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Pollination ecology and reproductive success in isolated populations of flowering plants: *Primula apennina* Widmer, *Dictamnus albus* L. and *Convolvulus lineatus* L.

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1. Introduction

1.1 Plant breeding systems

Breeding system is a key trait that affect different aspects of plants' life: individual fitness, the degree of plant dependence on pollinators, the response of plant reproduction to disturbance and environmental shifts, and eventually population genetics. Breeding systems range from enforced outbreeding, characteristic of dioecious plants (i.e., with distinctive male and female individuals) or of plants with a genetic self-incompatibility system, to agamospermy (Lloyd & Schoen 1992). Deposition of self-pollen within a plant may lead to deleterious effects on reproductive success, and have costs on both male and female fitness. Inbred offspring are usually less fit than outbred progeny, and inbreeding depression is generally indicated as a main selective force that shapes the evolution of plant mating strategies (Charlesworth & Charlesworth 1987).

Geitonogamy involves transfer of pollen between flowers of a same plant. It has the ecological properties of cross-fertilization and the genetic properties of self-fertilization (Lloyd & Schoen 1992). Whenever anthesis proceeds asynchronously in multi-flowering species, a certain amount of intra-plant pollination may occur. In self-compatible plant species, self-pollen deposited on stigmas may be in competition with cross-pollen, and reduce the production of cross-fertilized seeds (Vaughton & Ramsey 2010). Furthermore, the production of self-fertilized seeds can incur low seedlings recruitment and survival due to inbreeding depression, reducing female fitness (Owen et al. 2007).

Pollen discounting (i.e., self-pollination by which pollen is unavailable for outcrossing) decreases the amount of pollen available to fertilize other individuals, compromising fitness through the male function (Brunet 2005). As a result, geitonogamy can affect the overall genetic structure of the populations (de Jong et al. 1993).

In plants with hermaphroditic flowers, where male and female sexual organs are contained in a same flower, different features have evolved to promote cross pollination. Self-incompatibility system (SI) is a genetically determined pre-zygotic barrier to self-fertilization, which reduces risks of inbreeding and optimizes the potential for outbreeding afforded by insect pollination. Three types of genetic SI have been described: sporophytic (SSI), gametophytic (GSI) and ovarian (LSI). In SSI the incompatibility phenotype of the pollen is determined by the diploid genome of the plant that produced it, and it is recognized at the stigmatic level of the receipt plant; in GSI the incompatibility phenotype of the pollen is determined by its own haploid genome, and it is recognized at the stylar level (Hiscock & McInnis 2003). In LSI (late-acting self-incompatibility), the incompatibility phenotype is recognized at the ovarian level, before or after ovule is reached (Seavey & Bawa 1976).

In addition to genetic self-incompatibility, temporal and spatial separation of sexual functions are among non-physiological traits that can reduce the likelihood of self-fertilization (de Jong et al. 1993; Barrett 2002). Sexual structures can function at different times (dichogamy): anthers can expose vital pollen before stigma receptivity (protandry) or, otherwise, stigma can become receptive before anthers dehiscence (protoginy).

Anthers and stigma can also be placed at different heights inside flowers (herkogamy). In this way, it is unlikely to achieve self-pollination, and outcross fertilization might be higher. A typical form of herkogamy is "heterostily", where different morph-types show a reciprocal positioning of anthers and stigma, namely "distylous" and "tristylous" if sexual organs are placed on two or three levels, respectively. These mechanisms should increase the likelyhood of precise pollen transfer (Barrett 2002).

1.1.1 Distyly

Distyly is a form of heterostyly, a genetically-determined floral polymorphism in which floral morphs differ in style and stamens length, with long-styled "pin" flowers (P), where anthers are attached within the corolla tube, and short-styled "thrum" flowers (T), with anthers positioned near the mouth of the corolla (Darwin 1877; Ganders 1979; Barrett 1990). Besides sex-organ reciprocity, heterostylous plants usually have a heteromorphic incompatibility system that prevents selfing and intramorph mating (Barrett 2002), nonetheless several exceptions are known (Riveros et al. 1995; Brys et al. 2008a; Weller 2009). In distylous plants these traits are under a supergene control, which also determines other floral features, such as differences in pollen grain size and number between long-styled and short-styled flowers (Ganders 1979; Mazer & Hultgard 1993; Wolff & Liede-Schumann 2007).

Pollen size is generally negatively correlated to grain number (Ganders 1979; Cruden & Miller-Ward 1981): it has been recently demonstrated that larger size of thrum pollen grains is correlated to stigma depth, for the initial autotrophic pollen-tube growth phase, rather than with style length (Cruden 2009). It has been hypothesized that, in distylous species, selection has favoured increased pollen production by pins to compensate for unequal pollen flow (Ganders 1979); recently several studies reported that thrum stigmas capture less total pollen than pins (Stone & Thomson 1994; Matsumura & Washitani 2000; Nishihiro et al. 2000; Brys et al. 2008a).

Physiological and morphological traits of distylous species therefore contribute to promote intermorph pollen transfer, inducing high outcrossing rates. At the same time, heteromorphic incompatibility, by preventing intramorph mating, restricts outcrossed mating opportunities within a population (Barrett 2002; Thompson & Arroyo 2009).

Disassortative mating in distylous plants is expected to result in the equilibrium of morph frequencies (Ganders 1979; Heuch 1979), the so-called isoplethy (1:1 morph ratio). However, deviations from the 1:1 equilibrium ratio have been observed in natural populations, due to stochastic processes, pin advantage, or niche differentiation between morphs (Arroyo & Dafni 1995; Kéry et al. 2003; Van Rossum et al. 2006), with higher rates in small populations (Baker et al. 2000a; Endels et al. 2002; Shao et al. 2008; Brys et al 2008b). Biased morph ratio may affect the transfer of compatible pollen to the stigmas (Garcìa-Robledo & Mora 2007), and in isolated populations it could lead to Allee effect and increased genetic drift (Byers & Meagher 1992; Washitani 1996; Brys et al. 2007).

1.1.2 Resource allocation to sexual functions

Plants invest energetic resources in different structures such as storage roots or rhizomes, other vegetative parts (stems, leaves) and flowers (perianth, nectar, pollen and ovules). Allocation may vary in time, for instance different amounts of resources might be invested to pollen grains or ovules in early or late flowering season (Baker et al. 2005). Sexual allocation in angiosperms can be subject to many selective factors, such as self-fertilization (Greef et al. 2001) or plant size-dependent allocation in relation to geitonogamous selfing (de Jong et al. 1999).

In hermaphrodite flowers, resource allocation to sexual reproduction can be represented primarily by male and female sexual functions, which mainly are expressed through pollen and ovules production.

Different theories have been proposed to investigate the complex interactions and equilibria that rule sex allocation (Charlesworth & Charlesworth 1981; Charnov 1982), but the estimation of energetic resources allocation, costs and trade-offs between sexual functions remains difficult to analyse (Charlesworth & Morgan 1991).

In relation to male function, a trade-off between number and size of pollen grains produced has been observed in some distylous species. This energetic compromise is suggested to be in relation with an optimization of maternal resources and fertilization success (Cruden & Miller Ward 1981).

In general, floral specialization can be driven by selection through male function: when visits by insects increase male fitness while minimizing pollen loss, plants can specialize on different pollinators (Muchhala et al. 2010).

Resource allocation to female function may be more dependent to available resources and positively related to plant size, but differences might be also found among populations due to genetic or ecological conditions (Sánchez-Lafuente 2007; Andrieu et al. 2007).

The analysis of both pollen and ovule number might be useful to indirectly assess the type of breeding system of plants. Cruden (1977) proposed pollen:ovule ratio as an index of pollination efficiency; the higher is the ratio, the higher is the number of pollen grains required to achieve successfull pollination, hence the lower is the efficiency. A decrease in P:O’s is also correlated to the evolutionary shift in breeding systems from xenogamy to cleistogamy (Cruden 1977).

1.2 Plant – pollinator interactions

1.2.1 Floral rewards

Plant-pollinator system co-evolved as a mutualism in which plants offer essential rewards that promote repeated visits by insects, which eventually can lead to pollination. Pollinators give an unwilling fundamental service to plants in return for vital nutriments and other non-nutritive rewards.

Animals mainly seek nectar and pollen during their foraging bouts; other minor nutritive rewards include among others oils, resins and gums (Neff & Simpson 2005).

The vast majority of zoophilous flowers function as nectar flowers (Westerkamp 1996; Pacini & Nicolson 2007). At mean latitudes Hymenoptera and Lepidoptera are the main insect Orders that forage for nectar. Nectar is a complex solution which is mostly drinked by insects for their own consumption, as a source of energy and water. Its main components are in fact water and carbohydrates (mainly sucrose, glucose, fructose), which influence nectar concentration and energy values; other components are amino acids, proteins, lipids and organic acids (Nicolson & Thornburg 2007).

Pollen is a main source of proteins and nitrogen: it is directly eaten by Coleopterans, while bees collect it primarily as larval food (Westerkamp 1996). From the plant point of view, pollen is the vehicle for male gametes, and is usually transported inadvertitely by flower visitors. In fact pollen that is actively collected by bees and stored in specific body parts as corbiculae or legs-scopae, is commonly not available for pollination

(Westerkamp 1996). The patterns of rewards clearly influence visitors and pollinators behaviour, and consequently plant sexual reproduction.

1.2.2 Pollinators behaviour and insect-mediated geitonogamy

In dichogamous species with earlier maturation of the male phase (protandry), within-flower pollination is avoided. Nonetheless, if flowers develop asynchronously and anthesis proceeds from the basis toward the top of the raceme (i.e., the male phase being in an upper position compared to the female phase), in an entomophilous plant geitonogamy could be spread through the action of a pollen vector, depending on its foraging behaviour (de Jong et al. 1993; Carlson & Harms 2006). If insects forage downward in the inflorescence, pollen might be transported from dehisced anthers of upper flowers to receptive stigmas on lower flowers, resulting in inter-flowers inbreeding. Otherwise, with bottom to top visits on the raceme, pollination among flowers will hypothetically be null. Moreover, if a pollinator departs from upper flowers of a plant and alights on lower flowers of a different individual, geitonogamy might be minimized by outcross pollen deposition on female-phase flowers, and outcrossing enhanced by more pollen export from male-phase flowers to different plants (Best & Bierzychudek 1982; de Jong et al. 1993; Jordan & Harder 2006).

As pollinator behaviour is influenced by reward patterns, in species with sexual segregation the differential production of nectar between male- and female-phase can be regarded as an evolutionary trait that can contribute to reduce the level of self-fertilization (Pyke 1978; Waddington & Heinrich 1979).

Depending on floral arrangement and development, gender-biased nectar production may result in a vertical gradient of reward along the raceme (Carlson & Harms 2006).

This pattern can influence the amount of pollen gathered by pollinators and the degree of geitonogamy, maximizing pollen dispersal by inducing insects to start foraging at the bottom of the inflorescence and leave the plant before reaching the top empty flowers (Waddington & Heinrich 1979; Iwasa et al. 1995; Biernaske & Elle 2005).

The “marginal value theorem” (Charnov 1976) hypothesizes that animals optimize their foraging activities: past studies on bumblebees showed that their rules of movement seem to be optimally adapted, resulting in maximum net rate of energy gain (Pyke 1978, 1979, 1980).

1.2.2 Pollen limitation and reproductive effort

When plants receive inadequate pollen amounts or incompatible pollen types, pollen limitation occurs: the consequence is a reduction of the potential fruit or seed set. A reduction in seed production may have effects on the demographic structure of populations (e.g., low seed bank, low response to disturbance by re-establishing with seeds), and plants that are higly dependent on seed for propagation and survival can incur high risk for population persistence (Bond 1994).

Insufficient pollen quantity or incompatible pollen has been demonstrated to be a cause of reduced fecundity also in small plant populations (Byers 1995; Ågren 1996). When plants receive insufficient pollen, a sub-maximal fertilization of ovules occurs; in addition, if most pollen is not genetically compatible, the resulting seed set might be especially low.

It has been observed that there could be a pollen limitation but not a pollinator limitation (Wagenius & Lyon 2010), indicating that not all insect visitors can be efficient pollen vectors. For this reason, an integrated study from both pollinator and plant point of view is be more valuable.

Reproductive outcome (e.g. fruit:flower, seed:ovule ratios) has been shown to be positively correlated with population size (Brys et al. 2007) or population structure (Shao et al. 2008; Dauber et al. 2010). Moreover, indipendently of the actual size of a population, small or low-density patches of blooming plants may attract fewer pollinators, with increased likelihood of pollinator limitation (Sih & Balthus 1987; Ågren 1996; Ishihama et al. 2006). Furthermore, different ecological perturbations can act towards a disruption of the plant-pollinator interactions and lead to pollen limitation, like habitat fragmentation, loss of pollinators, resource availability and the presence of invasive plants (reviewed in Knight et al 2005).

With regard to the heterostylous system, pollen limitation has been found to occur in many species, where cross pollination may be obligate not only because of physiological self-incompatibility, but also because of structural traits (Thompson & Dommée 1993; Matsumura & Washitani 2000; Baker et al. 2000b; Waites & Ågren 2004; Brys et al. 2007, 2008b). Furthermore, as morph-types are mostly self-incompatible, the chance of cross pollination is actually reduced by half (at equilibrium): this could reduce the likelyhood of fertilization with compatible pollen type, and increase the chance of pollen limitation.

1.3 Isolated populations, habitat fragmentation and demographic consequences

Land use changes and other human activities are among the main causes that have led to a widespread destruction and fragmentation of the habitats of many plants (Vitousek et al. 1997; Turner et al. 2007). As a consequence, populations are often isolated (both spatially and/or ecologically), patches are reduced in size and remnant fragments are often in a matrix of unsuitable environments (Jennersten et al. 1992; Vitousek 1994). Isolation can also result from natural causes, such as long-term migration and colonization, or loss of natural habitat due to ecological changes or stochasticity, and eventually genetic differentiation could bring to divergence of species and to endemisms (Thompson 2005).

A reduction in populazion size and an increase in isolation related to fragmentation may be linked to increased inbreeding, decreased individual fitness, loss of genetic variation, and consequently to increased risk of population extinction (Murcia 1995; Kéry et al. 2000; Jacquemyn et al. 2002). Plant isolation and a decrease in population size could also lead to increased pollen limitation (Wagenius 2006; Wagenius & Lyon 2010). Connectivity between remnant populations through gene exchanges is considered as a key-factor for the persistence of viable populations, especially because pollination is the major component of gene flow for many plant species (Vekemans & Hardy 2004; Gonzalez-Varo et al. 2010; Van Rossum & Triest 2010). Pollen-mediated gene flow may be driven by different ecological factors, such as plant population size or density, heterospecific co-flowering species and habitat fragmentation (Albrecht et al. 2009; Aizen et al. 2002). Dependence on mutualists for reproduction in flowering species, although pollen transfer to stigmas is improved, has increased plant susceptibility to fragmentation (Bond 1994). In the long term, degradation and disruption of plant-pollinator mutualisms are expected to lead to decreases in the quantity and quality of seeds produced: this may be an early step in the demographic collapse of a plant population (Kéry et al. 2000; Aizen et al. 2002)

2. General purposes

Appropriate scientific information is critical for the assessment of species conservation status (Shimono & Washitani 2007; Gauthier et al. 2010) and for effective management and conservation plans of rare and threatened species (Aronne et al. 2004; Rodrigues et al. 2006; Rossi & Gentili 2008). Nevertheless, at present, data on the biology and ecology of plant species protected by laws or assigned to IUCN Red List of Threatened Species and of EU “Priority species”, and of flowering plants in general, are often missing.

This research focuses on reproductive biology and pollination ecology of entomophilous angiosperms, with particular concern to reproductive success in small and isolated populations of species that occur at their distribution limits or are endemic.

I considered three perennial herbs as model species: *Primula apennina* Widmer, *Dictamnus albus* L. and *Convolvulus lineatus* L.

I carried out field work on natural populations and performed laboratory analyses on specific critical aspects (resource allocation, pollen viability, stigmatic receptivity, physiological self-incompatibility, seed viability), through which I analysed different aspects related to plant fitness, such as production of viable seed, demographic structure of populations, type and efficiency of plant-pollinator system, and limiting factors.

3. *Primula apennina* Widmer

3.1 Species and study site

Primula apennina Widmer (Primulaceae) is a perennial hemicryptophyte with a very narrow geographic range and isolated populations. It belongs to sect. *Auricula*, endemic to Europe (Zhang et al. 2004), and its distribution range is restricted to the Northern Apennines (Italy), between Tuscany and Emilia-Romagna (Fig. 1), where it forms isolated populations along ca. 45 km of the crest (Alessandrini & Branchetti 1997; Crema et al. 2009).

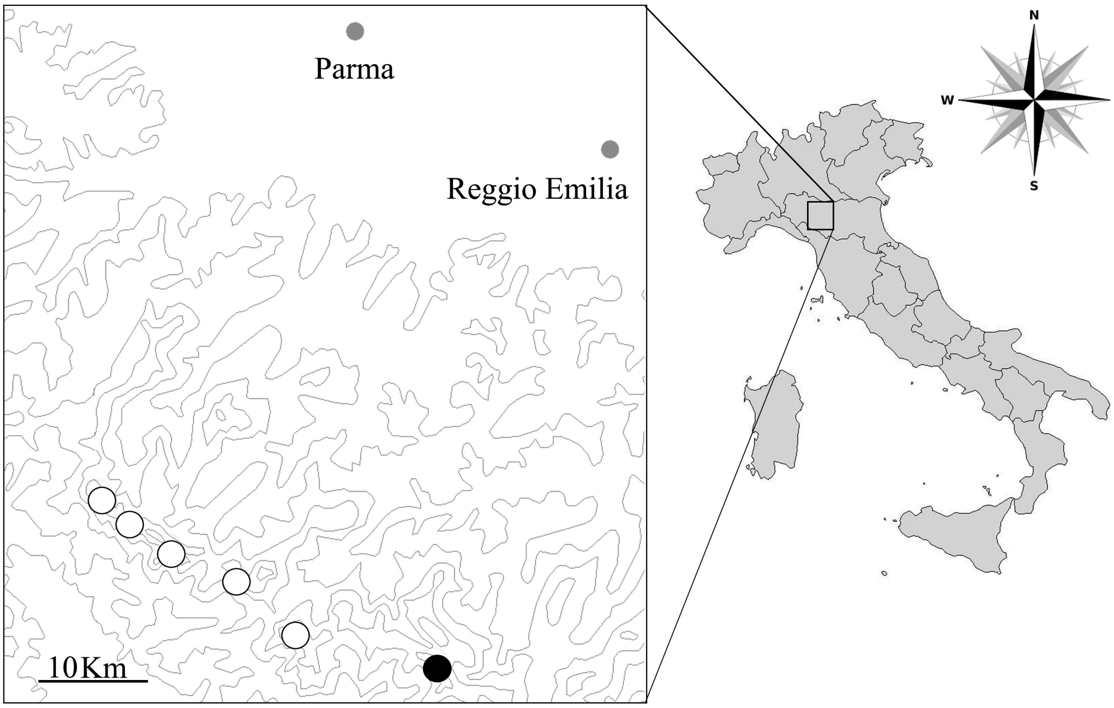

Fig. 1. Geographic location of *Primula apennina* in Northern Apennines (Italy). Contour interval is 250 m. Circles indicate the known populations; the black circle indicates the studied population (Mount Prado).

Like most species of its genus, *P. apennina* is distylous (Fig. 2a, b). It grows on acid soils (sandstone) and occurs in ledges and earthy crevices between 1400 and 2000 m above sea level (Zhang & Kadereit 2004; Fig. 2c). Short woody rhizomes assure vegetative propagation in the immediate proximity. Each rhizome produces each year one to several rosettes, some of them sterile and, usually, at least one fertile. Each fertile rosette develops a flowering stem bearing one to ten tubular pink flowers, blooming in May-June (Fig. 2d). Flower lifespan lasts for 5-7 days. Fruits are many-seeded capsules ripening from June to July.

Fig. 2. Long-styled (a) and short-styled (b) morphs of the distylous *P. apennina*; (c) typical habitat and (d) flowering and vegetative rosettes.

The study on *P. apennina* was carried out from 2006 to 2008 in the glacial cirque of Mount Prado (Reggio Emilia - Italy), in the national park “Parco Nazionale dell’Appennino Tosco-Emiliano”. The target population is located on steep sandstone sites with NW exposure, between 1770 and 1820 m above sea level (44°15’21” N – 10°24’23” E). Local climate is poorly documented: mean annual temperature is about

5℃; annual precipitation ranges from 1000 to 3500 mm, with an average of 2000 mm, in the form of snow from November to April (Panizza 1982; Rossetti 1988; Rossi et al. 2006). At the study site snow melts between May and the beginning of July, in relation with the topography and exposure (Ferrari et al. 2000).

P. apennina is of special interest for biodiversity conservation, being included in Appendix I of the Bern Convention (Convention on the Conservation of European Wildlife and Natural Habitats, 1979) and listed as "Priority species" in Annexe II and IV of the EC Habitats Directive (Council Directive 92/43/EEC on the Conservation of Natural Habitats and of Wild Fauna and Flora, 1992); it is also cited as "vulnerable" in the Red Book of Italian Plants (Conti et al. 1992, 1997) and locally protected in Emilia-Romagna (DPGR n. 664 1989) and Tuscany.

3.2 Aims

Main objectives of this investigation were to detect the differences in reproductive traits between morph-types and to study plant-pollinator system in an isolated population of the narrow endemic *P. apennina*.

In particular, I wanted to answer the following questions: i) does morph-ratio deviated from the 1:1 expected ratio at equilibrium? ii) are there any differences in sexual resource allocation and reproductive fitness between morphs? iii) is if female fitness limited by resource availabiliy? iv) which are the main pollinators? and v) is pollinator service a limiting factor to reproduction in the studied population?

3.3 Materials and methods

3.3.1 Demographic study

The target population is patchily distributed over an area of about 304,500 m^2, but it is possible to recognize two main sub-areas, separated by approximately 500 m, where plants grow generally compacted in small groups. Plants density varies greatly among patches (1.8 to 22.6 flowering stems m^{-2}), depending on ground morphology, with higher values associated to rocky crevices on mountain crests. Extent of occurrence of the studied population has been measured by the sum of minimum convex polygons (IUCN 2001), and it has been estimated to be about 152,000 m^2. An exact assessment of the number of individuals was not possible, due to the steep-rocky nature of the site: approximate estimate indicates an overall population size of few thousands individuals. According to the most up-dated distribution data (Fig. 1), this is the most isolated population of the species, being 13.8 km far from the nearest one.

To evaluate the proportion of morphs within the studied population, 5 plots were sampled across the whole population area, with size depending on ground features: flowering stems were counted, morph type (pin or thrum) was recorded, and morph frequency calculated for both flowering stems and flowers.

3.3.2 Sex allocation

The investment in pollen and ovules was analysed in order to describe morph specific traits and assess wether there were differences between long-styled and short-styled reproductive features. Pollen grain size was estimated by measuring the widest diameter in polar vision under an optical microscope (Nikon Eclipse E600), equipped with a digital micrometer. Measures were made on 50 pollen grains per morph type. To estimate mean ovule number per flower, seeds and unfertilized ovules were counted in 30 mature capsules of each morph. Pollen-ovule ratio (P:O) was computed considering the “legitimate” ratios, due to the peculiarity of distylous system corresponding to pollination (P_P:O_T and P_T:O_P, where P_P = pollen grains per pin flower, O_T = ovules per thrum flower, P_T = pollen grains per thrum flower, and O_P = ovules per pin flower). A total of 16 flower buds (8 per morph type) were transferred to the laboratory for ovules and pollen grains counting. To estimate the number of pollen grains I used the dilution method (Dafni et al. 2005) modified by Galloni et al. (2007): grains were counted with an optical microscope (Nikon Eclipse E600) equipped with a digital grid, and a manual counter; the figure obtained was multiplied by the dilution factor to obtain the total number of pollen grains per flower. In the same flower ovules per ovary were counted using a stereo microscope and a manual counter.

3.3.3 Mating system and female fitness

To detect the occurrence of spontaneous self-pollination, during the first year of study the flowering stems of 22 randomly chosen plants (with a total of 37 flowers) were covered with non-woven fabric at early anthesic stages, in order to prevent pollinator visits; 80 additional plants were randomly chosen, marked and monitored as controls.

To evaluate the reproductive fitness and assess whether there were significant differences between morphs, I considered seed set (seed:ovule ratio per capsule, hereafter indicated as S:O) and fruit set (Fruit:flower ratio, hereafter indicated as Fr:Fl) in open-pollinated plants over a three-year period (sample sizes are reported in Fig. 3 legend). I marked flowering rosettes with different tags for the long-styled and the short-styled morphs; before capsule dehiscence I collected all fruiting stems and brought them to the laboratory. All seeds and unfertilized ovules in each capsule were then counted. The relationships between female fitness components (number of flowers, fruits and seeds per flowering stem) were analysed. Effects of fitness components and morph type on reproductive success were also considered (see details in the paragraph 3.2.4 Data analysis).

3.3.4 Pollinator limitation

A pollen-augmentation experiment was carried out during full blooming period in 2008. Legitimate cross-pollinations (i.e. pin pollen on thrum stigmas, and vice versa) were performed on 6 short-styled and 7 long-styled plants randomly chosen in the population, with a total of 11 and 13 flowers respectively. Open pollinated plants (35 pin and 34 thrum plants, with 43 and 37 flowers respectively) were taken as controls. Due to the small size of the anthers and to the difficulty to reach the short style hidden in the corolla tube, a tiny brush was used to add pollen to stigmas; to enhance the probability of having vital pollen, mixtures of pollen (from two or three individuals) were applied. At the end of fruiting season all marked stems were harvested and brought to the laboratory for fruits, seeds and unfertilized ovules counting. Fruit and seed set for augmented vs. open-pollinated plants were then compared to test for pollinator limitation.

3.3.5 Data analysis

Normality of the data sets was tested using a Shapiro-Wilk test; where normality could not be achieved by appropriate transformations, non-parametric tests were performed.

Significant differences in the number of plants between morphs (i.e. deviations from the 1:1 morph ratio) in each plot and in all plots together were tested by a Chi-squared test.

Between-morph differences in following parameters were tested by using two-sample T-tests: i) pollen grains per flower, ii) pollen grains size, iii) seeds per capsule, iv) ovules per flower, and v) legitimate P:O ratios.

Effects of morph, year and their interaction on population means of S:O were analysed by a two-way analysis of variance (ANOVA). Differences in Fr:Fl between pin and thrum individuals were tested by non-parametric Mann-Whitney U-tests.

To analyse the possible relationships between i) the number of flowers and fruits per plant, and ii) the number of flowers and seeds per plant, Spearman's rank correlation coefficients were calculated for each morph. A mixed-model analysis of covariance (ANCOVA) was then used to analyse differences in the effects of flower number, fruit number and morph on reproductive success.

Mann-Whitney U-tests were used to test for differences in fruit set between open-pollinated controls and pollen augmented flowers; differences in seed set between controls and pollen supplemented flowers were analysed using a two-way analysis of variance (ANOVA), with morph and pollination treatment entered as fixed factors.

All statistical analyses were performed with R Development Core Team software ver. 2.10.0 (2009).

3.4 Results

3.4.1 Demographic study

In four plots no significant bias was found between morphs in the number of individuals (Table I). In one plot there was a bias toward thrum plants ($\chi^2 = 5.765$, $p = 0.02$); however, the number of pin and thrum flowers did not significantly differ in any of the sampled areas. Considering all plots together, there was no dominance of one morph over the other neither for plants (136 pin, 133 thrum), nor for flowers (273 pin, 283 thrum) (χ^2= 0.033 and 0.179 respectively, $p > 0.1$).

Plot	Surface (m^2)	Thrum plants	Pin plants	χ^2	Thrum flowers	Pin flowers	χ^2
		Number(%)	Number(%)		Number(%)	Number(%)	
1	1.55	17 (48.6)	18 (51.4)	0.028	52 (53.1)	46 (46.9)	0.367
2	9.75	14 (48.3)	15 (51.7)	0.034	31 (48.4)	33 (51.6)	0.063
3	59.4	56 (42.4)	76 (57.6)	3.03	127 (47.9)	138 (52.1)	0.457
4	18.8	24 (70.6)	10 (29.4)	5.765*	36 (56.2)	28 (43.8)	1
5	17.7	22 (56.4)	17 (43.6)	0.641	37 (56.9)	28 (43.1)	1.246

Table I. Demographic data from 5 plots census. Absolute number, percentage and chi-squared for thrum and pin morphs are reported. * p=0.02.

3.4.2 Sex allocation, reproductive success and fitness components

In the long-styled morph mean pollen production was almost two times greater than in the short-styled morph (mean ± s.e.: 91795 ± 11919 and 47436 ± 9708, respectively); an opposite trend was found for pollen size, being greater for the thrum morph (n = 50, mean ± s.e.: 17.12 ± 0.12 μm) than for the pin morph (n = 50, mean ± s.e.: 11.85 ± 0.98 μm). Significant differences between morphs were detected both for pollen production and for pollen grains size (grain number: t: 2.89, $p = 0.01$; grain size: t: 34.39, $p < 0.001$), while ovule number (mean ± s.e.: pin: 51.0 ± 2.0, thrum: 49.1 ± 2.1) did not significantly differ between morphs (t: 0.65, $p > 0.1$).

Mean values of legitimate P:O ratios differed significantly between morphs, P_P:O_T resulting 2.2 times greater than reciprocal P_T:O_P (mean ± s.e.: P_P:O_T = 1851 ± 240; P_T:O_P = 824 ± 169; t: 3.49, $p < 0.01$). Since the mean ovule number was not significanty different between morphs, the difference between ratios results from the higher number of pollen grains produced by pin flowers compared to thrum flowers.

None of the plants protected from insect visits set any fruit, indicating that spontaneous selfing is prevented in both short- and long-styled flowers.

In plants open to free-pollination, seed set over the three years of study did not show any specific trend in either pin or thrum type (Fig. 3). Considering years separately, no significant differences between morph types were found.

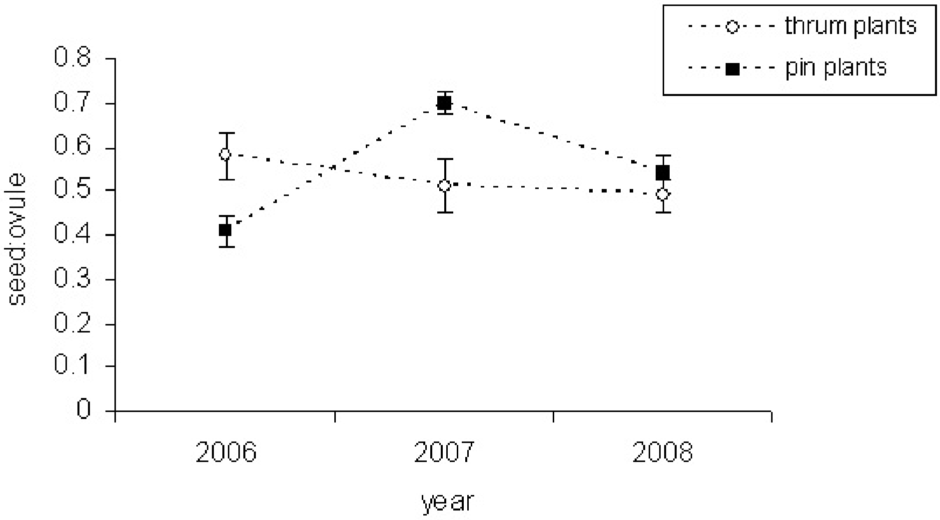

Fig. 3. Mean (± s.e.) seed:ovule ratio in short- and long-styled morphs over the three-years study period (sample sizes in 2006, 2007, 2008: thrum plants = 30, 21, 37; pin plants = 46, 33, 43).

S:O differed significantly between years, with significant effect year*morph, due to fluctuations between 2006 and 2007 (Table II). I found a significant difference in fruit set between morphs in one year (2007: U = 230, $p < 0.05$). Considering the entire three-year period neither seed set, nor fruit set showed consistent differences between morphs.

Source of variation	*df*	SS	F-value	p
Year	2	0.7087	5.8795	0.003 **
Morph	1	0.0006	0.0105	0.919
Year*morph	2	1.0760	8.9267	< 0.001 ***
Residuals	204	12.2953		

Table II. Analysis of variance (ANOVA) for seed set in short- and long-styled plants in the three-years study period.

Values of Fr:Fl per plant, regardless of morph type, showed a bimodal distribution, with a high percentage of non-fruiting plants (33%), and high proportion of plants with all flowers developed into fruits (43%) (Fig. 4).

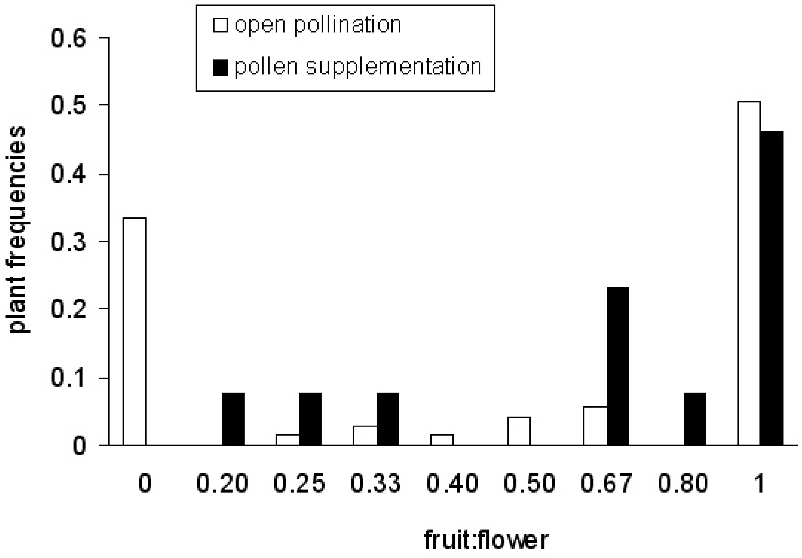

Fig. 4. Frequencies distribution of open-pollinated and pollen augmented plants by fruit set.

All reproductive components considered for the Spearman’s rank correlation analysis were highly positively related to different stages of the reproductive cycle. Number of fruits per plant and seeds per plant were found to correlate with number of flowers per plant both in long-styled ($\rho = 0.34$ and $\rho = 0.35$, $p < 0.001$) and short-styled ($\rho = 0.41$ and $\rho = 0.34$, $p < 0.001$) morphs.

The mixed-model analysis of covariance (ANCOVA) did not reveal significant interaction between flowers and morph for the number of fruits per plant, nor for the number of seeds per plant (Table III).

Source	*df*	SS	F-value	p
n. fruits:plant				
Flowers on plants	1	72.293	93.89	< 0.001***
Morph	1	1.012	1.31	0.253
Flowers*morph	1	0.484	0.63	0.429
Residuals	198	152.462		
n. seeds:plant				
Flowers on plants	1	170349	134.52	< 0.001***
Morph	1	702	0.55	0.458
Flowers*morph	1	4551	3.59	0.059
Residuals	198	250732		

Table III. Mixed-model analysis of covariance (ANCOVA): effects of flower number, fruit number and morph type on reproductive success (n = 202 plants).

3.4.3 Pollinator limitation

P. apennina pollinators are very scarce at the study population, where I could not record any insect visit during monitoring intervals over the three years of study. Despite abundant pollinating fauna (*Bombus* spp.) observed on *Vaccinium myrtillus* plants some 50-100 m below the population study, any of these insects were observed on a *Primula* flower. The diurnal Sphingidae *Macroglossum stellatarum* (Linnaeus 1758) was observed visiting flowers on very rare occasions; moreover, I sampled four individuals of a beetle species, identified as *Eusphalerum signatum* (Märkel 1857) ssp. *angulatum* (Luze 1911) (order: Coleoptera; family: Staphylinidae; subfamily: Omaliinae)[1], inside the corolla tube of some flowers.

No significant differences were found in fruit set between open-pollinated controls and pollen augmented flowers in both morphs (U = 114, p > 0.1 and U = 73, p = 0.1 for the long- and the short- styled morphs, respectively).

To estimate the total effect of pollination treatments, pin and thrum plants were clustered before analysis: no significant differences were found between augmented plants and controls (U = 376.5, p > 0.1).

The distribution of plant frequencies by fruit set in controls reflected the bimodal trend found over the three-year study period; a high number of plants did not set any fruit (33%), likewise a high percentage of plants (50%) showed a 100 percent fruiting success (Fig. 4). Otherwise, none of the hand-pollinated plants failed to set fruits; more than 75% of these plants set fruits for at least two-thirds of the flowers, 46% of wich showed a 100% fruit set (Fig. 4).

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[1] Voucher specimens are conserved at the Department of Biology, University of Bologna.

Mean S:O values (± s.e.) were slightly greater in pollen augmented flowers (0.62 ± 0.05 and 0.55 ± 0.09 for thrum and pin morphs, respectively) (Fig. 5); nevertheless, the two-way analysis of variance did not show a significant effect of morph (F = 0.22, p > 0.1), treatment (F = 1.36, p > 0.1) or their interaction (F = 0.98, p > 0.1) on seed set.

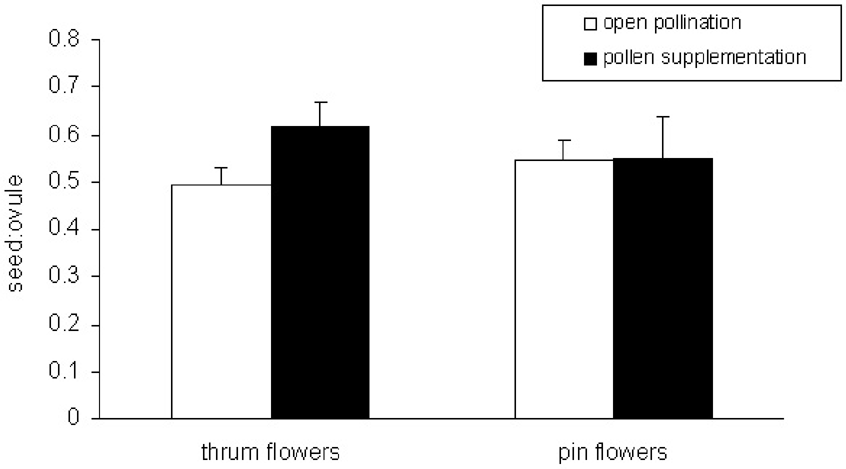

Fig. 5. Seed set (mean ± s.e.) in open-pollinated and in pollen augmented flowers.

3.5 Discussion

I investigated the reproductive strategy and outcome in *Primula apennina*, an andemic distylous species with a narrow distribution range and isolated populations.

The demographic analysis showed an overall equilibrium of morph frequencies in the study population, in contrast with results obtained for other distylous species (Ornduff 1980; Hicks et al. 1985; Pailler & Thompson, 1997). This can be ascribed to the comparable female fecundity of the two morph-types, and to the efficient pollination service observed in both long- and short-styled plants (see below). Moreover, the absence of statistically significant anisoplethy suggests the predominance of disassortative mating.

As a common trait found in other *Primula* and distylous species in general (Darwin 1877; Washitani et al. 1994; Richards 2003; Schlindwein & Medeiros 2006; Wolff & Liede-Schumann 2007), I found that in *P. apennina* the long-styled moprh produces more pollen of smaller size than the short-styled morph. As a consequence of this trade-off between size and number of pollen grains, a disassortative mating should result in a seed output biased toward thrum plants.

However, the trend observed in other distylous *Primula* species goes towards a pin reproductive advantage, especially in small populations under pollinator limitation (Washitani et al. 1994; Matsumura & Washitani 2000; Van Rossum et al. 2006). This counterintuitive fact is to ascribe mainly to a self or intra-morph pollen deposition, with subsequent enhanced selfing due to partial self-compatibility in the long-styled morph. In fact, relatively low self-incompatibility in the pin morph has been noted for several *Primula* and other distylous species (Ganders 1979). In addition, higher pin stigmas have been shown to contact more easily pollinators' bodies, and load more reciprocal pollen than short stigmas hidden inside the corolla tube (Stone & Thomson 1994; Matsumura & Washitani 2000; Nishihiro et al. 2000).

Nonetheless, I did not find a pin advantage: my study showed that in *P. apennina* spontaneous selfing is prevented in both morphs, and seed set did not significantly differ between morph, despite the two-fold larger number of pollen grains produced by the pin morph compared to the thrum morph. This result suggests that stigmatic pollen load should be the same in both long and short styles of *P. apennina*. considering the differences in pollen grain numbers but not in ovule number, a possible explanation for the similar reproductive output could be found in the peculiarity of distyly in relation with insects' features. Past studies showed no differences in pollen removal between long- and short-styled flowers by insect proboscis (Harder & Barrett 1993; Matsumura & Washitani 2002). In short-styled flowers, with anthers placed at the mouth of the corolla, pollen is more easily removed by insects, while in long-styled flowers the anthers, placed inside the corolla tube, produce more pollen grains compensating for their disadvantage in pollen removal (Matsumura & Washitani 2002). I Hypothesize a similar trend for the nectar seeker *M. Stellatarum*, which results in equal pollen amounts carried to both short and long styles.

The over production of pollen in pin morph is also reflected in legitimate P:O ratios (i.e., pollen of one morph type on ovules of the other type, and vice versa); the higher P.O value found for pin pollen can be related to a lower pollen transfer efficiency from long-styled to short-styled flowers (Cruden 1977).

Fruit set showed a bimodal distribution with two maxima, corresponding to 0 and 100 percent fruit per flower production, respectively. This finding suggests te existence of high pollination efficiency, and fits with a visit vs. non-visit pattern: when the pollinator visits a plant, it tends to pollinate all available flowers, while fruiting failure is associated with non-visitation by the pollinator.

Despite the very rare pollinator visits observed over the three-year study, they seem to provide a good pollination service, avoiding asymmetry in pollen flow between morphs; my results indicate no evidence of pollinator limitation in the studied population, neither for pin nor for thrum plants.

High seed production and the positive correlations found between different reproductive components, suggest that resource limitation, that could affect female fitness, does not play a significant role in limiting the output of pin or thrum plants.

Ecology and behaviour of pollinators can explain both the high levels of intra-population genetic diversity and the low genetic distance found among populations of *P. apennina* despite their remarkable geographic isolation (Crema et al. 2009). The diurnal hawkmoth *Macroglossum stellatarum*, with its long distance flight ability (Bertaccini et al. 1994) may provide long-distance pollen dispersal, as well as intra-population pollination service, with subsequent inter-population genetic flow. The small beetle *Eusphalerum signatum* ssp. *angulatum* may contribute mainly to intra-population genetic flow, through short-range movements (Zanetti 1987). Both *M. Stellatarum* and *E. signatum* do not stop their activities with adverse climatic conditions, which are characteristic of the mountainsides where *P. apennina* grows: this behaviour guarantees successful pollination of the short-lived flowers.

My results show no evidence of imminent threats to *P. apennina* population persistence at the study site. Nevertheless, the strict dependence on one or very few pollinator species for reproductive success, as found in this study, could be a factor leading to increased risk of plant extinction (Bond 1994), as the loss of mutualistic insects implies the lack of pollen vector, which is especially vital for the reproduction of distylous species. Even so, being a K-strategist, this species has the intrinsic potentiality to overcome temporary limitations due to fluctuations of pollinator populations.

Since *P. apennina* lives on the upper parts of the mountains (crests and mountaintops), no future habitat will be suitable in front of long-term climate warming (Parmesan & Yohe 2003; Parolo & Rossi 2007). Global warming could also act toward a disruption of plant-pollinators interactions (Memmott et al. 2007). All these features are key factors and should be considered in population viability analysis in the long-term period.

4. *Dictamnus albus* L.

4.1 Species and study site

Dictamnus albus L. (Rutaceae) is a long-lived perennial herb, characterized by thick storage roots. The potential lifespan of an individual is estimate to be at least 30 years (Jäger et al. 1997). Each individual produces one (rarely two or three) stem that bears many pentamerous and slightly zygomorphic white-purple flowers, on a long and loose raceme (Fig. 6a). Stamens are arranged in two whorls; the nectary is placed at the base of the gynophore, below the ovary (Weryszko-Chmielewska et al. 2001). Flowering begins after 5-7 years (Jäger et al. 1997) and occurs between the end of April and May. Fruits are capsules composed of 5 carpels with a loculicide opening (Fig. 6b, c); the black pear-shaped seeds are dispersed by autochory, due to increasing turgor pressure, with a maximum dispersal distance of approximately 4 m (Frey 2000).

Fig. 6. Flowering stem of D. albus (a); star-shaped fruit (b) and ripened fruit with seeds (c).

Like most species of the Rutaceae, plants of *D. albus* are characterized by oils, which are found both in leaves and in oil glands disposed throughout the stem and flowers. Secondary chemistry of the genus is unique within Rutaceae: *Dictamnus* has limonoids instead of coumarins, and special quinolones (Da Silva et al. 1988). In italian plants the main components have been found to be germacrene D, phytol, limonene, y-terpinene and citronellol (Tirillini et al. 2002).

D. albus is found at the fringes between xerothermic woodlands and (semi)natural grasslands, or within open oak forests, in the southern warm-temperate regions of Europe and Central and Eastern Asia (Hensen & Oberprieler 2005). The species has been designated as “vulnerable” in several European countries (Schnittler & Gunther 1999), and is locally protected across Europe. In Italy it is protected at regional level.

At present time, populations of *D. albus* are declining due to land-use changes, and suitable habitats are rare, often separated from each other by significant distances, and usually surrounded by agricultural landscapes (see Hensen & Wesche 2006).

In Italy, *D. albus* reaches its distribution limit: Emilia-Romagna is the southernmost region with a relevant presence of the species, even if in isolated populations (Alessandrini & Bonafede 1996, Conti et al. 2005).

The study population occurs at the edges of a Downy Oak forest (*Quercus pubescens*) of about 50 x 50 m (168 m above sea level; 44°25’ 11” N, 11°23’54” E), in the Natural Park “Parco dei Gessi Bolognesi e Calanchi dell’Abbadessa” (Bologna, Italy).

4.2 Aims

Main objectives of this investigation were to study the breeding system of *D. albus*, with special regard to mechanisms that could prevent self-pollination and promote cross-pollination between individuals.

I specifically wanted i) to assess plant reproductive success, ii) to analyse nectar production along the stem, to identify whether a gradient of reward could be found, iii) to describe insects’ foraging behaviour and quantify their movements on the racemes, iv) to evaluate the presence of pollinator limitation in the studied population, and v) to study population demography in relation to different habitat conditions.

The analysis of pollinator movements on flowering stems was performed to indirectly assess the contribution of insects to geitonogamy. In fact, despite the current literature concerning observations about upward movements of pollinators among flowers on a stem (Pyke 1978, 1979; Corbet et al. 1981; Best & Bierzychudek 1982; Haynes & Mesler 1984; Jordan & Harder 2006; Zhang et al. 2006), and quantification of geitonogamy by means of pollen transfer by pollinators (de Jong et al. 1993; Barrett et al. 1994; Karron et al. 2009), there has not been an attempt to infer plant inbreeding potential from pollinators behaviour. Moreover, previous studies considered either the relationships between plant species and one main pollinator species, usually bumblebees (Pyke 1978; Best & Bierzychudek 1982; Pappers et al. 1999; Klinkhamer & van der Lught 2004), or pollinator response to nectar distribution on artificial inflorescences (Waddington & Heinrich 1979; Cartar & Abrahams 1996; Biernaskie et al. 2002; Ferdy & Smithson 2002; Jordan & Harder 2006); here I consider the whole plant-pollinators system.

Pollinator foraging behaviour was analysed using an original statistic method.

4.3 Materials & methods

4.3.1 Phenology and flower characteristics

Flower phenology was studied in May 2007. Male and female functions development was observed every 24 hours on five flowers from two different plants; morphological aspect and maturity stage of anthers and style were recorded. With respect to male function, a qualitative pollen viability test (DAB test, Dafni et al. 2005) was performed on freshly dehisced and almost empty anthers (n = 10 flowers).

Stigma receptivity was assessed by observing the germination of pollen grains *in vivo*. Two different treatments were performed: i) hand-cross pollination on downward bending styles (n = 7), and ii) hand-self pollination on upward bending styles (n = 15). Open pollinated flowers were chosen as controls (n = 13). Gynoecia were collected 24 hours after the treatments, fixed in FPA solution (40 % formalin, 5; propionic acid, 5; 50 % ethanol, 90) and brought to the laboratory; styles were mounted on microscope slides, stained with 0.1 % aniline blue and observed by fluorescence microscope (Nikon Eclipse E600). Pollen germination and pollen tubes development were taken into consideration for analyses.

The relationship between phenological stage and flower position in the inflorescence was analysed in 5 plants. Flowers were numbered from bottom to top, and five developmental stages were considered: I) perianth open, anthers closed and stigma unreceptive, II) one to five anthers dehiscent, stigma unreceptive, III) six to nine anthers dehiscent, stigma unreceptive, IV) all anthers open, stigma unreceptive, V) end of male phase, stigma receptive (Fig. 7a, b, c). The flower position along the stem was normalized as “flower position : number of flowers on the stem”, to avoid effects due to different inflorescence size.

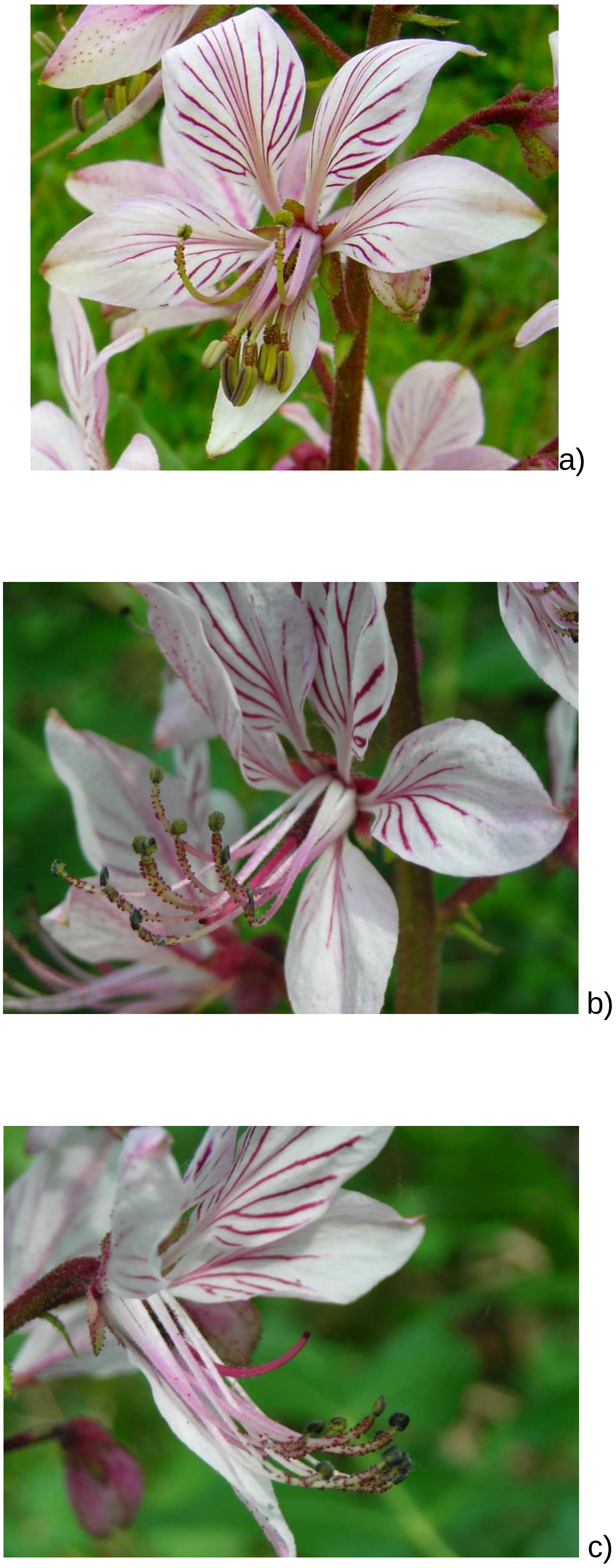

Fig. 7. Different phenological stages of flower development. (a) Stage II, (b) stage IV, and (c) stage V (see text for details).

In order to evaluate the number of pollen grains per flower, I marked five flower buds and collected two closed anthers from each flower (one per whorl); I then applied the dilution method (Dafni et al. 2005) modified by Galloni et al. (2007). Values obtained for each whorl were averaged to obtain mean pollen grain number per flower. Mean ovule number was assessed by collecting unopened fruits (n = 57) and counting seeds and unfertilized ovules. Average flower number per stem was calculated on a sample of 34 plants.

4.3.2 Reproductive success

In order to determine *D. albus* breeding system, in 2007 different treatments were performed on separate plants. Non-manipulated flowers, where open-pollination was allowed, were chosen as controls (C; 12 plants, 129 flowers). To detect agamospermy, I removed styles prior to flower opening (12 plants, 135 flowers). To assess the degree of self-compatibility and the extent of autonomous self-pollination, I performed two treatments: i) spontaneous selfing (SS), in which pollinators visits were excluded (12 plants, 104 flowers), and ii) hand-self pollination (HS), in which receptive stigmas were brushed directly with dehiscent anthers excised from different flowers of the same stem (10 plants, 69 flowers). All plants were bagged with pierced plastic bags before flower opening, and bags were removed once fruits began to ripe. Fruits were collected prior to opening, brought to the laboratory, and the number of seeds and unfertilized ovules counted.

In 2007, a germination test on seeds from open pollinated flowers (C; n = 30) and self-pollinated flowers (SS + HS; n = 26) was performed. Seeds were sown in pots with a soil mixture of 50 % clay, 35 % organic matter, and 15 % sand, similar to the soil in the study area. Pots were placed in the Bologna Botanical Garden, and left in natural conditions. Germination was observed in 2008 and 2009.

4.3.3 Nectar analysis

Nectar analyses were performed in 2009; flowers were sampled only once and not removed from the plants. Nectar volumes were estimated using Drummond Microcaps® (0.5, 1, 3 or 5 µl, Drummond Scientific Co., U.S.A.). Nectar concentration, expressed as % on a w/w basis of an equivalent sucrose solution, was measured by hand held refractometers EBS45-03 and EBS45-05 (Bellingham & Stanley Eclipse, Bellingham + Stanley LTD., U.K.). Since the refractometers were not temperature compensated, the International temperature Correction for °Brix scale was applied.

Nectar standing crop (i.e., the amount of nectar in a flower exposed to pollinators at a given moment; Pacini & Nepi 2007) was eveluated three times a day (10:30 AM, 14:30 and 17:00 PM; n = 55, 52, and 54 flowers, respectively) over 2 days in nine different plants. Stages of flower development were noted before sampling.

The amount and quality of nectar along the stem were studied on five plants bagged the day before analysis, by sampling nectar from all open flowers; measures were repeated for 3 days roughly at the same intervals as for standing crop analysis (sample sizes are given in Table IV).

To evaluate pollinators' nectar uptake in the five stages of flower development (previously described), nectar volumes were compared between open (nectar standing crop) and bagged flowers (sample sizes are given in Table IV). Gender biased nectar production was analysed in bagged flowers, by considering mean values in both male and female flower stages.

The total amount of sugar per flower was calculated to estimate nectar energy values, according to Bolten et al. (1979). Per cent values of sugar were converted to mg of sugar per µl using Galetto's exponential regression, and nectar energy value was then calculated by the product of total sugar content x 4 cal/mg (Galetto & Bernardello 2005).

4.3.4 Flower visitors and pollinators

To assess the spectrum and behaviour of visitors and pollinators, I modified the protocol used for the European ALARM Project (Westphal et al. 2008). Four observation units, of 6 plants each, were chosen in the study population. Observations were repeated in two flowering seasons (April-May, 2007 and 2008). Surveys were performed twice a day (AM and PM) during suitable weather conditions for pollinators (minimum of 15℃, low wind, no rain); each survey consisted in four 15 min observation periods, alternated to 15 min breaks. At each visit I recorded the type of floral reward collected (nectar or pollen), the number of visits to a plot, the number of flowers visited per plant, and the sequence of flowers visited on a given plant; I also reported if the insect touched the receptive stigma (i.e., upward bending) or not, in order to identify potential pollinators. After each observation survey, I spent the next 30 min collecting all visiting insects for identification. Specimens were then identified to the genus or species level, and conserved at the BES Department, University of Bologna. Relative abundance of visitors and potential pollinators was then estimated.

4.3.5 Pollinator importance

Pollen loads were analysed from sampled insects, to evaluate the fidelity of potential pollinators. Pollen baskets were excluded from counts, as this pollen is unlikely to be available for pollination. Pollen grains were removed by systematically dabbing a section (5 mm diameter) of fuchsin jelly on each insect body (sample sizes are given in Fig. 3); then the jelly was placed on microscope slides, melted, mounted with a cover glass and sealed with nail varnish. I analysed 100 grains per slide, or all grains on the slide if less than 100, under optical microscope; the relative abundance of *D. albus* pollen on total pollen load was then evaluated, using pollen grains taken from mature but still closed anthers as reference.

In order to evaluate the relative importance of pollinators, the relative abundance and the degree of fidelity of taxa that touched receptive stigmas was considered. Taxa with less than 2 specimens were not considered. For each taxon I calculated an index of pollinator importance (PI) as a measure of its pollination ability (*sensu* Gibson et al. 2006), by multiplying its relative abundance (percentage of visits on total visits) by its fidelity (percentage of *D. albus* pollen grains carried).

4.3.6 Pollinators directionality

To study pollinators' movements among flowers on a given plant, flowers were numbered in ascending order from bottom to top. For each pollinator observed during surveys I recorded the sequence of visits, and performed a linear regression between the total number of visited flowers and the sequence of visits. Taxa with only one observation were excluded. Subsequently, for each pollinator taxon I calculated the mean angular coefficient (*b*) and the mean standard error. The linear regression describes the patterns of movement along the stem as follows:

- $b = 1$: upward visits on consecutive flowers;
- $b > 1$: upward visits on consecutive and/or non-consecutive flowers;
- $0 < b < 1$: upward and downward movements;
- $b \approx 0$: random visits;
- $b < 0$: downward movements.

The value $b = 1$ indicates an even pace from bottom to top of the inflorescence, and is therefore assumed as the threshold beyond which geitonogamy does not play a significant role. The more the b value exceeds 1, the lower is the probability of geitonogamy.

For each pollinator species I then calculated a modified Student's t, replacing μ with 1 at numerator;

$$t = (b - 1) / s_b$$

where b is the mean angular coefficient, and s_b is the standard error of b.

As a result, it is possible to assess if b differs significantly from 1 (threshold) by the p-level associated to each t value.

4.3.7 Pollinator limitation

To assess the degree of pollinator limitation in the studied population, a supplementary hand-pollination experiment was performed in 2007 and 2008. Pollen was transferred by brushing receptive stigmas (i.e., when stiles bended upwards) directly with dehiscent anthers from two different plants for two consecutive days, to increase the probability of successfull pollination; anthers were collected from plants at least 25 m away from manipulated ones, to avoid genetic similarities.

In 2007, 43 flowers (11 plants) were pollen-augmented, and 129 flowers (12 plants) were randomly chosen as controls. In 2008, 60 flowers (21 plants) were hand-pollinated with cross-pollen, and 127 flowers (27 plants) were marked as controls.

All manipulated flowering racemes were enclosed in pierced nylon bags at the beginning of fruiting to avoid the loss of seeds. After about 1 month fruits where counted, and mature capsules were collected before opening; the number of developed seeds and undeveloped ovules was counted in the laboratory using a dissecting microscope, and seed set (mean seed/ovule per capsule) was calculated.

4.3.8 Demographic study

To study the population demographic structure, 2 permanent quadrats of 3x3 metres were chosen and analysed for 3 consecutive years, from 2008 to 2010. Quadrats were set in different ecological conditions, one at the edge of the wood, the other inside a mature part of the wood (i.e., with less sun light availability). This is supposed to cover the most common features of the habitat of *Dictamnus albus*. Quadrats were monitored each year in the same period (18-25 of June), at the end of flowering season.

Five developmental stages were considered, based on field observations and previous studies (Jäger et al. 1997): plants developed from seed the same year of observation (i.e., seedlings; P), young plants with simple leaves (P2), sub-adult plants with at least one composed leaf with three leaflets (i.e., juveniles; GT), vegetative adult plants with composed leaves (A), and fruiting adult plants (F; note that all plants that showed flowers set at least one fruit). Seedlings were easy to recognize, as they displayed a typical whorl with four verticillate leaves.

Moreover, yearly natural fluctuations in flowering have been studied from 2006 to 2010 in a permanent area of 500 m^2 at the fringe of the Downy-oak wood (*Quercus pubescens*).

4.3.9 Statistical analysis

The relationship between flower development and its position in the stem was analysed through analysis of covariance. Data of reproductive success and nectar were not normally distributed, and appropriate transformations were not useful to achieve normality. Hence, differences among fruit and seed sets, and among nectar volumes were detected using a non-parametric Kruskal-Wallis test. Post-hoc pairwise comparisons were performed by using Mann-Whitney U-tests. K-W tests were performed with PAST Version 1.92 (Hammer et al. 2001). Analysis of covariance, linear regressions and Student’s *t* tests were performed with R Statistics Version 2.10.1 (R Development Core Team 2009). Means (± s.e.) are given.

4.4 Results

4.4.1 Phenology and flower characteristics

Total lifespan of a single flower was 5.75 ± 0.63 days. Flowers were found to be protandrous: the male phase started the second day of anthesis, and lasted 3.20 ± 0.20 days. At flower opening both stamens and style were bending downward, then upper lateral stamens started to bend upward, followed by anthers dehiscence; the other stamens sequentially followed the same pattern, those at lower position being the last ones. Pollen was viable from early stages of anthers development, after upward bending of the stamens (over 80 % of viable pollen grains the day of anther dehiscence). Viability dropped rapidly: the percentage of viable grains after the first day of pollen exposure ranged approximately from 50 % to 5 %. However, due to the sequential stamens development, in a single flower viable pollen was available through the overall male phase.

The female phase started approximately the fourth day of anthesis, after upward bending of the style, when anthers were almost empty. This stage lasted 2.70 ± 0.75 days. Stamens were always longer than the style, so that anthers were spatially separated from the receptive stigma.

High percentages of pollen germination were observed in upward bending styles, for both hand-self pollinated flowers (79 %) and open pollinated controls (91 %). All germinated pollen tubes exceeded three-fourths of the style lenght, and 60 % of them reached the ovary. None of the pollen grains observed in downward bending styles developed pollen tubes (Fig. 8).

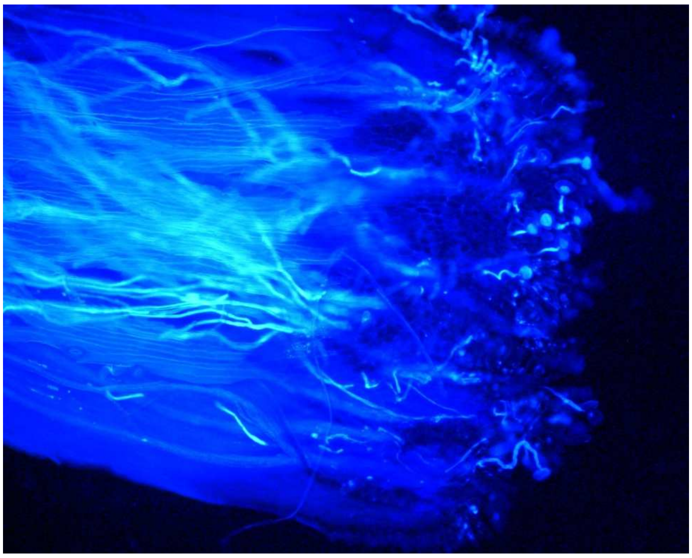

Fig. 8. Pollen grains with developed pollen tubes on stigma and through the style, under fluorescent light.

The analysis of covariance showed a strict dependence of flower development on flower position in the stem (Sum of squares = 73.7, *df* = 1, F = 173.0, $p < 0.0001$), with older flowers at the bottom of the inflorescence.

All monitored plants produced a single inflorescence: floral display ranged from 2 to 22 flowers (mean ± s.e.: 8.88 ± 0.86). Mean pollen grain number per flower was 177850 ± 11276, mean ovule number per fruit was 14.63 ± 0.24, and the average number of ovules per carpel was 2.93 ± 0.05.

4.4.2 Reproductive success

The test for agamospermy clearly indicated that production of seeds without fertilization is not possible in *D. albus*, as none of the manipulated flowers developed into fruits.

Overall, open pollinated flowers resulted in a fruit set (fruit:flower) of 0.24 ± 0.05, and in a seed set (seed:ovule) per capsule of 0.53 ± 0.04.

Although hand-self pollinations revealed self-compatibility (fruit:flower = 0.31 ± 0.09), spontaneous selfing was very low (fruit:flower = 0.05 ± 0.02). Nevertheless, seed set per capsule obtained by hand-self pollinated flowers (0.22 ± 0.04) was similar to that of spontaneous self pollinations (0.16 ± 0.04) (Fig. 9).

The non-parametric Kruskal-Wallis analysis of variance showed significant differences among treatments for fruit set (H = 10.42, $p < 0.01$). Post-hoc pairwise comparisons pointed out a significant difference between spontaneous selfing and controls (M-W U-test, p = 0.002), and between hand-self pollination and spontaneous selfing (M-W U-test, p = 0.02) (Fig. 9).

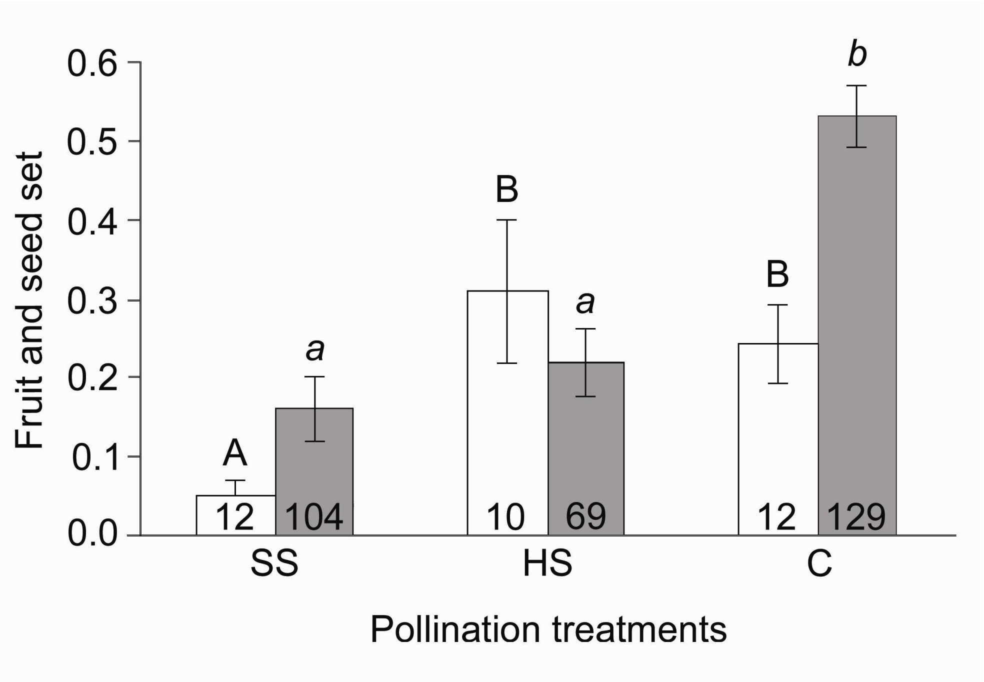

Fig. 9. Fruit:flower (open bars, capital letters) and seed:ovule per carpel (filled bars, italic letters) in experimental pollination treatments (SS = spontaneous selfing, HS = hand-self pollination), and open pollinated controls (C) of *Dictamnus albus* (mean ± SE). Different letters indicate significant differences between treatments after Mann-Whitney post-hoc pairwise comparisons. Sample sizes are shown inside bars (number of plants and flowers).

Statistical differences among treatments were also found for seed set in developed fruits (K-W, H = 22.31, $p < 0.001$), but there was no difference between the two self-pollination treatments (SS and HS; M-W U-test, $p = 0.47$); highly significant differences were found between spontaneous selfing and controls (M-W U-test, $p = 0.002$), and between hand-self pollinated flowers and controls (M-W U-test, $p < 0.001$) (Fig. 9).

The seed germination test showed low germination rate after self-pollination treatment: only 2 of the 26 sowed seeds developed into seedlings. A higher germination rate (40 %) was recorded for control seeds, with 12 seedlings developed from 30 seeds. All seedlings emerged the first year of observations.

4.4.3 Nectar analysis

Mean nectar standing crop was 0.98 ± 0.12 µl at 10:30 h, 0.75 ± 0.07 µl at 14:30 h and 0.82 ± 0.10 µl at 17:30 h, with no significant differences between intervals. Nectar concentration was generally high, ranging from 25.3 to 76.4 %.

Considering flower phenological stages, mean nectar concentration (%) ranged between 51.26 ± 2.90 and 61.32 ± 1.12 in open flowers, and between 56.56 ± 1.83 and 62.65 ± 1.71 in bagged flowers (Table IV).

	Unbagged flowers				Bagged flowers			
Class of flower development	n (n)	Nectar volume (µl)	Sucrose (%)	Energy (mg of sucrose)	n	Nectar volume (µl)	Sucrose (%)	Energy (mg of sucrose)
I	15 (14)	0.82 ± 0.16	51.26 ± 2.90	0.54 ± 0.09	6	0.47 ± 0.07	61.06 ± 2.66	0.38 ± 0.06
II	62 (60)	0.87 ± 0.08	56.29 ± 1.09	0.63 ± 0.06	8	1.10 ± 0.31	62.65 ± 1.71	0.87 ± 0.23
III	12 (11)	0.85 ± 0.13	61.32 ± 1.12	0.71 ± 0.09	10	1.60 ± 0.30	58.33 ± 1.20	1.72 ± 0.33
IV	35 (32)	0.78 ± 0.14	56.37 ± 1.42	0.61 ± 0.10	7	2.87 ± 0.82	56.56 ± 1.83	2.05 ± 0.59
V	34 (30)	0.83 ± 0.14	53.29 ± 1.60	0.58 ± 0.09	12	2.50 ± 0.28	59.38 ± 1.66	1.84 ± 0.25

Table IV. Nectar analysis in unbagged and bagged flowers in five classes of flower development (see text for details). Mean (± SE) values of nectar volume, percentage of sucrose, and milligrams of sucrose are given. n = number of flowers; (n) = number of flowers used for sucrose and energy analysis (empty flowers excluded).

Unbagged flowers presented a constant nectar volume during the whole lifespan, whereas bagged ones showed an increase of nectar amount through stages of flower development (Fig. 10). Moreover, when I consider mean nectar volumes through the overall male phase (stages II to IV) and in the female phase (stage V), these were respectively 1.79 ± 0.30 µl (n = 25) and 2.50 ± 0.28 µl (n = 12), indicating that flowers produce, on average, more nectar in the female phase.

Nectar volume differed significantly among open and bagged flowers (K-W, H = 49.11, $p < 0.0001$): post-hoc pairwise comparisons showed significant difference in stage III (M-W U-test, $p < 0.02$), and highly significant differences in stages IV and V (M-W U-test, $p < 0.001$). A low production of nectar was observed in withered flowers, after initial stages of ovary development.

Energy values followed the same trend of nectar volume in both open and bagged flowers (Table IV).

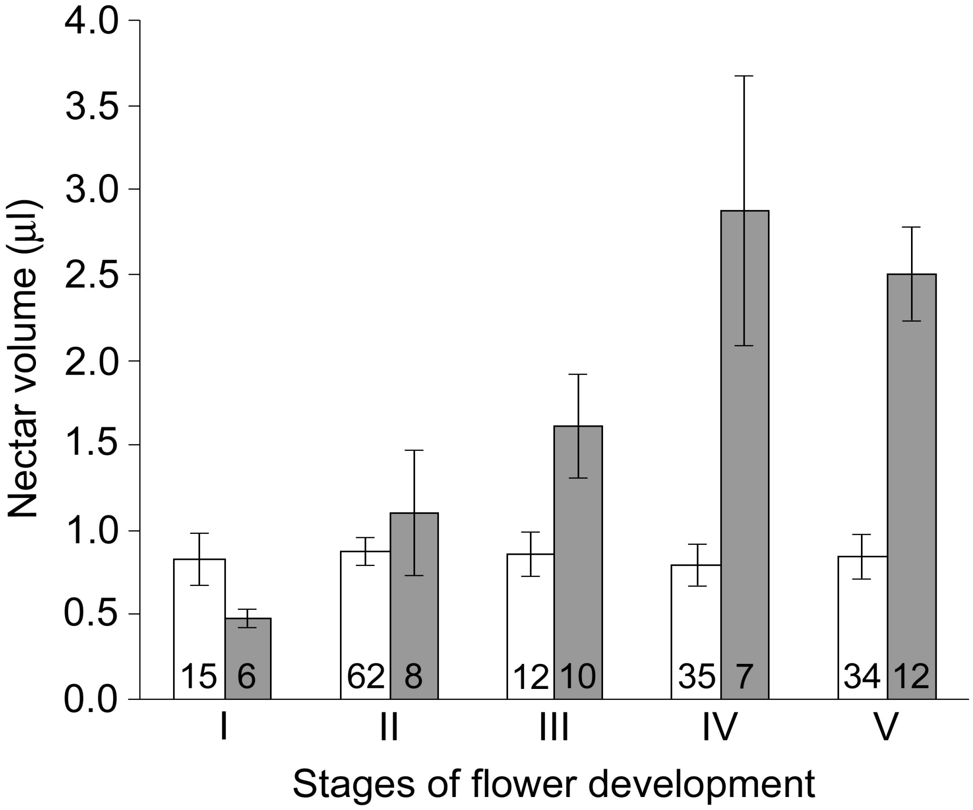

Fig. 10. Nectar volume (mean ± SE) in unbagged (open bars) and bagged flowers (filled bars) during flower development (male phase: stages II to IV, female phase: stage V; see text for details). Sample sizes are shown inside bars.

4.4.4 Flower visitors and pollinators

Total time of insects' observations was 10 hours. The majority of visitors were bees (Hymenoptera, superfamily Apoidea), and the most represented family was Apidae; only one species of Diptera was observed (Table V). Visitor species were observed seeking either pollen or nectar, with the exception of *Megachile sp.*: this bee collected one or the other reward in different foraging bouts, with distinct behaviour. Insects feeding on pollen (*Lasioglossum* sp., *Syrphus* sp., and *Megachile* sp.) alighted on dehiscent anthers and did not touch any other floral part. Some *Syrphus* individuals have been observed while collecting oil from glandular hairs on stamens. The frequency of visits to a plot and the number of flowers visited per plant varied quite widely among taxa (Table V).

Insect	Reward	Approaches to a plot (%)	Flowers visited/Plant (mean ± SE)	Contact with receptive stigma
Hymenoptera				
Andrenidae				
Andrena sp.	Ne	4 (3.54)	1 (n=4)	+
Apidae				
Apis mellifera	Ne	30 (23.62)	2.17 ± 0.19 (n=65)	+
Ceratina sp.	Ne	4 (3.54)	2.17 ± 0.60 (n=6)	-
Habropoda tarsata	Ne	7 (6.19)	5.90 ± 1.02 (n=7)	+
Bombus spp.	Ne	9 (7.96)	3.61 ± 0.46 (n=18)	+
Halictidae				
Lasioglossum sp.	Po	55 (48.67)	1.44 ± 0.11 (n=55)	-
Megachilidae				
Megachile sp.	Ne/Po	12 (9.45)	3.45 ± 0.36 (n=29)	+
Diptera				
Syrphidae				
Syrphus sp.	Po	6 (5.31)	1.5 ± 0.22 (n=6)	-

Table V. Data on insect behaviour from observation surveys. Ne = nectar, Po = pollen, + = insect contacts receptive stigma, - = insect does not contact receptive stigma, n = cumulative number of visited plants.

The most abundant genus of visitors was *Lasioglossum* (23.4 %), followed by *Bombus* (18.8 %), *Apis* (12.5 %), and *Megachile* (10.9 %) (Fig. 11a, b, c).

Fig. 11. Pollinators and visitors on *D. albus* flowers. (a) *Apis mellifera*, (b) *Bombus* sp., (c) *Bombus pascuorum* and *Syrphus* sp.

Among visitors, five taxa (*Andrena* sp., *Apis mellifera*, *Habropoda tarsata*, *Bombus* spp. and *Megachile* sp.) touched receptive stigmas, mostly with the lower part of abdomen, while seeking nectar: they were so considered as potential pollinators (Fig. 12).

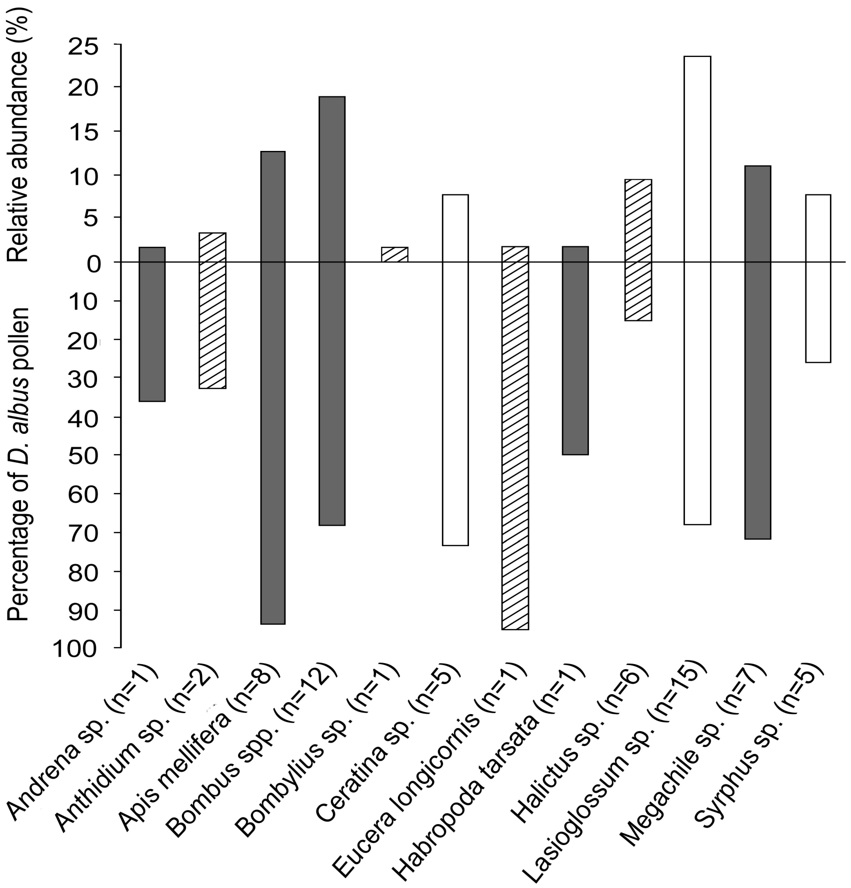

Fig. 12. Relative abundance (%) of visitors and percentage of *Dictamnus albus* pollen on total insects' pollen load. Pollen baskets were excluded from counts, where occurring. Filled bars: insects touch receptive stigma; open bars: insects do not touch receptive stigma; dashed bars: data on behaviour not available. n = sample size.

4.4.5 Pollinator importance

All sampled insects carried *D. albus* pollen, except for the Diptera *Bombylius* sp.: among them, only two taxa carried less then 30 % of specific pollen (*Syrphus* sp. and *Halictus* sp.). *Eucera longicornis* and *Apis mellifera* showed an almost monospecific *D. albus* pollen load (95 % and 94 %, respectively), followed by *Ceratina* sp., *Megachile* sp., and *Bombus* spp. (74 %, 72 %, and 68 %, respectively). High amounts of specific pollen loads were also found on the pollen feeder *Lasioglossum* sp. (68 %). Insect behaviour (contact with receptive stigma) and pollen load analysis allowed the detection of potential pollinators among visitors (Fig. 12).

In relation to pollinator importance, the genus *Bombus* showed the highest PI value (PI = 0.13) (Table VI). *Apis mellifera* exhibited a slightly lower value (PI = 0.12), and *Megachile* the lowest one (PI = 0.08).

Insect	n	Relative abundance	Pollen fidelity	PI
Apis mellifera	8	0.13	0.94	0.12
Bombus spp.	11	0.19	0.68	0.13
Megachile sp.	7	0.11	0.72	0.08

Table VI. Indexes of pollinator importance (PI) of the three main pollinator taxa. n = sample size.

4.4.6 Pollinators directionality

For two taxa mean angular coefficients were greater than 1: *Bombus* species, which showed the highest value (*b* = 1.87, and *A. Mellifera* (*b* = 1.41) (Table VII). By contrast, the *b* value for *Megachile* was close to zero (*b* = 0.24).

Mean angular coefficient was significantly greater than 1 only for *Bombus* spp. (t = 2.35, p = 0.02); however, *A. Mellifera* showed a close to significant positive value
(t = 1.64, p = 0.05). the *b* value for *Megachile* was significantly lower than 1 (t = -2-22, p = 0.02).

Insect	n	*b*	Modified Student's *t*	p-value
Apis mellifera	36	1.41 ± 0.35	1.64	0.05
Bombus spp.	10	1.87 ± 0.53	2.35	0.02
Megachile sp.	19	0.24 ± 0.48	- 2.22	0.02

Table VII. Angular coefficient (*b*) values (mean ± SE), respective modified Student's *t* values, and associated p-values for the three main pollinator taxa. n = sample size.

4.4.7 Pollinator limitation

There was a great increase in productivity from 2007 to 2008 both for fruit and seed set. In open pollinated plants, fruit set was more than three times greater in 2008 than in the previous year (2007: Fr:fi = 0.24 ± 0.05; 2008: Fr:fi = 0.93 ± 0.04), and seed set increased four times from one year to the other (2007: S:O = 0.12 ± 0.03; 2008: S:O = 0.48 ± 0.05).

The same trend was found in pollen augmented flowers for both fruit set (2007: Fr:Fi = 0.41± 0.06; 2008: Fr:Fi = 0.88 ± 0.06) and seed set (2007: S:O = 0.24 ± 0.04; 2008: S:O = 0.59 ± 0.05), with less increase between years (Fig. 13).

Statistical analyses revealed a pollen limitation only in the first year of manipulations: both fruit set (Fr:Fi t = 2.15, p =0.04) and seed set (S:O t = 2.35, p = 0.03) were significantly higher in hand-pollinated flowers than in open pollinated controls in 2007. Differently, no significant differences were highlighted neither in fruit set (Fr:Fi t = 0.87, p > 0.1) nor in seed set (S:O t = 1.71, p = 0.09) in 2008.

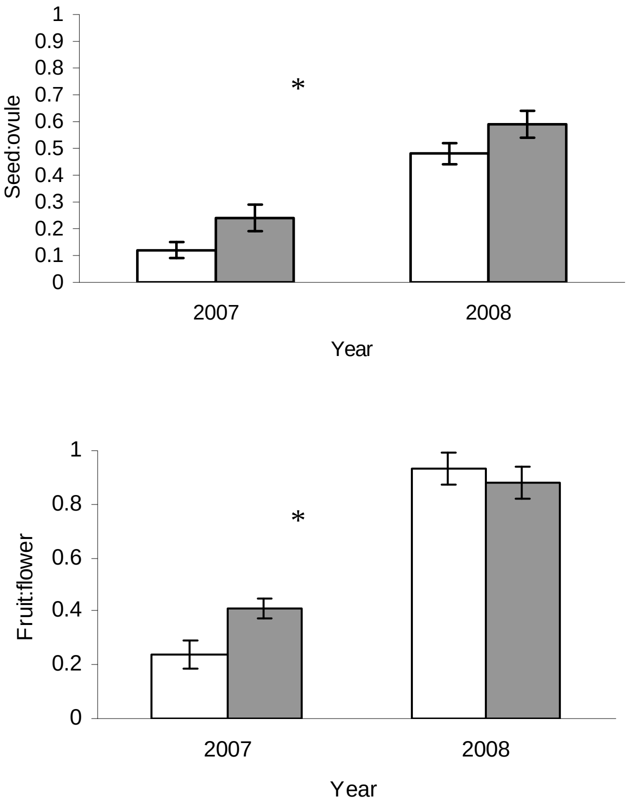

Fig. 13. Fruit and seed set (mean ± s.e.) in pollen supplementation experiments, in two consecutive years. Empty bars = open-pollinated controls; filled bars = hand-augmented flowers. * = significant differences after Mann-Whitney comparisons.

4.4.8 Demographic study

The total number of plants was almost ten times greater at the wood edge than inside the mature wood, in each year of study. At the edge, the range varied from 208 in 2009, to 239 in 2010, with 215 plants in 2008. In mature wood, the range varied from 25 in 2009 and 2010, to 27 in 2008.

Even though absolute numbers were very different between permanent quadrats, the distribution of relative abundances was comparable for three of the five classes. The most abuntant classes were always young (P2) and adult (A) plants (Fig. 14). Young plants (P2) were present with percentages from 40 % in mature wood (2009), to 49 %

at the edge (2008). Percentages of adult plants (A) were slightly higher in the mature wood, ranging from 33 % in 2008 to 48 % in 2009.

The greatest and most important difference between areas lies in the number of seedlings and in the number of fruiting plants. No fruiting or flowering plants were found in the mature wood during the 3 years of study. In the same plot, no seedlings were found in 2008 and 2009, one in 2010. By contrast, at the wood-edge fruiting plants were present, even if always in low percentages (absolute numbers: 9 in 2008, 1 in 2009, and 11 in 2010), and seedlings were abundant (absolute numbers: 18 in 2008, 19 in 2009, and 13 in 2010) (Fig. 14).

The number of flowering plants in the permanent area of 500 m^2 was highly variable among years. There was a maximum in 2006, with 509 flowering stems; in 2007 and 2008 there were 43 and 380 stems, respectively; in 2009 it was observed a minimum of 28 flowering plants, while in 2010 they were 475.

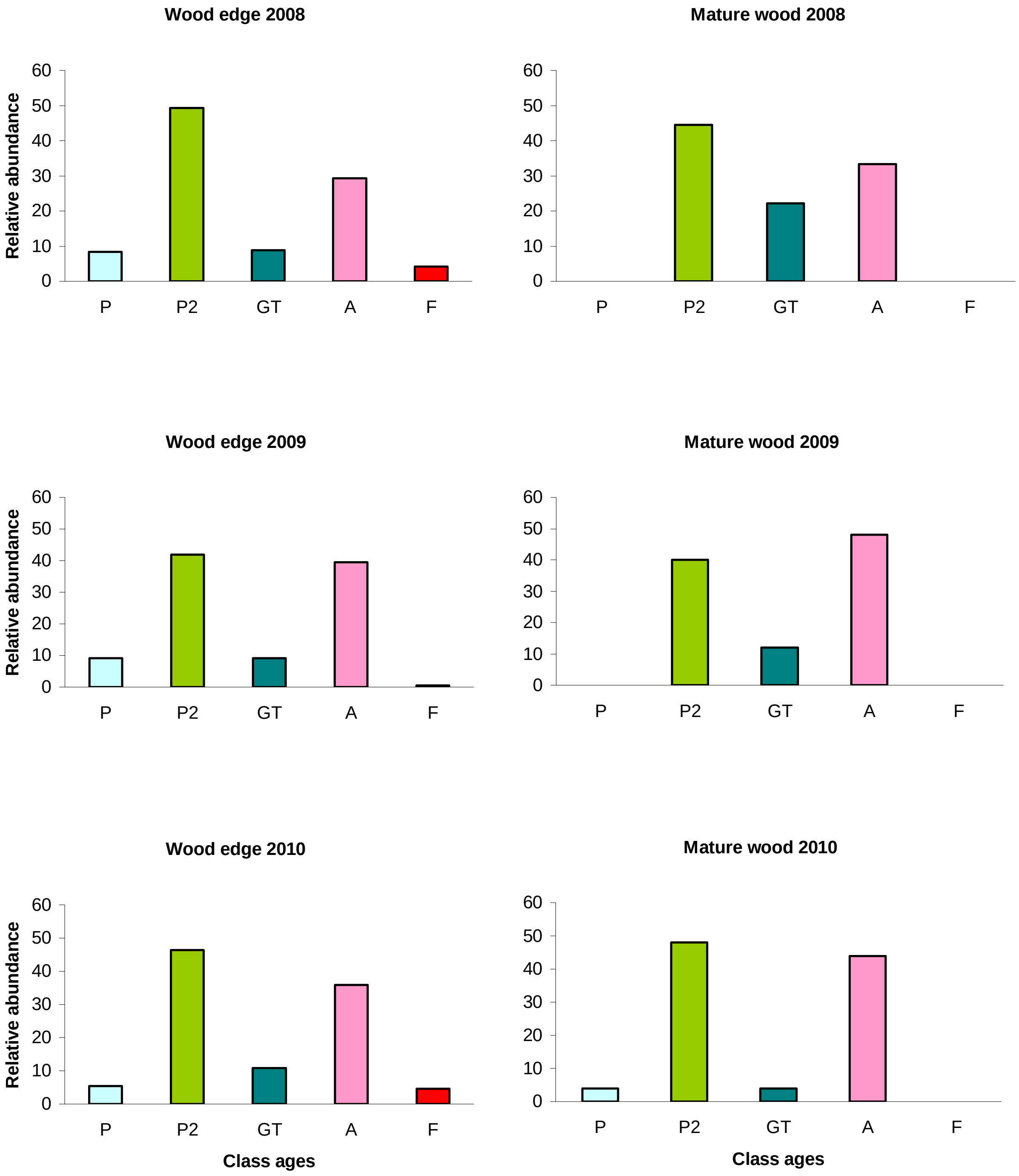

Fig. 14. Demography analysis on *D. albus* at the wood edge and in the mature wood during three years. Relative abundance of class ages (P = seedlings; P2 = young plants with simple leaves; GT = juveniles; A = vegetative adult plants with composed leaves; F = fruiting adult plants).

4.5 Discussion

I analysed the plant-pollinator system from both plant and pollinators point of view. My findings indicate that nectar distribution along the raceme and pollinators' behaviour act toward a reduction of geitonogamy on the plant side, while principal pollinating insects' rules of movement within the inflorescence maximize their net rate of energy intake. Moreover, I use a modified statistic method to analyse patterns of pollinator movements, by means of observations in natural conditions. This approach allows an indirect estimation of the extent of geitonogamy in plants with simple racemose vertical inflorescences.

The breeding system of *Dictamnus albus* has been analysed with field experiments in a natural population. Seed is produced only by sexual reproduction, as flowers where stigma was excided did not produce any apomictic seed. The species is self-compatible, as shown by manipulations; the growth of pollen tubes after self-pollination indicates a lack of barriers to self-fertilization acting at the stigmatic or stylar level, which implies absence of sporophytic or gametophytic self-incompatibility systems (Haring et al. 1990). The relatively high fruit set in both hand-selfed flowers and open-pollinated controls, confirms the potential self-compatibility. Nevertheless, the low seed production after self-pollination compared with controls suggests the existence of a late-acting self-incompatibility system, or of deleterious recessives, which results in inbreeding depression (Seavey & Bawa 1986). Furthermore, inbreeding depression is likely involved in the low germination rate, recorded for self-fertilized seeds compared to controls, which can be explained with a reduced fitness of selfed-flowers (Ellstrand & Elam 1993; Fenner & Thompson 2005).

My observations show that in *D. albus* flowers are protandrous and the receptive stigma is spatially separated from anthers. The joint action of dichogamy and herkogamy prevents spontaneous selfing within the same flower. Results demonstrate also that autonomous self-pollen transfer among flowers in a plant is negligible. Nevertheless, if insect-mediated, geitonogamy is theoretically possible within the flower-rich raceme, and it should bring deleterious effects on plant fitness: the self-pollen deposited by pollinators is no longer available for outcrossing, decreasing male gametophyte fitness (Brunet 2005) and gene flow through the population; it might also cause stigma clogging or compete with outcross pollen for fecundation (Ehlers 1999; Vaughton & Ramsey 2010).

Anthesis proceeds sequentially in the inflorescence, so that during full plant blooming the older flowers are found at the bottom and the younger at the top.

Nectar standing crop analysis and pollinator surveys indicate that nectar is the main reward. The experiment on bagged racemes shows that nectar production is higher during the last phases of flower maturation, which coincide mainly with female stage. This gender-biased nectar production, as well as pollinator guilds, are in agreement with data found for other species with the same pattern of nectar presentation (reviewed in Carlson & Harms 2006).

The higher energy values found in the flowers at the basis of the raceme, might work together with nectar amount to attract pollinators toward “female” flowers, after they have already functioned as male gametophyte donors. Pollinator nectar uptake is in fact higher during last flowering stages, as shown by the comparison between nectar volumes in bagged and open flowers.

Among the wide spectrum of visitors observed on *D. albus* inflorescences, it is possible to recognize two pollen robbers and only few effective pollinator species, which forage mainly for nectar. Similar patterns have been documented in other insect-pollinated species, where a restricted number of abundant pollinators dominate the diverse visitor assemblage (Thompson 2005).

Syrphus sp. and *Lasioglossum* sp. feed directly on dehiscent anthers without touching any other floral part. Since *Syrphus* individuals were scarce and visited less than two flowers on the same stem per flight, their potential negative effect on male fitness due to pollen wastage is supposed to be not significant. By contrast, the higher frequency and longer time visit of *Lasioglossum* (pers. obs.) might have detrimental effects on the male fitness component by increasing pollen discounting.

Indexes of pollinator importance (PI) underline the major role played by *Bombus* spp. and *Apis mellifera* as male gametophyte transporters. As shown by analyses, they forage following a precise pattern, starting to feed on lower (i.e., older) flowers, and then visiting few upper (i.e., younger) flowers on the raceme. In this way, there is a high probability for them to encounter functionally male flowers after female ones, depleting the level of geitonogamy.

However, a certain degree of geitonogamy might be considered, since I observed that megachilids do not follow any precise pattern of movements along the stem, but rather they forage at random among flowers. These bees do not follow any gradient of nectar during their visits, probably because they usually forage for pollen as a main reward for larval food provisioning, while nectar is used for self-feeding or for dampen pollen in nest cells (Stephen et al. 1969). Past studies demonstrated that solitary bees pollination could have negative impact on seed set (Franzen & Larsson 2009). In the studied case, the short foraging bout and low PI index of *Megachile*, together with the major pollinating role played by bumblebees and honeybees, should limit its contribution to geitonogamous pollination.

A previous study showed a significant positive relationship between the highest PI index in a given pollinator assemblage, and fruit set (Galloni et al. 2007). Therefore, the most important pollinators, with their upward movements, should increase outcrossing and plant fitness, and counteract downward movements made by *Megachilidae* visitors.

The measure of pollinators' behaviour on a plant that I propose, obtained by combining field observations with statistical analyses, can be used as an indirect method to assess the magnitude of geitonogamy in other species with flowers arranged on a simple vertical inflorescence (e.g., *Aconitum*, *Delphinium*, *Campanula*, *Digitalis*). It could be also used as a simple and cheap means prior to more specific tests (e.g., genetic markers, genotyping progeny).

Considering the perspective of pollinators, one main response can be recognized for the most effective bees. Bumblebees and honeybees visit few flowers upwards on the inflorescence, following the decreasing gradient of nectar reward. This behaviour is consistent with the declining reward hypothesis, which predicts a visitation pattern from more to less rewarding flowers and an early departure from the plant when the energy intake rate lowers under a certain level (Best & Bierzychudek 1982; Carlson & Harms 2006). Such rule of movement is likely to result in the maximum net rate of energy gain, and can be considered as an adaptation to the pattern of nectar presentation (Charnov 1976; Pyke 1978, 1979, 1984).

With regard to the plant point of view, my data are consistent with the inbreeding avoidance hypothesis. According to this theory, in plants with flowers arranged in a single raceme, gender-biased nectar production has to be considered as an adaptation to promote otucrossing, and decrease geitonogamy and pollen wastage (Pyke 1978; Carlson & Harms 2006).

When an insect moves from a plant to another, the first flower visited can undergo only outcross pollination; more visits on a same plant will reduce the amount of cross pollen available for pollination, while increasing the possibility of self-pollen deposition, that is expected to increase with plant size (de Jong et al. 1993; Barrett et al. 1994; Karron et al. 2009).

But intra-inflorescence self-pollen transfer might be prevented by *D. albus* protandry and by the sequential pattern of pollinator visits. Main pollinators visit only a small fraction of the available flowers on a plant, following nectar gradient (i.e., in the same order of flower maturation). Since it has been demonstrated that most pollen grains are deposited on the first few flowers (Thomson & Plowright 1980), geitonogamy results unlikely and pollen export is maximized (Iwasa et al. 1995).

When inbred, plants of *D. albus* produce fewer offspring with lower fitness compared to outcrossed. This finding again supports the hypothesis of inbreeding avoidance, which is based on the prediction that costs of inbreeding through within-plant pollen movement are significant (Carlson & Harms 2006).

I can therefore conclude that in the perennial *Dictamnus albus*, both plant features and pollinators behaviour promote outcrossing and strongly limit intra-plant pollination.

In addition to analyses on pollinators behaviour, I observed the presence of pollen limitation in 2007. Reduced seet set can be a consequence of low pollen quantity or quality, and can also occur after sufficient pollinator visits (Byers 1995; Wagenius & Lyon 2010). However, the very low flowering in 2007 may have caused reduced fruit and seed set, due to insufficient visits by insects; in fact, decreases in plant population size or density may increase pollen limitation and bring to reproductive failure due to Allee effect (Groom 1998; Forsyth 2003; Knight et al. 2005).

Populations of a long-lived plant like *D. albus* can survive to sporadical events of pollinator limitation without major negative consequences, but since I observed a very low seed production after self-pollination, negative genetic consequences may arise as a consequence of selfing. Moreover, plants recruitment from seed may be negatively affected if pollen limitation occurs for several years, by reduction of soil seed bank.

Variation in reproductive success of animal-pollinated plants might be due to high spatial and temporal variability in pollinator populations and pollination services (Thompson 2001; Petanidou et al. 2008; Albrecht et al: 2009). High fluctuations in the number of flowering plants observed in the selected area may influence the attractiveness to pollinators, with negative consequences on reproductive output in years with a very low flowering.

Pollinator abundance may be negatively affected by forest closure, with negative impact on population structure (Grundel et al. 2010). The abandon of agro-sylvo-pastoral activities in the last century has led to a landscape change in large parts of Europe, with a subsequent spread and colonisation by shrubs and trees, and the closure of oak woodlands (Debussche et al. 1999; Andrieu et al. 2007), indicated as one of the major threats to plants biodiversity (Thompson 2005).

The studied *D. albus* population is found at Downy Oak wood edges and in clearings, but plants are also present under thick canopy, in mature woods. A main difference observed between the two environmental conditions is related to the total number of plants, indipendently of their age. Plants at the wood fringes were ten times more abundant than inside the closed wood in all years. The other difference, more important for population persistence in the long term, is the exclusive presence of vegetative plants inside the wood. Here, flowering may be prevented by the low light that reaches the ground, which does not allow an acquirement of sufficient resources by adult individuals (Levine & Feller 2004; Mayberry & Elle 2009). Moreover, only one seedling was observed in three years inside the wood: since there has not been flowering in the area in the previous two years, its appearance might be due most likely to seed dormancy or to dissemination by animals.

Growth rates and survival in the shade are critical life history traits for many species, which can be influenced by changes in wood stand conditions (Endels et al. 2005; Lehtila et al. 2006) and can influence the maintaining of a popluation (Andrieu et al. 2007; Abe et al. 2008).

In general, I did not observe a demographic transition: during the three years of study, in any situation the distribution of age stages was similar. This might be due to low forest dynamics and stable environmental conditions, even if a general spread of shrubs has been observed during years (pers. obs.); more years of observations could be helpful in highlighting any changes.

Flowering and seedling recruitment clearly indicated wood fringes as the optimal environment for *D. albus*. Another fact that suggests wood edges as the most favourable habitat for development and persistence of *D. albus* populations, is that plants stop to grow within about 10 metres from the wood limits (pers. obs.). This could be due to poor dispersal into this habitat and/or reduced germination (Andrieu et al. 2007), and/or low seedling survival, probably because of too much light exposure, lack of moisture in the soil or other factors.

Several environmental factors determine population dynamics through vital rates such as reproduction, recruitment, growth and survival. For efficient conservation management, it is important to know which habitat types can maintain populations in the long period, how transition rates vary in relation with habitat and how this variation affects population dynamics (Lehtila et al. 2006; Abe et al. 2008).

Long-lived species can persist to some habitat deterioration, but population survival can be negatively affected in the long period and eventually face extinction if environmental conditions deteriorate further (Lehtila et al. 2006; Mayberry & Elle 2010). For this reason, future management actions, such as artificial clearings inside the wood, might be considered to maintain present populations and to enable long time persistence of the species.

5. *Convolvulus lineatus* L.

5.1 Species and study site

Convolvulus lineatus (Convolvulaceae) is a mediterranean plant that is found between 0 – 500 m a.s.l.; typical habitats are open with dry-rocky soils, and meso-hygrophilic basophil meadows (Tutin et al. 1972; Pignatti 1982). The species reaches its northern distribution limits in France, where it is protected by regional law in Provence-Alpes-Côte d'Azur, Auvergne, Pays de la Loire and Poitou-Charentes; it is also found in Languedoc-Roussillon and on Corsica (Tela Botanica). In France, natural populations are usually isolated because of habitat fragmentation due to human activities.

C. lineatus is a perennial hemicryptophyte, charachterized by woody stocks and procumbent herbaceous stems. Rhizomes assure vegetative propagation in the immediate proximity, and plants often form small clonal patches (Fig. 15a, b).

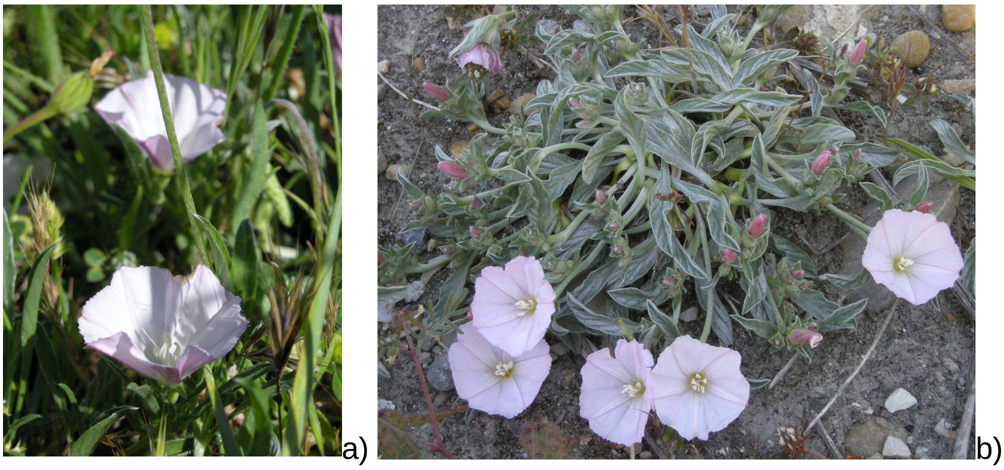

Fig. 15. Flowers (a) and a clonal patch with flowers and floral buds (b) of *C. lineatus*.

Leaves are linear to elliptical or oblanceolate, typically silver-sericeous on both pages. One to many pedunculate flowers are found at the end of the stems; white to pink corollas are 12-25 mm wide. Flowers have been observed to be protandrous (pers. obs.). Flowering occurs between mid-May and June. Fruits are pubescent capsules, each capsule usually bearing one (but up to three) seed; fruiting occurs in July and fruits fall on the ground when mature. Epizoochory is also reported as a dissemination strategy (Tela Botanica).

This study was conducted in 2010 in 4 isolated populations near the Mediterranean coast in Southern France. Both western populations, Gruissan (LR1) and Séte (LR2), are situated in Languedoc-Roussillon; the two eastern populations, Sollac (PACA1) and Massilia (PACA2), are located in Provence-Alpes-Côte d'Azur. Gruissan is the widest population of the study, within which I considered two big patches (for a total of 146 m^2) separated by several metres. At Séte, I considered the whole population (158 m^2), in which there was a clear separation between two patches. At the site of Sollac, plants were found along a path large around 4 m, for a total of 132 m^2. Observations of a marked discontinuity in floral phenology was suggestive of the presence of two major clonal patches: plants from the two ends were chosen for inter-patch pollinations. The population of Massilia occurs in a protected site, inside an enclosure, and it's the smaller of the study (31 m^2) (Fig. 16). It was not possible to detect patches at more than 5 m from each other. In all populations, plants presented a scattered distribution, with individuals clustered together in small patches. Approximate distance between populations is shown in Fig. 17.

Fig. 16. Warning sign at the protected site at Massilia, in the Provence-Alpes-Côte d'Azur region, Southern France.

5.2 Aims

Main objectives of this investigation were i) to assess the conservation status of a protected population at Massilia, where in 2009 I observed a complete absence of seed production, and ii) to evaluate the effects of pollination distance on reproductive success. As in the other studied sites *C. lineatus* presented a certain reproductive effort, the starting hypothesis was that in Massilia there could be a lack of compatible pollen receipt.

In four target populations, intra- and inter-population experimental pollinations were performed to detect the presence of clones or incompatibility types in the nearby surroundings of plants, and in general to evaluate the effects of pollination distance on reproductive success.

5.3 Materials and methods

5.3.1 Experimental pollinations

In order to evaluate the relationships between pollination distance among plants and reproductive success in isolated populations, in May 2010 dehiscent anthers were collected from 3-6 different plants, and hand-cross pollinations by three different pollination treatments were performed as follows:

i) pollinations with pollen taken from plants within 5 m from the maternal plant (hereafter, within-patch);

ii) pollinations with pollen taken from plants more than 5 metres distant (hereafter, between-patch);

iii) pollinations with pollen taken from plants belonging to different populations (hereafter, inter-population; pollen taken minimum 3 km apart).

Pollination treatments performed for each population are reported in Fig. 17.

Only in one population, Massilia (PACA2), it was not possible to perform inter-patch pollinations, due to its small dimension and uniform distribution (i.e., only one patch of the species occurs in the site).

One additional population has been taken as pollen donor for inter-population pollinations.

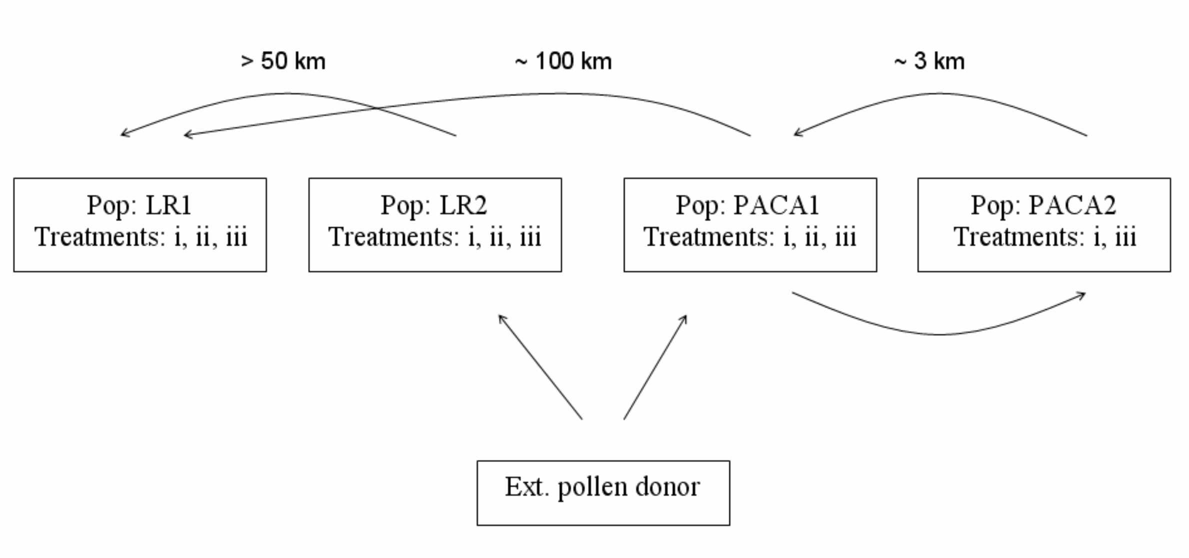

Fig. 17. Diagram of experimental pollinations. “Treatments” indicates which manipulation has been performed in each population: i) within-patch, ii) between-patch, and iii) inter-population pollinations. Arrows indicate pollen source and the direction of manual pollinations. Approximate distances (km) between populations are indicated upon arrows. Extended names of populations are given in the text. “Ext. pollen donor” is an additional population used for inter-population pollinations.

At least two dehiscent anthers (from different plants) were brushed directly on protruding curved stigmas. For pollinations between populations more than 5 km far, anthers were collected the day before and stored in the fridge. Manipulated flowers were marked with plastic tags of different colours for each treatment. Sample sizes are shown in histograms (Fig. 18). Fruits were counted when mature, and collected to avoid genetic contamination by seeds originated by fertilization with pollen from different populations; mean fruit:flower ratios were then calculated. Among-treatment comparisons were made to detect any difference in reproductive success in relation with pollination distance. Chi-squared tests of independence were used to assess differences among treatments; a *x+1* transformation has been performed for the population of Gruissan due to a total absence of fruit production after within-patch pollinations.

5.4 Results

5.4.1 Experimental pollinations

Mean fruit set was quite variable in controls among populations. Fruit set was very low in 2 populations (Séte: Fr:fl = 0.03 ± 0.01; Massilia: Fr:fl = 0.04 ± 0.01), while it was higher in the others (Gruissan: Fr:fl = 0.25 ± 0.05; Sollac: Fr:fl = 0.27 ± 0.02).

In the experimental pollinations at the population of Séte (LR2), the overall reproductive success was very low. I found only one fruit for all the 75 hand-pollinated flowers (in between-patch pollinations; Fr:fl = 0.03 ± 0.03).

In Gruissan (LR1) there were significant differences among treatments ($\chi^2 = 12.63$, $p < 0.01$): differences were found for within and inter-population pollinations ($\chi^2 = 8.15$, $p < 0.01$), and for between and inter-population pollinations ($\chi^2 = 6.24$, $p < 0.02$). No differences were found in within – between-patch comparisons (Fig. 18).

In Sollac (PACA1) I found significant differences among treatments ($\chi^2 = 6.52$, $p < 0.05$): chi-squared showed differences in within – between-patch ($\chi^2 = 5.59$, $p < 0.02$) and in within – inter-population pollinations ($\chi^2 = 5.53$, $p < 0.02$), but not in between – inter-population pollinations.

In Massilia (PACA2), where only within – inter-population comparison was possible, the chi-squared test showed significant differences between treatments ($\chi^2 = 4.82$, $p < 0.05$).

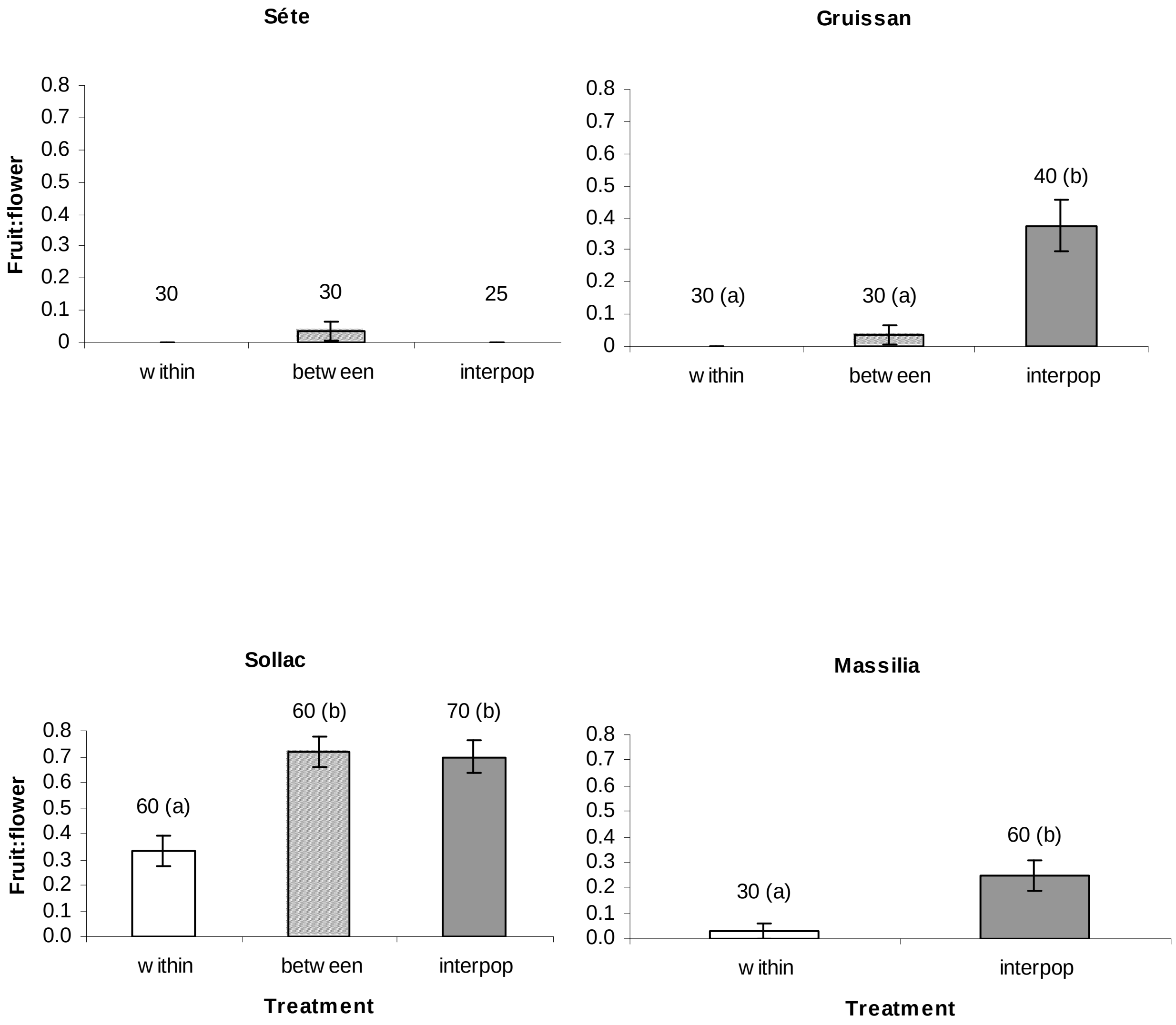

Fig. 18. Fruit set (mean ± s.e.) in the 3 experimental pollination treatments. Within = within-patch pollination (< 5 m); between = between-patch pollinations (> 5 m); interpop = inter-population pollinations. Sample size (i.e. number of pollinated flowers) is given above the columns. Different letters indicate significant differences between treatments, after chi-squared tests.

5.5 Discussion

The population of Séte must be excluded from the discussion, since the almost total failure of fruit set does not allow a meaningful set of conclusions.

A certain amount of the low fruit set observed in the populations of Séte and Massilia could be due to problems connected with low pollen viability (Petanidou et al. 2001). However, I sampled anthers from different plants, and the pollen collected from distant populations was stored for only one night in the fridge; moreover, comparisons with controls in the same populations show similar patterns of productivity to intra-population manipulations. Hence, I suppose that a bias due to pollen viability might be of minor importance.

Considering the three populations that set fruit, a general trend can be observed: an increase in the distance of pollination, from within-patch to between-patch and between-population, leads to a general increase in fruit reproductive success. This suggests that an incompatibility type occurs in the close surroundings of the small clonal patches, and that its presence reduces fecundity due to a deficit in outcross pollen.

In a clonal, protandrous and likely self-incompatible species like *C. lineatus*, the amount of compatible pollen arriving on a stigma, and subsequently seed set, may be limited not only by the quantity of pollen receipt, but also by a paucity or absence of compatible genotypes from nearby potential mates (Charpentier et al. 2000; Wagenius et al. 2007).

I also observed high amounts of floral herbivory (especially anthers predation), which could limit reproductive output and act along, or in contrast, with pollinators as a selective pressure on reproductive traits (Thompson 2005). Previous studies reported distinct results for different species: Herrera (2000) observed non-additive fitness consequences of exposure to herbivory and pollination; by contrast, Lavergne et al. (2005) found additive effects because of negative consequences of herbivory even in absence of pollinators, due to autonomous self-pollination of the studied species. Hence, to understand the level at which reproductive limitation is affected by insufficient outcross pollen and/or by herbivory and quantify the role of pollinator-mediated selection, an experiment that segregates effects of interaction is necessary.

During field work, I observed few small insects visiting open flowers. They were mainly coleopterans and orthopterans, which fed for pollen or just laid in the corolla (Fig. 19). In either way, insects came in contact with pollen on different body parts and touched stigmas during their movements, so they can be considered as potential pollinators. As a consequence of the small dimensions of insects and therefore of their short range movements, I assume that intra-patch pollination might be the most likely situation in the studied populations, especially in Gruissan and Sollac.

Fig. 19. Small insects on open flowers of *C. lineatus*: a coleopteran eating pollen from dehiscent anthers (a) and an orthopteran (b).

Reduced sexual reproduction may have negative consequences in the short term for the population dynamics, since it could affect recruitment potential from seeds. In a fragmented landscape, reduced reproduction can also decrease the opportunity to colonize unoccupied patches. Vegetative propagation could allow a persistence of *C. lineatus* populations, but in the long term the absence of genetic variability, on which natural selection could act upon, might compromise the ability of small genetically-isolated populations to respond to environmental changes.

The Mediterranean region has faced thousand years of human activities, which impact has modified the spatial configuration of habitats in the landscape (Thompson 2005), and the habitat of many species (e.g., Römermann et al. 2005; Buisson et al. 2006; Andrieu et al. 2009; González-Varo et al. 2010), including *C. lineatus,* is now fragmented. Compatibly with suitable habitat remnants and land-use destinations, different practical actions could be put in use to increase plants fitness and population persistence.

An increase in the number of populations could ensure a genetic flow between isolated remnants, and increase visits by pollinators (Van Rossum & Triest 2010). Moreover, connectivity of small isolated populations can be highly correlated with progeny performance by enhanced outcrossing rates (González-Varo et al. 2010). Finally, since no outbreeding depression has been observed after inter-population pollinations, it is possible to make an action of genetic rescue using distant populations as seed donors. This could ensure reproduction by seeds and long-term persistence especially for the smaller or more threathened populations, for which any natural gene flow could not be expected. However, the specific case of Massilia appears to have no long-term survival prospects. In fact, even if it is a protected population, it is surrounded by non-suitable habitat; if a restoration of the site will not be performed, there would not be chance for further colonization and spread of the population.

6. General conclusions

As showed by this investigation, several factors can influence plant reproduction and distribution, pollination success and the interactions between plants and pollinator fauna.

In a fragmented habitat, pollination specialization *per se* does not involve a higher vulnerability: specialized plants are not attended to necessarily face a higher risk than generalists (Aizen et al. 2002, Ashworth et al. 2004). In *Primula apennina*, where population are highly spatially isolated, despite the only two potential pollinator taxa found in the studied population, there was not evidence for pollinator limitation, indicating a high efficiency in pollen transfer.

However, as discussed above, other factors can act together with isolation towards a disruption of the plant-pollinator system, with subsequent detrimental effects at both individual and population level in the short- or long-period.

Isolation can be due to natural causes, as for *P. apennina*, whose populations find suitable habitat only on the top of the mountains, separated from each other by large unsuitable valleys; it can also be due to human-induced habitat alteration and fragmentation, as for *D. albus* and *C. Lineatus*.

In each case, the absence of genetic flow between populations may be a threat to their persistence, and pollinator limitation might increase the risk at local level. However, spatial isolation does not always coincide with functional isolation: if pollinators are efficient and able to cover long distances, risks connected with isolation may be overcome, and long-term survival assured. This is the case of *P. apennina*, where long-flights of a diurnal moth assure functional connection, with subsequent gene flow among distant populations. By contrast, an opposite situation was found for *C. lineatus*, where spatial and functionial isolation coincided: the small-range movements of pollinators do not allow pollen transport among populations, which are genetically isolated to each other.

In relation to reproductive success, reduced reproduction might have negative consequences in short, medium and long term, affecting plant and population persistence (Kéry et al. 2000). Even in species with a potential for self-pollination, a negative response to habitat fragmentation is likely (Aizen et al. 2002), with subsequent detrimental effects in isolated populations. Nevertheless, when plant species are mainly outcrossers and present mechanisms that prevent self-pollination, risks linked to isolation may be reduced. Pollinator movements in response to nectar distribution on plants of *D. albus* could be an example of co-evoluted mechanism that prevents self-pollination while promoting outcrossing.

Conservation actions aimed to reduce the degree of populations isolation can be necessary to preserve ecological dynamics. Small isolated remnants can be less visited by pollinators; connectivity can be ensured by population introduction and reintroduction (Van Rossum & Triest 2010), and by reinforcing reduced populations, with genetic rescue if there is no evidence of outbreeding depression. Experimental pollinations performed between long-distance isolated populations of *C. lineatus* show that practical actions can be a choice to ensure long-term survival, especially in small remnants. In fact, habitat fragmentation can have negative effects on mating patterns and genetic diversity of plant populations, and subsequently on offspring fitness as a result of inbreeding depression (Cascante et al. 2002; Aguilar et al. 2008). Moreover, performance and genetic diversity of progenies in fragmented small populations can be influenced more by outcrossing rates than by genetic diversity of adult plants (González-Varo et al. 2010). Enhancing connectivity and assuring high outcrossing rates is thus fundamental for the production of viable seedlings and for the future of plant populations.

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