by Dudash (1990), and Goodwilie *et al.* (2005) indicated that field conditions increase the level of inbreeding depression; on the contrary, Carr and Eubanks (2002) suggested that inbreeding depression decreases in field conditions; while Chang and Rausher (1999) and Angeloni *et al.* (2011) found that there were not significant differences with the results of the two study sites. In our experiment, inbreeding depression was observed only in the germination rate parameter in the field experiment, but, at the same time, the cumulative inbreeding depression was higher than in the *ex-situ* study. From this point of view, our results differ from those obtained in the previous experiment carried out in *ex-situ* conditions (Nebot *et al.* 2016; [Chapter 1]), which indicated that there was not inbreeding depression at this stage, and are in agreement with Dudash (1990) and Goodwillie (2005), suggesting that field conditions increase the level of inbreeding depression.

2.5 Conclusions

This study suggests that the narrow distribution of *Dianthus morisianus* is not caused by the breeding system. The species is self-compatible and has a mixed mating system without problems of pollen limitation nor inbreeding depression. These results indicate that neither the genetic

background (Angeloni *et al.* 2011) nor the limited reproduction (Alonso *et al.* 2010) are threats to the species, as suggested by the *ex situ* experiment. The decline of pollinators, as observed in the present study, could be a more important problem for this species. This factor highlights the need and the importance of knowing which are the specific pollinators; currently, there is a lack of knowledge in this area which we hope to fulfull in the next studies.

Another important finding is the fact that the results obtained in the experiments carried out in the natural population are very similar to those obtained *ex-situ*. This indicates that *ex-situ* studies can provide accurate information on the breeding system of narrow endemic species and can be a good substitute for *in situ* studies, which could potentially have an adverse effect on the usually small and vulnerable natural populations.

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CHAPTER 3

COMPARISON AMONG A REINTRODUCED AND A NATURAL POPULATION OF THE THREATENED NARROW ENDEMIC *Dianthus morisianus* Vals. (CARYOPHYLLACEAE)

3 Chapter III. Comparison among a reintroduced and a natural population of the threatened narrow endemic *Dianthus morisianus* (Caryophyllaceae).

3.1 Introduction

During the last decades, biodiversity loss has been increasing rapidly as a consequence of human action (Leakey and Lewin 1996). Narrow endemic species usually have few, small and fragmented populations (Wilcock and Neiland 2002; Larrinaga *et al.* 2014) mainly as a result of habitat destruction (Baillie *et al.* 2004). Therefore, the establishment of new populations, the increase of the occupied area or the number of individuals of the existing populations can increase the prospects of survival (Pavlik 1996; Luijten *et al.* 2002). Reintroductions, understood as the establishment or reinforcement of new or existing populations to increase the survival prospects of a species by increasing population size and genetic diversity, or by representing specific demographic groups or stages (Pavlik 1996; Godefroid *et al.* 2011), have become well established as a technique to conserve threatened plant populations (Maunder 1992; Maschinski *et al.* 2007; IUCN 2013; Cogoni *et al.* 2013). Reintroduction actions are not the simple planting of new individuals in nature; these actions entail subsequent studies during the following years aimed at verifying the reintroduction success and at identifying the possible factors that can cause the action to fail. The persistence of a population depends on many factors; among others, the species' longevity, mating system and dispersal ability (Picó and van Groenendel 2007), but also on the presence of inbreeding depression, heterosis, outbreeding depression (Weisenberger *et al.* 2014), pollen limitation, and on pollinators availability (Ashman *et al.* 2004). When planning reintroductions, it is important to take into account the genetic diversity of the introduced plants, and the presence of efficient pollinators, which maintain the outcrossing (Kearns *et al.* 1998). Obtaining information about the breeding system and the ecology of the species is also essential in such circumstances (Schemske *et al.* 1994; Mckay *et al.* 2005).

During the last decades, several reintroductions have been carried out, but not all these studies have taken into account the correct data when analysing the reintroduction success (Menges 2008). The definition of "reintroduction success" has changed during the last decades, evolving from the simple concept of "population with recruitment" (*i.e.*, the population can maintain itself) (Pavlik 1996; Sutter 1996) to the more complete, current definition, *i.e.*, the population is able to persist and reproduce, is characterized by a high survival rate, generates new individuals which are able to

flower and produce fruits (Godefroid *et al.* 2011). Nowadays, there are very few studies that analyse the ability of transplants to flower and set fruit (Menges 2008; Tyndall and Groller 2006; Fenu *et al.* 2016), and there are even fewer that evaluate the long-term success after the reintroduction (Menges 2008; Godefroid *et al.* 2011). Getting information on the reintroduction success at least 10 years after the reintroduction action is important because, as demonstrated by Drayton and Primark (2012), a high success during the first years does not imply success in the long-term, although this is also variable depending on the plant life cycle and habitat.

In the last years, the number of studies that highlighted the importance to compare wild and reintroduced populations has increased (*e.g.*, Bell 2003; Maschinski and Duquesnel 2007; Colas 2008; Guerrant 2013; Menges *et al.* 2016); but, even so, this number is still very low.

In order to reduce its extinction risk, a conservation project was founded in 2007 by the Autonomous Region of Sardinia, which entailed the realization of a reintroduction and its fencing. This reintroduction was carried out in an area, managed by the public administration institution Ente Foreste della Sardegna (see Fig. 1C of the general introduction; Cogoni *et al.* 2013). This first reintroduction of *D. morisianus* was conducted on 2010 with 113 one-year-old plants in a protected site 150 meters away from the natural population. These plants came from seeds harvested during 2008 and 2009 in the natural population. A second reintroduction was carried out in 2012 with 25 plants of three-years-old in an unfenced area 1.3 kilometers far from the natural population and 1.05 kilometers from the first reintroduction. These reintroductions were studied to compare some aspects of their reintroduction success (Fenu *et al.* 2016).

In this chapter, we compared the reproductive biology of the offsprings of the reintroduced population with the results obtained in the natural population [chapter 2], with the aim to evaluate the success of the first reintroduction. In particular, the aims were 1) to deepen the knowledge of the success of the first reintroduction; 2) to analyse the female reproductive success of both the natural and the reintroduced populations, 3) to investigate the flowering phenology of the offsprings; and 4) to obtain information on the breeding system of offsprings of the reintroduced population, in order to determine the presence of inbreeding depression, pollen limitation and the pollinator dependence of the population.

3.2 Material and methods

3.2.1 Plant material

Dianthus morisianus is a threatened narrow endemic species which grows only in one natural population on established sand dunes in Portixeddu (south-western Sardinia; Bacchetta *et al.* 2010).

The habitat has been highly modified and fragmented by afforestations with pinus tree which were carried out in the past to stabilize dunes, as well as by urbanization, grazing, livestock and farming. The small population size and the limited seedling recruitment (Cogoni *et al.* 2012) make *D. morisianus* potentially prone to extinction; this species is actually considered as one of the most threatened of the island (Bacchetta *et al.* 2012) and is categorized as Critically Endangered in the IUCN Global Red Lists (Fenu *et al.* 2013). Previous investigations conducted on *ex situ* plants (Nebot *et al.* 2016 [chapter 1]) and in the natural population [chapter 2] demonstrated that *D. morisianus* is a self-compatible species, with mixed mating system and that it does not have neither problems of breeding system nor high inbreeding depression.

The phenology and reproductive studies were carried out on 50 offsprings of the reintroduced population five years after the reintroduction (2015), while the female reproductive success was investigated during 2016. In this study, 146 plants in the natural population and 182 plants in the reintroduced population (82 reintroduced and 100 offsprings) were randomly selected.

3.2.2 Phenology and breeding system

To analyse the phenology of the offsprings of the reintroduced plants in the reintroduction, open flowers were observed daily from the start of the flowering (14th of May) until plants stopped to produce new flowers (10th of June). Each day, the new open flowers were tagged with a univocal code in order to recognize the studied flowers. At the end of the flowering, we were able to analyse the flowering duration at the level of the individual plants and of the population, the flowering phenology, the synchrony of each plant (S_i) and the synchrony at the population level (Z). We used the formula proposed by Augspurger (1983), which takes into account the relation of the duration of the flowering on each plant (f_i), the number of studied plants (n), and the number of days in which each plant flowers at the same time as the others (e_j). The level of synchrony of each plant was analysed as: $S_i = [1/(n-1)](1/f_i)\sum e_{j\neq i}$, and the synchrony of the population as: $Z = (1/n) * \sum S_i$.

To characterize the female reproductive success of the populations, prior to flowering, we selected the plants of the natural population, the reintroduced ones and the offsprings and measured the number of stems, the height of the taller stem (cm; hereafter "height"), the number of buds per stem, the total number of buds per plant (buds) and the distance of the nearest plant (dm; "distance"). When the first flowers opened, we measured the diameter of the corolla (mm; diameter) of two flowers per plant and classified the colour of the petals into white, light pink, pink, dark pink, or fuchsia. Two weeks after the pollination event, the total formed fruits and the number of

fruits affected by herbivory and parasitism were counted and the two tagged fruits per plant were harvested. All harvested fruits corresponded to tagged and measured flowers. For each fruit, the number of ovules and seeds were counted and weighted; the weight of 10 seeds was used. We divided the plants in these three groups due to in a precedent study Fenu *et al*. 2016 observed that the reintroduced plants each year increase the number of stems and in the natural population most of the plants present low values even if some of them can be elder than the reintroduced plants.

The breeding system of the offsprings of the reintroduced population was investigated by applying five pollination treatments: 1) autonomous self-pollination: flowers were bagged prior to anthesis with fine mesh bags and nothing was done until the time of harvesting (n= 17 on 14 plants); 2) geitonogamy: emasculated and bagged flowers were pollinated with pollen from another flower of the same plant (n = 25 on 20 plants); 3) xenogamy: emasculated and bagged flowers were pollinated with pollen from another plant of the same population (n = 38 on 32 plants); 4) supplementary pollination: flowers were left without bag to open pollination, and pollen from another plant was added manually (n = 11 on 9 plants); and 5) control: flowers were left for open pollination without applying any treatment (n = 33 on 30 plants). To make sure that the seeds did not disperse in the field during the fruit dispersal period, tea bags were put on all treated flowers at the time of fruiting.

At maturity, the fruits of the different pollination treatments were harvested. In the laboratory, the number of ovules, aborted seeds and mature seeds were counted for each fruit; mature seeds were then weighted. This data allowed us to evaluate the reproductive success of each treatment by fruit set, seed/ovule ratio, and seed mass. These results were then compared with those obtained in the natural population in order to verify if the different conditions had affected the flower phenology, the breeding system, the level of inbreeding depression, pollen limitation and pollinator dependence (see chapter two). We also calculated the Autonomous Self-pollination Index (SFI), and the Self Compatible Index (SCI) according to Lloyd and Schoen (1992). The SFI gave us information on the ability of the plants to produce seeds in the absence of pollinators; the following formula was used: SFI = seed/ovule of autonomous self-pollination / seed/ovule of the xenogamy treatment. SCI showed the level of self-compatibility of the species and it was obtained through the formula: seed/ovule of geitonogamy / seed/ovule of xenogamy. Moreover, the selfing rate (S) was calculated using the formula proposed by Charlesworth and Charlesworth (1987): S = (xenogamy–control) / (xenogamy–geitonogamy). In our case study, the formula was modified following Aizen and Basilio (1995), who used the ovule ratio of geitonogamy treatment instead that of the autonomous self-pollination. Values between 0 and 0.5 indicate null or partial self-fertility or self-

incompatibility, while values until 1 indicate preference of self-fertile or self-compatible. Additionally, the Pollen Limitation Index (PL) was calculated following Tamura and Kudo (2000) through the formula: PL = 1 - (control/supplementary). Values near 0 indicate absence of PL, while values near 1 indicate high pollen limitation. Finally, the inbreeding depression (ID) was calculated following the formula proposed by Charlesworth and Charlesworth (1987): $\delta = 1 - (W_s/W_o)$, where W_s is the mean seed/ovule ratio of the geitonogamy treatment and W_o the mean seed/ovule ratio of the xenogamy treatment. The cumulative level of inbreeding depression of all the analysed parameters was assessed following the formula of Husband and Schemske (1996): $\delta = 1 - [(W_{sa}/W_{oa})\times(W_{sb}/W_{ob})\times(W_{sc}/W_{oc})\times(W_{sd}/W_{od})\times(W_{se}/W_{oe})\times(W_{sf}/W_{of})]$.

3.2.3 Germination tests

After conducting the laboratory experiments, seeds were sown in the reintroduction area. To investigate the germination ability associated with each pollination treatment, seeds were buried in the sand using biodegradable peat pots at a depth of 1 cm (Cogoni *et al.* 2012); 30 seeds per pollination treatment were distributed individually in each pot following an ordered distribution. Seeds were then covered with sifted sand to ensure that the germinated seeds belonged to our study. Seeds were sown in September 2015 and the germination rate and mortality rate of the seedlings were recorded until April 2016. At this point, the results were compared among pollination treatments and between populations (natural vs. reintroduced) in order to verify if there were any differences.

3.2.4 Statistical analyses

Generalized Linear Mixed Models (GLMMs; Bolker *et al.* 2009) were used to test the effects of the five different pollination treatments on the female reproductive success of *D. morisianus* in both the natural and the reintroduced population, as well as to compare them. The dataset regarding the natural population was retrieved from a previous study and consisted of data collected in two different years (2014-2015) which were considered jointly (2015) for the purpose of this analysis since there were not any significant differences between 2014 and 2015 (see chapter 2). The different parameters characterizing the reproductive success (fruit set, seed number, seed set, aborted seeds and seed weight, germination rate, mortality rate, and time to germination) were analysed as response variables, the different pollination treatments as fixed factor while the population was considered a random factor. The GLMM analyses were carried out using the package *lme4* version 1.1-12 (Bates *et al.* 2015) with the function *lmer().* The current version of *lmer* does not provide F-tests for fixed effect, therefore we compared our model with the null model to test the significance of the fixed effect (Faraway 2005). Significant differences ($P < 0.05$) in the

ANOVA indicate that the pollination treatment influenced the analysed reproductive success parameter. As for the *post hoc* contrasts, we used the package *lsmeans* version 2.24 (Russell and Lenth 2016) with the function *lsmeans()* to verify if there were any differences among treatments.

The data obtained from the measurements conducted to characterize the female reproductive success in the natural and reintroduced populations were compared by General Linear Model (GLM) in order to test the differences among the natural plants, reintroduced ones and the offspringsof the reintroduced population. In the natural population, we obtained the number of stems per plant, the number of buds, the height of the tallest stalk, the distance to the nearest plant and the herbivory rate, but not the number of fruits, which is the variable that bears the effects of all the other variables. We assumed a Gaussian distributions with identity link when the data was normally distributed, a Poisson distributions with a log function when the data were not normal, and a binomial distribution with a logit link function for proportions. The presence of significant differences among the different response variables were checked through the function *glht()* of the package *multcomp* version 1.4-6 (Hothorn *et al.* 2008) using multiple comparisons of means with Tukey contrasts.

To analyse the female reproductive success, structural equation modeling (SEMs) were used to investigate the hypothetical relationships among the data (Shipley 2016). Since SEM analyses need a high number of data, the low number of fruits harvested in the natural population (due to a high rate of herbivory; table 1) did not allow us to carry out this analysis in the natural plants. To estimate the standardized path coefficients, we used the maximum likelihood method (Grace *et al.* 2012). The normality of variables was verified with a Shapiro test; whenever possible, those with non-normality were log transformed in order to accomplish the multivariate normality, otherwise they were left without any transformation. To analyse the co-linearity among variables, we applied the variation inflation factors (VIFs) for each variable (Petraitis *et al.*1996) using the package *usdm* version 1.1-15 (Naimi 2015) and the function *vifcor()*. To test the goodness-of-fit test, we applied the likelihood χ^2; since this shows some deviance when data do not accomplish the multivariate normality and when sample size is small (Bollen 1989), we also used the Goodness of Fit Index (GFI) and Bentler and Bonett's normed-fit index (NFI). A significant goodness-of-fit test indicates that the model is poor at describing the observed covariance among variables, while a non significant value indicates that the predicted covariance pattern is not distinguishable from that observed (Hayduk 1987). As for the GFI and the NFI, values range from 0 to 1, and values > 0.9 indicate an acceptable fit of the model for the studied data (Bollen 1989).

We constructed two models in order to obtain the female reproductive success: model 1 tested the parameters at the level of plants and model 2 tested some parameters of the flowers. Each model was conducted three times, the first one to analyse all the measured plants of the reintroduced population (A), the second one to test only the reintroduced plants (B), and the third one to test the offsprings of the reintroduced population (C). The variable seed set was removed from model 1, as indicated in the VIF analysis. Finally, we used seven observations and six parameters for model 1 and five observations and four parameters in model 2, the models fulfilling one of the assumptions were then identified to conduct the SEM analyses.

To design our path diagrams we hypothesized that the more stems the plant had, the more buds it might produce, as observed in other studies (*e.g.*, Sandring and Ågren 2009; Larrinaga 2014); we also expected that the number of fruits was highly correlated with the number of buds. We specified that the fruit set could be affected by the distance of the nearest plant and by the height of the plant. Higher plants could attract more pollinators (Ehrlén *et al.* 2002); also, the proximity of plants could increase flower display (Hegland *et al.* 2009) but, at the same time, this could negatively affect the production of fruits or seeds in the presence of inbreeding depression. We also considered that herbivory occurs in plants with more fruits. Since there are numerous studies that indicate that pollinators may prefer particular flower traits (Gómez *et al.* 2007, Dudash *et al.* 2011), in the model 2 we assumed that the larger the diameter of the flower, the higher the number of pollinators it could attract. As for the colour of the flower, we were not sure if this trait could affect the number of seeds, since the species is pollinated by both diurnal and nocturnal pollinators (see chapter 4). We also included the number of ovules per fruit in the model. To conduct these analyses we used the *Lavaan* package version 0.5-22 (Rossel 2012) with the function *sem().* All the statistical analyses were carried out using R 3.3.1 (R Core Team 2016).

3.3 Results

3.3.1 Phenology

During the analysis of the proportion of open flowers in the two populations in 2015 we observed that, in the natural population, the number of open flowers increased gradually, whereas in the offsprings of the reintroduced population it increased more rapidly (Fig. 1A). The natural population showed only one peak, while the offsprings of the reintroduced plants showed some fluctuations during the peak and a higher proportion of open flowers at the end of flowering with respect to the natural population. The blooming lasted 29 days in both populations, but in the reintroduced one it was delayed by one day as compared to the natural one (see chapter 2). The mean duration of flowering per plant in the reintroduced population was 17 days, while that of the natural one was 18 (see chapter 2). The temperatures were slightly lower in the reintroduced than in the natural population (Fig. 1B), but they were characterized by the same pattern. The mean synchrony per plant in the reintroduced population was 0.74 ± 0.08 and 0.74 at the population level, being lower than in the natural population (0.81 ± 0.08 per plant and 0.83 for the population, see chapter 2).

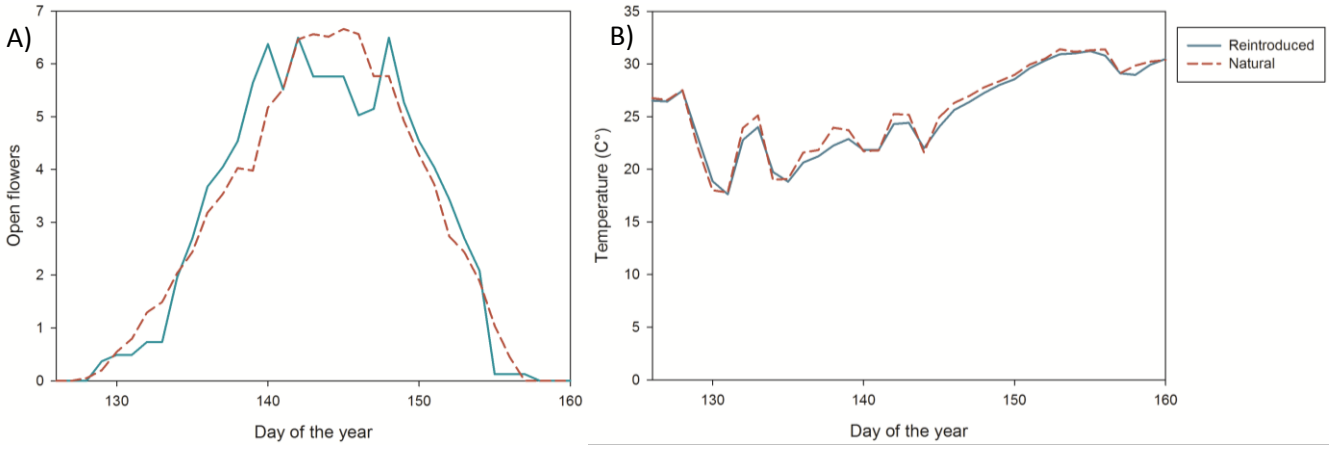

Figure 1. A) Flowering phenology and B) mean temperatures of the natural and reintroduced populations of *D. morisianus*. The X-axis indicates the day of the year from the 6th of May (day number 126) to the 9th of June (day number 160).

The number of stems, buds per stems, buds, and height varied significantly among the two populations, and also among the three groups of plants (Table 1). The reintroduced plants produced three times more stems than natural ones but fewer buds per stalk than the offsprings of the reintroduced population and the natural plants; the number of buds per plant was also much higher in the reintroduced population. The highest plants were the reintroduced ones, followed by the offssprings and the natural ones. The distance of the nearest plant in the natural population was lower than in the plants growing in the reintroduced population, but they did not show significant differences. The diameter of the flowers was bigger in natural plants but there were not significant

differences neither among groups of plants nor among populations. The number of fruits was higher in the reintroduced plants, but the fruit set did not show significant differences. The seed weight, number of seeds per fruit and seed set did not show significant differences neither among reintroduced plants nor the offsprings ones. The number of ovules was the only parameter that varied significantly between the two groups of plants, the offsprings ones being those with more ovules. The distribution of the colours of the flowers followed the same pattern in the three groups of studied plants: pink was the predominant colour (50%), followed by light pink (30%), dark pink (15%), white (3%) and fuchsia (2%). The number of fruits, fruit set, mean seed weight, number of seeds, seed set and ovules were not studied in the natural population due to the insufficient data available as a result of animals eating the stems and leaving only the basal leaves.

Table 1. Mean and standard deviation of different reproductive variables of *Dianthus morisianus* in the natural and reintroduced populations. The data of the reintroduced population are divided into introduced plants and established plants. The last column shows the mean values of the three groups of plants. We also reported the minimum and maximum values of each parameter. Different letters indicate significant differences at $P < 0.05$.

	Natural	Reintroduction			Total
		Reintroduced plants	Offsprings	Mean reintroduced population	
Number of stems	5.1 ± 4.58^{ad} (1-36)	15.73 ± 0.88^{b} (1-66)	6.02 ± 4.44^{c} (1-20)	10.20 ± 10.56^{e} (1-66)	8.45 ± 9.28 (1-66)
Number buds/stems	2.13 ± 1.02^{ad} (0.8-8)	2.48 ± 0.88^{b} (1-5.64)	2.93 ± 1.07^{c} (1-6)	2.74 ± 1.02^{e} (1-6)	2.53 ± 1.05 (0.8-8)
Number of buds	10.19 ± 9.09^{ad} (1-72)	39.96 ± 10.03^{b} (3-198)	16.22 ± 13.20^{c} (2-74)	26.41 ± 29.33^{e} (2-198)	20.63 ± 25.37 (1-198)
Height plant (cm)	36.25 ± 10.34^{ad} (5-68)	45.48 ± 0.31^{b} (21-67)	41.72 ± 8.87^{c} (27-71)	43.31 ± 9.53^{e} (21-71)	40.92 ± 10.42 (5-71)
Distance (cm)	77.85 ± 90.63^{a} (8-520)	65.50 ± 34.40^{b} (8-120)	95.70 ± 133.00^{a} (13-770)	67.98 ± 84.91^{c} (8-770)	73.89 ± 91.62 (8-770)
Diameter (mm)	23.15 ± 3.46^{a} (15.23-35.98)	22.34 ± 3.30^{b} (12.49-28.73)	22.58 ± 3.74^{c} (9.04-30.45)	22.97 ± 3.562^{c} (9.04-30.45)	22.66 ± 3.51 (9.04-35.98)
Number of fruits	-	33.03 ± 35.21^{a} (1-157)	13.88 ± 13.79^{b} (1-70)	22.52 ± 27.21 (1-157)	-
Fruit set (%)	-	73.80 ± 27.39^{a} (10.71-100)	74.33 ± 28.14^{a} (5.26-100)	74.09 ± 27.71 (5.26-100)	-
Mean weight of 10 seeds (mg)	-	12.60 ± 8.40^{a} (6.96-46.04)	12.70 ± 5.90^{a} (6.84-48.76)	12.70 ± 7.20 (6.84-48.76)	-
Number of seeds per fruit	-	34.73 ± 15.61^{a} (5-76)	33.38 ± 13.87^{a} (8-69)	34.26 ± 15.07 (5-76)	-
Seed/ovule	-	44.79 ± 23.774^{a} (6.47-98.3)	43.88 ± 20.45^{a} (10.35-89.24)	44.28 ± 22.02 (6.47-98.3)	-
Number of ovules per fruit	-	77.93 ± 13.47^{a} (44-108)	81.38 ± 15.05^{b} (37-112)	78.23 ± 14.30 (37-112)	-
Herbivory (plants)	83.31%^{a}	9.23%	26.19%	18.79%^{b}	59.54%
Parasitism (fruits)	-	25.76%	24.07%	-	-

3.3.2 Breeding system

Autonomous self-pollination was the treatment with the lowest values of fruit set, seed/ovule ratio, number of seeds and the one with the highest mortality rate (Table 2), showing significant differences with respect to the other pollination treatments (Table 2), but not among populations (Table 3). The supplementary was the treatment with the highest fruit set, number of seeds, seed/ovule ratio and seed weight, but there were not significant differences with respect to control, xenogamy, and geitonogamy. Only the fruit set, seed/ovule ratio and mortality rate showed significant differences among treatments (Table 2); more specifically, between autonomous self-pollination and the other pollination treatments (Table 2). When we compared the results of the natural population with those of the reintroduced one, we found significant differences among populations only in the seed weight, mortality rate and time to germinate parameters (Table 3). The seeds produced by offsprings of the reintroduced population plants were heavier, had a lower mortality rate and germinated for a longer period than those from the natural plants (Table 2 and 3, see chapter 2). After analyse the effect of the individual on the response variables, we only obtained a significant effect in the weight of seeds (Table 3).

Table 2. Mean and standard deviation of fruit set (%), seed number, seed/ovule ratio, seed weight (mg), and germination and mortality rate (%) obtained in the five pollination treatments. Different letters indicate significant differencess ($P< 0.05$).

	Fruit set	Seed number	Seed weight (mg)	Seed/ovule ratio	Germination rate	Mortality rate
Autonomous self-pollination	41.12^{a}	5.43 ± 2.93^{a}	12.503 ± 2.904^{a}	8.90 ± 5.21^{a}	66.66^{a}	0^{a}
Geitonogamy	80.00^{b}	30.21 ± 16.85^{b}	10.068 ± 3.234^{a}	45.44 ± 18.79^{b}	88.92^{a}	0^{a}
Xenogamy	84.21^{b}	33.97 ± 19.49^{b}	11.004 ± 5.013^{a}	48.18 ± 26.23^{b}	52.60^{a}	40.00^{c}
Supplementary pollination	90.90^{b}	39.40 ± 21.33^{b}	11.598 ± 3.197^{a}	58.98 ± 31.59^{b}	65.00^{a}	23.07^{b}
Control	84.85^{b}	33.65 ± 17.21^{b}	13.568 ± 4.597^{a}	46.35 ± 19.94^{b}	65.00^{a}	15.38^{b}

Table 3. Statistical results of the GLMMs carried out to analyse the fixed (treatment) and random (population) effects on the studied parameters. The significant differences among the two populations are indicated in bold.

Measure	Factor	d.f.	Chi-square	*P*>chi-square	Random
Fruit set	Treatment	4	63.706	**<0.001**	Residual>population Residual>individual
Seed/ovule	Treatment	4	63.293	**<0.001**	Residual>population Residual>individual
Aborted seed	Treatment	4	6.314	0.1769	Residual>population Residual>individual
Seed weight	Treatment	4	5.224	0.265	**Residual<population** **Residual<individual**
Germination rate	Treatment	4	55.067	0.459	Residual>population
Mortality rate	Treatment	4	247.280	**<0.010**	**Residual<population**
Time to germination	Treatment	4	2.7208	0.743	**Residual<population**

In the reintroduced population, the value of the pollen limitation was 0.22, very similar to that obtained in the natural population (0.24, see chapter 2), thus indicating that the species is not pollen-limited. The other parameters correlated with the breeding system, SCI and SFI, with values of 0.94 (the same value was recorded in the natural population) and 0.18 (0.21 in the natural population, see chapter 2), respectively. The studied plants had a selfing rate of 0.66; this parameter was very different from that obtained in the natural population (-0.13, see chapter 2).

3.3.3 Germination tests

As shown in Fig. 2, in the reintroduced population seeds germinated from October to April, being November the month with the highest germination rate in all treatments with the exception of the autonomous pollination. Most of the seeds in this treatment germinated from December to April, when the lowest germination rate was recorded in the other treatments. Fewer plants died in the reintroduced population than in the natural one, but without an identifiable pattern.

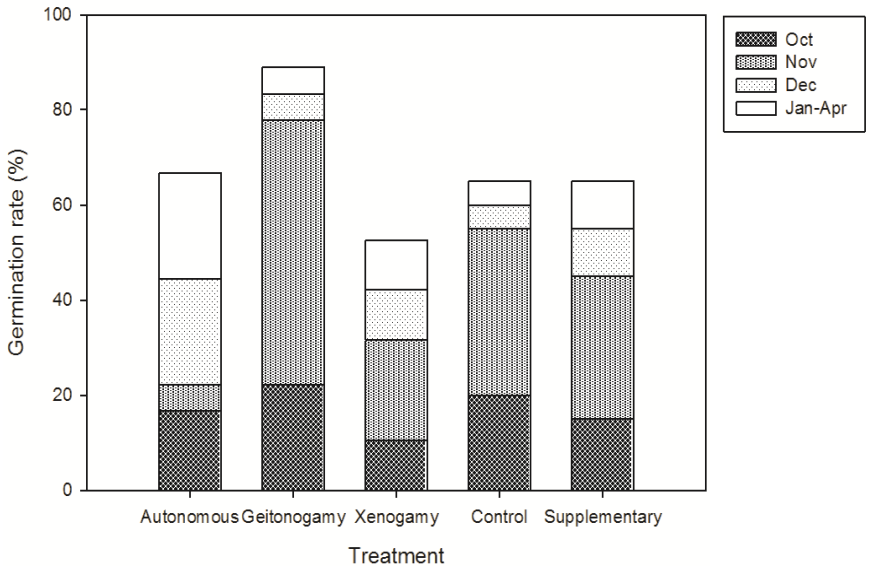

Figure 2. Germination rate of seeds from pollination treatments of *Dianthus morisianus* in the reintroduced population during six months. Each pattern indicates a different period of germination.

3.3.4 Inbreeding depression

The inbreeding depression was studied in six parameters (Table 4) and three of them (*i.e.*, seed weight, germination rate and time to germinate) showed negative inbreeding depression, therefore indicating the presence of outbreeding depression. The cumulative value of inbreeding depression was -0.66.

Table 4. Coefficients of inbreeding depression (δ) in different traits of *Dianthus morisianus* obtained in the natural and the reintroduced populations.

Plant traits	**δ**	
	Natural	Reintroduced
Fruit set (%)	0.01	0.05
Seeds/fruit (n)	-0.08	0.12
Seed set (%)	0.11	0.06
Seed weight 10 seeds (g)	0.12	-0.25
Germination rate field (%)	0.29	-0.66
Time to germinate	-0.06	-0.23

3.3.5 Path analysis

The model 1 obtained for the reintroduced population (Fig.3, 1A) was associated with a GFI and NFI = 0.90, indicating that the model provides a good fit compared to a null model that assumes independence among all variables; however, the χ^2 showed significant differences (χ^2 = 144.57, d.f. = 9, P< 0). Diagram 1A (Fig. 3) displays the magnitude of the direct effects, being buds the main direct effect on fruits, and stems the main indirect effect (Table 5). It was not possible to apply model 1 to the natural population because of the high herbivore rate, as explained above. The second diagram (Fig. 3, 1B) shows the results of the reintroduced plants. In this case, there were four important factors which influenced fruit production, namely buds and fruit set as direct effects and height and stems as indirect effects (Table 5). The last diagram (Fig. 3, 1C) displays the data of the offsprings of the reintroduced population. In this diagram, the main direct effect was also the number of buds and fruit set, while the indirect effect of the height decreased (Table 5). Model 1 applied on the diagrams B and C provided values of GFI and NFI of 0.88 and 0.91 for the reintroduced plants and 0.58 and 0.75 for offsprings, respectively. According to the GFI and NFI of the offsprings, the data did not fit very well with the model. The χ^2 for diagrams B and C were 61.908, d.f. = 9, P = 0 for the reintroduced plants and 197.106, d.f. = 10, P = 0 for offsprings. The significance of χ^2 could be due to the fact that it assumes multinormality of the variables, large number of data and no missing data, while our dataset did not present normality, there were missing data and the number of values was low. As GFI and NFI indicated high fit of the model, we accepted them.

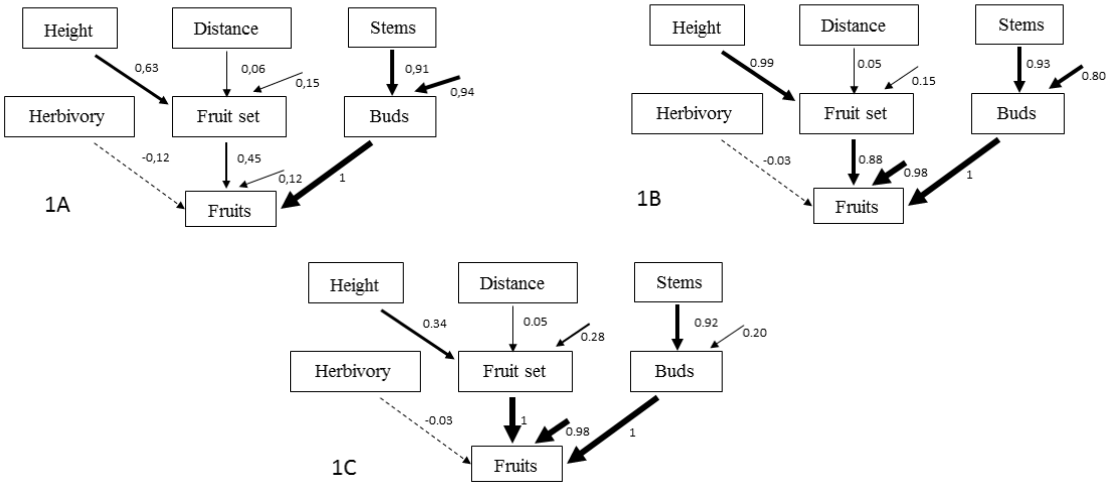

Figure 3. Path diagrams of model 1 for the plant parameters studied to evaluate the reproductive success of *Dianthus morisianus* in the reintroduced population (A), reintroduced plants (B), and established plants (C). Solid lines indicate a positive effects and discontinuous lines a negative effect. Arrow widths are proportional to path coefficients.

The model 2 obtained for the reintroduced population (Fig. 4, 2A) was associated with a GFI and NFI of 0.99 and 0.98, respectively, indicating that the data fit perfectly with the model. The χ^2 value was 0.49 (d.f. = 3, P = 0.92). In diagram 2A (Fig. 4) we can observe that the seed weight depends negatively on the number of produced seeds; the diameter of the corolla was the most influent indirect effect (Table 5). The direct effects for the reintroduced plants are shown in diagram 2B (Fig. 4); a high negative effect on the seed weight was observed and the most important indirect effect was also in this case the diameter of the corolla. Model 2 applied on diagram B was associated with values of GFI and NFI of 0.99 and 0.96, respectively, and χ^2 = 0.968, d.f. = 3, P = 0.81. In the last diagram (Fig. 4, 2C), there was a low effect of the number of formed seeds on their weight and there was no significant indirect effect (Table 5). This diagram had also high values of NFI and GFI (0.85 and 0.98, respectively) and the χ^2 value was 1.733 (d.f. = 3, P = 0.63).

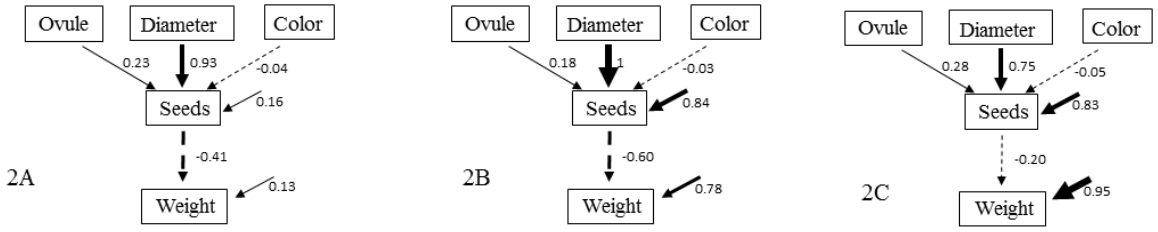

Figure 4. Path diagrams of model 2 for the studied flower parameters measured in the reintroduced population (A), in the reintroduced plants (B) and in the established ones (C). Solid lines indicate positive effects and discontinuous lines negative effect. Arrow widths are proportional to path coefficients.

The number of buds was the greatest direct effect on the number of fruits per plant in the three diagrams of model 1. The height of the stems was also important for the fruit set, but the effect varied in the different groups of plants, whereas the distance of the nearest plant did not have an effect. The number of seeds per fruit was highly affected by the diameter of the petals in the three diagrams and, to a lesser extent, by the number of ovules. The colour of the flower did not have an effect on the number of seeds per fruit. The direct effect of the number of seeds per fruit was negative for the seed weight (*i.e.*, the higher the number of seeds, the lighter they were), this effect also varied among the different diagrams. The number of stems and the diameter of the corolla were the main indirect effects on the number of fruits and the seed weight, respectively. The diameter was negatively correlated with the weight and only had a significant effect in diagrams A and B.

Table 5. Indirect effects for models 1 and 2 in the figure 3 and 4 for *Dianthus morisianus*.

Indirect effects	Fruits			Weight		
	A	B	C	A	B	C
Stems	0.91	0.93	0.92			
Buds	-	-	-			
Fruits	-	-	-			
Fruit set	-	-	-			
Height	0.29	0.87	0.34			
Distance	0.03	0.04	0.05			
Ovule				-0.09	-0.11	-0.06
Diameter				-0.38	-0.60	-0.01
Colour				0.02	0.03	0.01

3.4 Discussion

The flowering pattern varied a bit among the two populations, thus indicating that the flowering time is affected not only by the temperature, but also by other factors, as suggested by Arroyo (1990). The two populations displayed high values of synchrony and each day plants could exchange pollen with more than 70% of the plants of the population. The high synchrony, the same flowering period and their closeness (150 m away from each other) could facilitate the exchange of genes among populations as within each population (Goodwillie and Ness 2005) and attract more pollinators (Méndez and Diaz 2001). Higher values of synchrony could be adverse for the population in the presence of a low pollination rate (Valtueña *et al.* 2008). The manual and the control treatments indicate that the pollination rate is not low as we obtained high reproductive success, and that the population is able to attract pollinators. The offsprings were characterized by values of fruit set, seed/ovule ratio and germination rate that were very similar to those obtained in the natural population, therefore indicating that there was not a reduction of reproductive success in the reintroduced population. The results of this study and, in particular, the low values of SFI and PL, corroborate those obtained in the natural population (chapter 2) and confirm the fact that the species requires pollinators to achieve the same reproductive success of the control fruits. The pollen limitation obtained on the offsprings is a little higher than in the natural plants and could be caused by the presence of fewer pollinators (Ashman *et al.* 2004). Although we did not found high levels of pollen limitation, the supplementary treatment produced more fruits and seeds, the latter being heavier than in the control treatment. To understand its effect on the species are needed future studies. The number of seeds of the offsprings did not affect their weight, an opposite pattern to that observed in the natural population (chapter 2), this could indicate that there are not limiting resources. The reintroduction is located in an open sand area and consists of few plants, while the

natural population grows in an area occupied by numerous individuals and species. Contrarily to the natural population, which showed low levels of inbreeding depression in the germination rate, offsprings were characterized by high outbreeding depression at this stage. This is fairly common in reintroduced populations formed by a mix of seeds from different populations due to the presence of distantly related individuals (Bouzat 2010) with locally adapted alleles (Tallmon *et al.* 2004; Lofflin and Kephart 2005; Leinonen *et al.* 2011). However, in our case, the reintroduced plants came from the only existing natural population and therefore should have the same adaptations; this means that other factors might be responsible for this kind of pattern and further data is needed in order to understand it. The selfing rate was another parameter for which we recorded higher values in the reintroduced population: this could be caused by the fact that the reintroduced population is still young and there are few individuals, or it could be due to the high floral display per plant which cause pollinators to stay on the same plant instead of moving around the population (Culley *et al.* 1999). It is important to note that reintroduced plants were produced using a mix of seeds harvested in the natural population and where plants were planted the original position in the natural population was unknown. According to Edmands and Timmerman (2003) the outbreeding depression, just like the inbreeding depression, can be stronger in later stages, which is in accordance with our results. The high selfing rate and the presence of outbreeding depression could lead to the disappearance of crossing (Weller *et al.* 1998) but, since our plants produced more fruits and seeds by xenogamy, the negative effects of the outbreeding depression can be reduced. With the exception of the inbreeding depression, all the other parameters are in accordance with those obtained in the natural population.

The germination rate did not show significant differences neither among pollination treatments nor among populations. This could indicate that the climatic conditions in the two populations were very similar. We observed different germination patterns, but only the mortality rate was significantly different among pollination treatments in the reintroduced population. The germination time lasted three months more in the reintroduced population than in the natural one, this pattern could have been influenced by the fact that the reintroduced population is oriented to north and could benefit from a higher humidity than the natural one, which grows on a less inclined slope and is exposed to the sunlight during the whole day.

The reintroduced population consists of numerous offsprings (Fenu *et al.* 2016) and we used more than 100 of these (66.67% of the reproductive offsprings) when they were in the reproductive stage. These new plants were not only able to flower and produce fruits, but they were also taller and generated a significantly higher number of flowers than the natural ones. The reintroduced

plants are older than the offsprings and, as observed by Fenu *et al.* (2016), the number of stems and fruits per plant have been increasing each year; last year, for example, there has been an increase of 4.39 stems and 10.24 fruits with respect to the previous season. The offsprings were characterized by higher values than natural plants, even if the first ones were "younger". This could indicate that there is a higher availability of resources in the reintroduction area, which leads to higher size (Ollerton and Lack 1998). The number of fruits could not be recorded in the natural population because of the herbivory, but natural plants also produced a high fruit set before being eaten by animals (personal observation). The phenology of flowering is very important to attract pollinators (Forrest *et al.* 2010) and the high floral display of the reintroduced plants highly contributes to this task (Guitián and Sánchez 1992), therefore facilitating its reproductive success. Other factors that demonstrate that the reintroduction was successful were an increase of the area occupied by the population and the high dispersal ability; actually, we found newly offsprings which were more than 7 meters away from the offsprings and more than 10 away from the reintroduced ones. The occupied area increased not only downhill, but also around the entire perimeter of the reintroduced population. In the natural population plants were nearer to one another than in the offsprings, this pattern was expected because of the fact that the reintroduced population was planted only six years ago, therefore it is constituted by fewer and younger plants; even so there were not significant differences. At the level of flowers, there were neither significant differences among the reintroduced plants nor among the natural ones, which indicates that the parameters that have an effect at the plant level do not influence the production of seeds. The high number of produced seeds is advantageous considering the low establishment of seedlings observed in the natural population (Cogoni *et al.* 2012; Chapter 2).

The main direct factors that affect the number of fruits were the number of buds and the fruit set, while the height and the number of stems were the most important indirect effects. The low value of the height in diagram 1C could be due to the fact that data did not fit well with the model. Therefore, we can conclude that, in the reintroduced population, the size of the plant is very important to produce fruits. As expected, the number of seeds affected negatively the seed weight as a result of the distribution of the resources. The most important indirect effect was the diameter of the corolla: pollinators were attracted by the biggest corollas, regardless of their colour.

The high levels of herbivory observed in the natural population indicated us that this could be an important threaten for the species. We found also herbivory in the reintroduced population were plants are protected by a fence. This indicates that also small mammals eat *D. morisianus.* In future

studies should be studied the effect of the herbivory, due to even if eaten plants survives, the reproductive success is affected negatively.

3.5 References

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CHAPTER 4

POLLINATION ECOLOGY OF *Dianthus morisianus* Vals. (CARYOPHYLLACEAE): NECTAR PRODUCTION AND THE ROLE OF DIURNAL AND NOCTURNAL POLLINATORS

4 Chapter IV. Pollination ecology of *Dianthus morisianus* (Caryophyllaceae): Nectar production and the role of diurnal and nocturnal pollinators

4.1 Introduction

The genus *Dianthus* is one of most important within the Caryophyllaceae family and it is characterized by several types of breeding system, flower morphology and pollination syndromes (Kephart *et al.* 2006). Pollinator syndromes were defined by Faegri and van der Pijl (1979) as "a group of flower traits that have evolved in response to selective pressure forced by different pollinators". More recently, Fenster *et al.* (2004) defined it as "the suite of correlated floral traits associated with the attraction and utilization of specific groups of animals as pollinators". In the last decades, it has been accepted that the pollination syndrome cannot be determined only by floral morphology (Waser *et al.* 1996; Fenster *et al.* 2004; Ollerton *et al.* 2009). Currently, it is known that flowering phenology, length of lifespan, nectar composition and secretion patterns are also determinants for pollinator species (Baker and Baker, 1983; Galetto and Bernardello 2005; Bobrowiec and Oliveira 2012). Different works (*e.g.*, Freitas and Sazima 2006, Ollerton *et al.* 2009) reported that the pollination syndrome could be used as an hypothesis to design studies on pollination ecology, but not to identify the effective pollinators. An effective pollination depends on the characteristics of the flowers, on the pollinator morphology and on their behaviour (Armbruster *et al.* 2004, 2009). The main pollinator syndromes in the genus *Dianthus* are two, namely i) the diurnal syndrome, which is usually characterized by asynchronous flowering, pink or red corolla, continuously open flowers during both day and night and without changes in scent intensity during the day (Jürgens *et al.* 2002, 2012; Witt *et al.* 2013); and ii) the moth nocturnal syndrome, which is characterized by white corolla, synchronous flowering at night, long corolla tube, and intense scent emission in the evening/night (Young 2002). Even if there are clear differences between the two syndromes, the existence of several species that possess the characteristics of only one classical syndrome and that are pollinated by both diurnal and nocturnal pollinators is well documented (Amorim *et al.* 2013). *Dianthus sylvestris* is characterized by diurnal and nocturnal pollination being the main pollinators species of the families Noctuidae and Sphingidae. Also for *D. gratianopolitanus* the main pollinators belongs to Sphingidae and Noctuidae families but the primari mode of pollination is diurnal. (Will *et al.* 2013 and Kephart *et al.* 2005).

Most angiosperms depend on pollinators for reproduce, at least to some extent (Kearns and Inouye 1997; Young 2002; Blanché and Bosch 2007). Narrow endemic plants, which are usually restricted to small and fragmented populations (Alonso *et al.* 2010, Larrinaga *et al.* 2014) can be affected by a low plant density, which may reduce their attractiveness for pollinators. The degree of outcrossing/selfing in self-compatible species depends on the movement of pollinators and of the pollen they carry (Inouye *et al.* 1994). The pollinator behaviour is determined by several plant and floral traits as for example the nectar production rate (Biernaskie *et al.* 2002; Nicolson and Nepi 2005) and on nectar composition (Baker and Baker 1983; Bernardello *et al.* 1999) which in part determine the frequency and duration of pollinator visits. Floral nectar is widely known as being the key reward offered by animal-pollinated plants to their pollen vectors (Proctor *et al.* 1996). Pollinators can be estimated from measurements of nectar volume and solute concentration (Kearns and Inouye 1993; Dafni 2005), since each group of pollinators has a preferred concentration range (Galetto and Bernardello 2005). Nectar concentration is directly affected by the depth of the corolla; being lower in species with long corolla tubes than in those with short ones (Galetto and Bernardello 2005). The solute concentration in a flower can change with the humidity, the resorption of solutes, water evaporation and it can also be depleted by foraging animals (Corbet 2003). Nectar removal can modify the secreted volume, the sugar content, and also the concentration of the nectar (Galetto and Bernardello 1992, Bobrowiec and Oliveira 2012). In order to properly interpret the foraging behaviour of nectarivores, it is important to know the nectar secretion rate and the standing crop, intended as the quantity of nectar in a flower at a given time, which is usually expressed in terms of mass of sugar per flower (Corbet 2003). Nectar is secreted with particular rhythms throughout the lifespan of a flower, therefore, the knowledge of the nectar production dynamics is fundamental to understand plant-animal relationships (Galetto and Bernardello 2004). Increases in nectar availability favour pollinator attraction, thus promoting a high number of floral visits (Longo and Fisher 2006) and increasing pollen flow (Fisogni et al. 2001).

Day and night-pollinated species involve complementary pollination systems taking advantage of the diurnal or nocturnal pollinations (Maruyama *et al.* 2010) to increase reproductive success. This suggests that these species may have evolved specific mechanisms to maximize the visits of some pollinator suits without reducing the complete spectra of visitors. Plants may also change the scent and nectar composition throughout the day, as diurnal and nocturnal floral visitors likely have different olfactory abilities and scent preferences (Miyake *et al.* 1998; Dötterl *et al.* 2012). A mixed pollination system might allow plants to achieve a stable seed production when unpredictable

conditions or unstable pollinator abundance causes variation in the pollination success (Gómez and Zamora 1996).

As indicated by Kephart *et al.* (2006), there are numerous species of Caryophyllaceae which are characterized by both diurnal and nocturnal pollination. Because of this, it is important to identify the pollinators in all time slots of the day, paying particular attention to crepuscular and nocturnal pollinators, which are often understudied due to the difficulties associated with carrying out the experiments at night. Most insect-pollinated plants are generalists; this means that the pollination effectiveness of different insect species is similar (co-pollinators) (Ollerton *et al.* 2009; Cruz-Neto *et al.* 2011). Knowledge of the degree of specialization vs. generalization between the pollinators and the plant species dependent on them is very important in conservation biology, especially for threatened species (Kearns 1998; Blanché and Bosch 2007; Martinell *et al.* 2010). Plant species that have a highly specialized interaction with their pollinators are thought to be more susceptible to habitat fragmentation (Bond 1994; Ghazoul 2005). Habitat destruction not only affects the distribution of plant species, but it can also affect pollinators (Aizen and Feinsinger 2003; Aguilar *et al.* 2006). Small populations may be less attractive for insects (Ågren 1996; Cresswell *et al.* 2002); following fragmentation, pollinator behavior may change (Lázaro *et al.* 2008) and, as a result, insect pollination may decrease, thus affecting pollen flow (Ashworth *et al.* 2004; Ghazoul 2005). All these effects end up influencing the reproductive success of the species. When trying to identify the most efficient group of pollinators for a given plant, it is important to do exclusion experiments in order to understand the respective contribution of diurnal and nocturnal pollinator species (Young 2002; Giménez-Benavides *et al.* 2007; Ortega-Baes *et al.* 2011). In the Mediterranean basin, the most common nocturnal pollinators in nocturnal and crepuscular hours are moths and hawkmoths (Martinell *et al.* 2010). Plants with lepidopterans as primary visitors have as nocturnal pollinators both Noctuidea (moths) and Sphingidae (hawkmoths). *Hadena* Schrank, the most common pollinator for most moth-pollinated plants, is represented by more than 145 species feeding almost exclusively on Caryophillaceae hosts (Troubridge and Crabo 2002); these moths not only gather nectar, but also pollinate and oviposit on Caryophyllaceae hosts (Dötterl *et al*. 2006; Kephart *et al.* 2006). The insects that lay their eggs on the same flowers that they pollinate to rear offspring on the seeds constitute nursery pollination systems (Dufay and Anstett 2003). These flowers usually have a long tubular corolla, therefore only insects with long proboscides can reach the nectar cumulated at the bottom (Biere and Honders *et al.* 2006). The interaction between *Hadena* moths and some Caryopyllaceae plant species is well known. Moths in these systems can be effective both as seed predators and as pollinators (Westerbergh 2004). Plant-insect interactions

in nursery pollination systems involve both mutualistic and antagonistic aspects because the pollinators' offspring consume female reproductive structures of the plant during their development (Dufay and Anstett 2003). It is important the presence of an equilibrium between the mutualism and antagonism of this interactions in order to obtain a positive effect. On the other hand, the generalist pollination can counteracts the negative effects of ovoposition.

Pollen flow between flowers is greatly influenced by the spatial distribution of plants, floral characteristics, efficiency, and behaviour of pollinators (Barret 2003). Most of the pollen is usually deposited at short distances on the first few visited flowers and can be increased by high floral densities (Harder and Barret 1996; Flanagan *et al.* 2009) and by plant population size (Karron *et al.* 1995; Waites and Ågren 2004); only occasionally it is dispersed over long ranges (Van Rossum *et al.* 2011). Butterflies and large moths spend more time moving between patches and travel longer distances in search of pollen or nectar (Miyake and Yahara 1998). In contrast, some diurnal pollinators, such as bees, tend to forage within patches (Altizer *et al.* 1998). Hoverflies (Syrphidae) and bees flies (Bombylidae) are also common visitors in many Caryophyllaceae (Kephart *et al.* 2006), while syrphids are usually observed as pollen-collecting generalists and poor pollinators (Larson *et al.* 2006). Finally, some floral visitors that appear to be pollinators are more properly classified as "pollen thieves" (Inouye 1980). They thieve pollen when the anthers have just opened and without pollinate flowers due to the immature stigmas.

Dianthus morisianus is a threatened narrow endemic species which grows only in one natural population on established sand dunes in Portixeddu (south-western Sardinia; Bacchetta *et al.* 2010). The habitat has been highly modified and fragmented by afforestations which were carried out to stabilize dunes, as well as by urbanization, grazing, livestock and farming. The small population size and the limited seedling recruitment (Cogoni *et al.* 2012) make *D. morisianus* potentially prone to extinction, it is actually considered one of the most threatened plants on the island (Bacchetta *et al.* 2012) and it is categorized as Critically Endangered in the IUCN Global Red Lists (Fenu *et al.* 2013).

In previous studies, we observed that the species is highly dependent on pollinators, therefore in this study we investigated the interactions between *D. morisianus* and its pollinators. The aims of this study were: 1) to investigate the presence of diurnal and nocturnal pollinators and their effectiveness to produce fruits and seeds, 2) to verify the presence of a generalist or a specialist pollination system, 3) to assess the nectar production of *D. morisianus*, and 4) to verify the presence

of nursery pollination (insects that lay their eggs on the same flowers that they pollinate to rear offspring on the seeds and it potentially impose large fitness costs on their plant hosts).

4.2 Material and methods

4.2.1 Study species and plant material

D. morisianus Vals. (Caryophyllaceae) is a self-compatible gynomonoecious plant (Nebot *et al.* 2016 [chapter 1]). It is a perennial species characterized by numerous woody stocks, erect stems and a basal rosette with thin and linear leaves. The stems bear terminal multi-flowered heads; with long corolla tube and the colour of the corolla is white to pink. Petals are characterized by 6–8 teeth, rounded and irregularly lobed. The species has 10 anthers and two styles of 14 mm long (Bacchetta *et al.* 2010) and its flowers open during the whole day (see chapters 1-3). The flowering season is from early May to late June, and ripe fruits can be found during June–July (Bacchetta *et al.* 2010).

To carry out this study, in 2016 we selected three different areas in the population in order to avoid interference among experiments; each area was used for one of the three experiments conducted in this work, *i.e.*, pollination exclusions, nectar production quantification and pollinator census.

4.2.2 Partial insect exclusion

In order to know the efficiency of diurnal and nocturnal pollinators, we used 40 flowers during the 2015 flowering peak and 90 in 2016. The first year we conducted 20 diurnal and 20 nocturnal pollinator exclusions, while in 2016 we used 30 plants for each treatment and added the control treatment; during 2015, our studies were affected by herbivory, while in 2016 we increased the number of replicates to ensure we had a sufficient number of data to carry out a meaningful statistical analysis. Each treatment was applied randomly on selected plants and only one flower per plant was used. The diurnal pollinator exclusion consisted in covering flowers with fine mesh bags in the last male phase (stage e, see chapter 1) during the day and unbag it during the night in order to allow night pollination. Conversely, the night exclusion flowers were bagged at night and unbagged in the morning in order to test the diurnal pollinators. The control flowers were always left unbagged and open-pollinated during the whole flower lifespan. Bags were removed and placed between 7:30-8:00 and 20:30-21:00 until petals withered. The time considered as being “night” included the sunrise, while the day time included the sunset. Mature fruits were harvested three weeks after pollination and taken to the laboratory, where we counted the number of mature and aborted seeds and the number unfertilized ovules of each fruit. The fruit set, seed/ovule ratio, and weight were used to compare the efficiency of the two groups of pollinators.

4.2.3 Nectar secretion pattern and sugar concentration

During the flowering peak, the nectar secretion dynamics, the quantity of sugars and their concentration, and the standing crop were analysed. In order to test the secretion pattern and the sugar concentration during day and night, 40 hermaphroditic flowers were bagged with fine mesh bags prior to anthesis. Once the flowers opened, continuous extractions were carried out twice a day until they withered. To test the standing crop, we selected 100 flowers in different flowering stages. The volume and sugar concentration were analysed in the early morning and before dusk to investigate the characteristics of the nectar for diurnal and nocturnal pollinators. To evaluate the pollinator nectar uptake, we compared the results of bagged flowers with those of the standing crop. Nectar was extracted with 1 µl microcapillars (microcaps, Drummond Microcaps®). Microcaps were introduced into the bottom of the calyx, taking care not to damage nectaries. Nectar volume was calculated by measuring the nectar column in the microcapillars with digital callipers (Top Cal 150 PW). After that, the extracted nectar was deposited on the centre of the prism of a hand-held Bellingham and Stanley refractometer (Eclipse model 45-81; 0–50%) and the sugar concentration was read immediately afterwards to minimize the evaporation of the drop. After each nectar removal, a new microcap was used in order to avoid incorrect results due to the obstruction of microcaps; each nectar extraction was followed by temperature and relative humidity (RH) measurements. We were not able to determine sugar concentrations in nectar volumes smaller than 0.2 µl. The amount of sugar (mg) in the nectar was quantified in the laboratory following the method proposed by Galetto and Bernardello (2005). All nectar extractions were carried out by the same person to ensure reproducibility among measurements. Morning extractions were conducted from 7:00 a.m to 10:00 a.m. with temperatures ranging from 15 to 37 ºC and RH from less than 20 to 74%, while evening extractions were performed from 18:30 p.m. to 21:00 p.m., with temperatures ranging from 15 to 25 ºC and humidity percentages from 33 to 81%.

4.2.4 Nectar accessibility

The nectar accessibility of the species was calculated by measuring the functional flower length (*i.e.*, the distance from the bottom of the calyx to the end of the floral tube; Jürgens 2006), which is an indication of the minimum length of the pollinator's proboscis.

4.2.5 Pollinators census

Insect pollinators and visitants were studied both during the day and at night. To study diurnal visitors and pollinators, we conducted focal observations: we selected different groups of plants (15-20 flowers each) which were observed during 15 minutes at different times of the day, from the 18th of May until the 4th of June; this period corresponded to the flowering peak. During each

census, we recorded the insect species, the frequency and duration of visits, the behaviour and the number of visited flowers. Censuses were conducted from 8:30 until dark. A total of 50 h of observations were conducted on diurnal census. During the experimental period, the sunrise was from 6:00 to 6:09 and the sunset from 20:36 to 20:49. At the beginning and at the end of each census, the temperature and the relative humidity were measured using a thermo hygrometer. The temperature and humidity varied from 16 to 34 ºC and the RH from less than 20 to 55 %.

Nocturnal visitors were studied by using light traps, headlamps and a video camera with red light. Each day we selected a different spot in the population in order not to influence the pollinators' behaviour and we captured the insects that were attracted by the light. The two observers carried a headlamp with red light, as suggested by Reynolds *et al.* (2012), to improve visibility without disturbing the moths' behaviour. 18 hours of nocturnal observations from 21:00 to 24:30 were conducted; during the census, the temperature and the humidity varied from 11 to 23 ºC and from 60 to 84%, respectively.

4.2.6 Statistical analyses

To analyse the results of pollination exclusions experiments, General Linear Mixed Models (GLMMs) tests were conducted using "treatment" as a fixed factor and "year" as a random factor. The analyses were carried out using the package lme4 version 1.1-12 (Bates *et al.* 2015) and the function *lmer()*. As indicated in chapter 2, we performed an ANOVA to test our model against the null model. The nectar volume, sugar concentration, and quantity of sugars were analysed by two-way ANOVA. We called "time" the moment of the extraction (morning/evening) and "phase" the phenological stage of the flower (a-i, see Fig. 3 chapter 1); temperature and humidity were included as covariates. Finally, we conducted a three-way ANOVA in order to compare the results of the standing crop with those of the bagged flowers, adding "treatment" to compare the nectar characteristics among bagged and unbagged flowers. The ANOVA were tested with Tukey's *post*-hoc comparisons. All the analysis were conducted using R 3.3.1 (R Core Team 2016).

4.3 Results

4.3.1 Partial pollination exclusion

This experiment was affected by herbivory and we lost 8 treated fruits (six from diurnal exclusions and two from nocturnal exclusions). During the analysis of the fruits, we found four fruits from the nocturnal and the control experiments with one caterpillar each one which had eaten all the seeds and ovules of the ovary.

The results of the experiment are reported in table 1; some of the values obtained in 2015 were higher than in 2016, but since there were not significant differences they were analysed jointly. All of the treatments produced fruits, being the control the treatment that produced more fruits, followed by diurnal and nocturnal exclusion. Both the number of fruits and the seed weight in the control were significantly different with respect to the values obtained in the nocturnal exclusions; significant differences were also observed between control and diurnal exclusion for the seed/ovule ratio variable (Table 1).

Table 1. Reproductive success of the pollination exclusion experiment; different letters and the bold indicate significant differences at $P < 0.05$.

	Control	Diurnal exclusion	Nocturnal exclusion	χ^2	d.f.	*P*	Random
Fruit set	100.00^{a}	92.00^{ab}	89.00^{b}	6.198	2	**0.045**	Residual>year
Seed/ovule ratio	54.58 ± 6.24^{a}	35.29 ± 3.87^{b}	45.13 ± 3.17^{ab}	12.427	2	**0.002**	Residual>year
Seed weight (mg)	13.40 ± 2.00^{a}	12.10 ± 2.00^{ab}	9.80 ± 2.00^{b}	7.865	2	**0.019**	Residual>year

4.3.2 Nectar

The mean accumulation of nectar in bagged flowers was of 0.86 ± 0.95 µl, with 26.87 ± 7.22% and 0.33 ± 0.29 mg of sugar, while unbagged flowers accumulated 0.66 ± 0.62 µl of nectar with 33.03 ± 8.93% and 0.32 ± 0.26 mg of sugar. The nectar quantity was higher during the female phase and, more specifically, when they were at stage h (when the styles are at their maximum receptivity moment; see Fig. 3, chapter 1). The results are reported in Fig. 1 and in the Table 1 of the appendix. When bagged flowers reached the female phase (stage f, Fig. 3, chapter 1) we were not able to obtain the nectar secretion in most of them, we therefore selected 20 additional flowers in their early female phase. Some flowers without nectar production were found.

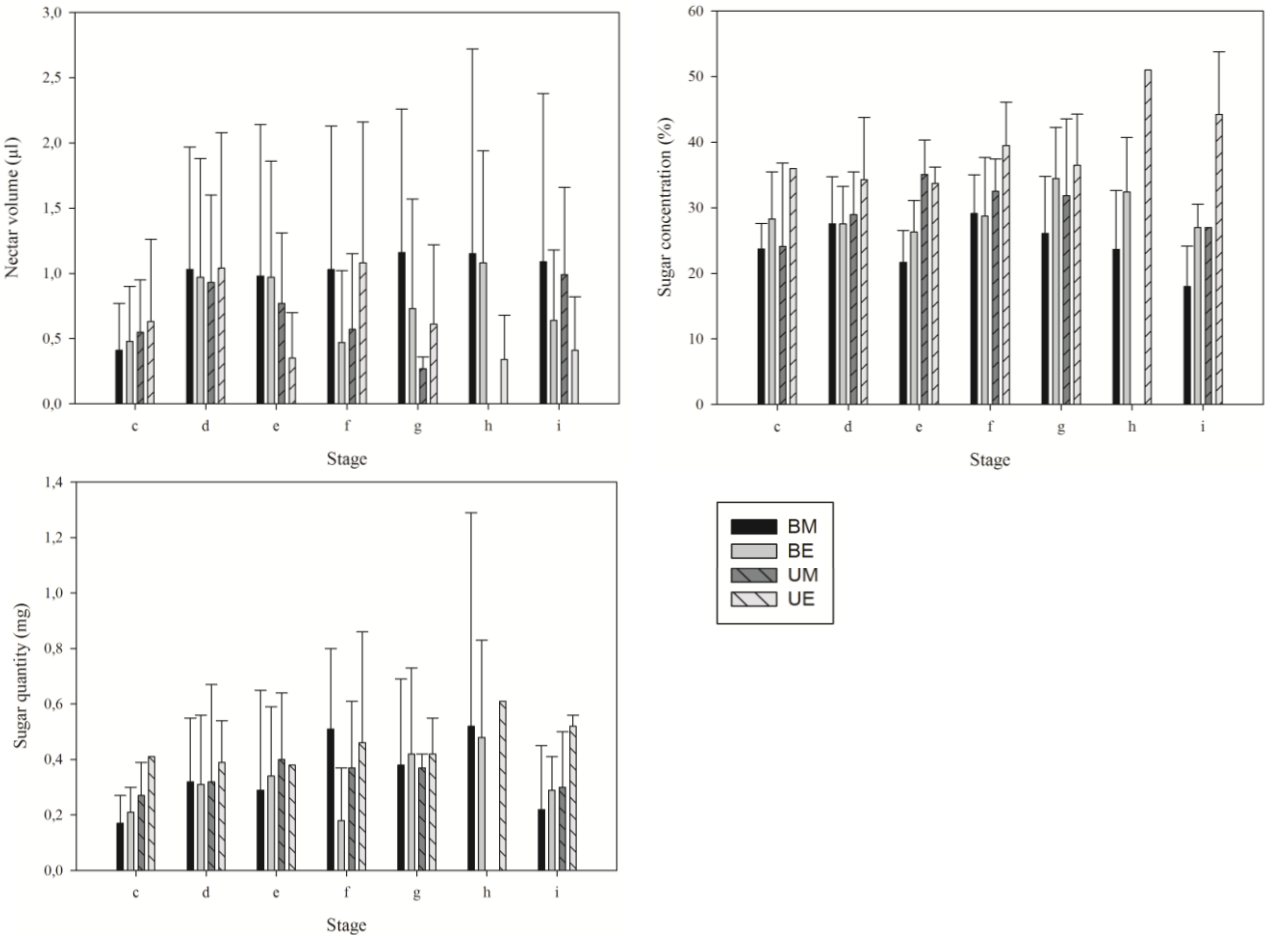

Figure 1. A) Nectar volume (mean ± SE), B) sugar concentration, and C) sugar quantity in morning-bagged flowers (BM; plain black bars), evening-bagged flowers (BE; plain light grey bars); morning unbagged (UM; striped dark grey bars) and evening unbagged flowers (UE, striped light grey bars) during flower development (male phase: stages c-e, female phase: stage f-i.

When analysing the standing crop, we observed that the volume of the nectar extracted in the morning and in the evening was not significantly different, but both sugar concentration and sugar mass were lower in almost all morning extractions, with significant differences (Table 2). Female and male stages did not show significant differences (Table 2). Neither the humidity nor the temperature had a significant effect on the flowers used in this experiment.

Table 2. Results of the two-way ANOVA analysing the volume, the concentration and the quantity of sugars of nectar extracted in the standing crop (unbagged flowers); values are significant at $P \leq 0.05$ and are indicated in bold.

	Nectar volume (µl)			Concentration (%)			Quantity of sugars (mg)		
	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*
Time	1	0.370	0.546	1	5.375	**<0.050**	1	5.520	**<0.050**
Phase	6	0.833	0.551	6	1.871	0.119	6	1.870	0.120
Temperature	1	4.930	**0.032**	1	0.490	0.489	1	1.135	0.295
Humidity	1	0.181	0.672	1	0.813	0.374	1	0.055	0.815
Phase:Time	5	0.566	0.725	5	0.673	0.647	5	0.704	0.624
Error	42			29			29		

As for bagged flowers, diurnal extractions showed a higher volume than nocturnal ones, with a lower sugar concentration and a higher quantity of sugars, but only the concentration values were significantly different (Table 3). When sorting the data by different gender stages, female stages produced a lower nectar volume, with lower concentration, and a lower quantity of sugars than male stages, but there were not significant differences (Table 3). Humidity significantly affected the quantity and the concentration of sugars, while temperature only affected the concentration. The concentration of sugars varied significantly when comparing the time of extraction and the phase of the flower.

Table 3. Results of ANOVA two-way analysing the nectar volume, concentration and quantity of sugars in the nectar extracted on bagged flowers; values are significant at $P \leq 0.05$ and are indicate in bold.

	Nectar volume (µl)			Concentration (%)			Quantity of sugars (mg)		
	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*
Time	1	0.005	0.941	1	8.246	**<0.010**	1	0.039	0.844
Phase	6	2.123	0.052	6	2.122	0.054	6	0.907	0.492
Temperature	1	1.328	0.250	1	4.644	**<0.050**	1	0.008	0.930
Humidity	1	5.583	**0.019**	1	9.447	**<0.010**	1	8.836	**<0.010**
Phase:Time	6	1.088	0.371	6	3.726	**<0.010**	6	1.626	0.144
Error	191			142			138		

When comparing the nectar extracted by accumulation and standing crop, we observed that, in the later case, we obtained a lower nectar volume, a higher sugar concentration with significant differences, and a higher quantity of sugars than in bagged flowers (Table 4). Morning nectar extractions in the standing crop were lower, with significantly lower sugar concentration than bagged flowers and variable sugars. On the contrary, in the evening extractions of the standing crop, we obtained a variable accumulation of nectar depending on the stage, significantly higher sugar concentrations and a lower quantity of sugars than in bagged flowers (Table 4). In the standing crop, male stages had the same nectar volume than female ones, but significantly lower sugar concentrations and quantity of sugars. There were not significant differences when analysing the interaction between time and treatment and between stage and treatment (Table 4). In morning extractions, we obtained a higher nectar volume in male stages than in female ones while, during the night, female stages turned out to have a higher nectar volume. Female flowers were characterized by a higher sugar concentration than male stages both in the morning and in the evening extractions. As for the quantity of sugars, it was higher at night for both male and female stages. Also in this case, the only variable with significant differences was the concentration of sugars (Table 4). In the comparison among the two treatments, the temperature did not show a significant effect, but the humidity did (Table 4). The comparison of stages:time:treatment did not

highlight significant differences in any of the variables. The temperature and the humidity were very changeable during the day, ranging respectively from 10 to 40 °C and from less 20% to more than 80% during the flowering period.

Table 4. Results of the three-way ANOVA comparing the volume, the concentration and the quantity of sugars of the nectar extracted from bagged and unbagged flowers; values are significant at $P \leq 0.05$ and are indicated in bold.

	Nectar volume (µl)			Concentration (%)			Quantity of sugars (mg)		
	d.f.	F	*P*	d.f.	F	*P*	d.f.	F	*P*
Time	1	0.003	0.957	1	10.963	**<0.010**	1	0.770	0.381
Stage	7	1.943	0.063	7	2.552	**<0.050**	7	1.651	0.124
Treatment	1	0.028	0.867	1	32.666	**<0.010**	1	0.608	0.436
Humidity	1	4.424	**<0.05**	1	6.952	**<0.010**	1	7.469	**<0.01**
Temperature	1	0.067	0.795	1	3.286	0.070	1	0.273	0.602
Stage:Time	7	0.785	0.600	7	2.892	**<0.010**	7	0.961	0.461
Stage:Treatment	5	0.487	0.785	5	1.968	0.085	5	0.727	0.604
Time:Treatment	1	0.443	0.506	1	1.742	0.188	1	1.046	0.307
Stage:Time:Treatment	4	0.999	0.408	4	1.216	0.305	4	1.448	0.220
Error	235			173			169		

4.3.3 Nectar accessibility

The average functional flower length of female flowers was 20.84 ± 0.75 mm, while it was 26.08 ± 1.63 mm in hermaphrodite flowers, meaning that pollinators must have a proboscis of at least 26 mm of length in order to reach the nectaries. Female flowers measured on average 26.49 ± 1.49 (n = 10) mm from the bottom of calyx to the end of styles, while hermaphrodite flowers showed a mean of 30.31 ± 2.03 mm (n = 20). The corolla of female flowers was also smaller than those of hermaphrodite ones (15.56 ± 1.56 mm and 24.09 ± 1.79 mm, respectively).

4.3.4 Pollinators

During the first days of the study, we did not observe any insect activity, neither in the studied plants, nor in the population. There were also other species flowering at the same time as *D. morisianus*, namely *Cistus salviifolia*, *Lavandula stoechas*, *Scabiosa maritima*, *S. niceensis* and *S. berguinottii*. The 79.27% of census had not any visit. *Macroglossum stellatarum* L. (Lepidoptera: Sphingidae) was observed as the main diurnal pollinator of *D. morisianus* (Table 2 of the appendix), this species was not observed while pollinating other species. *M. stellatarum* visited numerous flowers in different flowering stages and moved around plants; a maximum of two individuals at the same time and in the same area was observed. Sometimes, they returned to the same flowers after a short time, which suggested that they had not always removed all the available nectar. Other species were also observed as pollinators at the same time and in the same area, but

they moved around visiting many flowers of many different species. The other observed insects were: *Gonopterix cleopatra* (Lepidoptera: Pieridae), which gathered nectar of both *D. morisianus* and *S. maritime*; *Pieris rapae*. (Lepidoptera: Pieridae) and some Diptera (Syrphidae and Bombyliidae) (Table 2 appendix). We also observed *Bombus* in the population, but it rarely stood on *D. morisianus*; rather, it usually pollinated flowers of *C. salviifolius*, the few times that it stopped on *Dianthus* flowers, it inhibited the production of seeds due to style clogging with pollen from *Cistus* (personal observations). During the years of study, we observed several "pollen thieves" (*i.e., Oedemera* sp., and some Orthoptera, Brachycera and Diptera (Bombyliidae) species).

During the first nocturnal censuses the light of the moon helped to see nocturnal pollinators and their interaction with the plants but, at the same time, it caused the light traps to fail in attracting them and it led to a high radiance due to the reflection on the sand. When the rise of moon began to be delayed and the moon started to wane, the light trap started to attract some pollinators. During four days, a big hawkmoth was observed while pollinating several flowers of *D. morisianus* at the end of dusk; this made the capture difficult due to the lack of light. We also observed several Lepidoptera as visitors, namely Noctuidae (*Hadena* sp., *Heliothis* sp., *Autographa* sp.) and Sphingidae (Table 2 of the appendix). We were not able to capture all the observed pollinators and, in particular, the hawkmoths and the *Autographa* sp. individuals. The video camera only registered some crickets that were stealing pollen.

4.3.5 Nursery pollination

During the analysis of fruits we found 17 caterpillars, four of them belonged to the control and the nocturnal pollination treatments of the pollination exclusion experiment, and the others from studies of the chapter 3. The fruits containing caterpillars had a small hole on the mature capsule and the entire ovule and their seeds had been eaten. These caterpillars were reared in the laboratory but, since all the fruits were mature at that time, they were not suitable because caterpillars usually feed on immature seeds. We also found numerous fruits with a hole in the calyx, this was also made by caterpillars when moving to another fruit once they had finished to eat all seeds in the previous one.

4.4 Discussion

D. morisianus presented both, diurnal and nocturnal pollination, even if the flower did not correspond with the classical pollination syndromes. As observed in the pollination exclusion experiment, open pollinated flowers were those that were characterized by a higher reproductive success, higher fruit set, seed/ovule ratio, and weight, showing significant differences between

nocturnal pollinators on the seed set and diurnal pollinators on seed weight. These results suggest that *D. morisianus* is characterized by a mixed pollination system, with effective pollinators both during day and at night. This is in accordance with Kephart *et al.* (2006) and Amorim *et al.* (2013), who indicated that it is common for plant species with a classical diurnal pollination syndrome to be pollinated by both diurnal and nocturnal pollinators. There are also numerous Caryophyllaceae species with long-tube corolla which are visited by both diurnal and nocturnal insects (Friedrich 1979; Kephart *et al.* 2006). In *D. morisianus*, the main diurnal pollinator was *M. stellatarum* (Sphingidae), while, at night, we know that pollinators are moths (Noctuidae) or hawkmoths (Sphingidae)] but we can neither determine which group is the main pollinator [nor their efficiency. Our results are in accordance with Kephart *et al.* (2006) and Martinell *et al.* (2010), who reported that these two groups of pollinators are very common in the Caryophyllaceae family and are also the main nocturnal pollinators in the Mediterranean basin. *D. sylvestris* and *D. gratianopolitanus* are two examples of species which are also pollinated by both diurnal and nocturnal pollinators and by the same pollinator genera of *D. morisianus* (Collin *et al.* 2002; Edhardt 1990, respectively). We also observed different effective insect pollinator genera on *D. morisianus*; this could indicates that this species is characterized by a generalist pollination. As reported by Larson *et al.* (2006), syrphids were collectors but poor pollinators in our system. The functional flower length showed us that pollinators should have a proboscis longer than 26 mm, which was the case of the pollinators we observed. The presence of *S. niceensis* in the same area could cause competion for pollinators due to the fact that this species is also pollinated by Noctuids, as observed by Buide *et al.* (2015).

During the analysis of the fruits we found some larvae inside, thus indicating the presence of a nursery pollination system. We obtained nocturnal pollinated fruits without larvae, which suggested that those were pollinated by male *Hadena* or by different species (other than *Hadena* sp.), which pollinate without laying eggs inside the calyx. The presence of different pollinators genera and effective diurnal pollinators might reduce the negative effect of nursery pollination. These results indicate that *D. morisianus* could present a nursery pollination system with *Hadena* species, such as *D. sylvestris* with *H.compta* (Collin *et al.* 2002).

As reported by Proctor *et al.* (1996), nectar is the main reward for pollinators, and *D. morisianus* produced it continuously during its whole lifespan. The continuous secretion of nectar also demonstrates the presence of both nocturnal and diurnal pollinators (Galetto and Bernardello 2005). The amount of nectar was very variable, being higher in female phases and in the morning. When petals withered, flowers still contained nectar, thus indicating the absence of nectar resorption. The absence of nectar secretion in bagged flowers could be due to some damage in the

nectaries because of lack of practice (Cruz-Neto *et al.* 2015) or to the effect of successive extractions. Nectar production entails a cost for the plant, therefore it is possible that, after several extractions, flowers stopped their production (Bobrowiec and Oliveira 2012). The amount of nectar was not significantly different between bagged and unbagged flowers, this could suggest that the analysed non-bagged flowers had not been visited recently by pollinators. The different concentrations of sugars could be due to the fact that the extraction of nectar could modify the concentration of solutes, which increases as the amount of nectar available decreases (Corbet 2003). The study species has long corolla and low nectar concentrations and this is in accordance with studies that indicate that the long corolla tube directly influences the concentration of nectars by facilitating or avoiding the evaporation or condensation, and that deeper corollas are characterized by lower concentrations of solutes (Galetto and Bernardello 2005). The low concentration of nectar is a typical characteristic of hawkmoth-pollinated species: hawkmoths move among patches (Miyake and Yahara 1999), increasing the crossing of pollen around the whole population. The significant differences in the concentration of nectar solutes between day and night is another indication of the presence of diurnal and nocturnal pollinators, since they have different preferences (Miyake *et al.* 1998).

4.5 Conclusions

This is the first study on the pollinators of *Dianthus morisianus.* This species is characterized by a complementary pollination system with both diurnal and nocturnal effective pollinators, which demonstrates that the only natural population of this taxon is not currently affected by a lack of pollinators. Rather, this population is affected by habitat loss and fragmentation (Cogoni *et al.* 2013). If this threat continues to affect the area, it could have some adverse consequences on the behaviour and presence of pollinators (Aizen and Feinsinger 2003) and therefore on the reproductive success of *D. morisianus,* since this species is highly dependent on pollinators (see chapter 2). Also, while the presence of a generalist system does not increase the resilience to changes, a self-compatible system is widely known as being beneficial in this sense and it might increase the species' chances of survival in the long term (Aguilar *et al.* 2006). Future studies should be aimed at acquiring a through knowledge of the nocturnal pollination effectiveness, to study the production of nectar in order to better understand its characteristics, at understanding the relative contribution of the *Hadena* nursery pollination, of how this affects the reproductive success, and at verifying the possible presence of competition between *Silene* spp. and *D. morisianus* due to the two species co-occur and could share pollinators.

4.6 References

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General conclusions

Throughout this thesis, we achieved the following general conclusions:

1. The *ex-situ* studies of the reproductive biology of *Dianthus morisianus* showed that the species is self-compatible and is characterized by a mixed mating system, as well as by a high reproductive success by xenogamy and geitonogamy pollinations **(Chapter 1).**
2. Flowers are characterized by proterandry and asynchronous flowering, thus enabling the occurrence of geitonogamous pollination **(Chapter 2)**.
3. *Ex-situ* studies are useful in order to understand some aspects of the reproductive biology of threatened plants without disturbing the natural population **(Chapter1 and 2).**
4. Currently, the natural population is not affected neither by pollen limitation nor by high inbreeding depression **(Chapter 2).**
5. The species is highly dependent on pollinators, as observed in both the natural and the reintroduced populations **(Chapters 2 and 3)**. In the absence of pollinators, the species could auto-pollinate, although the reproductive success would be very low **(Chapters 2 and 3).**
6. Plants of the natural and reintroduced populations flowered at the same time, with a high flowering synchrony. This characteristic and the proximity between the two populations may facilitate the crossing of pollen between them **(Chapter 3).**
7. To this day, the first reintroduction has been successful. The offsprings of the reintroduced population behave like the natural ones in terms of reproductive success, which demonstrates that the conditions of the habitat of the reintroduced population are appropriate for the development of the species **(Chapter 3).**
8. *D. morisianus* has a mixed pollination syndromes, it is pollinated efficiently by both diurnal and nocturnal pollinators and its main pollinators are Noctuidae and Sphingidae. The existence of effective co-pollinators makes *D. morisianus* a generalist species **(Chapter 4).**
9. *D. morisianus* forms a nursery pollination system with *Hadena* species **(Chapter 4).**

With the results obtained we can conclude that the current narrow distribution of the species is not due to a low reproductive success. If the destruction of the habitat continues, the pollinators could modify its behavior and therefore the reproductive success of the species would be affected due to the high dependence on pollinators to obtain successful offspring. Nowadays, in order to preserve the species, conservation efforts should aim at preventing a further habitat reduction. In future studies should be studied the impacts of the herbivory and the possitive-negative balance of nursery pollination, and the effect of the habitat fragmentation on pollinator assemblage.

Appendix

Table 1. Nectar volume (µl), nectar concentration (%) and energy (cal) (mean ± SE) in bagged and unbagged flowers during flower development (male phase: stages 1-3, female phase: stages 4-7).

		Bagged flowers				Unbagged flowers			
Flower stage	Time	N	Nectar volume (µl)	Nectar concentration (%)	Energy (cal)	N	Nectar volume (µl)	Nectar concentration (%)	Energy (cal)
c	Morning	12	0.41 ± 0.36	23.71 ± 3.89	0.69 ± 0.40	4	0.55 ± 0.40	24.12 ± 12.70	-
	Evening	8	0.48 ± 0.42	28.3 ± 7.17	0.86 ± 0.39	1	0.63	36	0.26
d	Morning	40	1.03 ± 0.94	27.56 ± 7.21	1.28 ± 0.94	13	0.93 ± 0.67	28.95 ± 6.51	-
	Evening	36	0.97 ±0.90	27.74 ± 5.74	1.26 ± 1.01	5	1.04 ± 0.67	34.3 ± 9.46	0.35 ± 0.15
e	Morning	27	0.91 ± 1.16	21.7 ± 5.02	1.19 ± 1.05	6	0.77 ± 0.54	35.1 ± 5.25	-
	Evening	28	0.94 ± 0.88	26.31 ± 4.80	1.37 ± 1.09	4	0.35 ± 0.33	33.75 ± 2.47	0.24 ± 0.00
f	Morning	15	1.03 ± 1.10	29.12 ± 5.89	2.03 ± 0.93	3	0.57 ± 0.58	32.5 ± 4.95	-
	Evening	12	0.47 ± 0.55	28.72 ± 8.96	0.74 ± 0.74	7	1.08 ± 1.12	39.5 ± 6.62	0.68 ± 0.39
g	Morning	21	1.11 ± 1.17	25.13 ± 8.59	1.54 ± 1.28	10	0.27 ± 0.09	31.87 ± 11.72	-
	Evening	24	0.69 ± 0.88	33.26 ± 8.44	1.67 ± 1.36	3	0.61 ± 0.63	36.5 ± 7.78	0.35 ± 0.13
h	Morning	8	1.15 ± 1.57	23.67 ± 8.99	2.07 ± 3.09	0	-	-	-
	Evening	7	1.08 ± 0.86	32.41 ± 8.28	1.91 ± 1.39	1	0.34	<50	0.22
i	Morning	4	1.09 ± 1.13	18.00 ± 6.13	0.90 ± 0.92	2	0.99 ± 0.67	27.00 ± 0.00	-
	Evening	3	0.64 ± 0.54	27.00 ± 3.53	1.15 ± 0.50	2	0.41 ± 0.03	44.25 ± 9.55	0.22 ± 0.04

Table 2. Frequency of insects during the census. Each diurnal census lasted 15 minutes, while for the nocturnal, each day was counted as a census.

Insects	Activity period	Frequency	Resource used	Behaviour
Lepidoptera				
Sphingidae				
Macroglossum stellatarum	Diurnal	12.95	Nectar	Pollinator
Another sphingidae	Dusk, nocturnal	60.00	Nectar	Pollinator
Noctuidae				
Hadena spp	Nocturnal	20.00	n.a.	Pollinator
Heliothis sp.	Nocturnal	20.00	n.a.	n.a.
Autographa sp.	Dusk	10.00	Nectar	n.a.
Pieridae				
Gonopterix cleopatra	Diurnal	5.18	Nectar	Pollinator
Pieris rapae.	Diurnal	1.04	Nectar	Occasional pollinator
Diptera				
Bombyliidae				
Bombylius sp.	Diurnal	2.07	Pollen	Thief
Syrphidae	Diurnal	5.70	Pollen	Thief
Brachycera	Diurnal	1.55	Pollen	Thief
Coleoptera				
Oedemeridae				
Oedemera sp.	Diurnal	3.62	Pollen	Thief
Orthoptera				
Ensifera	Diurnal, nocturnal	2.59 diurnal+20.00 nocturnal	Pollen	Thief/pollinator

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