

# Pollen quality limitation in the Iberian critically endangered genus *Pseudomisopates* (Antirrhinaceae)

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**Abstract** Pollen limitation occurs when sexual reproduction is decreased due to inadequate pollen receipt. Limitation is usually associated with the quality and the quantity of pollen; still, most studies do not discriminate between the two. We used hand-pollination experiments and observations of floral visitors to determine the mating system and limitations to seed production of the Spanish endangered species *Pseudomisopates rivas-martinezii*. We tested for agamospermy, autogamy, obligated autogamy, cross-pollination and supplementation. The response variables considered were fruit set, seed set and viable seed set. Previous studies indicated that (i) the species exhibits extensive clonal growth, (ii) plants flower profusely in summer, (iii) seeds showed to be highly unviable, and (iv) no seedlings were observed in the field. We found that *P. rivas-martinezii* is

predominantly self-incompatible, has an unexpected generalized pollination system considering its occluded corolla, and is limited by qualitative rather than quantitative pollen limitation. These results indicate that, in addition to other presumed environmental factors, the low seed viability of this self-incompatible species is due to pollen quality limitation as a result of reduced mate availability.

**Keywords** *Pseudomisopates rivas-martinezii* · Conservation · Mating system · Pollen limitation · Pollen quality · Pollen quantity

## Introduction

Pollen limitation, a decrease in potential plant reproduction due to inadequate pollen transfer (Larson and Barrett 2000; Ashman et al. 2004; García-Camacho and Totland 2009), may enhance plant population decline (Ashman et al. 2004; Ward and Jonson 2005). Recent reviews have shown that pollen limitation is a common phenomenon in plants (Knight et al. 2005). Different analysis conducted on seed production studies reported that about 63% of the species investigated exhibited pollen limitation depending on sites or years (Burd 1994; Knight et al. 2005).

Pollen limitation in insect-pollinated species comprises two components: low pollen quantity (derived from visitation rate and efficiency) and low pollen

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quality (affected by self-fertilization, mating between genetically incompatible plants and the degree of relatedness between parents through inbreeding depression). Qualitative factors probably limit seed production as strongly as quantitative factors, since pollen vectors typically disperse pollen locally (Harder and Aizen 2010). However, many pollen supplementation experiments have been unable to distinguish between these two components (Aizen and Harder 2007).

Main factors provoking pollen limitation are: (1) low pollinator visiting frequency (Totland and Sottocornola 2001; Hegland and Totland 2008), (2) low pollinator abundance, (3) the dominance of inefficient pollinators (Elle and Carney 2003), (4) an impoverishment or shift of the pollinator assemblage when different pollinators have complementary effects on plant fitness (Klein et al. 2003; Gómez et al. 2007; González-Varo et al. 2009), (5) pollinator discrimination against particular plant species or flower traits (Fenster et al. 2004), such as floral design and display (Thompson 2001), (6) the size and density of the population which affects pollinator attraction and the genetic structure of the population which influences compatible mate availability (Hamrick et al. 1991), (7) limited pollen availability, low pollen-tube survival or zygote death (Busch and Schoen 2008).

The degree of reliance upon pollinators for reproduction may be vital for plant reproductive success. Under pollinator scarcity or low mate availability, self pollen is unable to produce seeds in obligate outcrossers; therefore, plants may not yield enough seeds to maintain a viable population. Small populations of many self-incompatible species are threatened for this reason (DeMauro 1993; Byers 1995). Furthermore, species that reproduce clonally as well as sexually may show further reduced sexual reproduction, due to the extension of clones. Under the same circumstances of mate availability, selfers may reach higher seed production (Totland and Schulte-Herbrüggen 2003) and suffer lower inbreeding than outcrossers (Husband and Schemske, 1996; Charlesworth and Charlesworth 1987). However, the reduction in offspring number and quality due to lowered mate availability or mating among close relatives can be compensated by higher quality offspring promoted by outcrossing (Vallejo-Marín and Uyenoyama 2004) and the reproductive compensation over multiple

breeding seasons in perennial outcrossers (Goodwillie 1999; Larson and Barrett 2000).

In this study, we test the occurrence of pollen limitation in *Pseudomisopates rivas-martinezii* (Antirrhinaceae). This species is catalogued as Critically Endangered following the IUCN criteria in the Red List of Spanish Vascular Flora (Martínez Rodríguez et al. 2004) and is one of the 19 monotypic genera endemic to Spain (Nieto Feliner 1999), of which seven are endangered. It shows extensive clonal growth and although plants flower profusely, seeds at many sites seemed to be unviable, as shown by preliminary germinability tests, and no seedlings have been observed in the field (Martínez Rodríguez et al. 2004). The specific objectives of this study were to determine the mating system and evaluate the limitations to seed production in *P. rivas-martinezii*. These were assessed by (1) characterizing floral visitors by means of surveys to determine their abundance, frequency of visits and behaviour; (2) investigating the effects of pollen limitation, in both quantity (pollinator limitation and mate availability) and quality (self compatibility and inbreeding depression) on fruit set, seed set, and viable seed set by performing a series of pollination treatments.

## Methods

### Study species

*Pseudomisopates rivas-martinezii* (Antirrhinaceae) is an endangered species and genus endemic to the Gredos region, in the Spanish Central Mountain System. Within this region, it is distributed in two main areas separated by 20 km, one in Gredos Mountain Range and the other in La Serrota Mountain Range, comprising seven and three known populations, respectively (Vargas and García 2008). The species occurs from 1,400 to 1,990 m.a.s.l., inhabiting clearings within *Cytisus oromediterraneus* shrublands. Plants reproduce vegetatively by an underground stolon network, with connections between ramets that can span over one metre. Flowers are apparently occluded (personate) and bloom during July and August. As considerable strength is required for pollinators to enter occluded flowers of the other closely related Antirrhinaceae species, it has been historically hypothesized that

bees are the only pollinators (Müller 1929; Sutton 1988), as they exhibit the most suitable body morphology and strength (Gottlieb et al. 2005; Vargas et al. 2010). Fruiting occurs during August and September. Fruits can contain between 1 and 24 seeds, which are dispersed by barochory.

### Mating system

Pollination treatments were performed in *La Serrota* population (one of the three populations located in *La Serrota* Mountain Range, Cepeda de la Mora, Ávila), during August 2007 and 2008. The occupancy area of this population is approximately 14,400 m<sup>2</sup>, with a ramet density of  $27 \pm 4.4$  ramets per square metre. The closest populations are located 1 and 2.5 km apart, and no isolated individuals have been found in between. A former study was conducted in 2007 in which six pollination treatments were used to determine the mating system of *P. rivas-martinezii* and the possible reproductive limitations due to pollen quality and quantity. These treatments tested for: (1) *agamospermy*, by emasculating and bagging flowers, (2) *spontaneous autogamy*, by bagging flowers, (3) *obligated autogamy*, by bagging flowers and hand-pollinating them with pollen from the same flower, (4) *cross-pollination*, by bagging flowers and hand-pollinating them with pollen from ca., 100 plants from *Lóbrega* population (*Gredos* Mountain Range, Candeleda, Ávila), located 20 km apart, (5) *pollen supplementation*, by supplementing unbagged flowers with pollen from ca., 100 plants located 5–10 m away, and (6) *control*, flowers left open for natural pollination. Twenty flowers from ten plants (two per plant) were selected for each treatment. In the summer 2008, attention was focused only on those treatments that had a clear effect the previous year, namely: *obligated autogamy*, *cross-pollination*, *pollen supplementation*, and *control*. Sample size was increased to 30 flowers from 30 plants per treatment (one per plant) to obtain a more robust estimation. The components of reproductive success used to determine the mating system and test pollen limitation were: (1) fruit set, which was the ratio of fruits to treated flowers, (2) seed set, which was the ratio of seeds to the average number of ovules, and (3) viable seed set, which was the ratio of viable seeds to the average number of ovules. The number of ovules ( $n = 22 \pm 3$ ) was obtained separately from a sample

of 20 flowers from *La Serrota*. Seed viability and hence an estimate of seed germination, was assessed in 2008 by means of a Tetrazolium test optimized for the species, following the Tetrazolium Testing Handbook (Peters 2000). Seeds were imbibed in water overnight at 21°C, then slightly cut and incubated in 1%TZ at 27°C for 72 h. Seeds were finally bisected and viability was accounted for by observing the red coloring of living tissues.

### Floral visitor assemblage

Surveys were carried out in five patches in *Los Conventos* population (*Gredos* Mountain Range, Hoyos del Espino, Ávila) during the flowering peak in August 2009. The occupancy area of this population is approximately 12,100 m<sup>2</sup>. The goal was to describe the type of insects visiting *P. rivas-martinezii* flowers, rather than to infer any relationship between floral visitor diversity and abundance, and pollen limitation intensity. All surveys were performed under similar favorable conditions (sun and no wind). Within each patch, 10 plants were chosen for each survey, in which the total number of open flowers and the number of flowers visited during 10-min periods were counted. Surveys were done between 10 h 40 and 18 h 35, totaling 2,560 min. Any insect observed making contact with anthers or stigma was recorded.

### Data analysis

Differences between treatments were analysed using Generalized Linear Models (GLM), including treatment and year as factors. First, the effect on fruit set, seed set and viable seed set was assessed by fitting these variables to a binomial distribution, using *logit* as the link function. The interaction *treatment\*year* was not significant for any of these models, therefore, data of both years were analysed together. Second, pairwise differences were assessed using post-hoc likelihood ratio tests based on  $\chi^2$  distributions. All analyses were performed using JMP version 7.

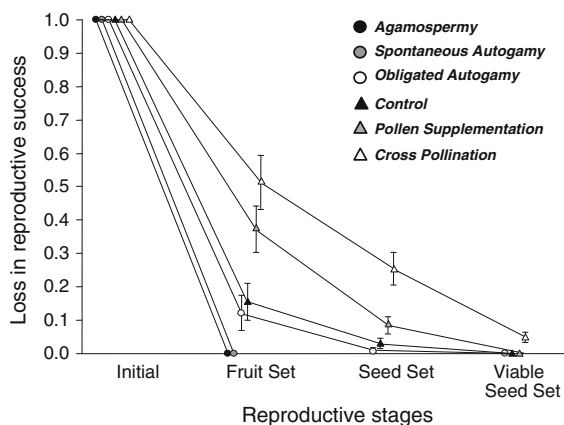
Two aspects of the floral visitor assemblage visiting *P. rivas-martinezii* flowers are considered in this study: abundance and diversity (Magurran 2004). Abundance of floral visitors was estimated by standardizing the number of visits per open flower and time unit (flowers visited per hour). Floral visitor

diversity was assessed by calculating richness ( $S_{\text{obs}}$ ), diversity, evenness, and dominance.  $S_{\text{obs}}$  was the number of species found visiting flowers in each patch; two asymptotic richness estimates, the incidence coverage estimator ( $S_{\text{ICE}}$ ) and the Michaelis–Menten index ( $S_{\text{MM}}$ ) were calculated using EstimateS software (Colwell 2005). Dominance was calculated as the relative abundance of the most abundant visiting species. Diversity was calculated by the Shannon–Wiener index and Hulbert’s PIE (Colwell 2005) by a randomization process using EcoSim (Gotelli and Entsminger 2004). Among-patch differences in floral visitor abundance and composition was tested using a permutational multivariate analysis of variance, PERMANOVA version 1.6 (Anderson 2001), on the basis of Bray–Curtis dissimilarities.

## Results

### Mating system

The experiments affected all fecundity estimates consistently. There were between-treatment differences in fruit set ( $\chi^2 = 39.975$ , 5 df,  $P < 0.001$ ), seed set ( $\chi^2 = 490.799$ , 5 df,  $P < 0.001$ ), and viable seed set ( $\chi^2 = 81.328$ , 3 df,  $P < 0.001$ ) (Fig. 1; Table 1).



**Fig. 1** Reproductive success at the different reproductive stages in *Pseudomisopates* as a result of pollination treatments in 2007 and 2008. Autogamy and spontaneous autogamy are only given for 2007. Obligated autogamy, cross-pollination, pollen supplementation and control are averages across the 2 years

Flowers treated for *agamospermy* and *spontaneous autogamy* in 2007 produced no fruits (Fig. 1; Table 1), being significantly different to all other treatments (Table 1). Flowers from *obligated autogamy* showed very low fecundity, producing 22% less fruits and 44% less seeds than controls, as well as no viable seeds (Fig. 1; Table S1). These differences were only significant in seed set (Table 1).

Cross-pollinated flowers showed the highest fecundity, producing 53% more fruits and 46% more seeds than control flowers (Fig. 1; Table S1). Although no differences were observed with *pollen supplementation* in fruit set, they differed in further seed stages (Table 1). Significant differences between *cross-pollination* and all other treatments were found at all stages (Table 1).

Pollen-supplemented flowers had higher fecundity than *control* flowers, producing 30% more fruits and 39% more seeds (Fig. 1; Table S1). However in 2007, pollen-supplemented flowers produced a similar fruit set to the *control* treatment; whereas in 2008, it showed a fourfold result compared to the *control* (Table S1). This difference was mainly due to the between-year variation in fruit set for the *control* treatment, which yielded a 68% lower fruit set the second year.

### Floral visitor assemblage

Insects from 6 orders, 16 families, and 26 species were recorded (Table 2). Most species belonged to the order Hymenoptera (12 species), although the most abundant species were Coleoptera. Excluding the three most dominant species (*Mylabris* sp., *Megachile* sp.1, and *Melighetes* sp.1), which comprised 63.7% of all visits, most species made less than 5% of visits. *Mylabris* sp. constituted 34.41% of visits, with an average visitation rate of  $7.01 \times 10^{-4}$  visits per flower per hour and an average of 2.1 flowers visited per plant. The solitary bee *Megachile* sp.1 carried out 18.43% of visits, with an average visitation rate of  $4.72 \times 10^{-5}$  visits per flower per hour and an average of eight flowers visited per plant. *Melighetes* sp.1 carried out 10.91% of visits, with an average visitation rate of  $2.39 \times 10^{-4}$  visits per flower per hour and an average of 1.5 flowers visited per plant.

On the day surveyed, the average number of flowers visited per plant was  $2.54 \pm 4.46$ , whereas

**Table 1** Results of the post-hoc  $\chi^2$  test comparing the effects of the pollination treatments on fruit set, seed set, and viable seed set in *Pseudomisopates*

|                        | Agamospermy | Spontaneous autogamy | Obligated autogamy     | Cross pollination | Pollen supplementation | Control |
|------------------------|-------------|----------------------|------------------------|-------------------|------------------------|---------|
| Fruit set              |             |                      |                        |                   |                        |         |
| Agamospermy            |             |                      |                        |                   |                        |         |
| Spontaneous autogamy   | 0           |                      |                        |                   |                        |         |
| Obligated autogamy     | 4.188*      | 4.188*               |                        |                   |                        |         |
| Cross pollination      | 21.522***   | 21.522***            | 14.929***              |                   |                        |         |
| Pollen supplementation | 14.464***   | 14.464***            | 7.144**                | 2.093             |                        |         |
| Control                | 6.245**     | 6.245**              | 0.465                  | 11.192***         | 4.295*                 |         |
| Seed set               |             |                      |                        |                   |                        |         |
| Agamospermy            |             |                      |                        |                   |                        |         |
| Spontaneous autogamy   | 0           |                      |                        |                   |                        |         |
| Obligated autogamy     | 7.983**     | 5.535*               |                        |                   |                        |         |
| Cross pollination      | 202.425***  | 141.750***           | 274.526***             |                   |                        |         |
| Pollen supplementation | 66.396***   | 45.697***            | 66.638***              | 101.342***        |                        |         |
| Control                | 21.935***   | 15.133***            | 8.464**                | 220.810***        | 32.115***              |         |
| Viable seed set        |             |                      |                        |                   |                        |         |
| Obligated autogamy     |             |                      |                        |                   |                        |         |
| Cross pollination      |             |                      | 44.469***              |                   |                        |         |
| Pollen supplementation |             |                      | 1.387                  | 36.905***         |                        |         |
| Control                |             |                      | $1.926 \times 10^{-8}$ | 44.469***         | 1.387                  |         |

Significance levels indicated as \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$

the proportion of flowers visited per plant was  $0.12 \pm 0.19$ . The average number of flights per insect within a plant was  $1.12 \pm 2.10$ . Abundance, as shown by the visitation rate was  $0.002 \pm 1.16$  visits per flower per hour. The observed species richness ( $S_{\text{obs}}$ ) in the population was 26 (95% CI = 20.8–31.19). The expected floral visitor richness according to asymptotic estimates was  $S_{\text{ICE}} = 41.54$  and  $S_{\text{MM}} = 40.6$ ; these values suggest that floral visitor assemblages were richer than inferred from our sampling. Hulbert's PIE index was 0.83 for the population (83% probability of two randomly selected flower visitors belonging to different species) and Shannon–Wiener  $H$  was 2.28. Dominance was 0.70, with few species scoring a relative abundance higher than 5%.

Patches did not vary significantly in floral visitor abundance (Kruskal–Wallis  $\chi^2 = 3.5378$ ; df = 4;  $P = 0.4721$ ). Pollination assemblage composition varied significantly among patches (PERMANOVA,  $F = 2.2078$ ,  $P = 0.01$ ). Although differences were mainly

due to slight variations in the abundance of *Mylabris* sp. and *Megachile* sp.1.

## Discussion

### Self-incompatibility

Pollination experiments showed that *P. rivas-martinezii* is predominantly self-incompatible and depends on pollen vectors for sexual reproduction. Indeed, fecundity after self-pollination and agamospermy was much lower than after cross-pollination or open pollination, and yielded no viable seeds (Fig. 1; Table 1). This result has been previously reported on the closely related *Antirrhinum* (Antirrhinaceae), in which many species are considered to be self-incompatible (Tseng 1938; Sutton 1988), but show different levels of self compatibility (East 1940; Mateu-Andrés and Segarra-Moragues 2004; Carrió et al. 2009). The total absence of fruits produced by

**Table 2** List of floral visitors of *Pseudomisopates* for *Los Conventos* population

| S. no | Order        | Family         | Species                         | Total | % Visit |
|-------|--------------|----------------|---------------------------------|-------|---------|
| 1     | Coleoptera   | Meloidae       | <i>Mylabris</i> sp.             | 224   | 34.41   |
| 2     |              | Mordellidae    | Mordellidae                     | 2     | 0.31    |
| 3     |              | Nitidulidae    | <i>Melighetes</i> sp.1          | 71    | 10.91   |
| 4     | Diptera      |                | <i>Melighetes</i> sp.2          | 1     | 0.15    |
| 5     |              | Bombyliidae    | <i>Hemipenthes</i> sp.          | 1     | 0.15    |
| 6     |              | Scathophagidae | Scathophagidae                  | 17    | 2.61    |
| 7     |              | Syrphidae      | <i>Eupeodes corollae</i>        | 3     | 0.46    |
| 8     |              | Miridae        | <i>Hadrodemus</i> sp.           | 2     | 0.31    |
| 9     | Hymenoptera  | Apidae         | <i>Anthidium</i> sp.            | 18    | 2.76    |
| 10    |              |                | <i>Amegilla</i> sp.             | 13    | 2       |
| 11    |              |                | <i>Anthophora</i> sp.           | 10    | 1.54    |
| 12    |              |                | <i>Bombus pascuorum</i>         | 2     | 0.31    |
| 13    |              | Chalcididae    | Chalcididae                     | 4     | 0.61    |
| 14    |              | Formicidae     | <i>Formica</i> sp.              | 2     | 0.31    |
| 15    |              | Halictidae     | <i>Halictus</i> sp.             | 28    | 4.3     |
| 16    |              |                | <i>Lasioglossum</i> sp.1        | 18    | 2.76    |
| 17    |              |                | <i>Lasioglossum</i> sp.2        | 14    | 2.15    |
| 18    |              | Megachilidae   | <i>Megachile</i> sp.1           | 120   | 18.43   |
| 19    |              |                | <i>Megachile</i> sp.2           | 28    | 4.3     |
| 20    |              |                | <i>Hoplitis</i> sp.             | 15    | 2.3     |
| 21    |              | Lepidoptera    | <i>Thymelicus</i> sp.           | 1     | 0.15    |
| 22    |              |                | <i>Lycanides boeticus</i>       | 4     | 0.61    |
| 23    |              |                | <i>Melanargia lachesis</i>      | 25    | 3.84    |
| 24    |              |                | <i>Erebia</i> sp.               | 2     | 0.31    |
| 25    | Thysanoptera | Sphingidae     | <i>Macroglossum stellatarum</i> | 21    | 3.23    |
| 26    |              | –              | <i>Gen</i> sp.                  | 5     | 0.77    |

agamospermy and spontaneous autogamy indicates that, as in other Antirrhineae species (Sutton 1988), this species is not apomictic, nor does it have a mechanism for spontaneous self-pollination.

#### Pollen quantity and quality limitation

Seed production in *P. rivas-martinezii* appears to be limited by the quantity of pollen grains reaching stigmas. This self-incompatible species depends on pollinator visits and is, therefore, subject to pollen quantity limitation, which is herein supported by the fact that flowers supplemented with pollen from the same population set significantly more fruits and seeds than controls (Fig. 1; Table 1). Furthermore, our control results showed that fruit set varied between the 2 years (Table S1), indicating that pollen

limitation fluctuates in time. The fourfold lower fruit set yielded by controls relative to supplementation in 2008 may suggest a decrease in number of pollinator visits, a lower load of compatible pollen than the previous year or less favorable environmental conditions. Moreover, the lower fruit set for most pollination treatments in 2008, indicates that environmental changes may be a major factor determining this fluctuation between the 2 years.

In addition, the low fecundity shown by self-pollinated flowers gives evidence for limited pollen quality. Flowers that were cross-pollinated with plants from a different population yielded the highest results (Fig. 1; Table 1). Differences between supplemented and out-crossed flowers were significant at the seed set and viable seed set stages, but not at the fruit set stage. These differences did not seem to

come from bagging, since fruit set was equivalent and no apparent additional damage to flowers was observed on unbagged flowers. Also supplemented flowers yielded higher results than controls which were not bagged either. These findings suggest that the main factor explaining reduced sexual reproduction could be due to limited mate availability promoted by clonal growth in the study area, which leads to low quality pollen reaching stigmas in this predominantly self-incompatible species. Other self-incompatible species such as *Centaurea corymbosa* (Hardy et al. 2004) or *Maianthemum bifolium* (Honnay et al. 2006) have shown that viable seed set is limited by mate availability. Furthermore, it is very unlikely that *La Serrota* population could naturally receive pollen grains from surrounding populations. Therefore, this study provides experimental evidence of a small population of a clonally reproducing, self-incompatible species that suffers from inefficient pollination and is threatened with extinction, as are many others under these circumstances (Les et al. 1991; Silvertown 2008).

The low viable seed set (10%) yielded by potentially high quality pollen used in cross-pollination, indicates that there must be other factors limiting seed viability. These could include limited resources, resource allocation between vegetative and reproductive growth and accumulation of deleterious mutations. Comparisons with other species may help understand the influence of these factors, even if results for *P. rivas-martinezii* could be very different in the absence of genetic problems. In addition, most studies do not account for seed viability, but only fruit set or seed set. In this sense, seed set can reach up to 49% with cross-pollination in *P. rivas-martinezii*, which is equivalent to that shown by other outcrossing perennials (Wiens 1984). Although many outcrossing perennials have shown even lower seed sets: *Linaria vulgaris* (1.6%) (Saner et al. 1994); *Decandra candensis* (2–7%), *Erythronium albidum* (6–11%) (Schemske et al. 1978) or *Genista versicolor* (25%) (Gómez and García 1997). In addition, seed viability is very variable across species, with *Linaria vulgaris* reaching up to 50% (Nadeau and King 1991) or *Veronica* spp. reaching up to 100% (Juan et al. 1995). Moreover, seed viability can also vary between years (Chambers 1989), and studies in progress have shown that this is the case in *P. rivas-martinezii* (M. E. Amat, unpublished).

Therefore, although a 10% is a very low value of viable seed set, it might be enough for a species that produces thousands of flowers and can compensate seed production across years.

### Pollinator limitation

Pollinator limitation may arise via low pollinator visitation rates and low pollinator efficiency (Gómez et al. 2007). Although an exhaustive survey was not performed for *La Serrota* population, a similar pollinator assemblage was observed, as expected from the equivalent ramet density and disposition of patches in both populations (Table S2). The visitation rate in *Los Conventos* population (0.0013) was low compared to other specialized Iberian species, such as some *Antirrhinum* species which showed 0.15 to 0.31 visits per hour (Vargas et al. 2010). In contrast, the generalist *Erysimum mediohispanicum* showed values between 33.3 and 95.5 visits per hour depending on the population (Gómez et al. 2007).

About 41% of visits were carried out by bees, which are very effective pollinators (Dafni et al. 2005), but their visitation rate was extremely low (below 0.0002 visits per flower per hour). Among low efficiency pollinators, beetles carried out about 46% of all visits. This low efficiency was dramatically exemplified by *Myiabris* sp. (which damaged 3.22% of flowers) and a number of different unidentified and unrecorded insect larvae. Low efficiency pollinators were as abundant as efficient pollinators. Therefore, efficient pollinator abundance may swamp out negative fitness effects of inefficient pollinators (Lau and Galloway 2004). Nevertheless, floral herbivory on plant reproduction should be further investigated, for it could be a relevant factor reducing seed production and pollinator visitation rates (Sánchez-Lafuente 2007).

The diversity in morphology, size, and behaviour of floral visitors suggested a generalized pollination system. This finding contradicts the previous idea that the occluded flower of *P. rivas-martinezii* would require specialized visitors as in other Antirrhineae species. For instance, in *Antirrhinum* species bees accounted for over 90% of visits (Vargas et al. 2010); pollinator assemblage of *Linaria aerurgineae* was composed mainly of bees (Herrera et al. 2009), and these were the only pollinators recorded in *L. vulgaris* (Stout et al. 2000) and *L. lilacina* (Sánchez-Lafuente

2007). The diverse floral visitor assemblage of *P. rivas-martinezii* indicates that the corolla is not fully occluded and that a type of semi-generalist personate flower can be assumed. Although opening the corolla to a more generalized pollinator assemblage may increase visitor diversity, extreme generalization may decrease overall efficiency (Gómez et al. 2007; Perfectti et al. 2009). The question remains as to whether additional factors affect visitation rates given that *P. rivas-martinezii* received a lower pollinator rate than *Antirrhinum* despite its semi-occluded flower.

#### Implications for conservation

Self-incompatible species may reduce seed production due to low pollinator efficiency (Schemske and Horvitz 1984; Vaughton and Ramsey 2010), mate availability (Byers 1995; Campbell and Husband 2007; Busch and Schoen 2008), and stigma clogging with self pollen (Proctor et al. 1996). In this sense, *P. rivas-martinezii* yields a very low quantity of seeds by hand self-pollination compared to pollen supplementation and hand cross-pollination and should be unable to produce enough self-seeds to maintain a viable population. Furthermore, mate availability may be extremely lowered by clones colonizing large surfaces and forming populations with a single or very few genotypes in clonally reproducing species (Charpentier et al. 2000). Studies in progress indicate that the spatial genetic structure created by clones in *P. rivas-martinezii* limits successful pollination (Amat et al., unpublished). Therefore, the balance and compensation across years between sexual and clonal reproduction will determine population viability, since it could be ultimately compromised by clonal reproduction alone.

It is important to conserve populations with high genetic diversity to allow successful cross pollination, increase the number of S alleles and allow adaptation to environmental stochasticity. Severe reductions in population size should be tackled by controlling excess grazing by large herbivores and maintaining habitat heterogeneity, with clearings that will allow the recovery and gene flow within populations. For this reason, reinforcements are not recommended before genetic data and knowledge on population dynamics are available for this regionally rare, but locally abundant species. Conservation

measures should focus on understanding the factors that enhance one kind of reproduction or the other and evaluating whether present conditions of the plant are able to assure its sustainability in the long run.

#### Concluding remarks

In conclusion, the low sexual reproductive success in *P. rivas-martinezii* is mainly a consequence of low mate availability (pollen quality) and secondarily of insufficient pollination (pollen quantity). Environmental stochasticity and resource limitation are other undergoing factors, as shown by the low seed viability and the variation in treatments across years. Although a priori floral morphology suggested a more specialized pollination system, a more generalized system can be inferred from the ample floral visitor assemblage observed on actual surveys, rather than on pollination syndromes (Aigner 2004). A low visitation rate of relevant pollinators, due to the long distance flights required to reach compatible mates, may be behind the causes of the observed pollen limitation. Further spatial genetic analysis would reveal the underlying mating opportunities among genotypes.

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# PLANT ECOLOGY

## Pollen quality limitation in the Iberian endangered genus *Pseudomisopates*

### (Antirrhinaceae)

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Table. S1. Fruit set, seed set and viable seed set resulting from pollination treatments.

<sup>a</sup> Initial number of flowers treated were 20 in 2007 and 30 in 2008. Flowers lost during the experiment are not included.

|                             | Agamospermy | Spontaneous<br>autogamy | Obligated autogamy |             |             | Cross pollination |             |             | Pollen<br>supplementation |             |             | Control     |             |             |
|-----------------------------|-------------|-------------------------|--------------------|-------------|-------------|-------------------|-------------|-------------|---------------------------|-------------|-------------|-------------|-------------|-------------|
|                             | <u>2007</u> | <u>2007</u>             | <u>2007</u>        | <u>2008</u> | <u>Mean</u> | <u>2007</u>       | <u>2008</u> | <u>Mean</u> | <u>2007</u>               | <u>2008</u> | <u>Mean</u> | <u>2007</u> | <u>2008</u> | <u>Mean</u> |
| N                           | 20          | 13                      | 11                 | 30          |             | 11                | 28          |             | 20                        | 30          |             | 16          | 30          |             |
| Fruit set                   | 0           | 0                       | 0.182              | 0.100       | 0.141       | 0.545             | 0.500       | 0.523       | 0.300                     | 0.400       | 0.350       | 0.313       | 0.100       | 0.207       |
| Seed set                    | 0           | 0                       | 0.136              | 0.061       | 0.099       | 0.477             | 0.503       | 0.490       | 0.258                     | 0.231       | 0.245       | 0.155       | 0.197       | 0.176       |
| Viable seed set             | -           | -                       | -                  | 0           | 0.000       | -                 | 0.097       | 0.097       | -                         | 0.004       | 0.004       | -           | 0.000       | 0.000       |
| No. of flowers <sup>a</sup> | 20          | 20                      | 11                 | 30          | 41          | 11                | 28          | 39          | 20                        | 30          | 50          | 16          | 30          | 46          |
| No. of fruits               | 0           | 0                       | 2                  | 3           | 5           | 6                 | 14          | 20          | 6                         | 12          | 18          | 5           | 3           | 8           |
| No. of seeds                | 0           | 0                       | 6                  | 4           | 10          | 63                | 155         | 218         | 34                        | 61          | 95          | 17          | 13          | 30          |
| No. viable seeds            | -           | -                       | -                  | 0           | 0           | -                 | 30          | 30          | -                         | 1           | 1           | -           | 0           | 0           |

Table S2. List of floral visitors of *Pseudomisopates* for a *La Serrota* population. This pilot study includes 510 minutes of observation, from 50 plants located in 5 patches.

|    | Order        | Family         | Species                  | Total | % Visit |
|----|--------------|----------------|--------------------------|-------|---------|
| 1  | Coleoptera   | Meloidae       | <i>Mylabris</i> sp.      | 24    | 32.00   |
| 2  |              | Nitidulidae    | <i>Melighetes</i> sp.1   | 28    | 37.33   |
| 3  |              |                | <i>Melighetes</i> sp.2   | 1     | 1.33    |
| 4  | Diptera      | Scathophagidae | <i>Scatophagidae</i>     | 3     | 4.00    |
| 5  | Heteroptera  | Miridae        | <i>Hadrodemus</i>        | 2     | 2.67    |
| 6  | Hymenoptera  | Chalcididae    | Chalcididae              | 7     | 9.33    |
| 7  |              | Formicidae     | <i>Formica</i> sp.       | 2     | 2.67    |
| 8  |              | Halictidae     | <i>Halictus</i> sp.      | 5     | 6.67    |
| 9  |              |                | <i>Lassioglossum</i> sp. | 1     | 1.33    |
| 10 | Thysanoptera | -              | <i>Gen</i> sp.           | 2     | 2.67    |